

**Autophagy in Head and Neck Cancer Associated Fibroblasts:
Biology and Therapy**

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is a devastating disease. Despite therapeutic advancements, little change in 5-year survival has been made for patients with HNSCC. To improve therapy, a better understanding of the underlying biology is needed. Recent results suggest that cancer-associated fibroblasts (CAFs) drive disease progression. CAFs comprise the most abundant microenvironment cell type in HNSCC, and robustly support the cancer. Yet, little is understood of the underlying biology of CAFs. We sought to investigate the biological mechanisms mediating CAF-facilitated HNSCC progression. To our surprise, CAFs demonstrate significant upregulation of autophagy compared to normal fibroblasts (NFs) from cancer-free patients. Autophagy is fundamentally involved in cell degradation, but emerging evidence suggests a role for autophagy in cellular secretion. Thus, we hypothesized that autophagy-dependent secretion of tumor-promoting factors by HNSCC-associated CAFs may explain their role in malignant development. In support of this hypothesis, we observed a reduction in CAF-facilitated HNSCC progression after blocking CAF autophagy. Assessment of CAF-conditioned media after autophagy blockade revealed levels of secreted IL-6 and IL-8, and other cytokines to be modulated by autophagy. We identify that HNSCC induces fibroblast autophagy through basic fibroblast growth factor, IL-6, and IL-8. Although autophagy is implicated in other cancers, little is known about autophagy inhibition in HNSCC. Thus, we assessed the therapeutic potential of targeting autophagy in HNSCC preclinical models. In a CAF-HNSCC mouse xenograft model, pharmacologic inhibition of Vps34, a key mediator of autophagy, enhanced the antitumor efficacy of cisplatin. Our results establish an oncogenic function of autophagy-dependent secretion in HNSCC stromal cells that promotes malignant progression.

Dedication

This work is dedicated to the patients who donated tissue for these studies.
I hope the experiments conducted and the results obtained will one day be of benefit to you.

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Chapter I - Introduction

Few maladies cause morbidity and mortality as great as head and neck cancer. Each year, 500,000 new patients are diagnosed globally with this disease (Torre et al., 2015). Both the global incidence and US incidence remain unchanged over the past three decades (Cancer Collaboration, 2017). The five-year survival rate is less than 50% (Braakhuis, Leemans, & Visser, 2014). Current treatment strategies founded in ablative surgery, cytotoxic chemotherapy and radiation cause patients to suffer significant morbidity. With dismal outcomes such as these, there is great need to develop better treatments for patients suffering from this disease.

Head and neck cancer comprises tumors originating from the mucosal surfaces of the oral cavity, pharynx and larynx. Of these, over 90% arise from proliferating squamous cells (HNSCC, head and neck squamous cell carcinoma). The remaining 10% develop primarily from salivary glands, and a small prevalence of sarcomas (Peng, Grogan, & Wang, 2014), paragangliomas (Pellitteri et al., 2004), and neuroblastomas (Carey et al., 2017). Alongside being the most prevalent, HNSCC is also the deadliest.

Etiologically, tobacco, alcohol, and human papilloma virus (HPV), comprise the primary risk factors for HNSCC. The non-viral causes, tobacco and alcohol, have long associated with HNSCC. Alcohol consumption of greater than 30 g ethanol/day (equivalent to three standard drinks (Kalinowski & Humphreys, 2016)) significantly increases the risk of oral cavity cancer (multivariable adjusted incidence rate ratio: 6.39) (Maasland, van den Brandt, Kremer, Goldbohm, & Schouten, 2014). Smoking tobacco increases risk for oro/hypopharyngeal cancer and laryngeal cancer (multivariable adjusted incidence rate ratio: 8.53 and 8.07, respectively) (Maasland et al., 2014). However, as global smoking rates decline (GBD Tobacco Collaborators, 2017), the landscape of non-viral oropharyngeal carcinoma has decreased by a remarkable 50% since 1988 (Chaturvedi et al., 2011).

In contrast, HPV positive oropharyngeal carcinoma incidence has increased by 225% in this same period (Chaturvedi et al., 2011). HPV Infection does not associate with tobacco or alcohol use, but rather promiscuous sexual history and marijuana use (Gillison et al., 2008). HPV has partiality for squamous epithelium in the head and neck, cervix, and anal regions. As a DNA virus, HPV incorporates into the host genome, and transforms the host cell with viral proteins E6 and E7 to inhibit the tumor suppressors, p53 and Rb, respectively (Vidal & Gillison, 2008). As such, these tumors present differently, and have a more favorable clinical outcome, than carcinogen-induced tumors.

Globally, other inciting factors include Betel (also known as Areca) nut chewing, Khat chewing, and Maté (Goldenberg et al., 2004). These all have a dose dependent effect on cancer incidence, and demonstrate mutagenic effects. Additionally, Epstein Barr virus can transform cells of the oropharynx, tonsil, and salivary gland, but most commonly affects the nasopharynx (Wheeler et al., 2014). However, incidence from these pales in comparison to tobacco, alcohol, and HPV.

The HNSCC Patient

HNSCC patients are primarily above the age of 40 and male, with a male to female ratio of 3:1-8:1 (Marur & Forastiere, 2016). Patient presentation, symptoms, and clinical outcomes vary by anatomic site. In the oral cavity, lesions often are self-discovered, as they are easily appreciable by physical exam. These lesions present as a thickening of the mucosa, a painless or painful mass, or ulcer, and are accompanied by dysphagia and odynophagia (Insalaco, 2017). Lesions of the larynx often are more difficult to discover, and present with hoarseness, shortness of breath, and stridor on exam (Insalaco, 2017). Tonsillar and base of tongue tumors commonly have no symptoms and grow to a large size before impacting speech, or restricting tongue

movement (Posner, 2016). Asymptomatic tumors lead to later detection, which confers a worse prognosis.

Prognostic factors at initial diagnosis include tumor staging, current tobacco use, and race (Fakhry et al., 2017). HNSCC tumor staging relies on TNM staging guidelines by the American Joint Committee on Cancer. The guidelines differentiate patient prognosis into categories ranging from 0 (best prognosis) to IV (worst prognosis) depending on tumor (T) characteristics, nodal (N) characteristics, and distant metastasis (M). Recently, the guidelines changed with the growing rate of HPV-positive HNSCC, and an appreciation for the different prognostic consequences of HPV-positivity. Now, p16 immunohistochemistry (IHC) is first used in oropharyngeal HNSCC staging considerations (Lydiatt et al., 2017). For all other sub-sites, and for p16 negative oropharyngeal tumors, the most significant variable in staging is the presence of metastasis (M). Distant metastasis places a patient at Stage IV regardless of T and N stage. Tumor size in centimeters at the greatest dimension determines T criteria. Number, symmetry (as in contralateral or ipsilateral lymph nodal involvement relative to primary site), and size of lymph nodes determines N criteria. These criteria adequately construct high predictive power for approximation of survival of individual patients (Groome, Schulze, Boysen, Hall, & Mackillop, 2001). Beyond TNM staging, other statistically significant risk factors include race and environment, with black non-Hispanics and current tobacco users having significantly worse outcomes (Fakhry et al., 2017). Overall, early detection provides better outcomes to patients. Yet, at diagnosis, over 40% of patients have advanced disease with regional node involvement (Marur & Forastiere, 2016).

Disease Progression

The TNM staging guidelines mirror the understood course of disease progression. Our current understanding of the disease course arises from histologic observations and a propensity for dysplastic lesions to transition into invasive carcinoma. To understand this, an understanding of the normal tissue architecture is helpful. In the head and neck, the mucosal architecture varies slightly by anatomic sub-site. The lining mucosa of the inner lips, cheeks, soft palate, floor of the mouth, undersurface of the tongue, and pharynx is nonkeratinized stratified squamous epithelium (Nahirney, 2013). The gingiva and hard palate are lightly keratinized (Nahirney, 2013). The dorsal tongue contains many papillae and differs slightly in architecture than other subsites

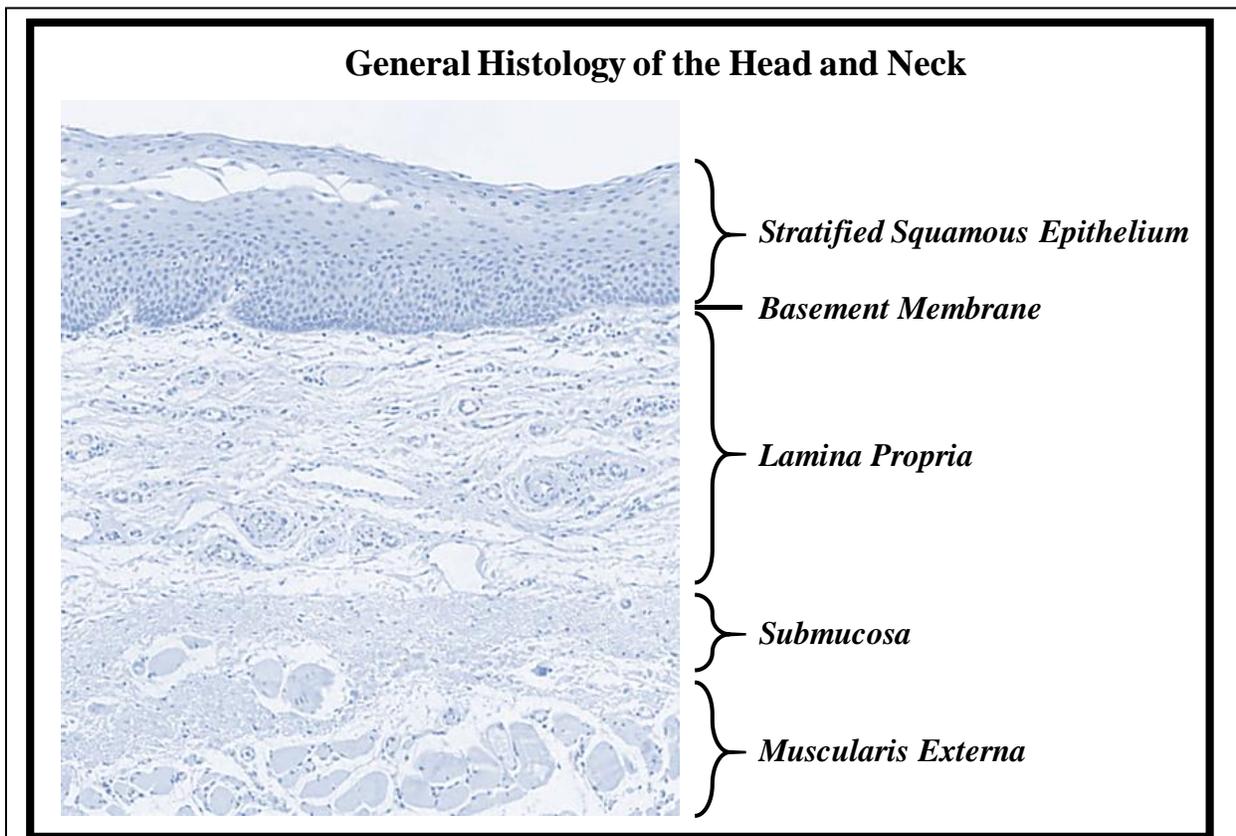


Figure 1.1-General Histologic Anatomy of the Head and Neck

Broadly, the head and neck epithelium follow the same anatomical pattern, with different regions having differing thicknesses of each mucosal layer. Light Microscopy (20X Magnification).

within the upper aerodigestive tract (Nahirney, 2013). However, broadly, the mucosa of the head and neck follow the same structural pattern (Figure 1.1).

Most superficially, the multilayered, nonkeratinized, stratified squamous epithelium grows from cuboidal basal cells resting on the basement membrane. Superficial squamous cells retain their nuclei, however, the density of nuclei decreases as cells migrate to the superficial surface. The epithelial basal cells attach to the basement membrane with keratins 5 and 14 anchoring to a hemidesmosome (Yancey, 2018). The basement membrane consists primarily of type IV collagen, laminins, and heparan sulfate proteoglycans (Yancey, 2018). This membrane divides the epithelium from the lamina propria. The lamina propria contains a loose, highly cellular connective tissue layer primarily containing fibroblasts. Small capillaries, lymph, and nerves extend through the lamina propria. Occasional papillae extend from the lamina propria into the stratified squamous epithelium to carry capillaries and lymph vessels (Nahirney, 2013). In subsites with musculature, the muscularis mucosa lies deep to the lamina propria and superficial to the submucosa, muscularis externa, and adventitia (Nahirney, 2013). Within the deep layer of the adventitia lie nerves and vasculature, including arteries, veins, and lymphatic channels. This structural pattern remains broadly consistent throughout the mucosal layers. At the superficial layer, carcinogens routinely encounter the stratified squamous epithelium leading to mutation and proliferation of these cells.

The architecture transitions during disease progression (Figure 1.2). HNSCC is thought to begin with an intraepithelial proliferation of squamous cells. This proliferation sometimes presents as leukoplakia, a white patch or plaque that cannot be removed by rubbing (Warnakulasuriya & Ariyawardana, 2016). Histologically, the squamous epithelium thickens in diameter, and the tissue may become dysplastic. Importantly, leukoplakia is a poor predictor for

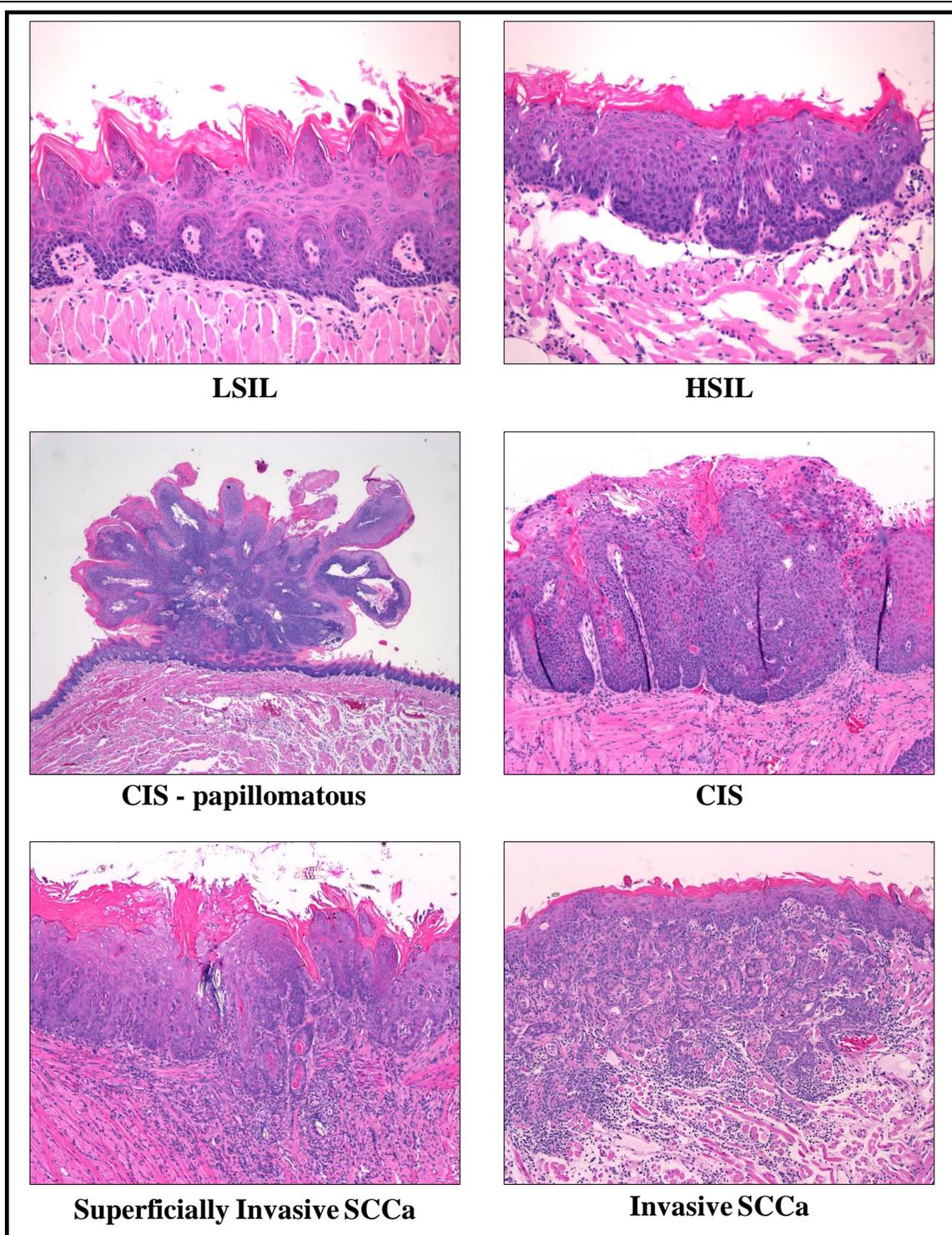


Figure 1.2 – Disease Progression of HNSCC

4-NQO murine model demonstrates progression of HNSCC from low grade squamous intraepithelial lesion (LSIL), high grade squamous intraepithelial lesion (HSIL), carcinoma in situ (CIS, of two types), and invasive squamous cell carcinoma (Invasive SCCa). Tongue with H&E stain (10 X CIS and SCCa; 20 X LSIL, HSIL).

HNSCC as less than 4% of leukoplakia patients progress to invasive cancer (Einhorn & Wersall, 1967). The predictor of leukoplakia progression appears to be the severity of dysplasia.

Dysplastic lesions are more common in the floor of the mouth, and least common in the retromolar space (Waldron & Shafer, 1975). This corresponds with commonality of HNSCC by subsite (Mashberg & Meyers, 1976). Severely dysplastic lesions are termed carcinoma in situ, which represents significant epithelial thickening and increased nuclei throughout the epithelial layer; yet, no histologically observable damage to the basement membrane or underlying structures occurs. As such, few cell types from deep layers are involved in early dysplastic lesions.

As carcinoma in situ progresses to invasive squamous cell carcinoma, the epithelial growth is thought to infiltrate the basement membrane. However, no direct molecular biology has demonstrated this transition. Histologic evidence demonstrates normal mucosa adjacent to the invasive tumor with a gradual transition of dysplasia. Molecular evidence demonstrates epithelial cells at the malignant surface take on a partial mesenchymal gene signature, that corresponds with high expression of MMP-10 and MMP-2 compared to the tumor as a whole (Puram et al., 2017). This would suggest these leading edge cells help to degrade the basement membrane. With this destruction of normal architecture, the epithelial cells begin to interact with many underlying cell types including fibroblasts, adipocytes, nerves, vasculature and numerous leukocytes. These microenvironment cells act as a double edge sword: working to both limit tumor progression yet also contributing to advanced disease. The proliferating epithelial cells modify the microenvironment. This occurs through direct modification of the extracellular matrix, and by secretion of numerous factors, which act upon almost all microenvironmental cell types. Although the epithelial cells harbor many mutations, the microenvironment cells are

understood to harbor few (Qiu et al., 2008). Yet, the activated carcinoma associated cells retain heritable patterns of activation markers, and a different expression profile compared to non-activated cells from cancer-free patients (Costea et al., 2013).

Beyond the initial presentation, HNSCC has a high propensity for recurrence. The majority of patients develop recurrent disease, and distant metastases occur in up to 30% of patients (Vermorken & Specenier, 2010). Carcinogenic risk factors of HNSCC facilitate field cancerization, where multiple primary tumors will develop within the same anatomic region (Slaughter, Southwick, & Smejkal, 1953). Molecular biology confirms this phenomenon as surrounding normal tissue around the carcinoma also has genetic alterations in the epithelia (Tabor et al., 2001). This facilitates a high rate of recurrence, as even adequately resected tumors and targeted radiotherapy cannot account for the dysplastic field of carcinogen exposure.

Overall, the five-year survival rate of patients at initial presentation is less than 50%. With such a dismal outcome, specialized treatment requires a multidisciplinary approach encompassing head and neck surgeons, radiation oncologists, and medical oncologists, alongside speech therapists, social workers, nutritionists and psychologists.

Current Therapy

Site, stage, and patient characteristics influence the treatment approach amongst the three treatment modalities of surgery, radiation, and chemotherapy. Primarily, surgery and radiation are used in early stage disease, while chemotherapy is used in combination with progressed stages. As the first and only therapeutic option available for many years, surgery has the longest record of accomplishment; thus, this established the head and neck surgeon as the primary leader of the care team. However, radiation and chemotherapy offer approaches to microscopic disease not identifiable by gross dissection. Additionally, for HPV+ tumors, radiotherapy is often used as

the primary treatment modality. Each therapeutic approach offers unique outcomes alongside distinctive morbidities.

Surgery

As the standard of care for nearly all head and neck malignancies, surgery provides excellent control of disease. Surgical approach and technique vary widely based on location of the tumor, and local aggressiveness (Lorenz, Couch, & Burkey, 2017). For examples, tumors of the oral cavity and oropharynx are approached transorally, whereas tumors of the hypopharynx and larynx, or regionally involved lymph nodes, are approached by neck dissection.

Considerations during surgery include cosmetic outcome, and retention of function. For example, the surgeon must consider impacts on the airway, swallowing and speech. Successful surgery largely depends on negative margins, with a preferred tumor-free margin of at least 3 mm (Lorenz et al., 2017). Reconstructive surgery, especially involving microvascular free flaps, has advanced considerably in the last 25 years, and thus offers the surgeon much latitude in establishing negative margins (Vermorken & Specenier, 2010). With negative margins and appropriate reconstruction, patients are unlikely to experience significant functional impacts.

However, morbidity increases with the extent of surgical procedures. Immediately post operation, complications include pulmonary embolism, hemorrhage and aspiration pneumonia (Vermorken & Specenier, 2010). Additionally, a comprehensive pain management plan is required following surgery. Depending on tumor location, a tracheostomy or gastrostomy tube may be considered, which can provide additional comfort (Lorenz et al., 2017). Additionally, although cosmesis is a concern to every surgeon, advanced disease is associated with large margins and can cause disfiguration. Additional reconstructive surgery provides palliation to these patients. Single modality treatment of surgery is appropriate for patients who present with

stage I or stage II disease, and in this population cure rates can reach up to 90% (Vermorken & Specenier, 2010). For patients with advanced disease, combination therapy is recommended.

Radiotherapy

Ionizing radiation damages molecules through direct ionization of target molecules and indirectly by generating hydroxyl radicals from water (Barcellos-Hoff, Park, & Wright, 2005). Ionizing radiation is delivered in fractionated doses of 2 Gy/fraction daily over 6-7 weeks to patients with high risk disease, or 1.6-1.8 Gy/fraction in low to immediate risk disease (National Comprehensive Cancer Network, 2017). In early stage disease, radiotherapy is appropriate as a single agent and can establish effective cure rates at 70-90% (Corvò, 2007). However, in advanced disease, radiation therapy only controls about 50% of tumors, and leaves the five-year survival rate at 30% (Corvò, 2007). Additionally, patients suffer long term-morbidity with complications arising from radiation, such as radiation-induced fibrosis, xerostomia, osteoradionecrosis, and trismus (Straub et al., 2015). The morbidity and mortality in patients with advanced disease undergoing radiation as a single modality therapy prompted studies in the combination of chemotherapy and radiotherapy.

Chemotherapy

Chemotherapy has developed extensively for head and neck patients over the last 40 years. Before the approval of cisplatin in 1978, patients only received palliative therapy with methotrexate and bleomycin (Hong & Bromer, 1983; Pitman, Miller, & Weichselbaum, 1978). Tumors responded to these cytotoxic agents, but these regimens offered no improvement in survival. Although cisplatin offered no improvement of response rate or overall survival compared to methotrexate as a single agent (Grose, Lehane, Dixon, Fletcher, & Stuckey, 1985), the arrival of cisplatin coincided with a number of combination agent trials. From these trials,

platinum containing combination therapies had the greatest response (Clavel et al., 1987). As such, these trials established cisplatin as a key therapeutic for HNSCC. Cisplatin only improves median survival compared to best supportive care by 1-2 months (Morton et al., 1985).

Additionally, amongst cytotoxic regimens, combination therapy with cisplatin only improves median survival by at most 25 days compared to single agent (Colevas, 2006). Thus, although cisplatin offered improved response rates, these regimens offered no improvement in survival at the cost of a plethora of side effects.

Of non-targeted chemotherapies, the best regimens consisted of cisplatin combined with either fluorouracil or a taxane. These regimens offer comparable response rates and survival, whether a taxane or fluorouracil are used (Sacco & Cohen, 2015). These offer a 30% response rate to patients, with a median overall survival of 6 to 8 months (Gibson et al., 2005). The advent of epidermal growth factor receptor (EGFR) targeted therapy with the monoclonal antibody cetuximab offered an improvement in survival, albeit just a 2-month extension compared to the cytotoxic regimen (Vermorken et al., 2008). However, remarkably, this was the first trial since cisplatin was introduced three-decades prior to demonstrate improved survival. As such, investigators initiated a number of trials targeting the EGFR pathway, such as additional monoclonal antibodies panitumumab (Vermorken et al., 2013) and zalutumumab (Machiels et al., 2011), and tyrosine kinase inhibitors, gefitinib (A. Argiris, Ghebremichael, et al., 2013), erlotinib (Soulieres et al., 2004), lapatinib (de Souza et al., 2012), afatinib (T. Y. Seiwert et al., 2014), dacomitinib (H. S. Kim et al., 2015). However, none of these studies demonstrate improved survival compared to cetuximab, and no significant improvements in side effect profile.

Since the addition of cetuximab to the clinical regimen 10 years ago, only recent developments in immunotherapy have improved survival. Head and neck cancers undergo

immune evasion by programmed death ligands (PD-L1 and PD-L2) on the cancer cell, which activate the T-cell suppressive receptor programmed death-1 (PD-1) (R. Ferris, 2015). Recently, nivolumab, a monoclonal antibody targeting PD-1, demonstrated remarkable results in improvement of survival relative to cetuximab, methotrexate or docetaxel (Robert L. Ferris et al., 2016). Results of another PD-1 antibody, pembrolizumab, corroborate this targeted strategy at improving survival and response (Tanguy Y. Seiwert et al., 2016). These recent reports demonstrate the clinical potential of targeting additional microenvironment cell types.

Thus, current treatment of HNSCC stratifies primarily by stage at diagnosis. Early stage tumors have a relatively excellent outcome by surgery and radiation alone. However, advanced stage tumors require extensive chemotherapy protocols alongside surgery and radiation. These protocols center around a cisplatin/docetaxel/cetuximab backbone, which offers, in combination with radiation and chemotherapy, a progression free survival of 7.1 months and an overall survival of 15.3 months (National Comprehensive Cancer Network, 2017). When the disease progresses despite platinum-containing chemotherapy, nivolumab offers a second-line agent (National Comprehensive Cancer Network, 2017). These regimens reflect more than 50 years of scientific work, and extend patient quality of life. However, the outcome of HNSCC with even the best clinical care is dismal. The best improvement in overall survival is measured in months of patient life, not years.

Preclinical Models-An Avenue to Improved Therapies

Overall, just three therapies have extended patient life in the past 70 years: cisplatin, cetuximab, and PD-1 inhibitors. The lack of effective therapeutics does not reflect the incredible responses observed in preclinical results. For example, the proteasomal inhibitor Bortezomib demonstrated fantastic results in *in vitro* and *in vivo* animal models (Sunwoo et al., 2001);

however, in the clinic, bortezomib resulted in early progression of HNSCC and trials were discontinued (Athanasios Argiris et al., 2011). Dissection of current preclinical models will likely reveal the biological mechanisms underlying the dearth of available therapeutics

The most widely used preclinical model is the HNSCC cell lines. These cells are derived from human explants by manual dissociation or enzymatic digestion of the specimen. Nearly every anatomic subsite of HNSCC has cell lines derived, and the abundance of available HNSCC cell lines contain nearly every pathogenic mutation (Lin et al., 2007). The *in vitro* techniques associated with understanding disease progression, such as proliferation, apoptosis, and invasion assessments are widely established, relatively inexpensive and useful for understanding the underlying mechanisms within the tumor cells. However, not all tumors can be cultured. The success rate of obtaining an epithelial only population from a patient explant is less than 30% (Owen et al., 2016). Additionally, pure cultures of HNSCC devoid of other cell types take on average 195 days to establish (Owen et al., 2016). This long length of time may confer selection pressures not applicable to patient biology. Further, the three-dimensional biology of the tumor is lost in the cultured cells. To model the three-dimensional environment, spheroid models allow for multidimensional analysis. Soft agar or low attachment plates make these models feasible. However, not all cell lines grow in soft agar, and many HNSCC cell lines will not associate in a three dimensional spheroid (Lin et al., 2007). This indicates that even three-dimensional systems cannot mimic the biology of every patient. These *in vitro* models are void of other cell types, vasculature, and physical pressures associated with a growing tumor.

To compensate for these deficiencies, animal models are employed to better include the dynamic microenvironment. Yet, no animal model perfectly impersonates the human disease. The biology of the human tumor is best described in xenograft murine models. These use human

tumor explants or cell lines inoculated into nude mice, carrying the Foxn1 mutation (Braakhuis, Sneeuwloper, & Snow, 1984; Rygaard & Povsen, 2007). Void of functioning T cells, these mice have an attenuated immune system that allows for the grafting of a human tumor. Ectopic implantation allows for clear assessments of tumor volume, but metastasis rarely occurs with subcutaneous flank tumors. The orthotopic floor of the mouth xenograft offers a model with more applicable metastatic characteristics. Orthotopic models offer opportunity for pulmonary metastasis and bone metastasis, alongside local invasion and vascular seeding (Dinesman, Haughey, Gates, Aufdemorte, & Von Hoff, 1990). Given the anatomic location, monitoring the progression of these tumors is difficult without imaging techniques. To provide a better understanding of the human microenvironment, patient derived xenografts (PDX) allow the direct implantation of a patient tumor, including all stromal components, into a murine host (Tentler et al., 2012). PDXs retain much of the genomic and proteomic characteristics of the original tumors (H. Li et al., 2016). However, drift occurs in PDXs that is not reflective of human HNSCC, for example there is increased expression of AKT and c-MYC (H. Li et al., 2016). PDX drift is attributed to the human stroma dissipating with successive passaging of the PDX, and an enhancement of murine stroma (H. Li et al., 2016). Additionally, all of these models develop in immune compromised athymic mice to allow growth of the xenograft.

Syngeneic, transgenic, and carcinogen-induced models offer a fully functional murine immune system. The SCC VII cell line, derived from a spontaneous C3H squamous cell carcinoma, is capable of implantation into a syngeneic host (O'Malley, Cope, Johnson, & Schwartz, 1997). In these mice, fully functional T-cells offer a dynamic microenvironment (O'Malley et al., 1997). Tumors have pulmonary metastasis and bone metastasis as observed in the orthotopic tumors (O'Malley et al., 1997). Yet, the inoculated tumors do not exhibit the

progression typical of this disease, and thus a true microenvironment is absent. To overcome this, transgenic models offer a mutation model similar to patient disease progression. Head and neck tumors have overexpressed cyclin D1, that leads to dysplastic lesions in the tongue, esophagus, and forestomach (Nakagawa et al., 1997). When Cyclin D1 overexpressing mice are crossed with p53 heterozygous mice, these dysplastic lesions give way to invasive squamous cell carcinoma in the oral cavity and esophagus (Opitz et al., 2002). Transgenic models of HNSCC are lacking compared to other tumor models due to the dearth of tissue-specific promoters within the anatomic regions affected by this disease. The best promoters to date are of keratin 5 and keratin 14, which are epithelial specific to the basement membrane and overexpressed in HNSCC (Caulin et al., 2004). Yet, most epithelial basement membranes express these keratins, making this a poor model for the head and neck. The lack of a specific promoter limits transgenic models. Carcinogen induced models offer a tissue specific insult, which leads to disease progression closely imitating the human course. The nitrate derivative, 4-nitroquinolone (4-NQO) acts as a mutagen, and can be delivered through the drinking water or direct painting of rodent tongues (X. H. Tang, Knudsen, Bemis, Tickoo, & Gudas, 2004). However, this technique takes at least 12 weeks to develop tumors, and imaging is required to monitor these tumors as they develop.

Overall, a great number of researchers and a large amount of resources have been invested in developing these preclinical models. Each has its own strengths and weaknesses. Sadly, few discoveries made in these models translate to better patient outcomes. This is true for both head and neck cancer and other cancers. The NCI has assessed the predictive value of cell lines, xenografts, and allograft models and have found no models that significantly correlate with

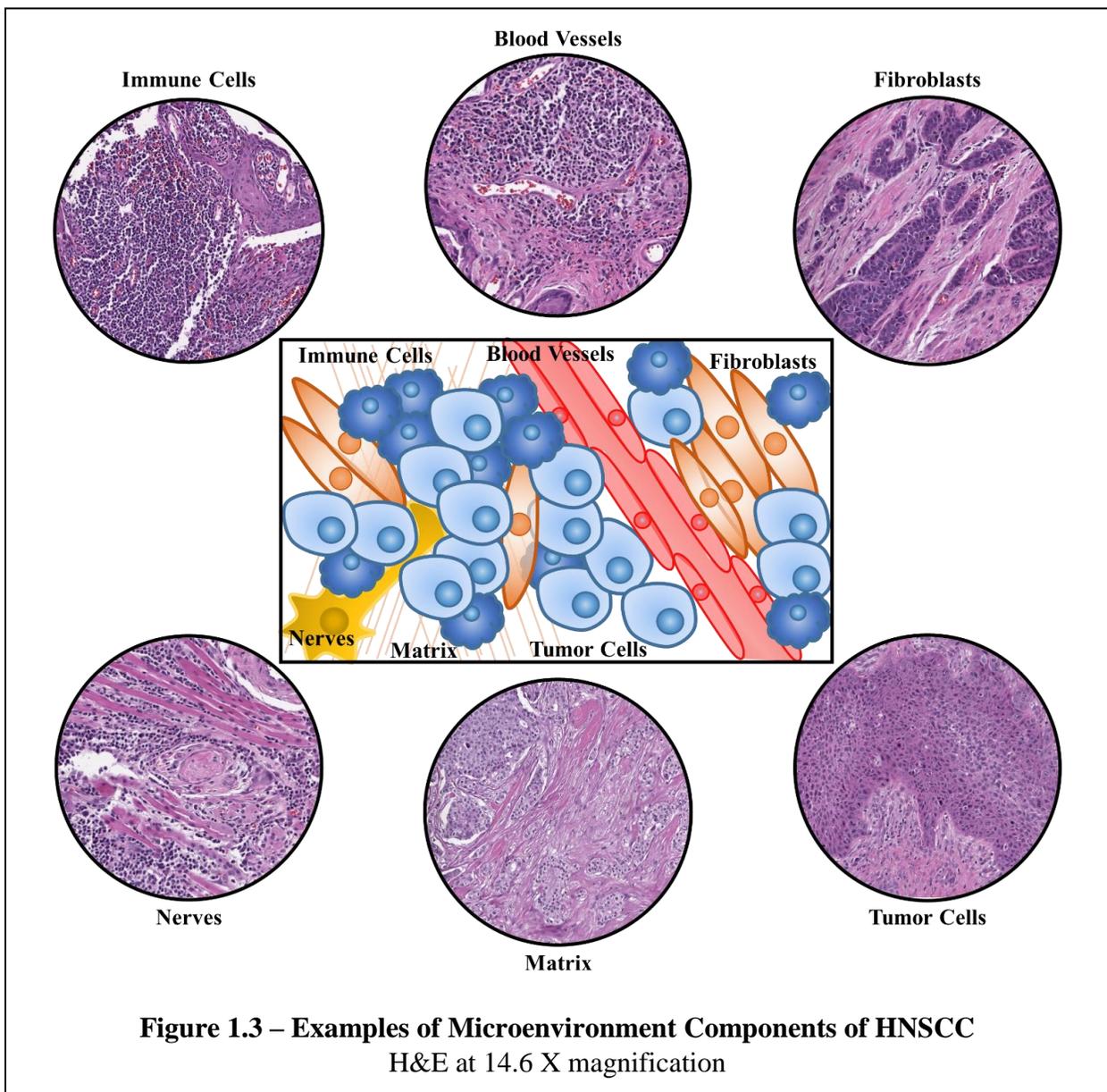
patient response in phase II studies, with the exception of non-small cell lung cancer (Voskoglou-Nomikos, Pater, & Seymour, 2003) (J. I. Johnson et al., 2001). So, what is lacking?

Perhaps, these poor outcomes occur because the human microenvironment is missing. The only microenvironment studied with current techniques is the murine microenvironment. Intriguingly, the best advancements in patient outcome modulate the tumor microenvironment. Surgery seeks to clear the tumor to a margin that retains a normal architecture. Radiation has the best long-term effects when a normal, non-fibrotic microenvironment develops. Additionally, despite decades of research focused on the rapidly proliferating cancer cells, all chemotherapeutic advancements in survival since cisplatin have acted upon the microenvironment. For example, cetuximab activates microenvironment NK cells to induce antibody-dependent cell cytotoxicity (Jie et al., 2015). Other methods of targeting EGFR within the cancer cell, such as gefitinib and erlotinib, have not extended survival. Additionally, nivolumab directly inhibits PD-1 on microenvironment T cells, and does not interact with the cancer cell (R. Ferris, 2015). Thus, it stands within reason that the microenvironmental influences on cancer progression likely equate and may surpass the contribution of cancer cells alone. New preclinical studies are needed which incorporate the patient microenvironment.

Microenvironment

The environment in which cancer develops dictates a great deal of disease progression. True of both the primary site and metastatic niches, the concept of the environmental influences on tumor growth has had attention even since Dr. Stephen Paget's seed-and-soil hypothesis regarding metastatic sites (Paget, 1989). However, despite decades of research, much is still to be understood about the microenvironment at both the primary site and metastatic niche. The vast number of cell types hampers clear understanding of the microenvironmental influence on

disease progression. Each cell dynamically regulates the neighboring cells, secreting hundreds of factors and altering cell profiles and extracellular matrix composition. Additionally, cells migrate in and out of the environment, physical pressures change, gas tensions alter, and blood flow is constantly changing. With intratumoral heterogeneity and the plethora of dynamics aforementioned, a perfect model of the microenvironment is not within reach of today's research tools. As such, research on the microenvironment pales relative to the vast amount learned about



the rapidly dividing carcinoma cells. Yet, the broad role of the microenvironment in disease progression has great appreciation.

The microenvironment consists of cellular and non-cellular components (Figure 1.3). Non-cellular components describe the extracellular matrix, physical pressures, gas tensions, and chemotactic gradients. The cellular components lead to the functional changes observed in the non-cellular components. The six broad microenvironmental cell types are: vascular endothelial cells (both blood and lymph), immune infiltrates, nerves, adipocytes, microbes, and fibroblasts.

Vasculature

The rapidly proliferating tumor requires a strong blood supply for nutrients and oxygen. As the tumor grows, oxygen tension decreases as distance from vasculature increases. Determined in lung adenocarcinoma, at a distance of 169 μm away from vasculature, low oxygen tension prompts tumor necrosis (Thomlinson & Gray, 1955). In hypoxic regions, hypoxia-inducible factors (HIF, particularly HIF1 α) are stimulated. These initiate transcriptional profiles that lead to two broad outcomes: (1) migration of carcinoma cells towards a more oxygen rich environment, and (2) the release of angiogenic cytokines. Of these, vascular endothelial growth factor (VEGF-A) is the most widely studied. VEGF-A promotes the proliferation of endothelial cells (Karl et al., 2007). Intriguingly, endothelial cells support the growth of HNSCC not only through providing a capillary supply, but also by secreting interleukin (IL)-6, IL-8 and epidermal growth factor (EGF) (Neiva et al., 2009). This symbiotic tumor-promoting relationship explains why VEGF, and HIF1 α expression correlate with poor clinical outcome (Hoogsteen, Marres, Bussink, van der Kogel, & Kaanders, 2007; Tse et al., 2007). However, despite a link to clinical outcomes, anti-angiogenic therapy in HNSCC has had limited clinical results, with no significant improvements in survival and increased adverse

events, such as bleeding (A. Argiris, Kotsakis, et al., 2013; Machiels et al., 2010; Williamson et al., 2010). Thus, intratumoral hypoxia and angiogenesis are important microenvironment characteristics in HNSCC; yet, how to target and modulate these phenomes still requires much research.

Although the tumor initiates a rich blood network, only half of malignancies are thought to spread through the blood vasculature (Maula et al., 2003). The paradox of a large number of circulating tumor cells (7 per 1000 peripheral blood mononuclear cells, equivalent to approximately 7000 circulating tumor cells per mL of peripheral blood (Weller et al., 2014)), and the dearth of lung metastasis (the first capillary bed encountered by veins) in early disease, indicates the importance of other vasculature for metastatic spread. The cervical lymph nodes are most often the first site of HNSCC metastasis, and thus highlight the role for intratumoral lymph in disease progression (Leemans, Tiwari, Nauta, Waal, & Snow, 1994). Additionally, intratumoral lymph density significantly correlates with clinical outcome (Franchi, Gallo, Massi, Baroni, & Santucci, 2004). Lymph endothelial cells are activated by VEGF-C, and VEGF-D, as well as other microenvironment factors which stimulate both the carcinoma and vascular endothelial cells including: fibroblast growth factor (FGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF)(Adams & Alitalo, 2007). The vasculature, both blood and lymph, provide vehicles by which cancer cells may spread to distant organs through disease progression.

Microbes

The role of the microbiome in cancer progression gathers great attention. The vast number of microbes contributes to the poor understanding of the role of these in HNSCC progression, and the bulk of microbiome work in HNSCC focuses on characterizing the

microbiome. The oral microbiome includes bacteria, viruses, archaea, and eukaryotes, and contains more than 1000 different microbes (Dewhirst et al., 2010). Tobacco and alcohol change the microbiome, while patients with an HPV infection have a microbiome that clusters with the normal population by 16S ribosomal RNA profiling (Leclercq et al., 2014; J. Wu et al., 2016). Broadly, patients with HNSCC have different taxa represented compared to healthy individuals (Schmidt et al., 2014). Most notably, fusobacteria increase in HNSCC patients (Gong et al., 2014; H. Wang et al., 2017). Targeting the microbiome to alter disease progression is a focus of current study, and no therapeutics target the microbial populations directly, to date.

Immune Infiltrates

The most widely studied component of the microenvironment, immune cells actively work to diminish the growing epithelial lesion. The immune microenvironment closely intertwines with disease progression. This is exemplified by observations that cancer occurs at sites of chronic inflammation, and yet evades the immune system. This is important for clinical outcomes as patients with high levels of intratumoral immune infiltrate have a better outcome than those whose tumors evade the immune system (Mandal et al., 2016). The immune contributions to the tumor microenvironment are vast, as this is a dynamic population of many cells, all which uniquely modify their environment. Out of the many immune cell types, T cells and dendritic cells compose the primary infiltrates in HNSCC; whereas NK cells and neutrophils have less abundance (Becht et al., 2016). Successful tumor progression only occurs if the cancer evades destruction by the immune system.

An immune suppressed microenvironment develops through active evasion of the cancer cells. Cancer cells reduce their immunogenicity, and secrete suppressive factors to establish this immunocompromised microenvironment. Typically, the immune system recognizes altered and

damaged cells by presentation of neoantigens in the human leukocyte antigen (HLA) complex to T-cells. However, tumor cells reduce HLA expression, leading to evasion from T-cells. NK cells actively seek for “missing self” cells that do not express HLA; however, carcinoma cells secrete cytokines such as transforming growth factor- β (TGF- β) to limit NK activation (Viel et al., 2016). Additionally, cancer cells express a number of immune checkpoints, such as PD-L1 and CD80. These establish inhibitory signals on activated immune cells, particularly T-cells, to diminish an immune response (R. Ferris, 2015). By reducing their immunogenicity, secreting immunosuppressive factors, and expressing inhibitory ligands, HNSCC evades the adaptive immune response.

The innate immune cells within the microenvironment paradoxically increase tumor growth and immune suppression. Dendritic cells within the microenvironment typically present antigen to activate an immune response. However, TGF- β and IL-10 within the microenvironment contribute to dendritic cell suppression, which leads to the secretion of factors to differentiate T-cells into regulatory T-cells (R. L. Ferris, Whiteside, & Ferrone, 2006). Additionally, tumor associated macrophages are widely present in HNSCC. Yet, these macrophages differentiate into the M2 subtype, which correspond with a suppressive milieu and increased angiogenesis (Costa et al., 2013; S. Y. Liu et al., 2008). Overall, a composed immune response would keep the tumor at bay. Yet, the tumor suppresses and evades the immune system, creating a dysregulated immune environment culpable of cancer progression.

Cell Populations in Low Abundance

Compared to other cell types, adipocytes and nervous system cells are poorly studied microenvironment components. Although these cells are abundant in the normal anatomy of the head and neck, understanding of these cells within the tumor microenvironment is lacking due to

difficulties with *in vitro* techniques. Adipocytes float in culture media due to their high lipid component, creating difficulties when comparing them with adherent cancer cells (Carswell, Lee, & Fried, 2012). Peripheral nerves in the head and neck arise from the cranial nerves and retain their nuclei within the brain. Although surgical specimens derived from HNSCC patients may contain axonal fragments, these fragments do not propagate without the nucleus. Despite these challenges, and low numbers of these cell types within the microenvironment, adipocytes and neurons contribute to tumor progression.

Few studies examine adipocytes within the HNSCC microenvironment. Yet, studies on other cancer types, primarily breast, indicate adipocytes function as tumor promoting cells within the microenvironment (Duong et al., 2017). Adipocytes in proximity to cancer cells have a tumor-promoting secretome, rich in hepatocyte growth factor, which is highly overexpressed in the HNSCC microenvironment (Dirat et al., 2011; Knowles et al., 2009; Rahimi, Saulnier, Nakamura, Park, & Elliott, 1994). The cytokines secreted from adipocytes increase cancer proliferation, migration and invasion, as well as angiogenesis. Additionally, adipocytes provide a reservoir for fatty acids necessary for rapid cell proliferation (Kwan et al., 2014; Y. Y. Wang et al., 2017). Intriguingly, prolonged exposure of adipocytes to cancer cells results in mature adipocytes losing their fatty acid content and exhibiting fibroblast-like morphology (Bochet et al., 2013). Histologically, when the tumor invades the surrounding adipose tissue, adipocytes decrease in number, and fibroblasts accumulate (Nieman, Romero, Van Houten, & Lengyel, 2013). Perhaps de-differentiation of adipocytes into fibroblasts occurs, but the mechanisms by which this occurs needs thorough investigation.

Neurons within the microenvironment contribute to pain associated with the tumor as well as a route for cancer metastasis. These neurons arise from cranial nerves V (trigeminal) and

VII (facial), both of which provide sensation. As such, HNSCC induces considerable pain attributed to cancer cells damaging the neuronal sheath, noxious stimuli within the microenvironment, and growth factors stimulating the proliferation of both the cancer and the nerve, such as nerve growth factor and brain-derived neurotrophic factor (Roh, Muelleman, Tawfik, & Thomas, 2015). Patients with perineural invasion within the HNSCC microenvironment have a worse prognosis, with higher rates of recurrence and a significantly lowered five-year survival (Fagan et al., 1998). As with adipocytes, a great deal more is yet to be understood with microenvironment neurons.

Fibroblasts

Fibroblasts comprise the most abundant cell type in the HNSCC microenvironment (Becht et al., 2016). Intriguingly, this is juxtaposed against the fact that regions susceptible to HNSCC have a relatively shallow lamina propria compared to other epithelial surfaces in the body. Nevertheless, these cancer-associated fibroblasts (CAFs) are in large abundance and considerably promote disease progression. CAFs take on an entirely different phenotype compared to normal fibroblasts (NFs) from the same anatomic location of cancer-free patients. Compared to NFs, CAFs promote HNSCC metastasis to distant organs, angiogenesis, and immune evasion. Despite the broad phenotypic differences and the clear role of CAFs in disease progression, the underlying biology of CAFs is poorly understood.

Perhaps assessing the role of fibroblasts in normal wound healing facilitates understanding these cells within the tumor microenvironment. In fact, cancer might be understood as a wound that does not heal (Dvorak, 1986). In normal tissue, fibroblasts exist to maintain the extracellular matrix. Upon injury, fibroblasts are summoned in increased numbers by a plethora of chemokines, including fibroblast growth factors (FGFs), platelet-derived growth

factor (PDGF), IL-1 β , and tumor necrosis factor alpha (TNF- α) (Eming, Martin, & Tomic-Canic, 2014). Fibroblasts migrate to the site of injury through integrin-mediated adhesion to collagen fibrils and fibronectin, and migration can be blocked by targeting each of these components (Guido & Tranquillo, 1993; Hsieh & Chen, 1983). At the site, fibroblasts differentiate into an activated phenotype (Hinz et al., 2007). This differentiation occurs in response to: (1) increased levels of TGF- β , (2) an altered extracellular matrix, and (3) mechanical stress within the environment (Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002). The activated fibroblasts are termed myofibroblasts, due to their contractile nature in conjunction with an accumulation of alpha-smooth muscle actin (α -SMA) within their cytoplasm (Gabbiani, Ryan, & Majne, 1971). These myofibroblasts secrete a number of matrix metalloproteinases (MMPs), which degrade the extracellular matrix, and tissue inhibitors of metalloproteinases (TIMPs) to keep the degradation in check (Soo et al., 2000). Additionally, myofibroblasts secrete many cytokines to initiate the epithelialization of the wound, including: KGF, EGF, and HGF among many others (Eming et al., 2014). After successful wound homeostasis, fibroblasts undergo apoptosis, returning to normal tissue abundance, however, the mechanisms by which this occurs are not clear (Desmouliere, Redard, Darby, & Gabbiani, 1995; H. Y. Zhang & Phan, 1999). Thus, fibroblasts promote normal wound homeostasis by infiltrating the site of the wound, degrading the aberrant extracellular matrix, and supporting the contraction and re-epithelialization of the wound.

However, when the wound is caused by rapidly proliferating epithelial cells, fibroblast-induced epithelialization makes the tumor worse. Our lab and others demonstrate CAFs to increase cancer cell proliferation by secretion of HGF (Knowles et al., 2009; Wheeler et al., 2014). Additionally, CAFs play a large role in promoting tumor metastasis. MMPs secreted by CAFs degrade the extracellular matrix and facilitate cancer cell invasion into underlying

structures and better access to vasculature (Lu, Takai, Weaver, & Werb, 2011). *In vitro* results demonstrate that squamous cells alone rarely invade into an extracellular matrix, but when the epithelial cells are seeded atop fibroblasts, there is significant invasion, with the fibroblasts leading the invasive front (Gaggioli et al., 2007). In animal models, HNSCC implanted with CAFs demonstrate increased metastasis to the lungs compared to HNSCC alone or HNSCC with NFs (Wheeler et al., 2014). Fibroblasts directly affect carcinoma cells to promote disease progression.

Additionally, CAFs modify other microenvironment components and cell types. Beyond modifying the extracellular matrix through MMPs and TIMPs, CAFs secrete vastly different collagens within the extracellular matrix which facilitate cancer progression (Sok et al., 2013). CAFs facilitate immune evasion by increased synthesis of prostaglandin E2, which inhibits T-cell proliferation (Alcolea et al., 2012; Harris, Padilla, Koumas, Ray, & Phipps, 2002). A number of cytokines secreted by CAFs activate angiogenesis within the tumor microenvironment, including HGF, IL-6 and IL-8 (Leef & Thomas, 2013). Additionally, preliminary results within our lab demonstrate CAFs mediate metabolic adaptations within the carcinoma cells. Overall, CAFs modulate nearly all hallmarks of cancer (Figure 1.4).

Despite such a dynamic contribution to the tumor microenvironment, mechanisms underlying sustained CAF activation remain largely enigmatic. Partially, limited understanding is a product of poor understanding of why HNSCC is highly fibrotic. Regions most often afflicted by HNSCC, for example the floor of the mouth, have a relatively shallow lamina propria with scant fibroblasts. Yet, HNSCC ranks among the tumors greatest in fibroblast abundance (Becht et al., 2016). The origins of the abundance of CAFs is unknown. CAFs may originate from rapid proliferation of tissue-resident fibroblasts, circulating mesenchymal stem cells, adipocytes,

endothelial cells, smooth muscle cells, or epithelial cells undergoing an epithelial to mesenchymal transition (Coen, Gabbiani, & Bochaton-Piallat, 2011; Humphreys et al., 2010; Labelle, Begum, & Hynes, 2011; Piera-Velazquez, Li, & Jimenez, 2011). Although there are a plethora of hypotheses, the origin and recruitment of CAFs to the tumor is poorly understood.

Beyond initial recruitment, the sustained activation of CAFs remains unclear. For example, CAFs grown in culture for many generations retain their tumor promoting affects compared to NFs, indicating a possible genetic or epigenetic change. However, mutations are

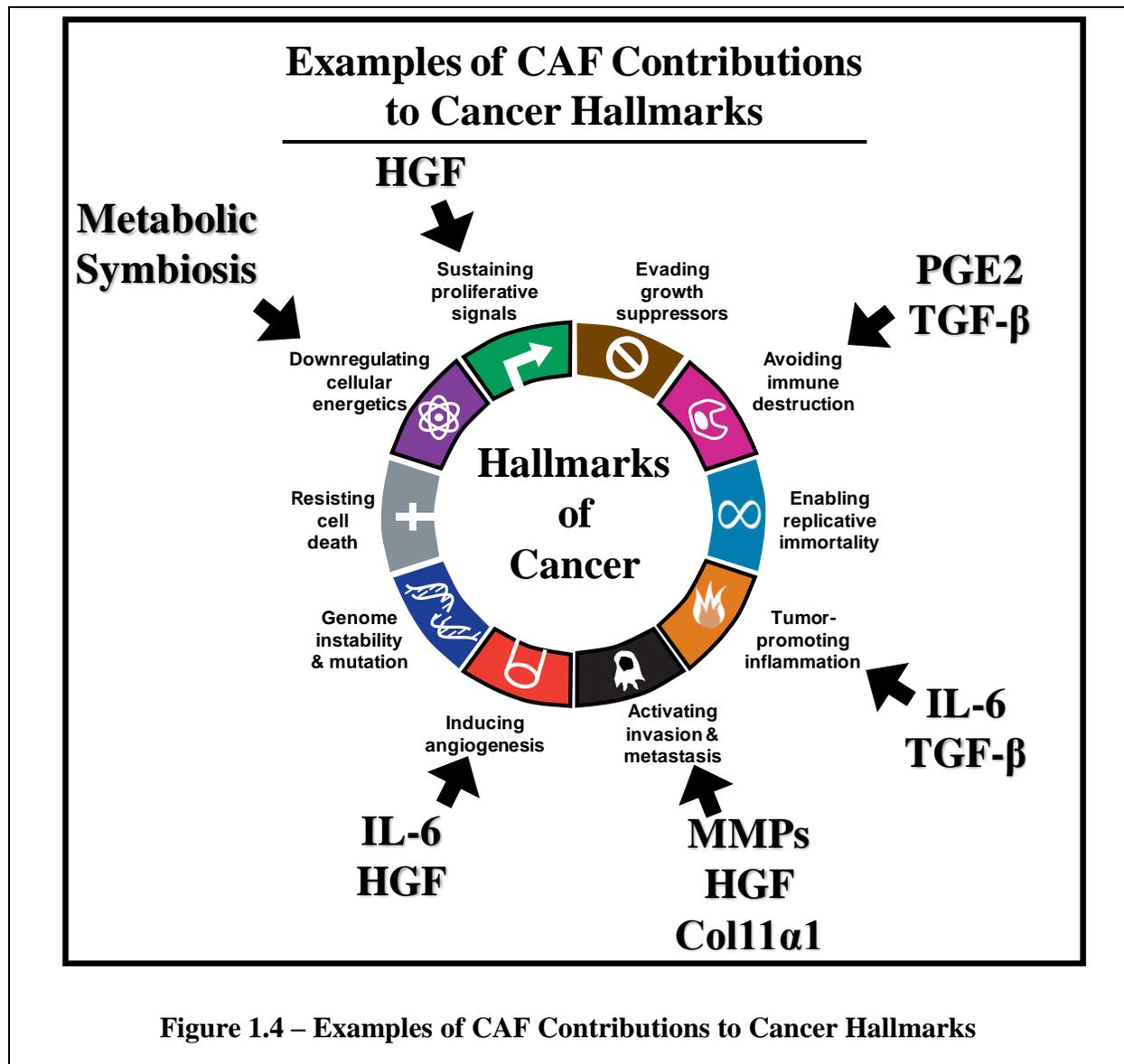


Figure 1.4 – Examples of CAF Contributions to Cancer Hallmarks

uncommon in CAFs (Qiu et al., 2008). Whereas epigenetic alterations are routinely studied in cancer, little is understood of myofibroblasts epigenetics, and no studies have been conducted on HNSCC CAFs. Hepatocytes exposed to TGF- β have a markedly different epigenetic signature, and this may be applicable to TGF- β activated CAFs (McDonald, Wu, Timp, Doi, & Feinberg, 2011). In colorectal cancer, CAFs display a global hypomethylation, with hypomethylation occurring in a stage dependent manner (Ling et al., 2016). Direct research into CAF epigenetics in HNSCC is lacking, but perhaps epigenetic changes account for the differing phenotype compared to NFs.

Additionally, although CAFs demonstrate markedly pronounced expression and secretion profiles compared to NFs, the underlying anatomy contributing to their secretory profile is understudied. These cells have a well-studied phenotype, which activates cancer, but the underlying machinery involved in producing this phenotype is not well known. CAFs comprise a large component of the HNSCC microenvironment, yet many questions remain about their underlying biology.

Perspectives

Overall, the microenvironment dictates tumor progression. However, microenvironmental cell types are not routinely included in HNSCC preclinical models. This may explain the dearth of therapeutics that have progressed from preclinical models to effective clinical trials.

The focus of this dissertation surrounds the aberrantly signaling fibroblasts. These are the most abundant microenvironmental cell type, but little is understood of the underlying biology. Phenotypically, CAFs are remarkably different from NFs. Previous results within our lab demonstrate CAFs promote proliferation and metastasis, and symbiotically communicate with the tumor (Wheeler et al., 2014). Despite these pronounced effects, little is known about the underlying biology. As such, we sought in this work to understand how CAFs promoted disease. What biological pathways within the cell prompted such dynamic differences in disease progression?

Our first observation was that CAFs appear astonishingly punctate when visualized by light microscopy (Figure 1.5). This led us to seek characterization of these vesicles. To our surprise, CAFs have a greater basal level of autophagosomes and autophagic flux compared to NFs.

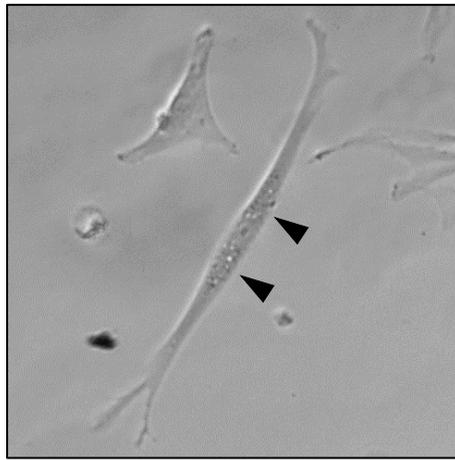


Figure 1.5-CAFs Have Punctate Vesicles throughout Cytoplasm

Representative light microscopy image of punctate vesicles (arrowheads) (200 X).

Chapter II-Autophagy

Autophagy, since its discovery, is predominantly known as a degradative process. Increased autophagosomes in nutrient deprived and stressed cells supports the concept that this pathway serves a pro-survival, metabolite-generating role. Mitochondrial structures identified within lysosomes led to the name “Self-Eating” (auto-, -phagy), further promoting the degradative connotation of this pathway.

The autophagic machinery is conserved across yeast and mammalian cells. Broadly, the pathway comprises the initiation of a double membrane vesicle, which sequesters cellular cargo and traffics toward degradation in the lysosome (vacuole in yeast) (Figure 2.1). A catalytic cascade initiates the pathway, and nutrient sensors AMPK and mTOR regulate this initiation. This cascade leads to enhancement of the double membrane vesicle, which incorporates the lipidated protein, LC3. Cargo receptors bind and carry autophagic cargo destined for the autophagosome, and facilitate the molecular tethering of cargo to LC3 on the autophagic membrane. The fusion of the autophagosome with the lysosome involves specialized SNARE complexes. This facilitates the delivery and degradation of autophagic cargo.

Autophagic Mechanism

Initiating Catalytic Cascade

The ULK1/Atg1 (unc-51-like kinase) complex exists as the upstream initiator of autophagy (Itakura & Mizushima, 2010; Matsuura, Tsukada, Wada, & Ohsumi, 1997). Nutrient sensing mediators, mTORC1 and AMPK, function to govern ULK1 activation. mTORC1, containing mTOR, raptor, and GβL, is sensitive to nutrient conditions within the cell, and is exquisitely sensitive to amino acid conditions (Wullschleger, Loewith, & Hall, 2006). Limited amino acid conditions repress mTORC1 activity (Sancak et al., 2008). mTORC1 functions to modulate autophagy during amino acid rich or amino acid deprived scenarios by negatively

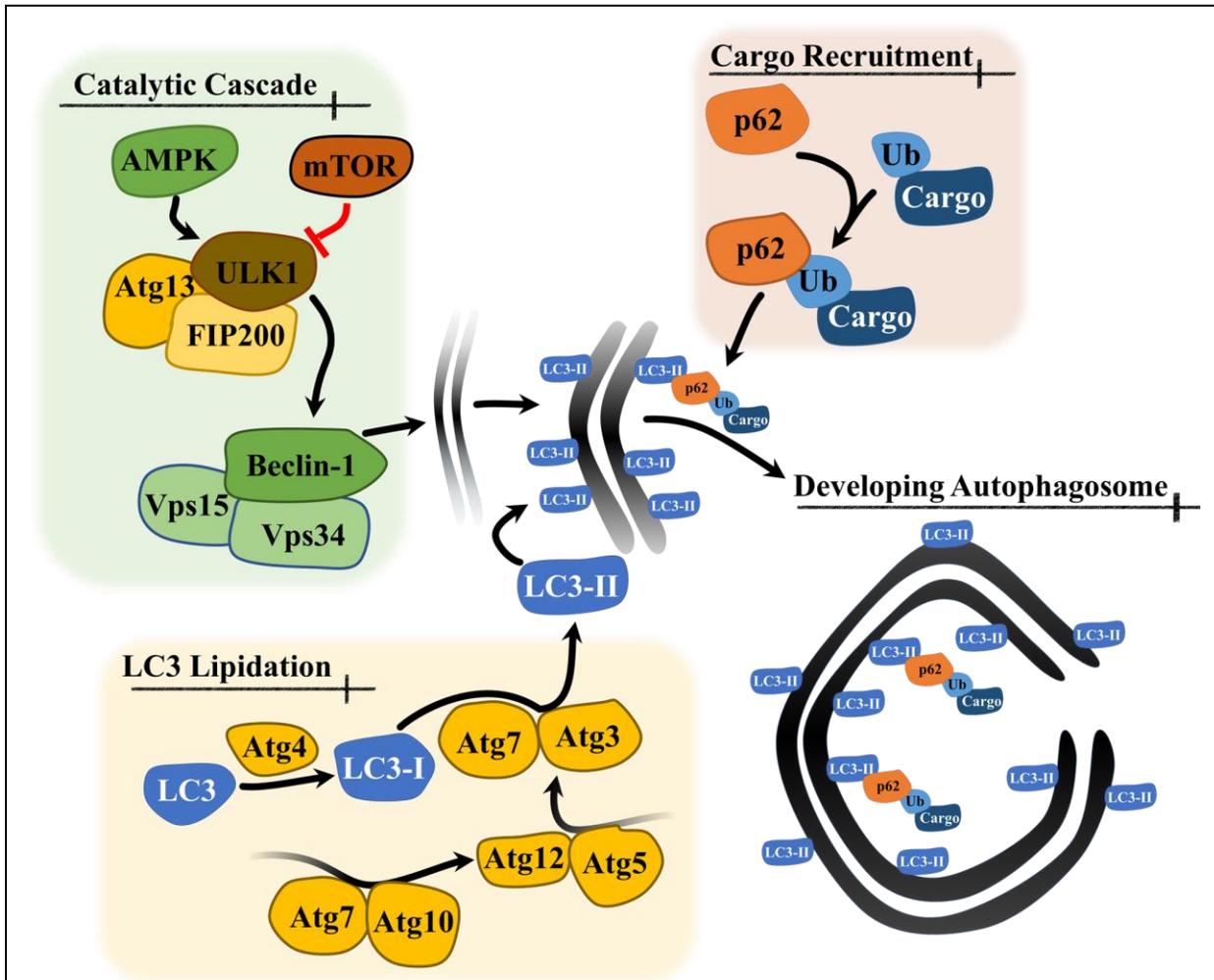


Figure 2.1 - Initiating Events in Autophagy

Three events occur to initiate the development of the autophagosome: (1) a catalytic cascade governed by the nutrient sensors, AMPK and mTOR, activates the Beclin-1 complex, which initiates the development of the double membrane structure; (2) LC3 is cleaved and lipidated in an E1/E2 ubiquitin-like conjugation system, and is incorporated into the double membrane on both inner and outer leaflets; (3) p62, or other cargo receptors, bind ubiquitinated cargo and tether the autophagic cargo to LC3-II within the autophagosome.

regulating ULK1. This occurs through raptor binding and consequential phosphorylation of ULK1 under rich amino acid scenarios, limiting ULK1 activity.

Conversely, AMPK activates ULK1. AMPK assesses glucose concentrations indirectly by measurement of AMP/ATP ratios. High concentrations of AMP implicate a low glucose environment and this enhances AMPK activity, which in turn, results in activating

phosphorylation of ULK1 (J. Kim, Kundu, Viollet, & Guan, 2011). Additionally, alongside the regulation of ULK1 by mTORC1 and AMPK, proteins Atg13 and FIP200 provide a scaffold to enhance ULK1 activity to its maximal level (Ganley et al., 2009). All said, low nutrient conditions of amino acids and glucose result in signaling cascades, which enhance ULK1 catalytic activity.

ULK1 mediates its pro-autophagic effects by phosphorylating, and thus activating, Beclin-1. Beclin-1 synchronizes an autophagy-initiating complex. The core components of this complex are Beclin-1, Vps15, and Vps34. Vps34 provides the primary agent of this complex's activity (Russell et al., 2013). As the only known class III PI3K, Vps34 regulates the pools of phosphatidylinositol 3-phosphate (PtdIns(3)P) surrounding the developing autophagosome (Volinia et al., 1995). This pool of PtdIns(3)P is advantageous for other autophagy related enzymes to complete the maturation steps. With rare exception (Zhou et al., 2010), Vps34, and the pool of PtdIns(3)P it provides, is necessary for autophagic flux (Kihara, Noda, Ishihara, & Ohsumi, 2001).

Despite a defined nutrient sensing catalytic cascade, the actual foundation of the lipid components of the autophagosome membrane is poorly understood. Speculation arises if this membrane initiates from the Golgi apparatus, mitochondria, recycling endosomes, the plasma membrane, secretory vesicles, or the endoplasmic reticulum, with the endoplasmic reticulum being the strongest candidate (reviewed in Shibutani & Yoshimori, 2014). Although the concrete origin is unknown, the developing autophagosome encompasses a cup-shaped double membrane structure, termed the isolation membrane or phagophore (Reunanen, Punnonen, & Hirsimäki, 1985). Through the remaining development of autophagy, this open-ended structure eventually closes to form a double-membrane vesicle termed the autophagosome.

Development of the LC3⁺ Autophagosome

LC3 (microtubule associated protein 1A/1B-light chain 3), the mammalian homologue of yeast Atg8, incorporates into the developing autophagosome. Originally proposed to regulate microtubule assembly (Mann & Hammarback, 1994), LC3 has become the most widely used marker of the autophagosome. By sucrose separation, LC3 demonstrates association with only autophagosomes and dense lysosomes, and not ER, Golgi, nuclear, plasma, or mitochondrial membranes (Kabeya et al., 2000). The unique association of LC3 with the autophagosome membrane and with the lysosome, the terminal destination of the autophagosome, has established its role as a specific autophagosome marker.

LC3 undergoes a series of modifications to incorporate this water-soluble protein into the lipid membrane of the autophagosome. Within six minutes of translation, LC3 is cleaved into the form LC3-I by Atg4 (Kabeya et al., 2000; Kabeya et al., 2004). Although LC3-I is ubiquitous throughout the cytosol, a physiological role for this isoform is poorly defined. During autophagic flux, an E1/E2 ubiquitin like conjugation system facilitates conjugation of LC3-I with PE (phosphatidylethanolamine) to form LC3-II (Kabeya et al., 2004). Atg7 comprises the E1 like protein, and Atg3 serves as the E2 like protein (Ichimura et al., 2000). Atg5 and Atg12 enable this E1/E2 LC3-II conjugation (Mizushima et al., 2001). Atg5 and Atg12 also conjugate through an E1/E2 conjugation system comprising Atg7 and Atg10 (Mizushima et al., 1998). The Atg12-Atg5 complex facilitates LC3 conjugation by enhancing the function of Atg3 (Hanada et al., 2007). Atg16 carries the Atg12-Atg5 complex to the developing membrane where the cleavage and lipidation of LC3 takes place (Mizushima et al., 2003). Through these cleavage and lipidation reactions, PE conjugates to LC3, which allows for incorporation into the

autophagosome. The newly lipidated form of LC3 provides a molecular anchor for autophagic cargo within the lysosome.

Although LC3 is the most widely used marker of the autophagosome, autophagy can still occur in LC3 knockout cells. However, in these cells, fusion with the lysosome is impaired, and there is an accumulation of autophagosomes (Padman, Nguyen, & Lazarou, 2017). This indicates LC3 is not necessary for development of the double-membrane autophagosome, but this protein is required for normal flux and final degradation of this pathway.

Cargo Recruitment

Cargo destined for the autophagosome can include whole organelles, protein aggregates, and even intracellular pathogens. Autophagy cargo receptors contain two broad domains, a cargo recognition motif, and a LC3-interacting region. Ubiquitin serves as the most common cargo recognition motif. Capable of marking proteins, organelles, and pathogens, ubiquitin serves as an adapter to bind these substrates to a cargo receptor. By proteomics, there are 33 proteins which could function as cargo receptors based upon their ability to interact with LC3 (Behrends, Sowa, Gygi, & Harper, 2010).

The best understood of these cargo receptors is SQSTM1 (also known as p62). SQSTM1 directly interacts with LC3, and is degraded under normal autophagic flux (Komatsu et al., 2007). The ubiquitin binding domain of SQSTM1 allows for the interaction of this protein with a large number of autophagic cargo destined for the autophagosome. Additionally, SQSTM1 has multiple other protein binding motifs which can allow for the adherence and trafficking of non-ubiquitinated proteins to either the autophagosome or proteasome (Moscat, Diaz-Meco, Albert, & Campuzano, 2006). The LC3-interacting region on SQSTM1 is crystalized, and serves as the paradigm autophagy cargo receptor (Ichimura et al., 2008).

Beyond SQSTM1, there are four other well-known autophagy cargo receptors. NBR1 (neighbor of BRCA1) has structural similarity to SQSTM1 in that it binds ubiquitin through a c-terminal UBA domain (Campbell et al., 1994). NDP52 (also known as CALCOCO2) has a non-canonical LC3 interacting region (von Muhlinen et al., 2012). This is especially important for the degradation of pathogens through autophagy. OPTN (optineurin) traffics proteins to LC3 in a ubiquitin independent manner by means of a coiled-coil domain (Korac et al., 2013). NIX (BCL2 and adenovirus E1B 19 kDa-interacting protein 3-like) has a BH3 domain and acts to recruit mitochondria to LC3 for autophagic degradation, also known as mitophagy (Sandoval et al., 2008). These cargo receptors allow for the selective recruitment of cargo to the developing autophagic membrane.

Degradative Mechanism

The fusion of autophagosome with lysosome follows an intricate recruitment strategy of mediating proteins to form a complex that melds the two lipid membranes (Figure 2.2). The pool of PtdIns(3)P generated by Vps34 recruits Ccz1-Mon1 (Caffeine, calcium and zinc 1-Monensin sensitivity protein 1) to the developing autophagosome (Hegedűs et al., 2016). This GEF (guanine nucleotide exchange factor) recruits the small GTPase Rab7 to the autophagosome. Rab7 facilitates tethering of the autophagosome with the lysosome by binding the HOPS (homotypic fusion and vacuole protein sorting) complex on the lysosome (Hegedűs et al., 2016). Additionally, PLEKHM1 binds the LIR of LC3 on the outer autophagosomal membrane and functions to accentuate HOPS-Rab7 recruitment (McEwan et al., 2015). With the autophagosome and lysosome near, the fusion of the two membranes is accomplished by a

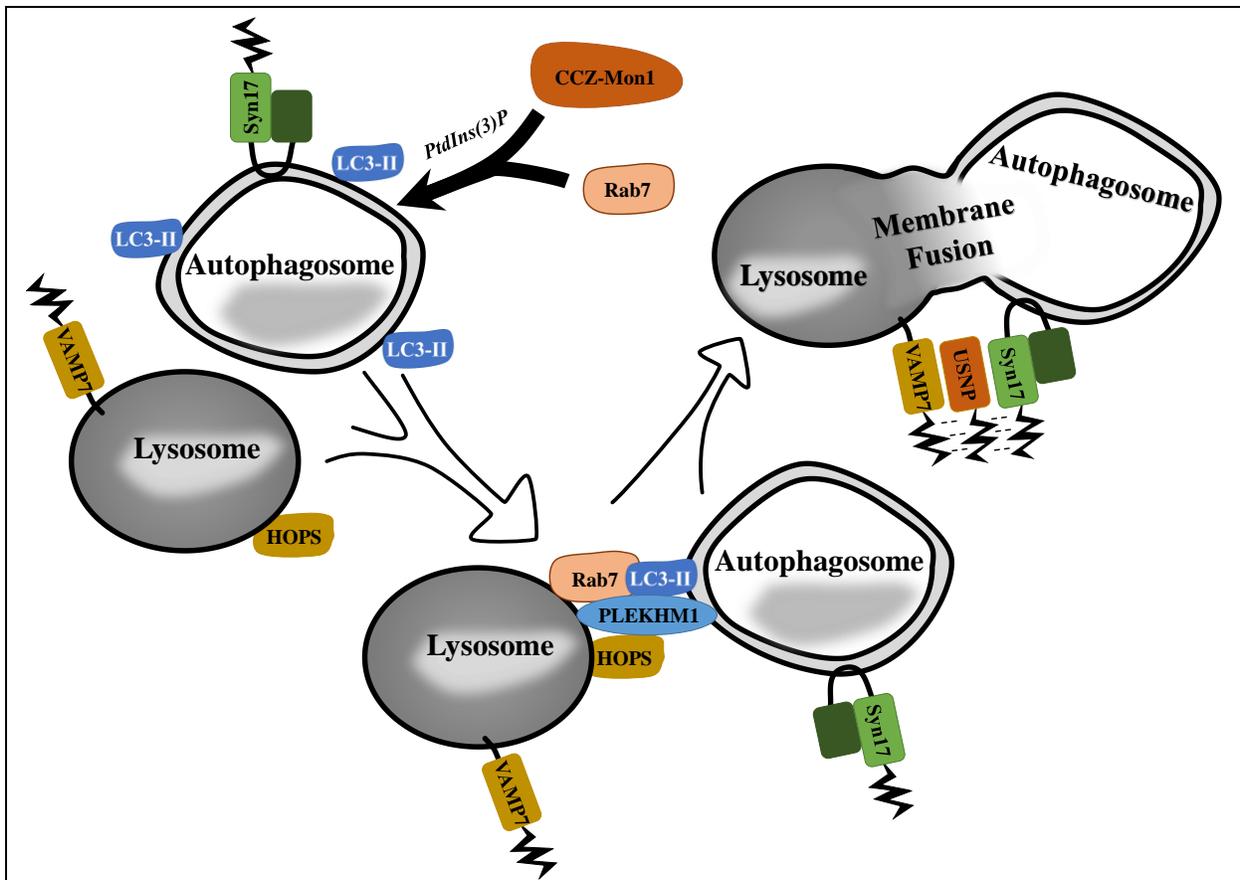


Figure 2.2 – Degradative Mechanism

Fusion of the lysosome and autophagosome is mediated by the recruitment of Rab7 to the autophagosome which subsequently binds the HOPS complex on the lysosome. The binding of these bring the Qa SNARE, Syn17, on the autophagosome to the R SNARE, VAMP7, on the Lysosome. The binding of these two with the Qbc SNARE, USNP, facilitates membrane fusion and the degradation of autophagosomal contents by the lysosome.

SNARE complex containing Syn17 (Syntaxin17, a Qa SNARE), USNP (SNAP-29 homologue ubiquitin, a Qbc SNARE), and VAMP7 on the lysosome (an R SNARE)(Itakura, Kishi-Itakura, & Mizushima, 2012; Takáts et al., 2013). This fusion allows for the disbursement of cargo and autophagic machinery into the lysosome.

As the main cellular organelle for degradation, the lysosome concludes degradative autophagy (Saftig & Klumperman, 2009). Lysosomal enzymes degrade both the autophagic molecular machinery and the cargo they carried. The function of these enzymes depends on a

high proton concentration and the resulting low pH. This pH gradient between the cytosol and the lysosome makes for an effective target at the termination step of autophagy, terming these agents as lysomotropic. Lysomotropic agents, such as the widely used chloroquine, disrupt the pH balance, which prevents the degradation of autophagic components and cargo, and an accumulation of the autophagy pathway intermediates. This pathway can also be perturbed by looking at the molecular mediators of autophagosome-lysosome fusion.

Orchestrating a passing of cellular components to the lysosome for degradation, autophagic machinery are nearly constant across species. With few exceptions, homologues of the molecular machinery exist in all living cells for the adaptation to microenvironmental influences. With an understanding of this machinery, experiments have perturbed this pathway and identified dramatic results in cellular secretion.

Autophagy-Dependent Secretion

Over the past 40 years, a subtle, but growing body of evidence points to an interesting function of this pathway in cellular secretion. Even in Noble Prize laureate Dr. Ohsumi's original report on autophagy in yeast, the outcome of increased autophagy under nutrient starvation conditions was enhanced secretion of labeled leucine (Takeshige, Baba, Tsuboi, Noda, & Ohsumi, 1992). Others have demonstrated the fusion of autophagosomes with the plasma membrane for the expulsion of cellular cargo (Schweers et al., 2007). Additionally, cells with enhanced basal autophagy have a distinct secretome compared to low autophagy cells (Kraya et al., 2015). With growing evidence, autophagy serves pleiotropic roles within the cell as both a degradative and secretory pathway.

Autophagy facilitates secretion in both normal physiology and pathology. Understanding autophagy-dependent secretion relies on an understanding of the autophagic molecular

machinery. Enhancement or attenuation of these components leads to consequential secretory alterations. Perturbation of this pathway has identified key signaling molecules and cytokines, such as IL-1 β and IL-6, which are altered with autophagy (Endo et al., 2017). Even entire viruses and bacteria are secreted through autophagic machinery labeled vesicles (Jackson et al., 2005). As such, this pathway affects many diseases ranging from cancer to viral infections, asthma, and Chron's disease. With surmounting evidence supporting the implications of autophagy-dependent secretion, and the lack of drugs targeting this pathway, biologists across disciplines will see benefit from the a new understanding.

Secretory Mechanism

Autophagy-dependent secretion stems from observations of the unconventionally secreted protein Acb1 (Acyl-CoA Binding Protein, *dictyostelium* homologue: AcbA). Functionally, Acb1 is released from pre-spore yeast cells to induce sporulation (Anjard & Loomis, 2005). Under autophagy enhancing conditions, such as low nitrogen concentrations, cells secrete increased levels of Acb1 (Duran, Anjard, Stefan, Loomis, & Malhotra, 2010). Following individual knockdown of autophagy components, Atg5, Atg7, Atg8, and Atg12, Acb1 secretion significantly decreased, despite constant Acb1 concentrations within the cell. This foundational report suggested a role for the autophagosome in secretion of extracellular proteins.

Intriguingly, Acb1 is a leaderless peptide, lacking a secretion signal sequence. Under conventional secretion, an N-terminal signal sequence directs proteins to the endoplasmic reticulum (ER) for folding, and then successive modification in the Golgi apparatus to facilitate secretion (Reviewed in (Rapoport, 2007)). This signal peptide consists of a positively charged amino terminus, a hydrophobic central region, and a cleavable carboxyl terminus, which allows for removal of the signal sequence in the ER. Bioinformatics estimates upwards of 30 percent of

all proteins have a secretory signal sequence. However, many proteins do not have this cleavable, targeting sequence, even though they are readily detected extracellularly. Notable examples include: macrophage migration inhibitory factor (Simons et al., 2011), tissue transglutaminase (Zemskov, Mikhailenko, Hsia, Zaritskaya, & Belkin, 2011), and IL-1 β (Dupont et al., 2011), among others.

With a foundation in Acb1, most mechanistic work on autophagy dependent secretion arises from studies interrogating IL-1 β secretion. The initial cloning of this protein identified IL-1 β lacked a secretion signal sequence (Auron et al., 1984). A connection with autophagy arose with the observation that IL-1 β secretion was enhanced following starvation of bone marrow-derived macrophages, similar to the enhancement of Acb1 secretion following nutrient starvation (Dupont et al., 2011). Knockdown of Atg5 demonstrated autophagy mediates the secretion of IL-1 β (Dupont et al., 2011). Since this finding, the molecular mechanisms involved in autophagy dependent secretion, including cargo recruitment, vesicle trafficking, and membrane release, have been primarily worked out using IL-1 β as the released protein of interest.

Cargo Recruitment

A modified autophagosome routes IL-1 β for extracellular secretion. This begins with cargo recruitment to the developing autophagosome. Mature IL-1 β binds to TRIM16 (Tri-partite motif 16, also known as ERBBP) (Munding et al., 2006). This IL-1 β -TRIM16 complex traffics to an autophagy sequestration membrane (Kimura et al., 2017). The sequestration membrane is not an autophagosome, but a shuttling membrane necessary for lipidation of LC3-I to LC3-II (M. Zhang, Kenny, Ge, Xu, & Schekman, 2015). Without TRIM16, IL-1 β cannot arrive at the sequestration membrane, or the resulting autophagosome (Kimura et al., 2017).

Vesicle Trafficking

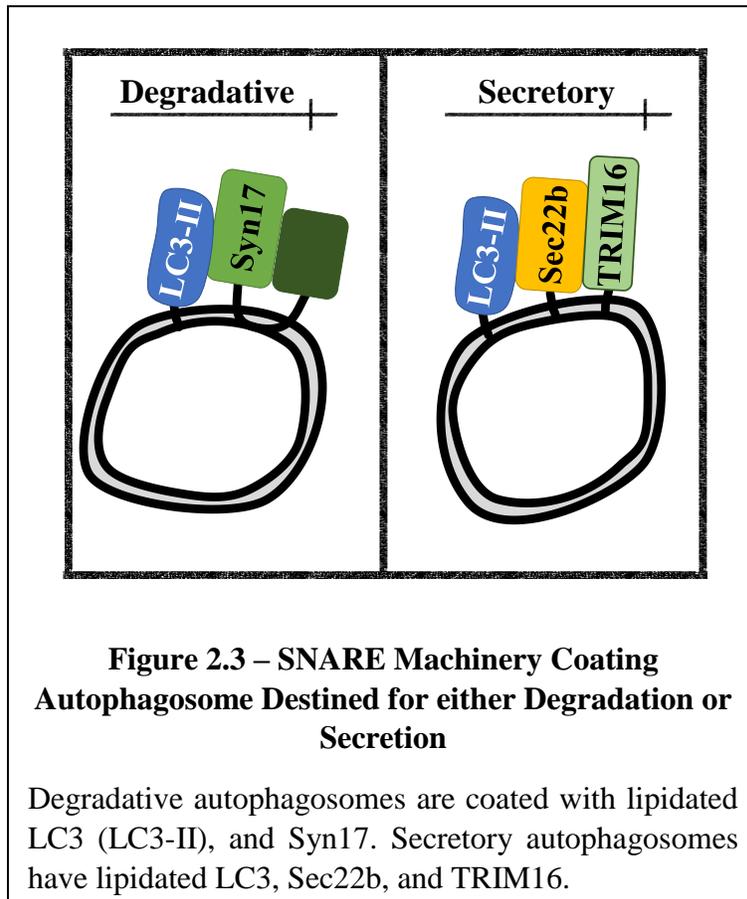
At the sequestration membrane, Sec22b binds the IL-1 β -TRIM16 complex. Sec22b consists of a longin domain (involved in protein transport to the plasma membrane) and a SNARE motif (Y. Liu, Flanagan, & Barlowe, 2004), with this SNARE motif critical to the vesicle fusion events involved in IL-1 β secretion. Originally identified as part of the vesicle fusion machinery involved in COP-II coated vesicle fusion in the ER-Golgi intermediate compartment (Mancias & Goldberg, 2007), Sec22b is of particular importance to autophagy. Upon knockdown of Sec22b, LC3 lipidation is decreased (Ge, Zhang, & Schekman, 2014). Paradoxically, Sec22b depletion leads to an increase in LC3-II levels by immunoblot and LC3 puncta by immunofluorescence (Kimura et al., 2017; Renna et al., 2011), but no overall differences in autophagic flux. Reconciling this finding, Sec22b depletion blocks trafficking of lysosomal proteases to the lysosome, thereby rendering the lysosome ineffective (Renna et al., 2011). With Sec22b depletion, IL-1 β secretion decreases (Kimura et al., 2017). Therefore, an autophagosome destined for secretion would have LC3-II, Sec22b, and TRIM16 on its cytosolic membrane.

Membrane Fusion

To fuse with the plasma membrane, the secretory autophagosome undergoes a SNARE mediated fusion event. SNARE proteins are tail anchored proteins, which mediate the fusion event (Y. A. Chen & Scheller, 2001). They assemble into a complex of Q (glutamine) and R (arginine) SNAREs across assembling membranes to join the acceptor and donor membranes (Y. Wang et al., 2016). The R-SNARE, Sec22b, on the secretory autophagosome binds to Qbc-SNAREs, SNAP-23 and SNAP-29 on the plasma membrane (Kimura et al., 2017). Together with Syntaxin-3 and Syntaxin-4 on the plasma membrane, these proteins mediate a SNARE complex allowing fusion of the secretory autophagosome with the plasma membrane (Kimura et al.,

2017). The fusion of the secretory autophagosome with the plasma membrane facilitates secretion of IL-1 β .

The modified autophagosome involved in IL-1 β secretion has characteristics similar to a degradative autophagosome, but differs in a few key cytosolic membrane elements to facilitate the trafficking to the plasma membrane. Similar to the degradative autophagosome, a secretory autophagosome has a double membrane labeled with LC3-II. Cargo recruitment in both secretion and degradation appear to rely on trafficking of cellular cargo to LC3. However, the destinations of the LC3⁺ double membrane vesicle differ based upon the SNARE machinery coating the cytosolic membrane (Figure 2.3). In a degradative autophagosome, Syntaxin17 allows for fusion with the lysosome. In a secretory autophagosome, Sec22b facilitates fusion with the plasma



membrane. These subtle differences in the cytosolic membrane proteins determine whether the contents are degraded or expelled.

Additionally, the role of the autophagolysosome in secretion remains unclear. Although the secretion of IL-1 β seems to bypass the lysosome, other secreted cargo depend upon lysosomal function. For instance, chloroquine inhibition of the lysosome alters the secretory profile of CAFs in a way that mimics Beclin-1 knockdown (New et al., 2017). Even IL-1 β seems to rely on the lysosome, as the same group that delineated the mechanism of autophagic secretion of IL-1 β also demonstrated in an earlier report that Bafilomycin A1, which inhibits the acidification of the lysosome, inhibits IL-1 β secretion (Dupont et al., 2011). Autophagy-dependent secreted cargo may all have the same route bypassing the lysosome, but an alternative pathway, which incorporates the lysosome, cannot be excluded based on the current data.

The TASCC (Tor associated spatial coupling compartment) provides an alternative mechanism for autophagy dependent secretion, and enhances IL-8 secretion. At the Golgi apparatus, the TASCC brings together mTORC1, rough ER, Golgi apparatus, autophagosomes and lysosomes (Narita et al., 2011). This creates a dynamic metabolism center functioning to sequester material through degradative autophagy to reconstitute new proteins for secretion. IL-8 and IL-6 were both identified in this compartment, and IL-8 mRNA was detected at the marginal regions (Narita et al., 2011). Under autophagy inhibition, the TASCC cannot form, preventing the translation of IL-8 and IL-6. This compartment identifies an alternative role of the autophagosome in secretion, as some secretory cargo are reliant upon a functioning degradative autophagy pathway, but not necessarily a secretory autophagosome.

Although the best understanding of autophagy dependent secretion comes from interrogation of the IL-1 β secretion, our understanding of the molecular mechanism is relatively

nascent and could potentially differ with different cargo chaperoned by the autophagosome. For instance, there seems to be cross-communication with the exosomal pathway. Exosomal fragments derived from prostate cancer cells demonstrate the presence of autophagy markers LC3, p62, and others (Hessvik et al., 2016). Within the cell, multivesicular bodies and exosomes are targeted to the autophagosome when autophagy increases (Fader, Sanchez, Furlan, & Colombo, 2008). This may allow for the fusion of membranes and the directing of an autophagosome to the plasma membrane. The mechanisms regulating the cross-communication between exosomes, multivesicular bodies, and secreted autophagosomes are ill-defined.

Despite some remaining questions, this mechanism of secretion is enthralling. Machinery once thought to be only involved in degradation now appear to have pleiotropic roles. Alongside this, over the last decade, the variety of cargo secreted has expanded to include everything from metabolites to full organelles.

Secreted Factors

Autophagy dependent secretion offers a wealth of factors extracellularly. These secreted components range from inflammatory mediators to granule contents. By perturbing the canonical autophagic machinery, researchers identified a number of components depend on autophagy for secretion. The list has expanded substantially from the initial identification of Acb1 secreted through an autophagy mechanism less than a decade ago.

Interleukins

The primary class of autophagy-dependent components are interleukins. As described above, IL-1 β has been extensively studied in relation to autophagic secretion, and provides the main understanding of the machinery involved in secretion. Similarly to IL-1 β , the IL-1 family member, IL-18, undergoes a similar secretory route, as inhibition of the autophagosome by

bafilomycin A1 or Atg5 knockdown attenuated IL-18 secretion (Dupont et al., 2011). Beyond the IL-1 family, other interleukins have demonstrated significant involvement with autophagy, notably IL-6 and IL-8.

IL-6 provides an interesting anecdote in the story of autophagy dependent secretion. Multiple groups observe the secretion of IL-6 depends upon autophagy. With Atg7 knockdown, IL-6 secretion reduces in pancreatic stellate cells (Endo et al., 2017), fibroblasts (Young et al., 2009), breast cancer cells (Maycotte, Jones, Goodall, Thorburn, & Thorburn, 2015), and human brain endothelial cells (Zhuang et al., 2017). Thus, IL-6 is secreted in an autophagy dependent mechanism. IL-6 then feeds back, and further enhances autophagy. Observed in pancreatic cancer cells (Kang et al., 2012), and fibroblasts (New et al., 2017), IL-6 provides a fascinating feed-forward loop accelerating autophagy dependent secretion.

Damage Response Mediators

Although primarily studied in a healthy cell population, autophagy-dependent secretion proves important even in apoptotic and necrotic cells. Necrotic cells selectively release HMGB1 (high mobility group box 1), whereas apoptotic cells retain this immune stimulus within their nuclei (Scaffidi, Misteli, & Bianchi, 2002). Upon knockdown of key autophagy proteins, Atg5, Atg7, and Atg12, HMGB1 secretion during necrosis ceases. Thus, autophagy dependent secretion mediates regulation of microenvironment damage responses.

Additionally, extracellular ATP release serves as a chemokine to mediate an immune response towards a damaged region. For example, following irradiation, ATP released from damaged cells, signals immune cells to the region. However, knockdown of Atg5 inhibits ATP release and a normal immune response cannot occur (Ko et al., 2014). Chemotherapy treated cells normally release ATP in a similar manner, and autophagy blockade attenuates ATP release

from pharmaceutically damaged cells (Michaud et al., 2011). Therefore, autophagy dependent secretion mediates appropriate damage responses by regulating HMGB1 and ATP from apoptotic or necrotic cells.

Secretory Granule Contents

In a variety of tissue types, secretory granules regulate tissue development and homeostasis. Autophagy dependent secretion facilitates a variety of these secretory granules from widely different tissue types.

Intestinal Paneth cells secrete a large number of antimicrobial proteins necessary for both innate defense and regulation of the microbiome. Dysregulation of these cells is one feature of Crohn's disease. Atg16 mutations predict susceptibility to Crohn's disease (Hampe et al., 2006). Dysregulation of Atg16 leads to dysfunctional granule exocytosis from Paneth cells (Cadwell et al., 2008). Disruption of normal granule exocytosis causes retention of key antimicrobials, such as lysozyme, resulting in an incompatible response to bacterial infection (Bel et al., 2017). Without autophagy-dependent secretion, normal Paneth cell secretions could not occur.

In endothelial cells, autophagy dependent secretion of secretory granules allows for homeostasis following vascular injury. VWF (von Willebrand factor) assembles long multimers, which, when tethered together, provide adhesion of circulating platelets and facilitates clotting. Weibel-Palade bodies contain VWF in endothelial cells. With impairment of autophagy, through knockdown of Atg5 or Atg7, Weibel-Palade bodies are retained intracellularly, VWF cannot be secreted, and there is impaired healing of the vessel wall (Torisu et al., 2013).

Secretory lysosomes have physiologic importance in tissue homeostasis and immune responses. For example, bone resorption relies on osteoclast-mediated secretion of lysosomal enzymes into an extracellular resorptive space. Once thought of as only a lysosome function,

when autophagy enzymes Atg5, Atg7, Atg4, and LC3 were knocked down or mutated, bone resorption dramatically decreased (DeSelm et al., 2011). This provides evidence of the involvement of autophagy dependent secretion in trafficking secretory lysosomes. This may occur in other myeloid derived secretory cells, such as natural killer cells, which granular contents are contained within a secretory lysosome (Lopez-Soto, Bravo-San Pedro, Kroemer, Galluzzi, & Gonzalez, 2017).

Furthermore, mast cells, components of the innate immune response, rely on autophagy dependent secretion for degranulation of secretory lysosomes. Mast cells play a crucial role in maintenance of the allergic response. Degranulation of these cells releases histamine and other cytokines into the microenvironment to mount an immune response. LC3-II localizes with secretory granules within mast cells, and is secreted with co-localized CD63, a secretory lysosome marker. Knockout of Atg7 results in impaired degranulation of mast cells, and an impaired anaphylaxis reaction (Ushio et al., 2011). Autophagic machinery prove necessary in the trafficking of granule components during immune response.

Ranging from intestinal cells, vasculature, osteoclasts, and immune cells, autophagy dependent secretion provides an essential homeostatic mechanism of granule release throughout an organism. Extensive characterization of the mechanisms of granule release compared to cytokine release remains to be studied. However, the core autophagic machinery prove essential to this secretory process.

Extracellular Matrix Components

Of note, an association of autophagy dependent secretion with extracellular matrix components has been observed. In pancreatic stellate cells, which synthesize the pancreatic stromal matrix, autophagy knockdown diminishes matrix synthesis. Upon knockdown of Atg7,

key matrix components are significantly reduced in expression (Endo et al., 2017). These include Collagen 1 α 1, fibronectin1, and periostin (Endo et al., 2017). The role of autophagy dependent secretion from fibroblasts of other tissues (New et al., 2017; Young et al., 2009) provides support for the concept that extracellular matrix synthesis occurs through an autophagy dependent secretory manner.

Role in Disease

With such a plethora of factors secreted, autophagy dependent secretion affects both normal physiology and pathophysiology. The understanding of autophagy dependent secretion in disease has grown primarily out of three disease classes: infection, neurodegeneration, and cancer.

Infection

Viruses hijack normal cell mechanisms to reproduce, and the autophagic machinery provide a fascinating example of this. Early on, picornaviruses, such as polio, were observed in double membrane vesicles, with an appearance similar to the autophagosome. Intriguingly, poliovirus components 2BC and 3A induce the formation of these double membrane structures (Suhy, Giddings, & Kirkegaard, 2000). 2BC directly modifies LC3, allowing its incorporation into the membrane (Taylor & Kirkegaard, 2007). Poliovirus then uses the autophagosome like membrane as a lipid source during viral replication. Once formed, the LC3 positive double membrane vesicles traffic the enclosed viral particles to the plasma membrane, where LC3 positive vesicles bleb off the host cell surface (Jackson et al., 2005). This creates a secreted autophagosome, coated with the host's own cellular components, but packed with poliovirus cargo (much like a Trojan horse). This method of non-lytic viral production significantly enhances infection and reproduction of the viruses.

Poliovirus transmission tightly intertwines with autophagy dependent secretion. With Beclin-1 overexpression, poliovirus transmission increases (Y. Chen et al., 2015). Accordingly, Beclin-1 knockdown significantly reduces poliovirus yield (Y. Chen et al., 2015). Pharmacologic inducers of autophagy, such as rapamycin, enhance poliovirus yield by more than three-fold (Jackson et al., 2005). Thus, autophagy dynamics correlate with poliovirus secretion.

Beyond poliovirus, other viruses undergo such autophagy dependent secretion. Rhinovirus and coxsackievirus also exit host cells in vesicles harboring LC3-II (Robinson et al., 2014). Varicella zoster virus exits the host in a single membrane vesicle harboring LC3, but also Rab11, an endocytic marker, which may indicate the convergence of the autophagy and endocytic pathways in varicella zoster expulsion (Buckingham, Jarosinski, Jackson, Carpenter, & Grose, 2016). Dengue virus induces LC3 puncta, in a manner that relies on Beclin-1 (Mateo et al., 2013). During dengue virus infection, beclin-1 inhibition significantly reduced extracellular virus (Mateo et al., 2013). Within the last decade, this method of non-lytic viral transmission has just been uncovered, and much work is still to be done with the exact mechanisms in the transmission of each viral type.

Ejected bacteria also rely on autophagy dependent secretion. *Mycobacteria tuberculosis* and *M. marinum* expel themselves from the host cell in an ejectosome (Hagedorn, Rohde, Russell, & Soldati, 2009). This is a vesicle decorated with LC3 protein that is expelled from the cell. The autophagic machinery are recruited to the distal pole of the ejectosome, and facilitate bacterial expulsion (Gerstenmaier et al., 2015). When Atg1 (ULK1), Atg5, Atg7, and Atg8 were knocked down, non-lytic cell-to-cell transmission of mycobacteria significantly reduced (Gerstenmaier et al., 2015). Thus, autophagy dependent secretion facilitates viral and bacterial transmission.

Neurodegeneration

Two neurodegenerative conditions, Alzheimer's and Parkinson's disease, have long been associated with defective autophagy. In both of these diseases, an intracellular inclusion body forms: amyloid beta aggregates in Alzheimer's disease, and α -synuclein inclusions in Parkinson's disease. The dysregulation of autophagy in these diseases was thought to prevent the degradation of these aggregates, which led to their abundance within the cell. However, recent evidence points to defective autophagy limiting their secretion, which results in intracellular accumulation.

In Alzheimer's disease, dystrophic neurites have an accumulation of autophagosomes (Nixon, 2007). These autophagosomes contain the bulk intracellular reservoir of amyloid beta (Yu et al., 2005). Although this finding led to the initial conclusion that the accumulation of amyloid beta and autophagosomes resulted from a dysfunctional autophagolysosome, Atg7 knockout transgenic mice have connected amyloid beta accumulation with a decreased secretory autophagy pathway. Atg7 knockout neurons had diminished amyloid beta secretion, and reconstitution of Atg7 restored the secretion of amyloid beta (Nilsson et al., 2013). Further, pharmacologic induction of autophagy with rapamycin enhanced amyloid beta secretion, while inhibition of autophagy with spautin-1 diminished secretion (Nilsson et al., 2013). Thus, autophagy influences secretion of amyloid beta in Alzheimer's disease.

In Parkinson's disease, α -synuclein aggregates accumulate within dopaminergic neurons (Luk et al., 2012). Both autophagy and the proteasome degrade α -synuclein. In neurons, p25, traffics α -synuclein to autophagosomes, while also preventing autophagosome lysosome fusion (Ejlerskov et al., 2013). This promotes secretion of α -synuclein containing autophagosomes (Ejlerskov et al., 2013). Upstream autophagy inhibitors, such as 3-methyladenine, attenuate α -

synuclein release (Ejlertskov et al., 2013). Thus, autophagy dependent secretion facilitates the secretion of α -synuclein in neurons.

Cancer

Relative to normal tissue, cancer tissue of almost all organ sites upregulates autophagic flux. Increased degradative autophagy provides a mechanism for renewal of damaged organelles and proteins in a metabolically active microenvironment. Increased autophagy also promotes cancer cell survival by facilitating therapy resistance (X. H. Ma et al., 2011). As discussed above, autophagy dependent secretion facilitates secretion of cancer-promoting factors, such as IL-1 β and IL-6. Within the microenvironment, both the cancer cells themselves and stromal supporting cells rely on autophagy dependent secretion for progression of the disease as well as therapy resistance.

The dynamics of autophagy dependent secretion in cancer cells is best exemplified by differing secretomes between cells of the same cancer site with differing basal rates of autophagic flux (Kraya et al., 2015). Genetically paired melanoma cells with differing basal levels of autophagy provides a unique material to study autophagy dependent secretomes (Kraya et al., 2015). Low autophagy cells had markedly reduced secreted levels of CXCL8, IL-1 β , LIF, FAM3C, and DKK3 compared to cells with high basal levels of autophagy (Kraya et al., 2015). Beclin-1 overexpression provided confirmation in these low autophagy cells that the factors secreted were indeed dependent upon autophagy. These factors support tumor progression, and allow for useful biomarkers in response to autophagy modulating cancer therapy.

Autophagy Dependent Secretion Summary

A secretory pathway conserved across yeast to mammals, autophagy-dependent secretion supplies a wide variety of cargo in both normal physiology and pathophysiology. Despite such

broad implications, our understanding of this pathway is in its infancy, with the molecular mediators only partially worked out with IL-1 β secretion. As such, few targeted inhibitors exist.

For example, hydroxychloroquine provides the lone clinically available autophagy inhibitor (Benoit Pasquier, 2016). This, and other lysomotropic inhibitors, destabilizes the lysosome and prevents autophagosome degradation. Although this may destabilize the TASC and prevent autophagy dependent secretion of IL-8 and IL-6, it also inhibits autophagic degradation useful for homeostatic maintenance of the cell. Alternatively, inhibitors such as SAR405 and Spautin-1 target autophagy upstream at the Beclin-1-VPS34 complex (J. Liu et al., 2011; Ronan et al., 2014). Effective at diminishing both secretory and degradative autophagy, these cannot differentiate between the two routes for the autophagosome.

Developing a selective route inhibitor, to better delineate the secretory and degradative routes, would provide details of the pathways and vesicle traffickers involved. The primary difference in the final destination relies on the differential expression of Syn17 or Sec22b on the cytosolic membrane. However, these two proteins function in multiple cellular pathways, and an inhibitor solely targeting either of these would be of little use. Perhaps proteomic approaches assessing carriers similar to TRIM16, unified cargo carriers that bind both LC3 and Sec22b, would be of use. By understanding the binding of cargo carriers to LC3 and trafficking intermediate Sec22, an appreciation of the exact cargo secreted through a secretory autophagosome could develop.

Overall, autophagy-dependent secretion proves essential in a wide variety of cellular processes with a plethora of factors secreted. Despite clear importance in both normal physiology and disease, there is much more to understand about the molecular mediators of this process. A

more clear understanding of the molecular mechanisms will facilitate therapeutic development to counteract disease and augment normal physiology.

A Role for Autophagy Dependent Secretion in the HNSCC Microenvironment?

CAFs derived from HNSCC patients have a punctate, vesicular architecture when viewed by light microscopy. Although these puncta typically characterize dying cells, CAFs proliferate quickly, and confer many factors to promote disease progression. Given the emerging understanding of autophagy dependent secretion, we hypothesized that HNSCC induces CAF autophagy, which facilitates tumor-promoting secreted factors. We sought evidence to determine autophagy characteristics in CAFs compared to NFs. We also assessed the role of HNSCC in CAF autophagy induction. Finally, given the dearth of current studies of autophagy inhibition in HNSCC, we assessed the utility of autophagy inhibitors in preclinical models. Our findings indicate a fascinating tumor-stroma symbiotic relationship centered around what is typically thought of a degradative pathway.

Chapter III-Materials and Methods

Cells and Reagents

HNSCC and tonsil or uvulopalatoplasty explants from cancer-free patients were collected with written consent from patients under the auspices of the University of Kansas Medical Center Biospecimen Repository Core Facility. All protocols for collection and use were approved by the Human Subject Committee at the University of Kansas Medical Center. Primary fibroblast explants were established using our previously described protocol (Wheeler et al., 2014), and all fibroblast lines used were cultured for no more than 12 passages. In all experiments, results presented are from fibroblasts derived from a minimum of 2 patient explants.

Well characterized HNSCC cell lines (UM-SCC-1 (a gift from Dr. Tom Carey, University of Michigan), OSC19, and HN5 (a gift from Dr. Jeff Myers, MD Anderson)), were used in this study (Lin et al., 2007). Established cell lines were authenticated by STR profiling at Johns Hopkins in 2015 using the Promega Geneprint 10 kit and analyzed using Genemapper v4.0 software. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Corning, Corning, NY) with 10% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO) without antibiotics. Cells were incubated at 37°C in the presence of 5% CO₂.

Chloroquine diphosphate salt, 4-nitroquinoline N-oxide (4-NQO), IL-6, IL-8 and bFGF were obtained from Sigma-Aldrich. SAR405 was obtained through APEX BIO (Houston, TX). Cisplatin was obtained from Fresenius Kabi (Lake Zurich, IL).

Antibodies used: LC3 A/B (#12741), Beclin-1 (#4122), phospho-p70 S6K (Thr389) (#9205), Stat3 (#9139), phospho-Stat3 (Tyr705) (#9145) from Cell Signaling (Danvers, MA); β -tubulin from Sigma-Aldrich; p62 (SQSTM1, M01) from Abnova (Taipei, Taiwan); Vimentin (#6260) from Santa Cruz Biotechnology (Dallas, TX); neutralizing antibodies, IL-6 (6708), IL-8

(6217), and secondary anti-rabbit IgG Dylight 680 (#35568), anti-rabbit IgG Dylight 488 (#35553), and anti-mouse IgG Dylight 800 (#35521) from ThermoFisher (Waltham, MA). Hoescht 33342 was used as a nuclear counter stain (ThermoFisher).

Primer sequences used: mTOR (F: GGCCGACTCAGTAGCAT; R: CGGGCACTCTGCTCTTT); SOX2 (F: ACGGAGCTGAAGCCGCC; R: CTTGACGCGGTCCGGGCT); β -ACTIN (F: AGGGGCCGGACTCGTCATACT; R: GGCGGCACCACCATGTACCCT), all obtained from ThermoFisher.

Control (#44236), siBECN1 (#29797), and siFGF-2 (#39446) siRNA was obtained from Santa Cruz Biotechnology.

Electron Microscopy

Tissues were processed at the KUMC Electron Microscopy research lab facility. Tissue samples were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer, and postfixed in 1.5% osmium tetroxide. Samples were embedded in resin-propylene oxide and allowed to cure. Tissues were sectioned using a diatome diamond knife on a Leica UC-7 ultra microtome at 80 nm thickness. Sections were collected on 200 mesh copper grids, and samples imaged using a J.E.O.L. JEM-1400 transmission electron microscope at 100KV.

Immunoblotting

Whole-cell lysates were extracted using RIPA lysis buffer and a mixture of protease and phosphatase inhibitors (Minitab, Roche). Lysates were sonicated on ice, debris removed by centrifugation, and supernatants stored at -80 °C. Sodium dodecyl sulfate-polyacrylamide 12% gels were used to separate proteins, and proteins were transferred to nitrocellulose membranes. Membranes were blocked with Odyssey blocking buffer (Li-Cor) in a 1:1 mixture with PBS 1% Tween-20 (PBST). Primary antibodies were incubated overnight in 1:1 blocking buffer to PBST.

Primary antibodies were detected using DyLight conjugated secondary antibodies. Protein bands were detected using Li-Cor odyssey protein imaging system and quantified using ImageJ software (v. 1.50i).

Immunofluorescent Imaging

Cells were plated at a low confluence (10,000 cells per well) in 8-well chamber slides (Thermo Fisher). Methanol (70%) was used to fix cells. Triton-x (0.5%) in PBS was used as a permeabilization buffer. Cells were blocked with a 2% Bovine Serum Albumin (BSA) solution. For paraffin sections, paraffin was removed by xylene, and sections were rehydrated by ethanol titration. Antigen was retrieved using sodium citrate solution. Both cells and tissue sections were then incubated overnight in primary antibody (1:100 concentration in 2% BSA) at 4 °C. Dylight (488-anti rabbit; 550-anti-mouse) conjugated secondary antibodies were used, and Hoescht staining following manufacturer's instructions was used for nuclear detection. Slides were mounted with coverslip in vectashield mounting media. Images were captured on a Nikon Eclipse TE2000 inverted microscope with a Photometrics Coolsnap HQ2 camera. LC3 puncta per cell of at least 30 cells in each experimental arm were identified by blinded observer at 20x magnification.

Conditioned Media Collection

CAFs (3×10^5 cells/well in 60mm dish) were plated in 10% FBS DMEM, and were treated with 20 μ M Chloroquine for 6 h or vehicle control (H_2O). Following drug treatment, cells were washed 2 times with serum free media, and then conditioned media was collected over 24 h in serum free DMEM. Following conditioned media collection, cell lysates were harvested for immunoblot analysis to confirm autophagy inhibition by assessing LC3 levels. Conditioned media supernatants were clarified by centrifugation and stored for no more than 2 weeks at 4°C.

CAFs (3×10^5 cells/well in 60mm dish) were plated in 10% FBS DMEM, and were transfected with 100 nM siBeclin-1, siATG7 or siControl (Santa Cruz Biotechnology) containing lipofectamine-2000 (ThermoFisher) liposomes in Opti-MEM for 4 h. Media was changed to 10% FBS DMEM overnight, and conditioned media collection began the next day for 24 h in serum free DMEM. Following conditioned media collection, cell lysates were harvested for immunoblot analysis to confirm siBECN knockdown. Conditioned media was clarified by centrifugation and stored for no more than 2 weeks at 4 °C.

Proliferation

HNSCC cells were seeded in triplicate (2000 cells/well, 96-well plate). After cells had adhered, various experimental conditions were applied for 72 h duration. Cell viability was assessed using CyQuant proliferation kit (Life Technologies) according to manufacturer's instructions. For irradiation experiments, plates were exposed to gamma radiation (J.L. Shepherd and Associates Mark I Model 68A cesium-137 source irradiator; dose rate = 2.9 Gy/min).

Invasion & Migration

Cell invasion and migration was assessed using the transwell Boyden chamber system. HNSCC cells were seeded in 8 μ m pore inserts for migration. For invasion, a layer of diluted (2 mg/mL) growth factor-reduced Matrigel (Corning) in DMEM was placed in the insert. HNSCC cells in serum-free media were seeded onto Matrigel. The inserts were placed in triplicate holding-wells containing treatment conditions for 24 h. Cells were also plated in experimental conditions in parallel to assess viability using CyQuant. The number of cells that moved to other side of membrane was counted after fixation and staining with Hema3 Kit (Fisher). The number of invading or migrating cells was normalized to cell viability.

Cytokine Array

Cytokine array (C5) was obtained from RayBiotech (Norcross, GA) and conditioned media from CAFs was analyzed following the manufacturer's instructions.

PCR

HNSCC cells were CFSE-(ThermoFisher)-labeled following manufacturer's protocol. CFSE-labeled HNSCC cells were co-cultured in a 1:1 ratio with unlabeled NFs for 72 h. Cells were harvested by trypsinization and FACS sorted using BD FACSAria IIIu. RNA was extracted from harvested cells using TRIzol reagent (Fisher) following the manufacturer's instructions. RNA was subjected to DNase digestion prior to cDNA preparation using the SuperScript First-Strand Synthesis System (Invitrogen). PCR products were resolved on agarose gel and imaged. Densitometric analyses were performed with ImageJ (v1.50i). PCR array (PAHS-176ZD, Qiagen) was used to identify differences between NFs and co-cultured NFs, and read using CFX96 Real-Time System (Biorad, Hercules, California).

Co-culture Proliferation Assay

HNSCC cells were CFSE-(ThermoFisher)-labeled following manufacturer's protocol. CFSE labeled HNSCC cells were co-cultured in 1:1 ratio with unlabeled CAFs for 72 h. Cells were harvested by trypsinization and labeled HNSCC cells were counted using Attune NxT Flow Cytometer (Life Technologies).

In vivo Experiments

All experiments were approved by the institutional review board at the University of Kansas Medical Center. To assess biomarker modulation by chloroquine, 100 μ L of HNSCC (UM-SCC-1, 0.5×10^6) alone or admixed with CAFs (0.5×10^6) were injected into the right flank of athymic male mice (n=3/group). After tumors were allowed to form, chloroquine was

administered by oral gavage (162 mg/kg) for three days (Zou et al., 2013). Tissue was processed for electron microscopy.

To assess autophagy inhibition in combination with cisplatin, 100 μ L of admixed HNSCC (UM-SCC-1, 0.5×10^6) and CAFs (0.5×10^6) were injected into the right flank of athymic female mice. Mice (n=9/group) were treated with cisplatin (3 mg/kg i.p. 1x/week), chloroquine (162 mg/kg oral gavage, 5 days/week) or SAR405 (50 μ L intratumoral injection of 10 μ M SAR405 in PBS, concentration determined based on *in vitro* IC₅₀, 5 days/week). Tumor diameters were measured by a blinded observer using Vernier calipers in two perpendicular dimensions as previously described (Wheeler et al., 2014). Tumors were excised and processed for electron microscopy.

To assess progression of autophagy in developing tumors, 4-NQO (100 ppm in sterile drinking water *ad libitum* (X. H. Tang et al., 2004)) was administered for 16 weeks to C3H mice. Mice were then given sterile drinking water for 3 weeks, and tongues were excised.

The Cancer Genome Atlas Data Analysis

TCGA head and neck cancer (HNSC) cohort gene expression RNAseq data downloaded using UCSC Xena Browser (<http://xena.ucsc.edu>). Expression levels of BECN1 or MAP1LC3B were designated as high or low in relation to median expression of gene-level transcription estimates ($\log_2(x+1)$ transformed RSEM normalized count). This was matched to clinical survivorship data from TCGA HNSC Phenotype data downloaded from UCSC Xena.

Statistical analysis

Data are reported as mean \pm standard error of mean (SEM). Non-parametric two-tailed Mann-Whitney U tests were used to assess significance in all experiments, and Kruskal Wallis test for comparison of multiple groups. For *in vivo* study, one-way analysis of variance test was

employed to assess the level of significance in tumor volumes between treatment arms. For TCGA survivorship comparison, log rank (Mantel-Cox) test assessed differences between curves. All statistical calculations were performed on Graphpad Prism Software (version 6.03), with significance determined by $p < 0.05$.

**Chapter IV-Autophagy Dependent Secretion in CAFs promotes
HNSCC Progression**

Rationale

The most recent and pronounced clinical responses in HNSCC therapy act upon the microenvironment. For example, cetuximab, the EGFR monoclonal antibody, activates antibody dependent cell-mediated cytotoxicity. Additionally, nivolumab and pembrolizumab, PD-1 targeted antibodies, activate an antitumoral T-cell response. Each of these therapies have made significant improvements in HNSCC outcomes by acting upon microenvironment cells, particularly immune cells; thereby, demonstrating the pronounced effects achieved by capitalizing on the tumor microenvironment. However, no current therapeutics attempt to modify the largest component of the HNSCC microenvironment, CAFs. Perhaps targeting communication between tumor and stroma may lead to novel therapeutics and better outcomes for HNSCC patients.

Our poor understanding of the underlying biology of CAFs contributes to the dearth of therapeutics targeting this largely abundant cell population. Past studies from our lab and others document the pronounced tumor promoting effects of these cells. CAFs significantly contribute to HNSCC proliferation by secreting a variety of cytokines, notably HGF (Knowles et al., 2009). Additionally, CAFs likely lead the cancer away from the primary site (Gaggioli et al., 2007). This CAF-mediated invasion leads to distant metastasis of the carcinoma cells (Wheeler et al., 2014). CAFs strongly promote cancer progression. However, despite broad documentation of CAFs enhancing HNSCC growth, secretory mechanisms contributing to this phenotype are unknown and not targeted.

As such, we undertook studies to delineate how CAFs differ from NFs. We demonstrate pronounced structural differences between CAFs and NFs. Strikingly, CAFs, despite their rapid proliferation *in vitro*, demonstrate markedly enhanced autophagosomes. Paradoxically, this

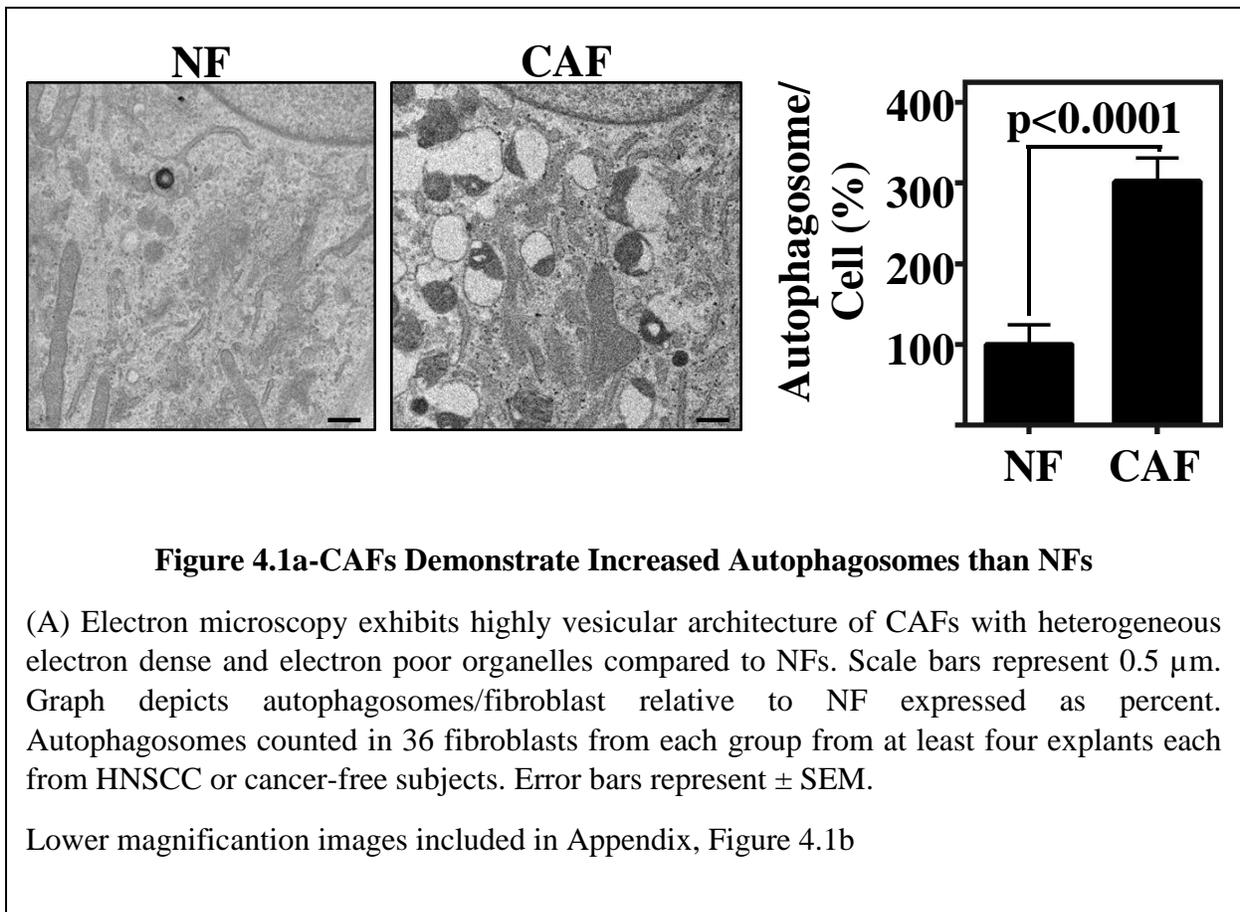
degradative pathway is highly upregulated in rapidly dividing, highly secretory CAFs. We characterize the surprising role for autophagy in cellular secretion, and provide evidence that CAF secretory autophagy enhances HNSCC progression.

Results

Given the broad secretory and tumor promoting differences between CAFs and NFs, we sought to understand the molecular mechanisms underlying the activated phenotype. As such, we undertook an ultrastructural investigation of CAFs compared to NFs. CAFs demonstrate a remarkably different architecture compared to NFs (Figure 4.1a, Appendix Figure 4.1b).

At a whole cell view, NFs have a stereotypically normal appearance. A well-defined filament network, appropriate mitochondria, scant lysosomes and peroxisomes, and proper endoplasmic reticulum are easily observable. However, CAFs demonstrate a disrupted cytoplasm, littered with hundreds of vesicles. These vesicles take on both electron dense and electron lucid appearances, and cover the majority of cytoplasmic berth. They have a heterogeneous appearance, and vary in size. Electron dense, heterogeneous vesicles bounded by a single membrane typically characterize damaged lysosomes (Douglas Kelly, 1984). Whereas, normal lysosomes have an electron dense, and rather uniform appearance. Many vesicles appear to contain smaller membrane bound vesicles, characteristic of multivesicular bodies, also termed secondary lysosomes (Douglas Kelly, 1984). Often, there are transitional forms of a lysosome and multivesicular body, but the function of these are unknown. Some vesicles appear to have both electron lucid and electron dense components within a single membrane. The electron dense component may be material indigestible by a lysosome, such as lipids, as lysosomes have a low abundance of lipases (Douglas Kelly, 1984). The multitude of lysosomes could indicate two

things: 1) that CAFs have increased cellular digestion; or 2) there is a blockade of digestion that leads to an increased number of lysosomes.



Intriguingly, many vesicles contain other cellular components. These are likely autophagosomes. Autophagosomes are characterized by (Figure 4.2):

- 1) Double Membrane Structure,
- 2) Cytoplasmic Cargo Enclosed, or in the process of engulfment by cup shaped structure,
- 3) Vesicular architecture, which differentiates from Golgi or endoplasmic reticulum,
- 4) A clear distinction from a mitochondria-which is characterized by an electron dense matrix, with a double membrane. The inner membrane of a mitochondria folds into the mitochondrial matrix in the form of many cristae. Although an autophagosome may engulf mitochondria, the inner membrane of the autophagosome does not form cristae, but circumscribes the vesicle.

Autophagosome Examples

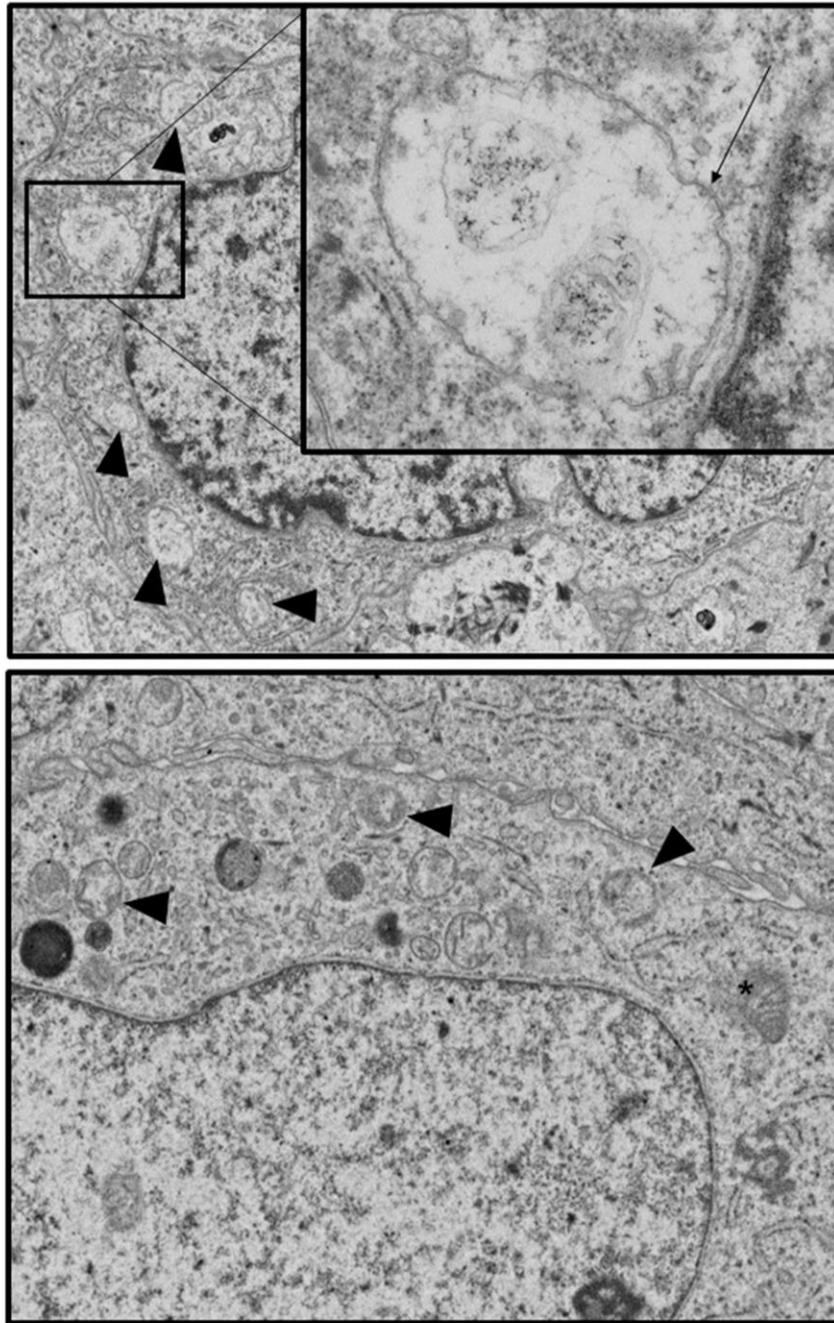


Figure 4.2 – Autophagosome Examples

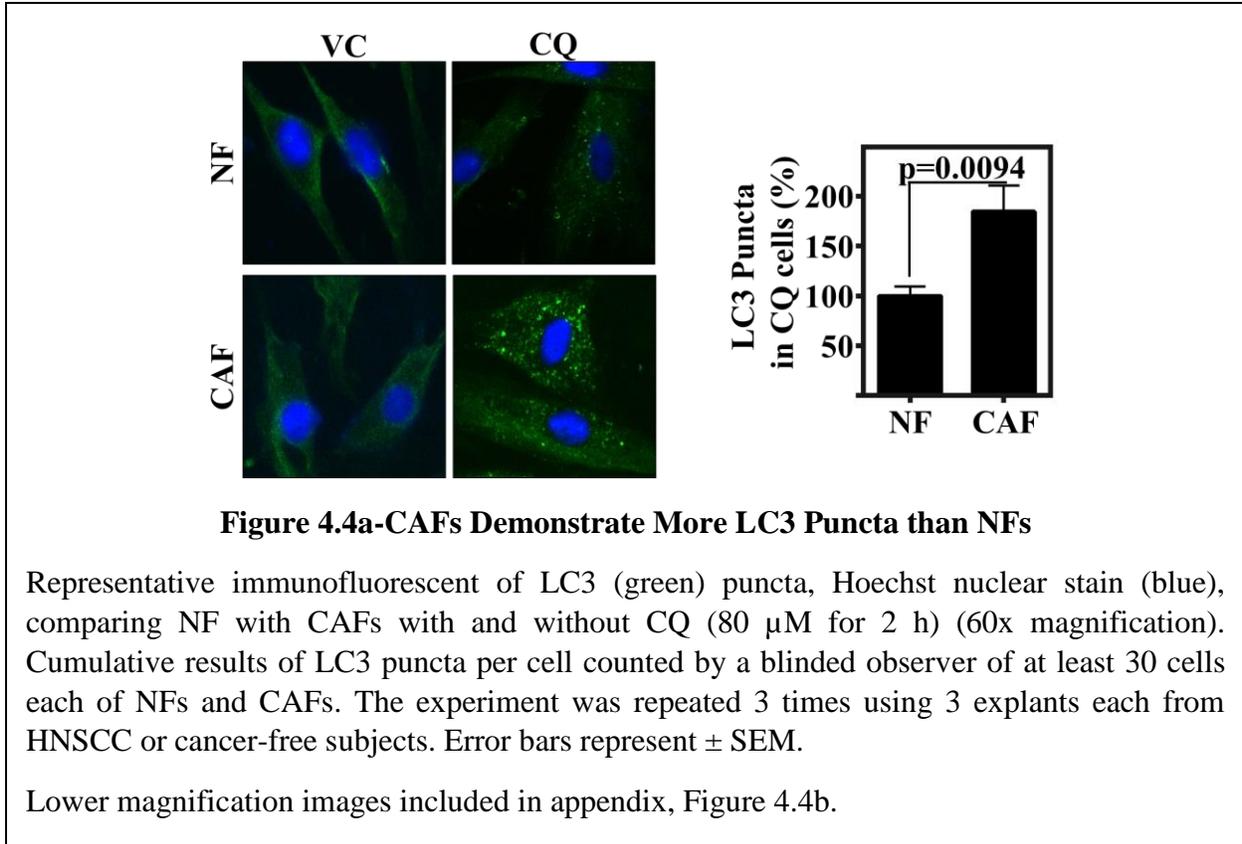
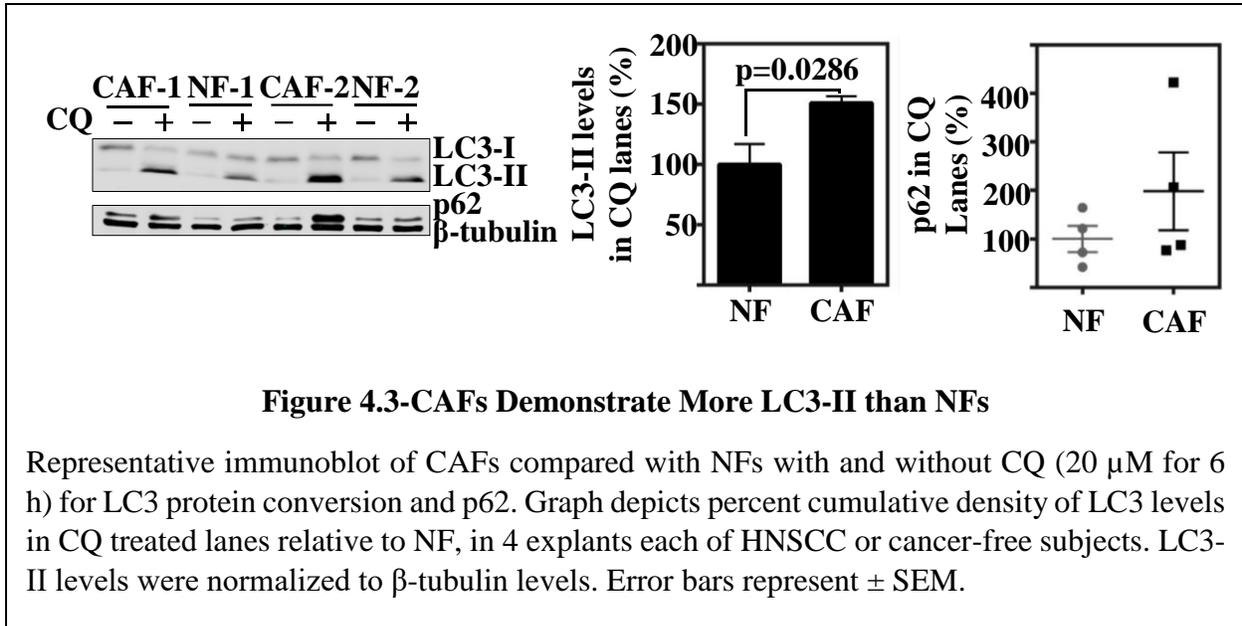
Autophagosomes (identified by arrowheads) were counted by blinded observer following these characteristics: 1) double membrane structure (Can be observed by arrow), 2) cytoplasmic cargo enclosed, 3) vesicular architecture to differentiate from Golgi or ER, and 4) clear distinction from mitochondria (asterisk). Images representative of two HNSCC tumor cells at 1200x magnification. Autophagosome inlet is at 12000X magnification.

By these criteria, CAFs demonstrate an abundance of autophagosomes, much greater than observable autophagosomes in NFs. The increased number of autophagosomes and lysosomes indicates that CAFs likely have increased autophagic flux compared to NFs.

Autophagic flux is characterized by the packaging of intracellular cargo into an autophagosome. Typically, cargo is sequestered by an autophagy cargo receptor. Sequestosome 1 (SQSTM1 or p62) is a paradigm cargo receptor. p62 binds ubiquitinated cargo, and traffics the material to the autophagosome. At the autophagosome, p62 binds LC3, which is cleaved and lipidated to the form LC3-II and is incorporated into the autophagosome membrane. LC3-II, the molecular tether of cargo to the autophagosome membrane, is the best marker of the autophagosome available. Increased autophagic flux yields increased LC3-II, but this is difficult to assess because LC3 and p62 are degraded in the lysosome. Thus, to accurately assess autophagy, a lysosomal enzyme inhibitor must be used to prevent the degradative step, allowing the autophagy intermediates to accumulate so that accurate assessment of total flux might be assessed. Chloroquine is a lysosomotropic agent that neutralizes the acidic pH of the lysosome, thereby rendering the lysosomal proteases ineffective. This allows for accumulation of LC3, p62, and any cellular cargo.

To confirm that the increased number of autophagosomes and lysosomes observed by TEM demonstrate increased flux, we assessed protein levels of LC3 and p62 by immunoblot (Figure 4.3). Chloroquine was used as a flux inhibitor. By immunoblot, CAFs have significantly increased LC3-II in chloroquine treated samples compared to NFs, indicating enhanced autophagic flux in CAFs. p62 was more pronounced in CAFs, but the differences between CAFs and NFs were not significant. This may indicate another cargo receptor might be involved, but

studies have not yet been conducted to characterize this. Thus, by immunoblot, we observe enhanced LC3-II in CAFs compared to NFs, which demonstrates enhanced autophagic flux.



Immunofluorescence allows for the direct visualization of autophagosomes within a cell by targeting LC3, and examining puncta formation. As LC3 accumulates on the inner and outer membranes of an autophagosome, punctate accumulations of fluorescence signal correspond with autophagosomes. This offers an alternative method to characterize autophagosomes in both CAFs and NFs. By immunofluorescence, CAFs demonstrate significantly more LC3 puncta in CQ treated samples (Figure 4.4b, Appendix Figure 4.4b). This confirms enhanced autophagic flux within these cells. Thus, by TEM, immunoblot, and IF, CAFs have an increased number of autophagosomes and autophagic flux.

As these are cultured cells, the question arose if the enhanced levels of autophagy could be due to the culture conditions, and might be a byproduct of the adherence of these cells to the plastic flask. Yet, if this was so, one would expect the same process to occur in NFs and CAFs as both these cell types are processed in the same way. These samples are obtained from a similar anatomic location from patients, digested using the same protocol, and passaged using the same technique. There are no differences between CAFs and NFs other than from which patient they were derived. Observing enhanced autophagy within CAFs is not likely a consequence of culturing the cells.

Nevertheless, we analyzed histologic specimens to confirm the *in vitro* observations. One caveat occurs by the fact that a lysosomal inhibitor cannot be used on histologic specimens, so true autophagic flux cannot be observed. Thus, increased numbers of autophagosomes might occur because of either increased autophagy or defective degradation. Nonetheless, assessment on histologic specimens was conducted to validate the observations observed in the cultured cell lines.

To assess autophagy through the disease process, mice received drinking water containing the carcinogen, 4-nitroquinolone (4-NQO). 4-NQO induces DNA damage, and thus causes dysplasia on the tongue surfaces, which develops into squamous cell carcinoma. Tongues were assessed *ex vivo* for autophagosomes throughout the disease process (Figure 4.5). In normal tongue samples, scant LC3 puncta were observed in the stroma. Specimens with low grade intraepithelial lesions demonstrated LC3 puncta in the stromal fibroblasts alongside a subtle increase in LC3 puncta within the epithelium. As the disease progressed, LC3 puncta became strongly apparent within the stromal fibroblasts. Fibroblasts in invasive squamous cell carcinoma

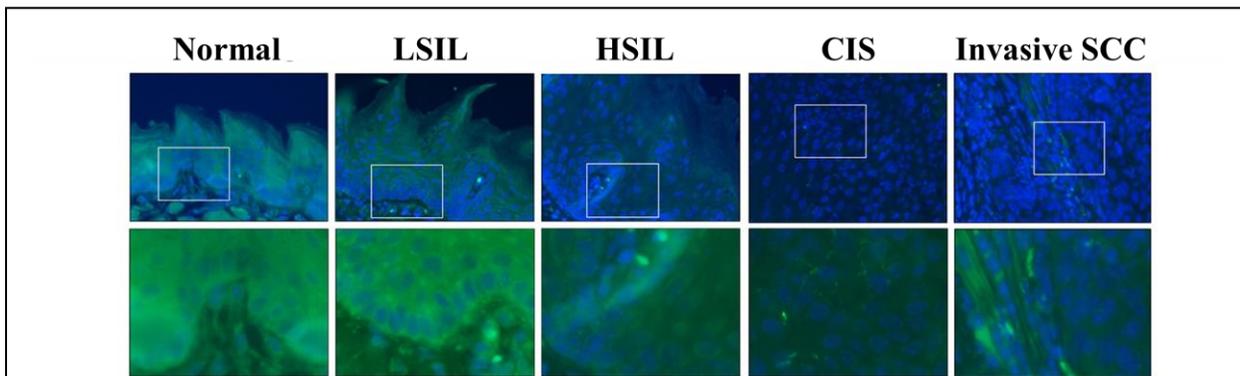


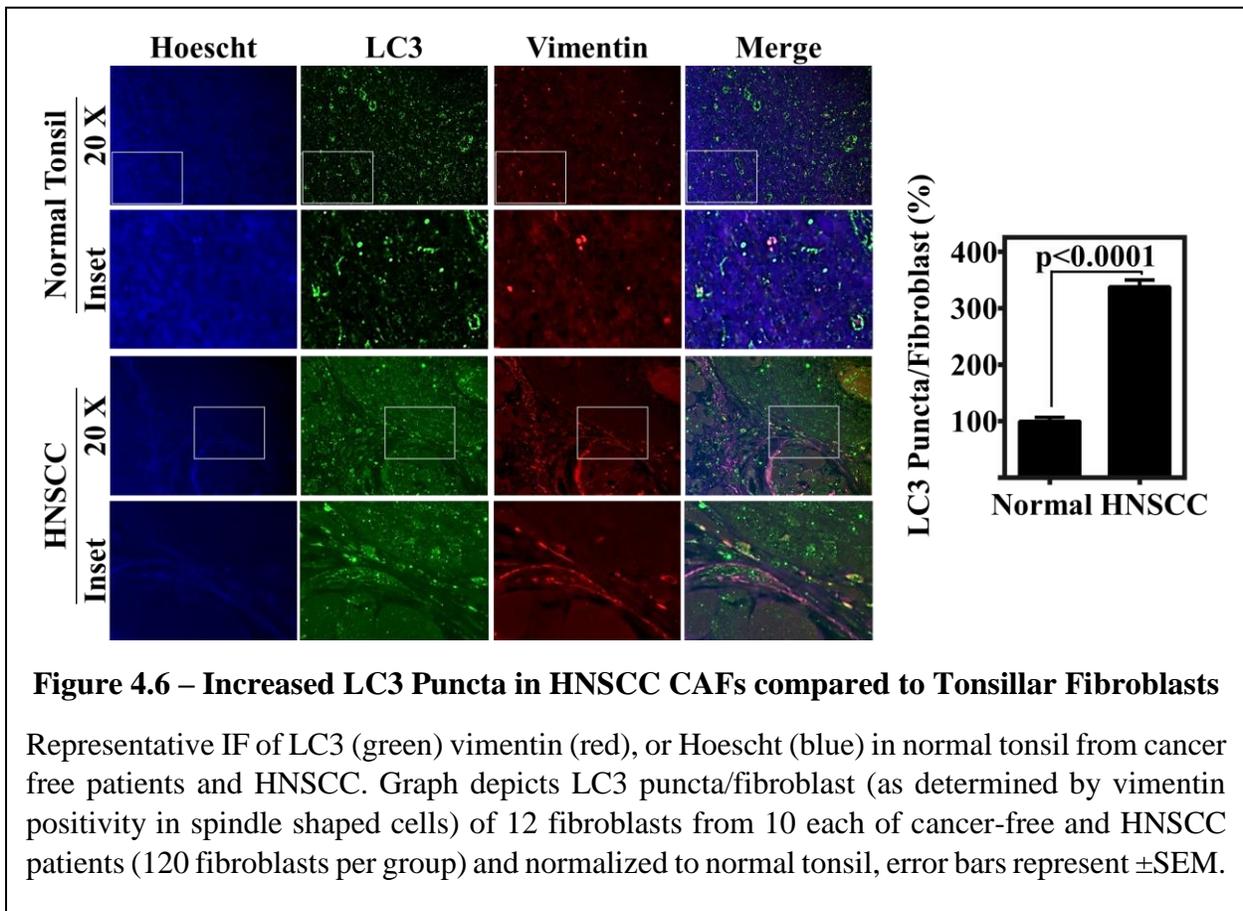
Figure 4.5 - LC3 Puncta Increases through Disease Progression

Representative IF images of LC3 (green) puncta in 4-NQO induced HNSCC model progression from normal tongue epithelium, low grade squamous intraepithelial lesion (LSIL), high grade squamous intraepithelial lesion (HSIL), carcinoma in situ (CIS), and invasive squamous cell carcinoma (Invasive SCC) at 20x magnification, top, and 60x magnification, bottom. Arrowheads depict LC3 puncta accumulation. Blue is nuclear stain. White box indicates area of 60X image.

specimens demonstrated significant LC3 puncta compared to fibroblasts resident in normal tongue epithelium. In a murine model, these data corroborate the *in vitro* findings from patient-derived CAFs and NFs.

Additionally, in patient specimens, LC3 puncta increase as the disease progresses (Figure 4.6). We assessed patient histology comparing normal tonsils and HNSCC specimens. In these specimens, vimentin, a marker of mesenchymal cells, delineated fibroblasts from squamous

cells. As vimentin would exist in all mesenchymal cells, and epithelial cells undergoing epithelial-to-mesenchymal transition also express vimentin, fibroblast morphology was also used to delineate fibroblasts from epithelial cells. In normal tonsillar specimens, fibroblasts had few LC3 puncta, and there was an overall low abundance of LC3 throughout the normal specimen. However, in HNSCC, there was robust LC3 puncta, and the fibroblasts demonstrated significant levels. Intriguingly, the increase in LC3 puncta occurs in both the fibroblasts and the epithelial cells, giving indication that LC3 puncta increase in both cell types through the disease process.



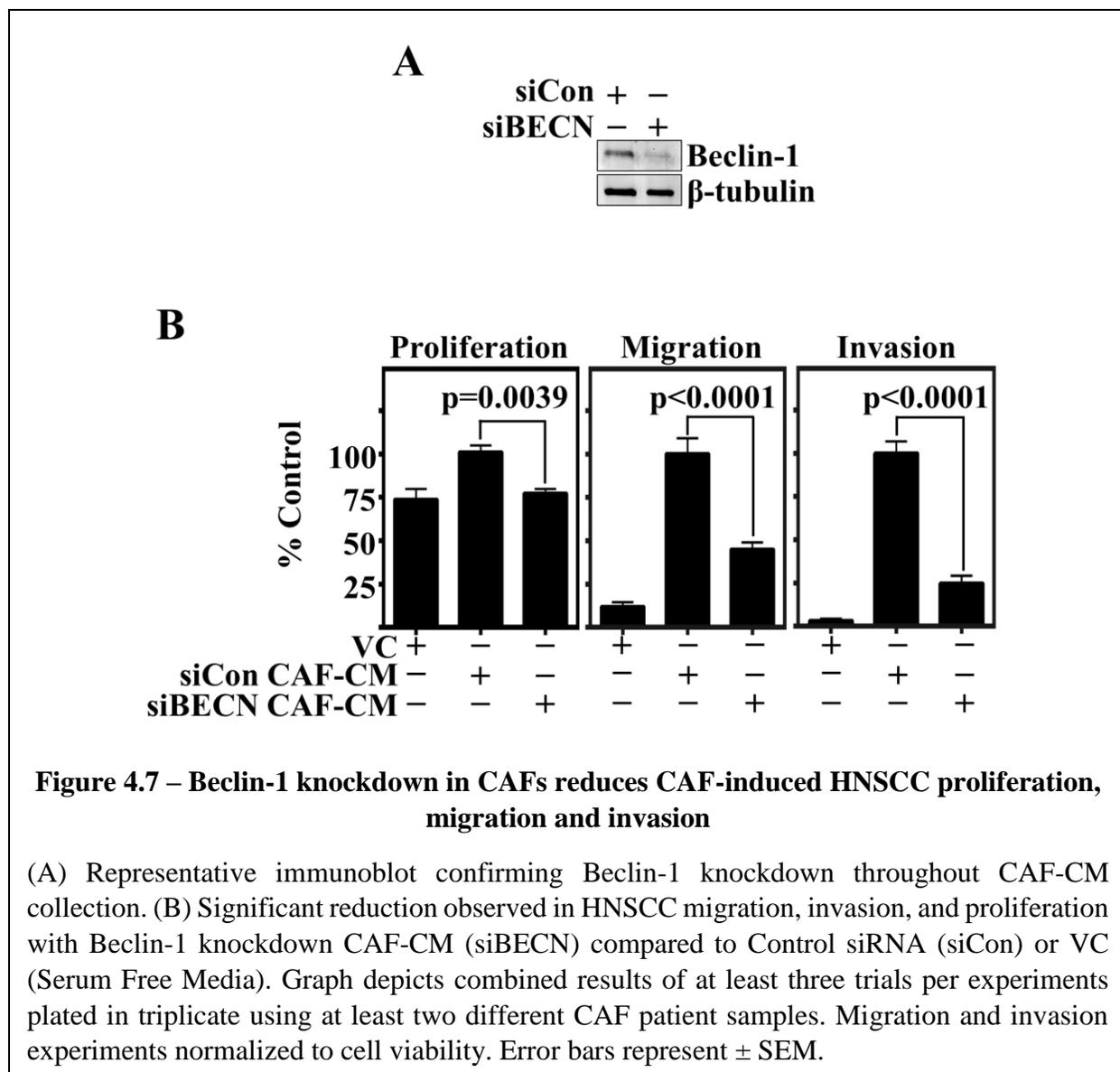
These data point to the conclusion that fibroblasts increase autophagic flux through the disease process. TEM, IF, and immunoblot of patient derived CAFs demonstrate increased autophagic flux. IF of both a murine model and patient-histologic specimens also demonstrate

enhanced LC3 puncta in CAFs through the disease process. Surprisingly, CAFs proliferate much faster than NFs. Autophagy as a self-eating mechanism usually coincides with quiescent, non-dividing cells. Yet, paradoxically, CAFs demonstrate enhanced autophagy. Perhaps autophagy has pleiotropic roles within these cells.

Autophagy dependent secretion provides a possible explanation for the role of enhanced autophagosomes within CAFs. Here, the autophagic cargo do not degrade within the lysosome, but are secreted. The exact mechanisms of this pathway are relatively understudied. CAF autophagy dependent secretion may underlie many of the tumor-promoting roles observed by our lab and others.

To assess the role of autophagy dependent secretion in tumor progression, we inhibited autophagy within CAFs and collected the secreted factors in CAF conditioned media (CAF-CM). Beclin-1 is the upstream initiator of both degradative and secretory autophagy. Levels of Beclin-1 correspond with autophagic flux. When Beclin-1 is inhibited, there is a significant decrease in autophagic flux. We collected Beclin-1 knockdown CAF-CM over 24 h, and assessed HNSCC proliferation, migration, and invasion. Substantiating previous reports, CAM-CM significantly increased HNSCC proliferation, migration, and invasion compared to serum-free basal media (SFM) (Figure 4.7). Intriguingly, Beclin-1 knockdown significantly attenuated CAM-CM contributions to proliferation, migration, and invasion. This indicates a role for Beclin-1 in cellular secretion.

To better delineate if this phenomenon is unique to Beclin-1 or to autophagy as a whole, we inhibited additional components of this pathway. Atg7 is necessary for the enzymatic processing of LC3-I to LC3-II. Inhibition of Atg7 prevents proper formation of an autophagosome. Atg7 knockdown CAF-CM demonstrates mitigation of CAF-CM contributions



to migration and invasion, corroborating the Beclin-1 findings (Figure 4.8). Both Beclin-1 and Atg7 modulating the ability of carcinoma cell lines to respond to conditioned media, indicates that autophagy likely has a secretory role within the CAFs.

Lysosomal degradation provides the final step in the autophagic pathway. The only inhibitor clinically approved to target autophagy, chloroquine (CQ), acts upon the lysosome. Thus, to assess the relevance of targeting autophagy-dependent secretion in patients, we inhibited autophagy in CAFs with CQ. To mitigate any effects of extracellular CQ, cells were extensively

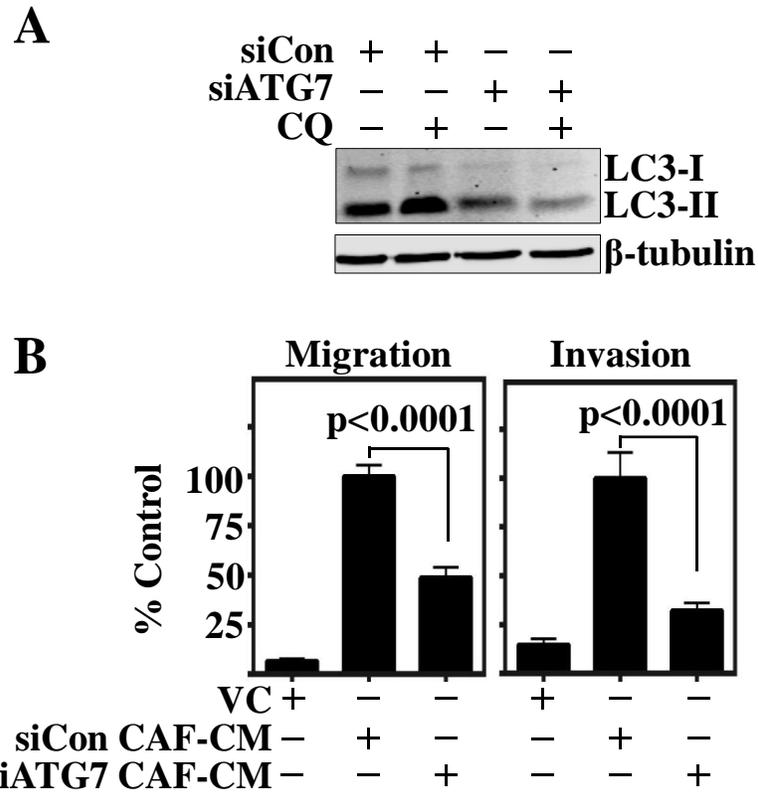


Figure 4.8 - Atg7 knockdown in CAFs reduces CAF-induced HNSCC migration and invasion

(A) Immunoblot confirms silencing of Atg7 (siATG7) by decreased LC3-II expression in CQ treated lane compared to control siRNA (siCon). (B) Significant reduction observed in HNSCC migration, invasion, in siATG7 compared to siCon. Graph depicts combined results of at least two trials per experiments plated in triplicate using two different CAF patient samples. Migration and invasion experiments are normalized to cell viability. Error bars represent \pm

washed following CQ incubation. LC3 levels following conditioned media collection demonstrate LC3-II accumulation in CQ treated cells compared to vehicle control treated cells (Figure 4.9A). This indicates CQ retained its effects through the 24 h CM collection period. Although, over the 24 h CM collection period, little change was observed in LC3-II levels

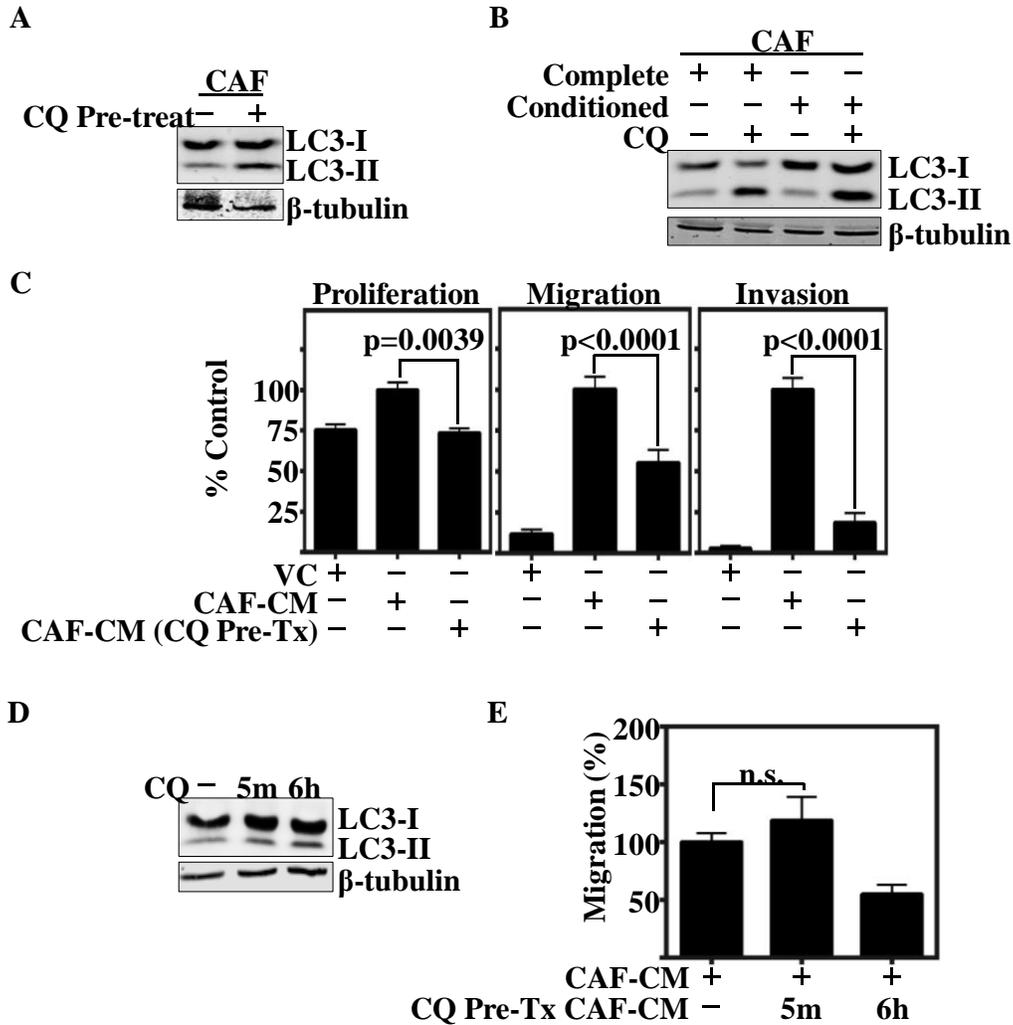


Figure 4.9 – CQ inhibits CAF-induced HNSCC Proliferation, Migration, and Invasion

(A) Representative immunoblot as validation of CQ autophagy inhibition throughout conditioned media (CM) collection. (B) Representative immunoblot of CAFs grown in complete media (10% FBS DMEM) or after 24 h of serum free media (mimicking conditioned media collection). CQ (20 μ M for 6 h) was applied at the end of the treatment period. No significant differences between complete media and conditioned media LC3-II levels demonstrates no increase in autophagy. (C) CAFs pretreated with CQ were washed extensively to remove excess chloroquine and then CM was collected with and without CQ pre-treatment. HNSCC (OSC19) migration, invasion, and proliferation are significantly reduced in CAF autophagy inhibited CM. Graph depicts cumulative results from three independent experiments including triplicate treatments, using CAFs derived from two HNSCC patients. Migration and invasion experiments were normalized to cell viability. Error bars represent \pm SEM. (D) 5 min CQ treatment (20 μ M) on CAFs did not significantly alter LC3-II conversion as assessed by immunoblot. (E) 5 min CQ treatment (20 μ M) of CAFs prior to CM collection had no difference in HNSCC (OSC19) migration. All error bars represent SEM.

between CAFs in complete media or serum free media (Figure 4.9B). CQ-inhibited CAF-CM mitigated CAF contributions to HNSCC proliferation, migration, and invasion (Figure 4.9C). To control for any effects of lingering CQ following extensive washing, a 5-minute treatment of CQ was applied and then washed off at the same dose as the typical 6-hour treatment. This 5-minute treatment did not increase LC3-II levels after conditioned media collection, compared to the robust increase observed in the 6 h treated cells, indicating a productive washing protocol (Figure 4.9D). No significant differences in HNSCC migration occurred with 5-minute CQ treatment, indicating minimal contribution of lingering CQ from CAF-CM (Figure 4.9E). Additionally, similar reductions were observed in multiple HNSCC cell lines. Overall, CQ treated CAF-CM produced similar effects to Beclin-1 and Atg7 knockdown, indicating a role for CAF autophagy dependent secretion in HNSCC proliferation, migration and invasion.

Of note, autophagy inhibition had little effect on CAF proliferation over the CM collection period. No differences in CAF number occur in autophagy-inhibited groups compared

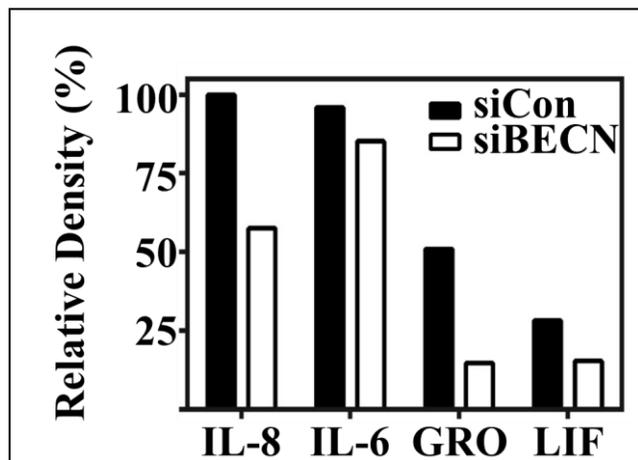


Figure 4.10 – CAF Cytokines Secreted in Beclin-1 Dependent Manner

Relative density of top 4 cytokines recognized on cytokine array of CAF-CM with Beclin-1 siRNA knockdown (siBECN) or Control siRNA (siCon).

to vehicle treated samples. This reflects the high IC₅₀ dose required of chloroquine to inhibit CAF proliferation. It is additionally important to note that CM collected under serum-free conditions may upregulate autophagic flux slightly. These findings substantiate the concept that CAF autophagy promulgates a secretory profile that enhances tumor progression.

CAF autophagy modulates a secretory phenotype responsible for HNSCC proliferation, migration and invasion. To understand which factors secreted by CAFs modify these HNSCC factors, we assessed Beclin-1 knockdown CM using a cytokine array. This array identified IL-8, IL-6, GRO, and LIF as the most pronounced factors secreted from CAFs (Figure 4.10). CAFs demonstrate significant reductions in IL-8, IL-6, and a plethora of other factors, under Beclin-1 inhibition. The findings of IL-8 and IL-6 reductions were quite intriguing because of past reports linking these to HNSCC progression. If these were responsible for the differences observed in HNSCC proliferation, migration, and invasion, reconstitution of these in CAF-CM would restore the reduction observed under autophagy knockdown.

Therefore, we assessed HNSCC migration following autophagy inhibition with and without IL-6 and IL-8. Reconstitution of these factors restored HNSCC migration to VC, control siRNA treated CAF-CM levels (Figure 4.11). This affirms the concept that these mediate, at least in part, the differences observed under CAF autophagy inhibition. Of note, the concentrations used of IL-6 and IL-8 correspond with reputable past reports, even though they are greater than what was observed in the ELISA. Nevertheless, the restored migration indicates the role of these factors in HNSCC migration, and the cytokine array and ELISA indicate the mitigation of these factors under beclin-1 inhibition.

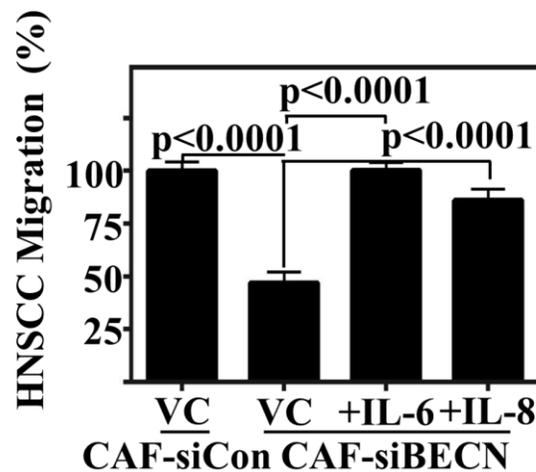


Figure 4.11 – Reconstitution of IL-6 and IL-8 Restore Autophagy Inhibition’s Effects on CAF-induced HNSCC Migration

Reconstitution of HNSCC (UM-SCC-1) migration in Beclin-1 knockdown CAF-CM with recombinant IL-6 (10 ng/mL) and IL-8 (80 ng/mL); data cumulative of two trials plated in duplicate using different CAF patient samples.

Discussion

CAFs inarguably contribute to HNSCC disease progression; however, the underlying biological processes involved are poorly understood. We undertook investigation to discover what processes account for such promotion of disease by this microenvironmental cell type. Our results demonstrate pronounced activation of autophagy in CAFs compared to NFs. Data supporting this interpretation come from 21 combined patient samples of both CAFs and NFs across four experimental methods, giving strong validation to this conclusion. Additionally, the 4-NQO murine model supports the concept that fibroblasts have enhanced autophagy through disease progression. This is the first observation of enhanced fibroblast autophagy from primary CAFs-derived from patient stroma, and corroborates recent observations of breast cancer cells

inducing autophagy in skin fibroblasts, and a *Drosophila melanogaster* tumor model where microenvironmental autophagy was observed (Katheder et al., 2017b; U. E. Martinez-Outschoorn et al., 2010).

While autophagy is conventionally a degradation pathway, recent reports of a role for autophagy in unconventional cellular secretion (Ponpuak et al., 2015) prompted us to investigate the role for CAF autophagy in secreting tumor-promoting factors. By collecting CAF conditioned media under autophagy inhibition through both upstream knockdown of Beclin-1, Atg7 and downstream lysosomal inhibition using chloroquine, we observed significant phenotypic differences in cancer progression *in vitro*. This indicated CAF autophagy modulates secreted factors important for tumor progression. Using a cytokine array, we identified IL-6 and IL-8 as being autophagy modulated in their secretion, consistent with previous observations of autophagy controlled secretion of these factors in other systems. IL-6 and IL-8 are elevated systemically in patients with HNSCC (Z. Chen et al., 1999), have been associated with resistance to targeted therapy (Fletcher et al., 2013), and are known to be secreted from stromal fibroblasts found in a number of cancer types (Nagasaki et al., 2014). This is the first report linking CAF tumor-promoting cytokine secretion with autophagy. Interestingly, IL-6, and IL-8 are among a plethora of factors associated with the senescence associated secretory phenotype (Coppe et al., 2008). We observed that primary CAF lines demonstrate rapid proliferation despite autophagy. Our data also demonstrate that knockdown of Beclin-1 mitigated secretory autophagy and consequently reduced the levels of IL-6 and IL-8 in CAFs. Thus, reduced IL-6 and IL-8 secretion on inhibition of autophagy coupled with the rapid proliferation of these fibroblasts leads us to the conclusion that this is not a replication induced senescent phenomenon. Recent reports indicate a role for autophagy controlled amino acid secretion by microenvironment cells of pancreatic

cancer (Sousa et al., 2016). Our data corroborate this finding and unveil a role for tumor microenvironment autophagy in altering secreted factors.

Intriguingly, autophagy dependent secretion seems to account for some but not all of the CAF-mediated effects on HNSCC migration and invasion. CAF secreted factors robustly increase migration and invasion, but autophagy inhibition only attenuates this increase by approximately 50 and 75%, respectively. This indicates other mechanisms of secretion are still involved as migration and invasion do not return to SFM conditions. In regards to proliferation, CAF autophagy inhibition decreased proliferation to basal levels with no significant difference in respect to SFM. Perhaps a more pronounced increase in proliferation may allow for resolution if canonical secretory mechanisms are involved in factors promoting cancer proliferation. Nevertheless, all of these techniques at assessing HNSCC progression indicate a role for factors secreted in an autophagy dependent manner.

Also of interest is the similarity of results obtained from CQ treated groups and RNA knockdown groups. Each method acted upon different stages of the autophagy pathway, Beclin-1 at the most upstream, Atg7 at a more intermediate step, and CQ at the degradative lysosome. Intriguingly, the role for the lysosome in secretory autophagy is unknown. The mechanism of secretory autophagy is poorly defined, and only investigated in light of IL-1 β as the secretory cargo (Kimura et al., 2017). In IL-1 β secretion, the lysosome is bypassed, and the secretory autophagosome is directed to the cell membrane. Yet, in other instances, lysosomal inhibitors, such as Bafilomycin A1, prevent IL-1 β secretion (Dupont et al., 2011). Additionally, NK cells and osteoclasts rely on secretory lysosomes to expel cytotoxic cargo, indicating a putative role for the lysosome in secretion (DeSelm et al., 2011; Lopez-Soto et al., 2017). Our data indicate

similar results independent of lysosomal function, indicating the secretory mechanism we observe likely depends on the lysosome.

A weakness of the current work is a lack of assessment of the secretome differences between Beclin-1 knockdown, Atg7 knockdown, and CQ treated CAFs. Additionally, future studies might better delineate the role for identified secretory autophagy mediators, such as TRIM16, and Sec22b (Kimura et al., 2017). Towards this end, the main cargo studied in other reports of secretory autophagy is IL-1 β , which CAF-secreted levels were too low of a concentration to appreciate by cytokine array, and an RNA difference was not appreciated on RNA sequencing. Future studies specifically targeting secretory autophagy, rather than both degradative and secretory, may decrease adverse effects, as degradative autophagy is important for normal cellular homeostasis.

Overall, CAFs demonstrate enhanced secretory autophagy compared to NFs. This significantly promotes HNSCC progression, and outlines a new avenue to direct therapy. Questions remain as to how HNSCC induces autophagy, and by what mechanism autophagic flux remains high in cultured CAFs. Additionally, although well studied in other cancers, the role of autophagy in HNSCC is under investigated. The patient histology specimens identify increased autophagosomes in squamous cells through disease progression; yet, few groups have assessed the role for autophagy inhibition in HNSCC. The results from CAFs indicate autophagy inhibition would likely modulate the microenvironment to prevent HNSCC progression.

**Chapter V-HNSCC Induces Autophagy in Cancer Associated
Fibroblasts**

Rationale

A large portion of late stage HNSCC tumors consist of fibroblasts. A greater ratio of fibroblasts to HNSCC correlates with HNSCC tumor volume (Bae et al., 2014). This is supported by laboratory findings that identify CAFs to promote HNSCC proliferation, migration, and invasion (Wheeler et al., 2014). Previous investigations into CAF-mediated HNSCC progression from our lab attribute much of this finding to CAF-secreted HGF. HNSCC does not secrete HGF, however, it is instead secreted by the tumor microenvironment. CAFs secrete HGF, which facilitates HNSCC progression (Knowles et al., 2009; Wheeler et al., 2014). However, HGF inhibition only partially decreases CAF-induced HNSCC proliferation, migration, and invasions. This demonstrates there must be other factors involved in CAF-mediated HNSCC progression.

The underlying secretory pathways mediating CAF-induced HNSCC progression are not well described. We demonstrate the role of autophagy-dependent secretion in mediating a large portion of CAF-induced HNSCC progression. Our previous results indicate that CAF-induced HNSCC proliferation, migration, and invasion are attenuated with CAF autophagy inhibition. HNSCC seems to regulate CAF autophagy, as CAFs have significantly greater autophagic flux than NFs. Yet, mechanisms by which autophagy induction occur in CAFs are unknown.

HNSCC symbiotically communicates with CAFs. HNSCC induces adaptation of NFs to CAFs. This occurs through a plethora of HNSCC secreted factors, which facilitate CAF adaptation. The best studied of these fibroblast activating factors is TGF- β (Kellermann et al.). TGF- β induces the myofibroblast phenotype, and blockade of this factor inhibits fibroblast activation as assessed by α -SMA. Although the role of TGF- β in inducing α -SMA is well described, the role for this factor in autophagy induction is unclear. In some cancers, TGF- β

induces autophagy (Kiyono et al., 2009). Yet, in fibrotic conditions, TGF- β limits autophagy (Patel et al., 2012). This dichotomy indicates other factors within the HNSCC microenvironment likely have a greater influence on the induction of autophagy in CAFs we observed in Chapter IV. Beyond TGF- β , many HNSCC factors contribute to fibroblast activation. For example, the fibroblast growth factor family retains its namesake by increasing fibroblast growth.

The FGF family consists of many soluble signaling ligands that bind to receptor tyrosine kinases (Ornitz & Itoh, 2015). Of these ligands, the FGF1 subfamily, consisting of acidic FGF and basic FGF (bFGF or FGF2) are the most well studied, with bFGF being the first FGF identified (Gospodarowicz, 1975). In HNSCC, bFGF is of interest as it binds FGFR2 expressed on the HNSCC cells in an autocrine signaling loop to cause disease progression (Marshall et al., 2011). The role for bFGF in modulating the surrounding stromal fibroblasts has been a focus of active study by our lab.

The induction of autophagy may arise from secreted factors such as TGF- β or bFGF, but may also come from other autophagy inducing stimuli. Autophagy induction focuses around two common themes – nutrient starvation and reactive oxygen species (ROS). Nutrient sensors have the most upstream effect on autophagy. mTOR recognizes amino acid conditions within the cell and serves as the molecular “brakes” on autophagy by means of inhibiting ULK1 activity (Sancak et al., 2008). AMPK responds to levels of glucose by assessing the ratio of AMP/ATP, and serves to activate ULK1 activity (J. Kim et al., 2011). Therefore, in conditions of low amino acids or low glucose, these two molecular mediators activate an ULK1 facilitated signaling cascade to promote autophagic flux. Beyond nutrient sensors, ROS serve as the other key autophagy inducers. Data supporting ROS induction of autophagy arise from studies on radiation biology. Ionizing radiation induces a number of ROS, alongside an increase in autophagy

(Chaurasia, Bhatt, Das, Dwarakanath, & Sharma, 2016). Although radiation has the capacity to ionize any molecule within the cell, the autophagy mediating effects are attributed to ROS (Scherz-Shouval et al., 2007). Exact molecular mechanisms of autophagy induction by ROS are unclear. ROS induce the activation of Atg4 (Scherz-Shouval et al., 2007); yet, this is not typically understood as a key upstream event in autophagy. Perhaps the widespread protein damage is sensed, and autophagic degradation is initiated by a yet unknown mechanism. A limitation in understanding ROS biology is that ROS are by nature short-lived molecules, and direct assessment of their role in autophagy induction is difficult to establish. Nevertheless, the role for ROS in inducing autophagy, even despite a murky mechanism, is well accepted. Thus, there may be a role for nutrient starvation and ROS in the microenvironment to induce CAF autophagy alongside a secreted factor.

The HNSCC microenvironment consists of both ROS and a low abundance of nutrients. The rapidly growing tumor quickly depletes the nutrient supply, and thus may contribute to autophagy induction in CAFs. Additionally, damaged mitochondria within the HNSCC cells produce an abundance of ROS that exist both intracellularly and in the extracellular space (Schaaf et al., 2013). Beyond this, the dysregulated phenotype of HNSCC cells produces an abundance of hydrogen peroxide into the microenvironment and this likely contributes to CAF autophagy induction. Additionally, cancer cells secrete a number of factors, but the association of these factors with autophagy induction is largely unknown.

Beyond head and neck cancer, investigations in other cancer models demonstrate the induction of fibroblast autophagy by cancer cells. The first report of this arose from the investigation of human foreskin fibroblast co-cultured with breast cancer cells (Ubaldo E. Martinez-Outschoorn et al., 2010). In this model, MCF7 cell-secreted hydrogen peroxide

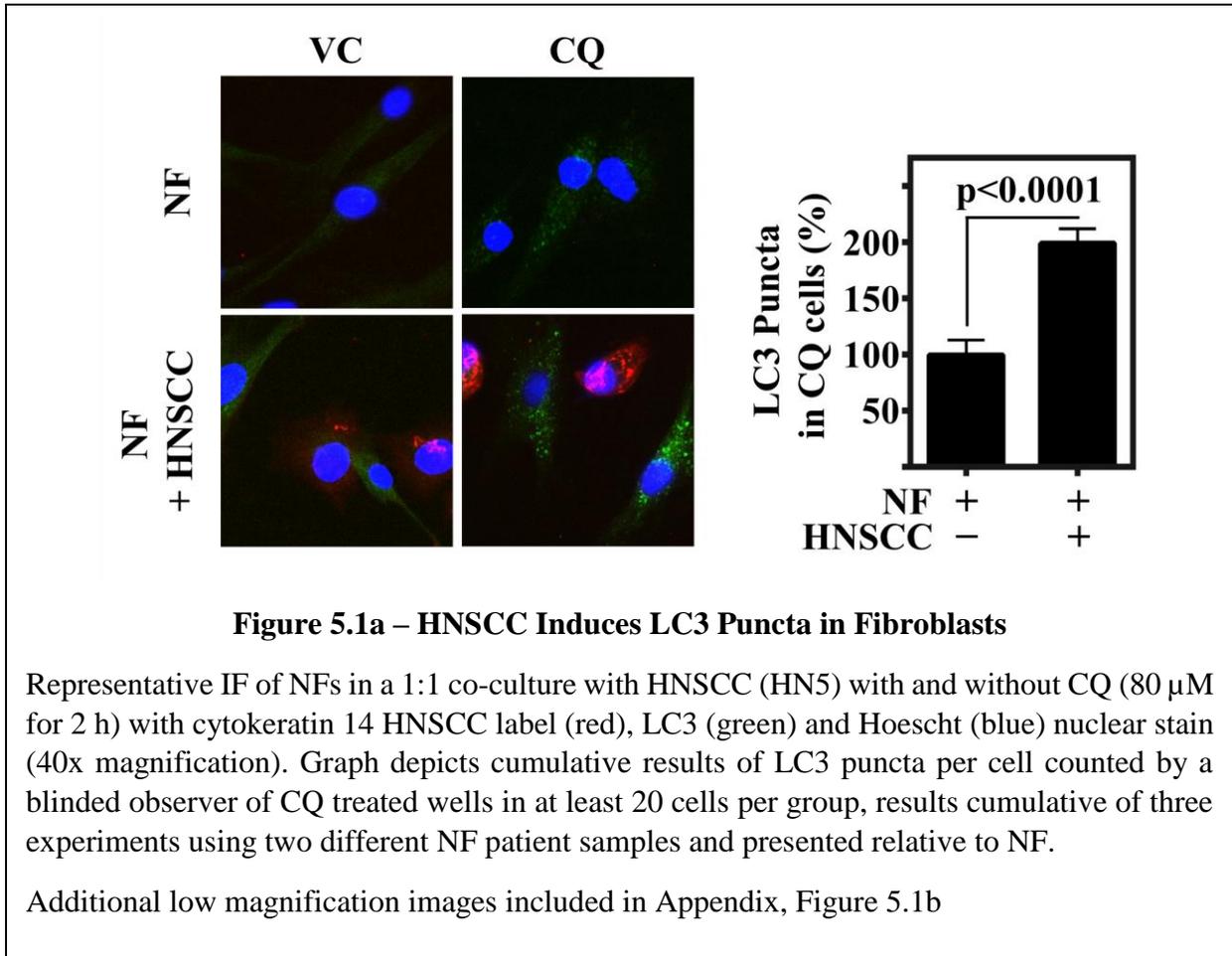
induced oxidative damage within co-cultured fibroblasts. This led to increased autophagy alongside a decrease in caveolin-1 expression. The role of autophagy induction in mediating breast cancer progression was not followed-up with by this or other groups. Recently, a mutant Ras *Drosophila* model also demonstrated pronounced microenvironment autophagy (Katheder et al., 2017a). This group attributed autophagy induction to STAT signaling within the microenvironment, which lead to ROS activation and stromal autophagy. Microenvironmental autophagy produced amino acids readily available for tumor growth. The ability of other cancer types to induce microenvironmental autophagy demonstrates the importance of understanding the underlying molecular mechanisms.

Thus, we undertook studies to understand the molecular mediators of autophagy induction in CAFs. We hypothesized HNSCC induce CAF autophagy through secreted factors. We observed HNSCC-secreted bFGF initiated a remarkable signaling cascade which led to pronounced autophagy within stromal CAFs. Factors secreted in an autophagy dependent manner fed-forward and potentiated autophagy-induction. This establishes both a HNSCC mediated and CAF potentiated mechanism for autophagy induction in stromal fibroblasts, which is critical for CAF-mediated HNSCC progression.

Results

To begin to understand the molecular mediators of fibroblast autophagy, we co-cultured NFs with HNSCC. NFs have a low basal level of autophagic flux, allowing for clear observation of autophagy induction. Cytokeratin 14, an epithelial cell marker found in basal cells and robustly expressed in squamous cell carcinoma, differentiates HNSCC from fibroblasts. Upon co-culture with HNSCC, NFs demonstrated a pronounced accumulation of LC3 puncta (Figure 5.1a, Appendix Figure 5.1b). This indicates the epithelial cell component of the

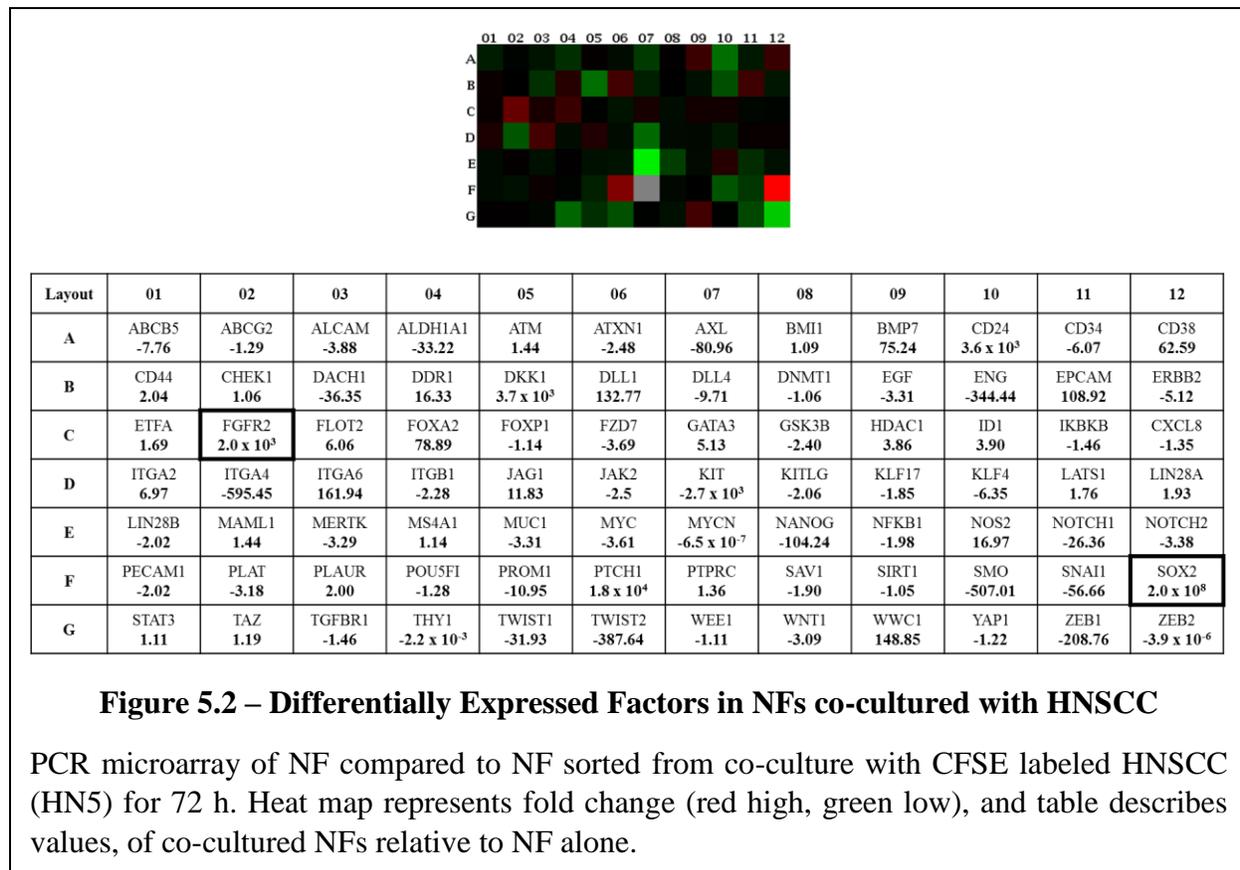
microenvironment supplies a strong influence on CAF autophagy, and we focused our studies on understanding the mechanisms between these cell types rather than other microenvironmental components.



Intriguingly, the low density of cells plated in co-culture helps to elucidate potential drivers of autophagy. For example, HNSCC induced NF autophagy in neighboring cells, but physical contact was not observable between each of these cell types. This indicates a secreted factor within the media initiates autophagic flux rather than direct cell-to-cell contact. We maintained a constant number of cells between experimental groups, and there was an abundance of nutrients in the media. This, alongside the short 24 h timeframe at which these observations

were made, indicates autophagic induction in NFs is not likely a result of nutrient deprivation. These observations point towards a secreted factor responsible for autophagy induction.

In an effort to elucidate activated pathways in co-cultured NFs, we used a PCR microarray to compare NFs in a homogenous culture and NFs derived from a HNSCC-NF culture (Figure 5.2). SOX2 demonstrated the greatest change between co-cultured NFs and NFs alone, with a robust increase in SOX2 in co-cultured NFs. SOX2 is well-known for its involvement in stem-cell maintenance, and as one of the four Yamanaka transcription factors mediating induction of pluripotent stem cells from fibroblasts (Takahashi & Yamanaka). Intriguingly, in these induced pluripotent stem (iPS) cells, autophagy is required to induce pluripotency (S. Wang et al., 2013).



SOX2 mediates the transient induction of autophagy in iPS cells (S. Wang et al., 2013). Ectopic expression of SOX2 leads to a dramatic increase in autophagy in mouse embryonic fibroblasts (MEFs). This coincided with a marked decrease in mTOR mRNA expression, indicating SOX2 transcriptionally repressed mTOR. SOX2 mediated mTOR repression lead to increased autophagic flux, as mTOR inhibits autophagy (S. Wang et al., 2013). Of note, Atg mRNA expression levels did not change despite an enhanced autophagic flux in iPS cells. SOX2 induction may be key to autophagy induction in co-cultured NFs.

Induction of SOX2 may occur through a variety of different stimuli. This includes Wnt signaling, hedgehog signaling, and PI3K signaling, all of which lead to increased SOX2 levels (Weina & Utikal, 2014). Of particular interest is the involvement of FGFR2 in SOX2 induction (Mansukhani, Ambrosetti, Holmes, Cornivelli, & Basilico, 2005). Not only is FGFR2 robustly expressed in the fibroblast compartment of HNSCC, it is highly upregulated in our PCR based array of co-cultured NFs compared to NFs alone (Figure 5.2). We hypothesized FGFR signaling is responsible for the increase in SOX2 observed in co-cultured NFs.

Fibroblast growth factors are the ligand for FGFR2 and bFGF is highly expressed in the tumor microenvironment. Results from our lab indicate a role for bFGF in the induction of CAF proliferation and migration. This lead us to assess the role bFGF may have in activating fibroblasts. However, in regards to autophagy, bFGF reduces autophagy in other systems, such as myocardiocytes (Z.-G. Wang et al., 2015) and neuronal cells (H. Y. Zhang et al., 2013). Nevertheless, the strong induction of FGFR2 in our co-culture system, and the activation of CAFs by bFGF observed by other studies within our lab, led us to hypothesize bFGF induces SOX2 which leads to an activation of fibroblast autophagy.

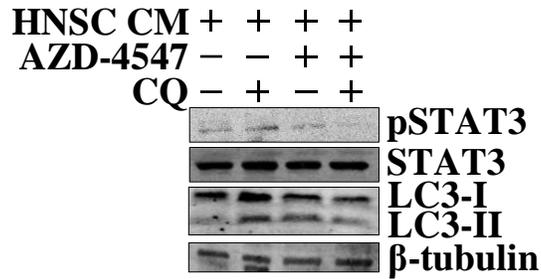


Figure 5.3 – Fibroblast STAT3 and LC3-II dependent on FGFR

Representative immunoblot of NF treated with HNSCC-conditioned media (CM), with and without FGFR inhibitor AZD4547 (2 μ M) for 24 h, and CQ (20 μ M for 6 h) of phospho-STAT3 and LC3.

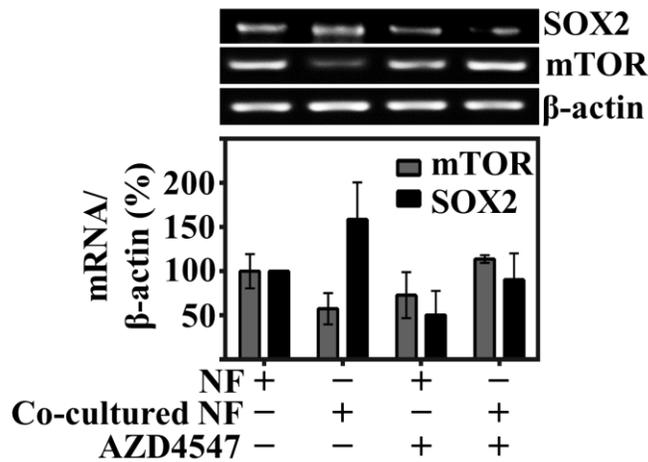


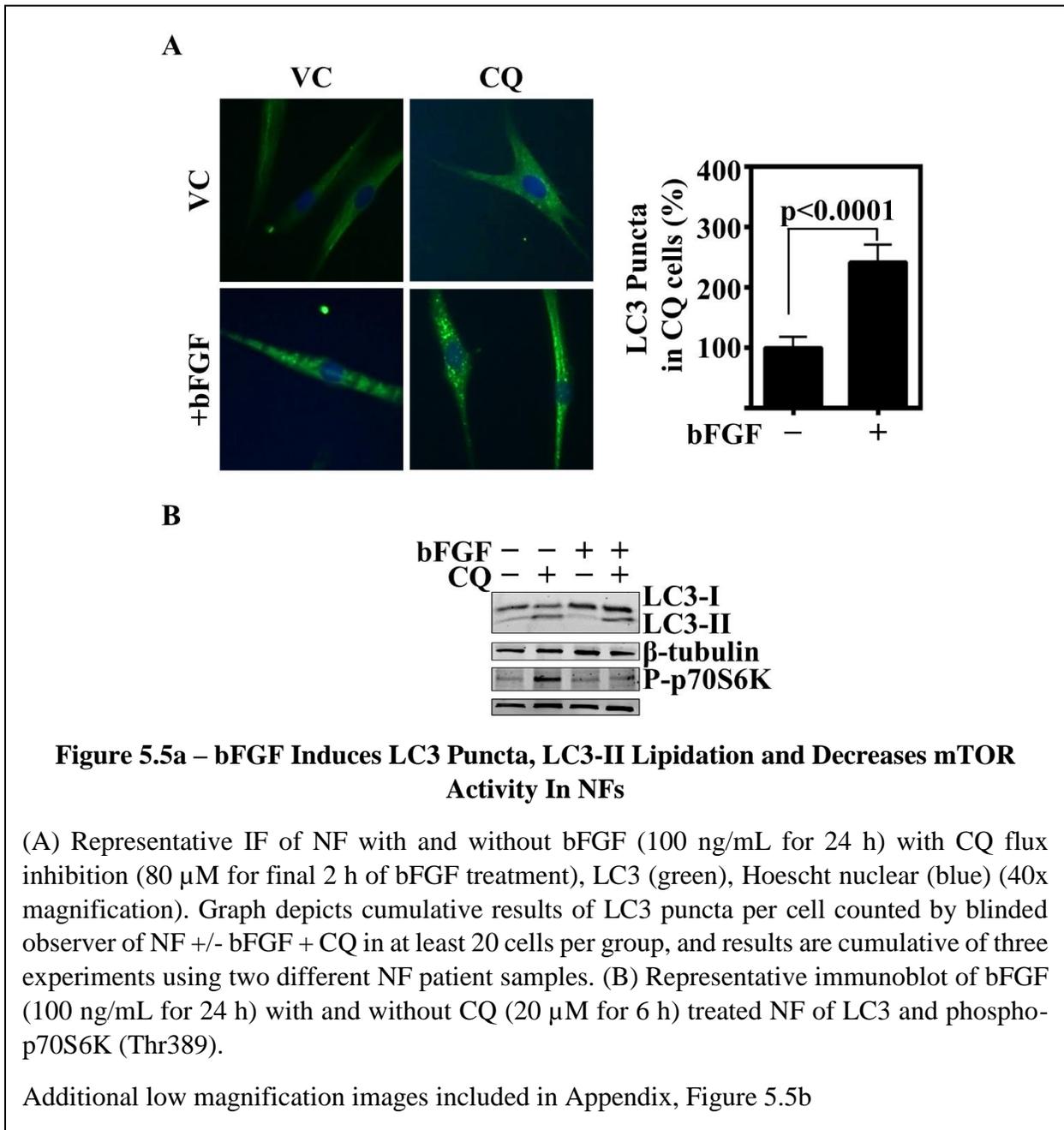
Figure 5.4 – HNSCC Induces SOX2 and Decreases mTOR in Fibroblasts through an FGFR Dependent Manner

Representative gel image of PCR products from NFs or NFs after sorting from co-culture with CFSE labeled HNSCC for 48 h with and without FGFR inhibitor AZD4547 (2 μ M). Graph depicts densitometric analyses of mTOR or SOX2 PCR product analyses relative to β -actin and normalized to NF alone; co-cultured NFs were sorted from CFSE labeled HNSCC (HN5) co-culture after 72 h with and without treatment using FGFR inhibitor AZD-4547 (2 μ M).

Therefore, we assessed a role for bFGF in inducing autophagy. bFGF serves as a ligand with high affinity for FGFR1 and FGFR2 (Ornitz & Itoh, 2015). AZD-4547 serves as a kinase inhibitor for these receptors (Gavine et al., 2012). Using HNSCC CM on NFs, AZD-4547 reduced levels of phosphorylated STAT3, a downstream mediator of FGFR2 signaling, (Figure 5.3). This reduction in STAT3 coincided with a decrease in LC3-II. This gives credence to the notion that FGFR mediates autophagy induction.

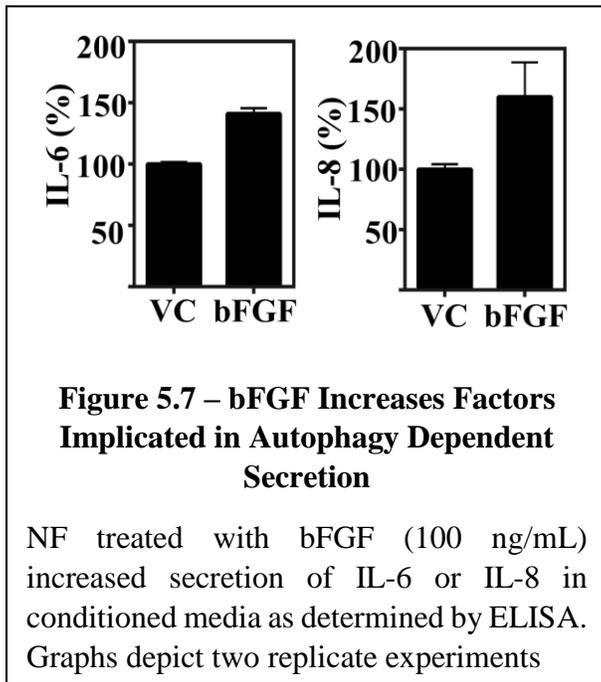
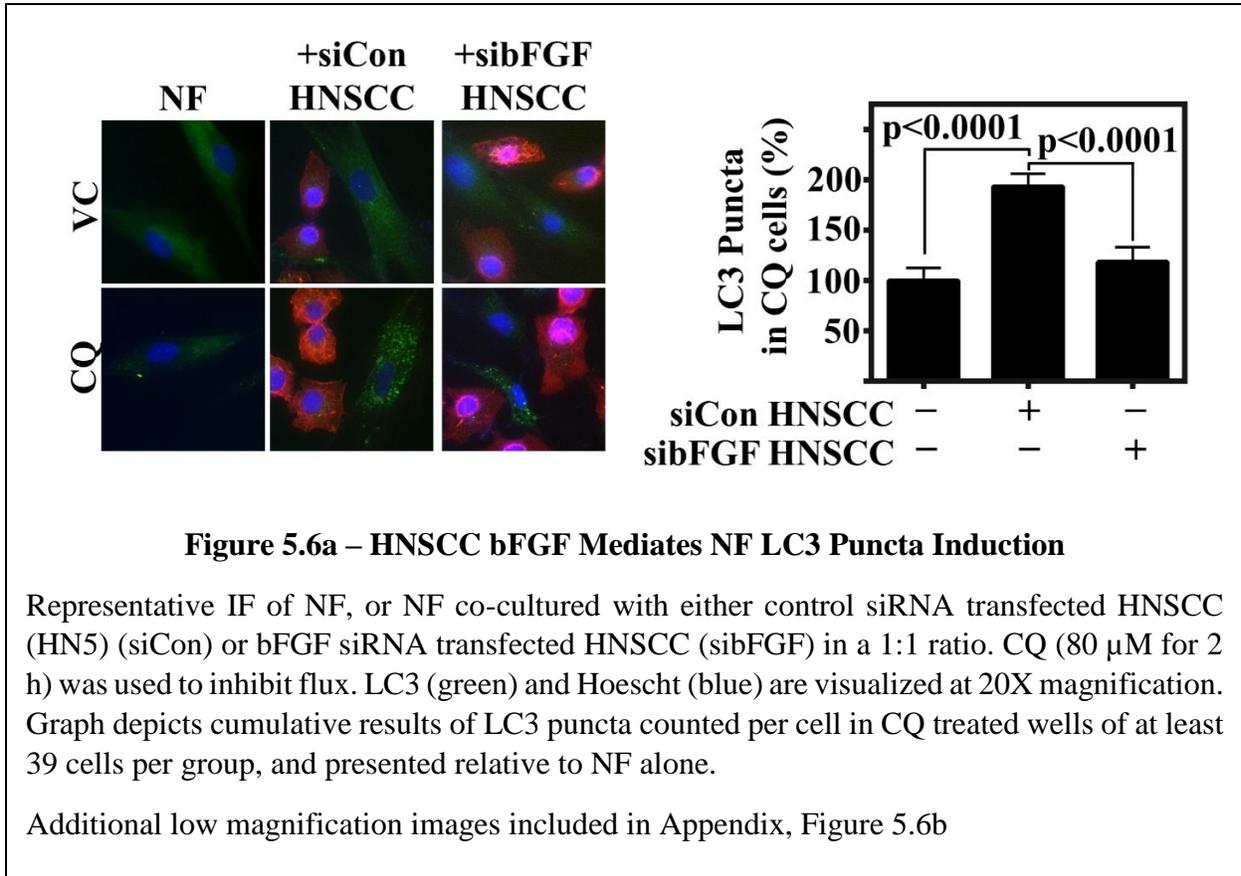
STAT3 bridges the connection between FGFR and SOX2. STAT3 directly binds to activated FGFR, and is phosphorylated to mediate its downstream effects (Dudka, Sweet, & Heath, 2010). STAT3 then directly binds the promoter of SOX2 to activate enhanced transcription (Zhao et al., 2015). HNSCC induced a marked increase in NF SOX2 levels (Figure 5.4). AZD-4547, a potent FGFR inhibitor, blocked the induction of SOX2. This implicates a role for FGFR in NF SOX2 induction by HNSCC.

SOX2 transcriptionally represses mTOR (S. Wang et al., 2013). This leads to an activation of autophagy. Supporting this concept, co-cultured NFs have repressed levels of mTOR mRNA (Figure 5.4). The suppression is relieved with AZD-4547. This indicates a role for FGFR in mediating both SOX2 overexpression and mTOR repression in the HNSCC-NF co-culture.



As the ligand for FGFR, bFGF is likely to induce the signaling cascade observed.

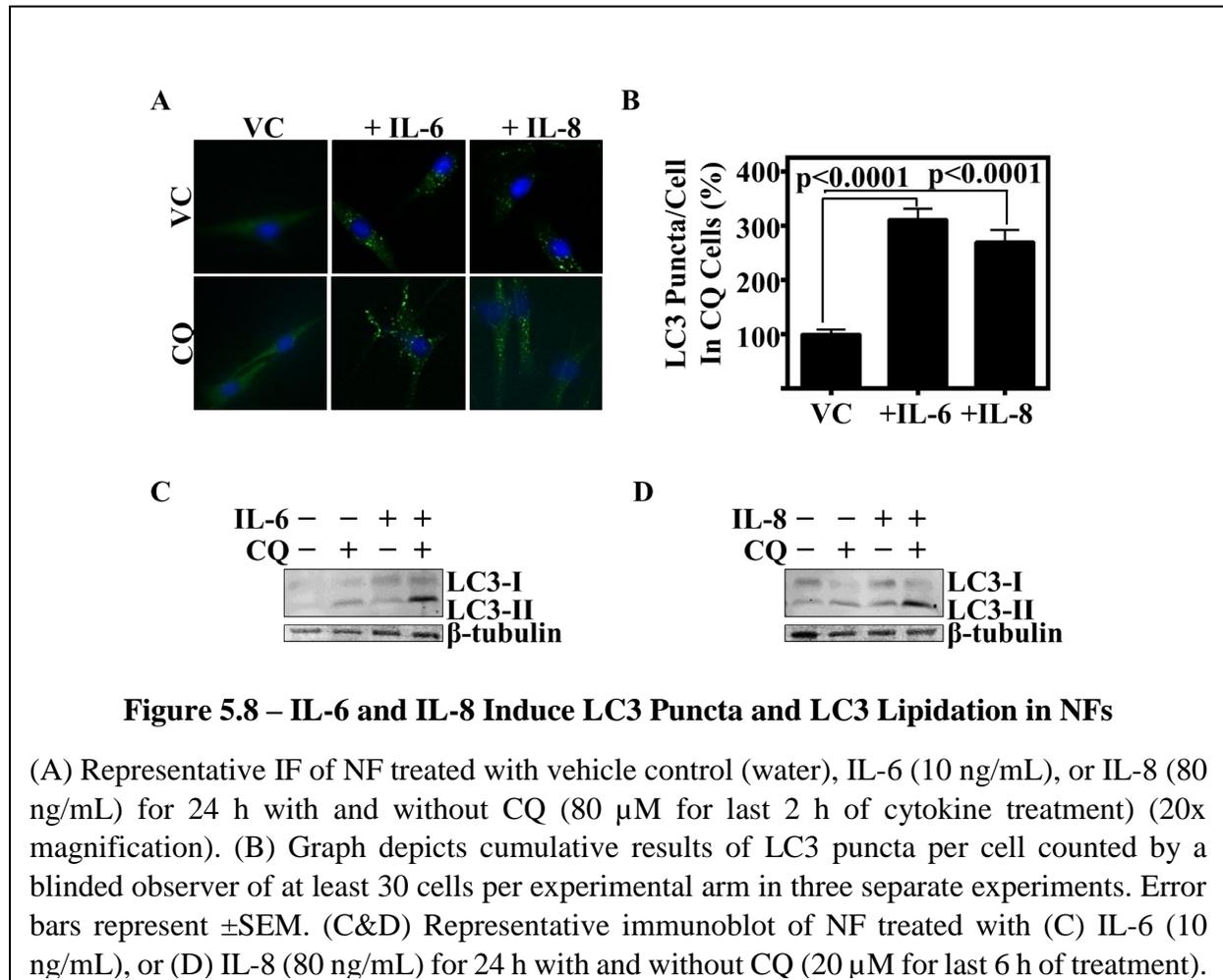
Exogenously added recombinant bFGF induces robust accumulation of autophagosomes in NFs



(Figure 5.5, Appendix Figure 5.5b). Additionally, recombinant bFGF induced an increase in LC3-II, and a decreased level of phosphorylated p70S6K, a surrogate marker for mTOR activity. This indicates bFGF diminishes mTOR function and increases NF autophagy. HNSCC secreted bFGF likely activates autophagic flux in surrounding fibroblasts. Thus, we silenced bFGF in HNSCC cells and then co-cultured with NFs. With bFGF silencing, HNSCC-mediated induction of LC3 puncta significantly reduced (Figure 5.6, Appendix Figure 5.6b). Thus, HNSCC secreted bFGF stimulates autophagic flux in NFs.

We previously demonstrated that autophagy-dependent secretion led to an increase in IL-6 and IL-8 from NFs. We hypothesized bFGF would increase secretion of IL-6 and IL-8 alongside its autophagy-inducing effects in these cells. Recombinant bFGF induced a marked increase in IL-6 and IL-8 (Figure 5.7). This demonstrates that FGF, alongside inducing autophagic flux, also leads to enhanced secretion of IL-6 and IL-8.

IL-6 has recently been implicated in autophagy induction of cancer cells in a mutant *Ras* *Drosophila* model (Katheder et al., 2017a). Additionally, IL-6 has been shown to enhance autophagy in prostate cancer cells. IL-6 and IL-8 are well understood to be in high levels within the HNSCC microenvironment (Curry et al., 2014). We hypothesized IL-6 and IL-8 may also induce autophagy in NFs in an autocrine manner. In a co-culture model, IL-6 and IL-8 neutralizing antibodies prevented HNSCC induction of LC3 puncta (Figure 5.8). Additionally, recombinant IL-6 and IL-8 induced significant accumulation of LC3 puncta in NFs. This was accompanied by significant LC3-II lipidation in the presence of autophagy flux inhibitor, CQ.

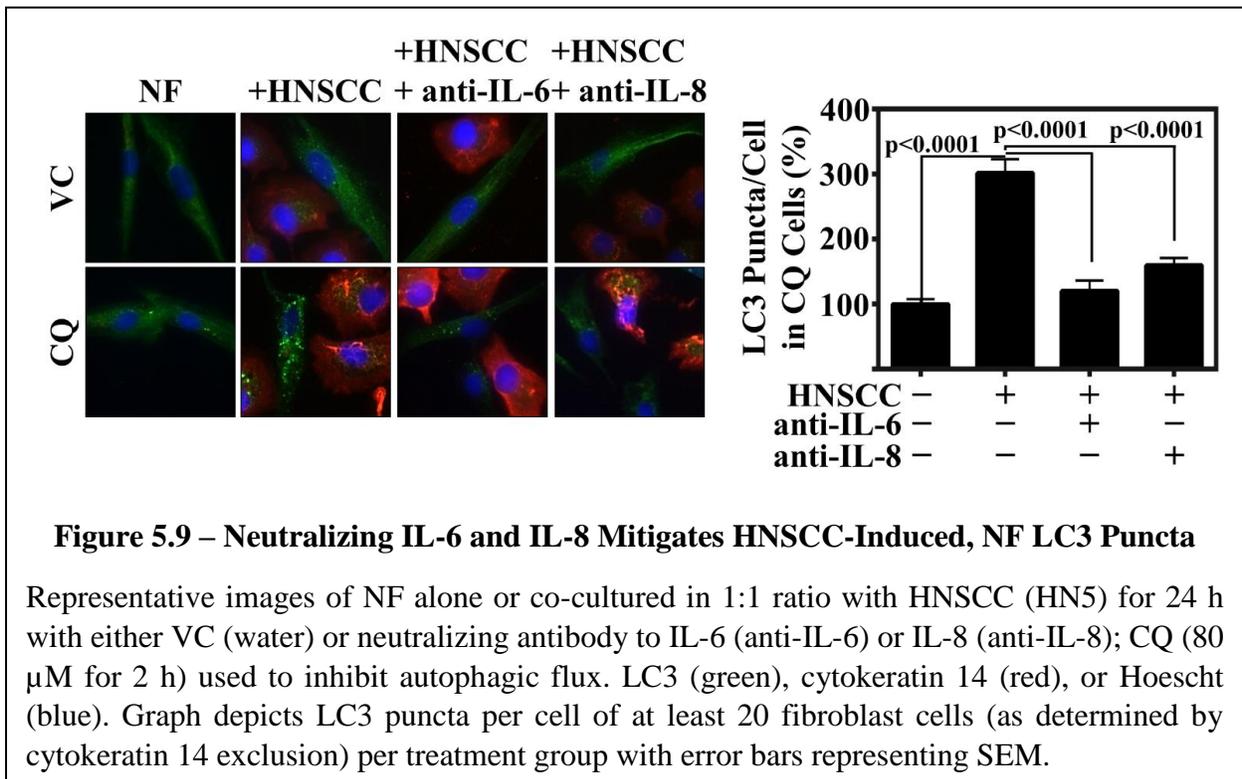


Neutralizing IL-6 and IL-8 mitigated HNSCC induced LC3 puncta in NF-HNSCC Co-culture

(Figure 5.9). Therefore, IL-6 and IL-8 induce a feed-forward mechanism to increase autophagy in CAFs.

These results indicate a mechanism for HNSCC induction of fibroblast autophagy dependent secretion. This occurs through bFGF activation of FGFR, which leads to phosphorylation of STAT3. STAT3 induces SOX2 levels, which act to transcriptionally repress mTOR. This repression is alleviated by a STAT3 inhibitor. mTOR inactivation leads to an increase in autophagic flux. This coincides with an increase in IL-6 and IL-8 secretion from fibroblasts. IL-6 and IL-8 feedback on the system to activate further, sustained autophagy in

fibroblasts. This reveals a targetable signaling loop of FGFR activation of CAFs, which leads to enhanced autophagy and secretion of IL-6 and IL-8 (Figure 5.10).



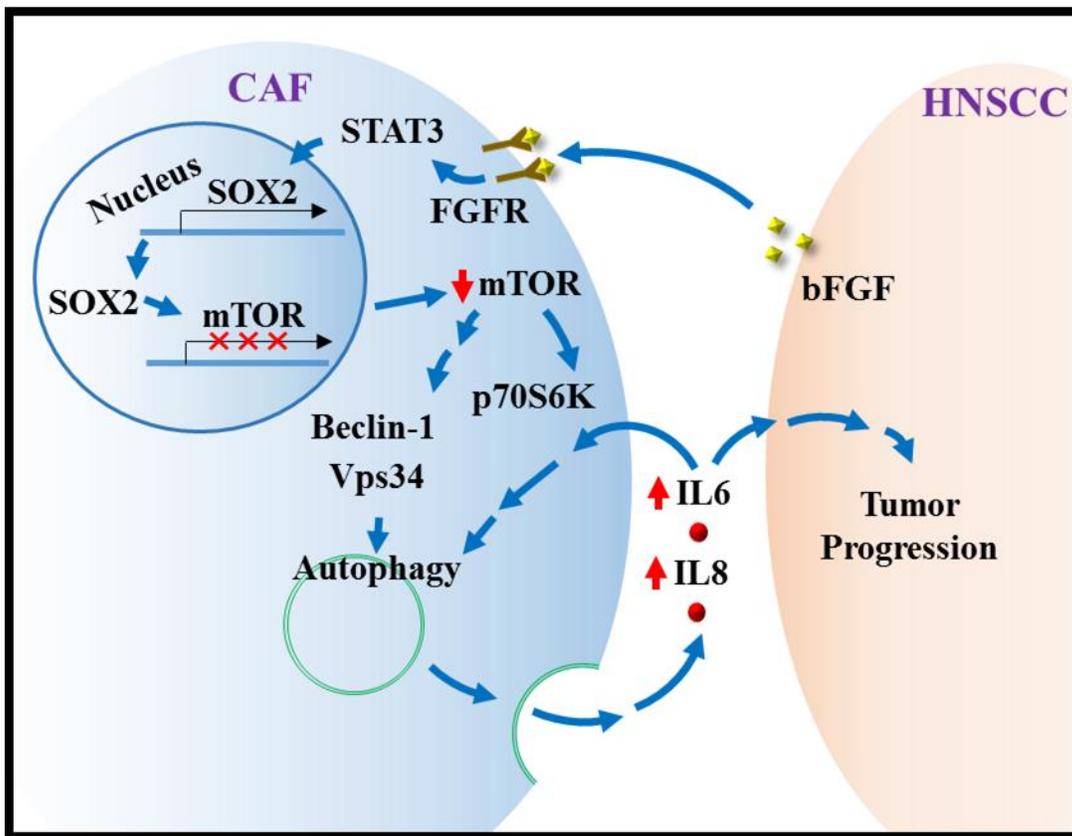


Figure 5.10 – CAF Autophagy Induction Mechanism

Schematic representation of the mechanism of autophagy induction in CAFs by HNSCC that facilitates HNSCC progression.

Discussion

CAFs significantly contribute to HNSCC disease progression. The molecular mechanisms regarding the cross-communication between CAFs and HNSCC cancer cells are largely unknown. Our results indicate HNSCC cancer cells induce CAF autophagy, which facilitates tumor-promoting secreted factors. This signaling loop will be useful in future therapeutic interventions for HNSCC, and also is likely applicable to other diseases with high levels of autophagy.

An important finding from these studies is the role of HNSCC in inducing autophagy. Although many diseases have either over-activated or diminished autophagy, the list of known autophagy inducers is relatively short. The current understanding revolves around cellular metabolic deficiency and ROS mediated damage. Our work characterizing a mechanism for mitogen activation of autophagy in microenvironment cells indicates future research opportunities to understand the role of secreted factors in autophagy induction. We observed bFGF, IL-6 and IL-8 all to have a pro-autophagic role within the fibroblasts. While the interest in the current work is to diminish autophagy to limit disease progression, other diseases might benefit from insights in autophagy induction. For example, neurodegenerative diseases such as Parkinson's and Alzheimer's disease are attributed to diminished autophagy. Perhaps the evidence supplied here might be useful for future studies in these and other diseases.

Additionally, the role for ROS and metabolic competition in HNSCC-mediated Activation of CAF autophagy is not discounted. Clearly, these stimuli are widely present within the HNSCC microenvironment. Cancer cells produce a large amount of ROS due to their damaged mitochondria (Liou & Storz, 2010). Additionally, the rapidly growing tumor creates a nutrient deprived microenvironment. Our results do not suggest that these factors are not present,

but rather adds additional detail to other microenvironmental stimuli involved in supporting autophagy-dependent secretion in CAFs.

Our results build upon recent evidence of SOX2 mediating an activation of autophagy (S. Wang et al., 2013). This occurred in MEFs, and was crucial step in the transition of MEFs to pluripotency. SOX2 is widely known as a stem-cell transcription factor. Surprisingly, autophagy is highly upregulated in stem-cells (Maycotte et al., 2015). Stem-cell autophagy upregulation is attributed to IL-6. The data presented support the concept of a transient stem-like phenotype in microenvironmental fibroblasts. Additional studies are needed to better define a stem-like phenotype and any associated functional consequences. However, CAFs do not seem to differentiate in culture. Patient-derived CAFs always maintain a fibroblast architecture.

The PCR based microarray identified other factors altered between NFs co-cultured with HNSCC and NFs alone. For example, PTCH1 was significantly upregulated. PTCH1 is a member of the hedgehog family. Histology demonstrates PTCH1 increases in HNSCC fibroblasts, whereas it is largely absent in normal tissue-associated fibroblasts (Ghosh et al., 2012). In regards to autophagy, hedgehog signaling impairs autophagy in *Drosophila* (Jimenez-Sanchez et al., 2012). However, despite an indication that PTCH1 is increased in our system, NFs clearly have increased autophagy in co-culture with HNSCC. In HNSCC, the role for PTCH1 in CAFs is unknown. Future studies might elucidate the importance of upregulation of this factor in surrounding fibroblasts. Additionally, MYCN, DKK1, and CD24 were significantly down-regulated in co-cultured fibroblasts. Each of these have been well-studied in regards to epithelial cells. However, no studies address the role for downregulation of these in HNSCC CAFs, or CAFs from other cancers. The PCR array provides many interesting findings for future studies.

The role of bFGF in stimulating autophagy and IL-6 and IL-8 secretion from NFs indicates a role in inhibiting this factor in HNSCC therapy. HNSCC have overexpressed and amplified FGFR (Daniele, Corral, Molife, & de Bono, 2012). Previously, a role for FGFR in autocrine signaling has been defined, but our results build on this to indicate an important role of FGF in the stroma. The pronounced results of FGFR signaling in cancer progression led to a number of current therapeutic trials in HNSCC and other solid tumors (Porta et al., 2017). Although these trials currently do not investigate autophagy, it will be quite interesting to assess autophagy modulation in the presence of FGFR inhibition in patients.

Sustained activation of autophagy in cultured CAFs is of interest for future studies. CAFs demonstrate high basal autophagy despite being cultured in the absence of cancer cells for 10-12 passages. This indicates a long-term, inheritable modification occurs. Perhaps the autocrine secretion of IL-6 and IL-8 are in a concentration adequate to sustain autophagy in culture. Additionally, evidence in other cell types indicates IL-6 can induce epigenetic modification by changing the localization of DNA methyltransferases (Hodge et al., 2007). However, our studies did not investigate epigenetic or genetic modifications in CAFs compared to NFs. Future studies should also investigate the effects of CAF-CM on NFs to see if HNSCC are essential. It is possible that autophagy induction could occur in an HNSCC-independent manner.

Also of interest is the role of autophagy in bFGF secretion. bFGF is an unconventionally secreted peptide, without a secretion signal sequence. Evidence suggests it passes through the cell membrane, but a role for autophagy-dependent secretion has not been assessed. In our RNAseq analysis, we observed a significant reduction of bFGF mRNA following Beclin-1 silencing. Perhaps, bFGF is modulated in an autophagy-dependent manner.

Overall, these results shed light on the activation of CAFs in the tumor microenvironment. bFGF initiates a signaling cascade, which leads to decreased transcription of mTOR. This repression of mTOR removes the molecular brakes on autophagy and stimulates an autophagy-dependent secretome. These results demonstrate the important role of HNSCC in modulating the tumor microenvironment. IL-6 and IL-8 also demonstrate the role CAFs have in maintaining their own activation.

**Chapter VI - Autophagy inhibition potentiates standard-of-care therapy
in HNSCC**

Rationale

Autophagy has a complex role in cancer (White & DiPaola, 2009). In some cases, autophagy protects against tumors. For example, Beclin-1 heterozygosity leads to an increase in spontaneous tumors, particularly lung cancer, liver cancers, and lymphomas (Qu et al., 2003). Additionally, Atg5 and Atg7 deficiencies lead to increased liver tumors (Takamura et al., 2011). Thus, autophagy acts to prevent tumorigenesis. However, autophagy also promotes tumor progression in late stages tumors. Metabolic stress within the tumor would typically lead to apoptosis of the cells. However, autophagy acts to promote cell survival, and restricts cell-death. Targeting autophagy in this scenario would promote cell death. These dichotomous interactions of autophagy-on one hand preventing tumor progression, while on the other facilitating tumor survival-makes dissecting the interactions of autophagy in cancer quite perplexing.

Our results indicate microenvironment autophagy contributes to HNSCC progression. The data previously demonstrated support a role for autophagy-dependent secretion in the tumor microenvironment. CAFs have significantly more autophagy than NFs, and this increase in autophagy coincides with the secretion of factors important for tumor progression. Other reports in different cancers support the idea of targeting an autophagic microenvironment (Katheder et al., 2017a). This gives credence to inhibiting autophagy in late stage tumors with a high degree of activated CAFs.

Besides a role for autophagy in the microenvironment, HNSCC has increased autophagy within the cancer cells. Our data indicate a role for autophagy in the cancer cells themselves. For example, HNSCC widely expresses LC3 puncta in our IF studies of HNSCC compared to normal tonsil histology. Additionally, cell lines of HNSCC robustly express LC3 puncta. Other groups confirm the increase of autophagy in HNSCC. This coincides with observations that patients

with high levels of LC3 puncta and p62 associate with a poor prognosis in oral cancer (J. L. Liu et al., 2014). Increased LC3 puncta associating with worse prognosis is documented in other HNSCC sub sites (J. Y. Tang et al., 2013). Despite these findings, investigations are lacking as to the therapeutic potential of autophagy inhibition in HNSCC.

Other solid tumors upregulate autophagy (White & DiPaola, 2009). In these tumors, targeting autophagy appears to have a therapeutic potential. This was first demonstrated with autophagy inhibition using CQ in a murine lymphoma model. Mice given 60 mg/kg qd of CQ alone had significantly less tumor growth (Amaravadi et al., 2007). This provides evidence for autophagy as a single therapeutic. Current paradigms suggest cancer cells upregulate autophagy as a mechanism to degrade toxic products that develop within the damaged cellular system. This would include damaged organelles, missense proteins, and to control for immune system attacks, such as the destruction of granzyme through autophagy. As such, combination therapy of inhibiting two degradative systems within the cell, such as the lysosome and proteasome, would produce pronounced effects. Demonstrations in colon cancer highlight the potential for dual proteasome-autophagy inhibition (Ding et al., 2009). When misfolded protein degradation is inhibited, rapid cell death initiates. This demonstrates cancer cells require a high basal level of autophagy for survival.

Many current therapeutics also induce autophagy. This is true of nearly all cytotoxic drugs where cancerous cells upregulate autophagy as a survival mechanism (Shen et al., 2011). Therefore, combination therapy targeting autophagy alongside current therapy demonstrates effectiveness in a variety of tumors. For example, autophagy inhibition using chloroquine potentiates the effects of 5-FU in colon cancer models (Sasaki et al., 2010). Also, autophagy inhibition improves cisplatin cytotoxicity in cervical cancer cells, another tumor with strong

correlation to HPV status (Xu et al., 2012). These studies demonstrate targeting autophagy alongside current therapeutics used for HNSCC would likely potentiate effects.

Chloroquine (CQ) has been widely used to target autophagy. CQ has been prescribed for the treatment of malaria and rheumatoid arthritis for many years. It has a well accepted safety profile, and can be taken for many years when living in an endemic area of malaria, or with the chronic condition of rheumatoid arthritis. However, retinal toxicity occurs after taking CQ for 10 years. This led to the development of hydroxychloroquine (HCQ), which has the same chemical structure but with the addition of just one hydroxyl group. HCQ and CQ have the same mechanism, but HCQ does not have retinal toxicity. To substantiate the broad safety profile, suicide attempts with hydroxychloroquine are ineffective (Gunja et al., 2009). This indicates that large doses of 20 g can be achieved, and have few adverse effects (Gunja et al., 2009). A phase I dose-escalation study in dogs demonstrates safety of combining HCQ with chemotherapy and provided evidence that this treatment had potential benefit with a 93% overall response rate. These studies demonstrate the relative safety of CQ administration, and potential utility in cancer therapy.

With a well-tolerated drug, and multiple cancers harboring high levels of autophagy, clinical trials assessing autophagy inhibition in multiple cancers have begun. The first clinical trial assessed the role of CQ in glioblastoma (Briceno, Reyes, & Sotelo, 2003). In this study, the median survival difference was 33 months in the combined CQ/standard therapy group compared to 11 months in the standard therapy alone group. Although this study was small, with only 18 patients, the robust response cannot be overlooked. This led to the need to further assess autophagy inhibition in the clinic.

Current published studies have been primarily dose finding in nature. As such, these studies have been relatively small, with a focus on biomarkers useful for determining doses of autophagy needed to have an effect. The most investigated peripheral marker is peripheral blood mononuclear cells. Adequate levels of HCQ result in buildup of autophagic vacuoles within these cells. At low doses-600 mg qd-no effect on peripheral blood mononuclear cells is observed (Mahalingam et al., 2014). At high doses, such as 1200 mg qd, peripheral blood cells demonstrate a significant increase in autophagic vacuoles (Rangwala et al., 2014). This high dose associates with a strong antitumor response in melanoma patients (Rangwala et al., 2014). In these trials, direct assessment of autophagy inhibition within the tumor did not occur, leaving an open question as to if intratumoral doses reached an adequate level. Of note, in the dog study, HCQ reached adequate concentrations to inhibit autophagy intratumorally; yet, a 100-fold difference in HCQ levels intratumorally was observed compared to plasma levels (Barnard et al., 2014). These preliminary studies indicate that the HCQ maximum tolerated dose (MTD) varied with the combined therapy. For example, Vorinostat, a histone deacetylase inhibitor, had a MTD of 400 mg qd (Mahalingam et al., 2014); whereas, the MTD with an mTOR inhibitor reached 600 mg bd (Rangwala et al., 2014). Dose limiting toxicities in these trials were primarily fatigue and gastrointestinal toxicity.

With an understanding of tolerated doses, additional trials investigated the antitumor utility of autophagy inhibition using CQ. Despite the pronounced effects observed in early glioblastoma trials, not all trials demonstrate pronounced results. In a glioblastoma phase II trial with more patients (n=76), and CQ delivered at 600 mg qd, no significant differences were observed in treatment groups (Rosenfeld et al., 2014). However, myelosuppression limited increased doses; and at the dose selected, autophagy inhibition was not consistently achieved

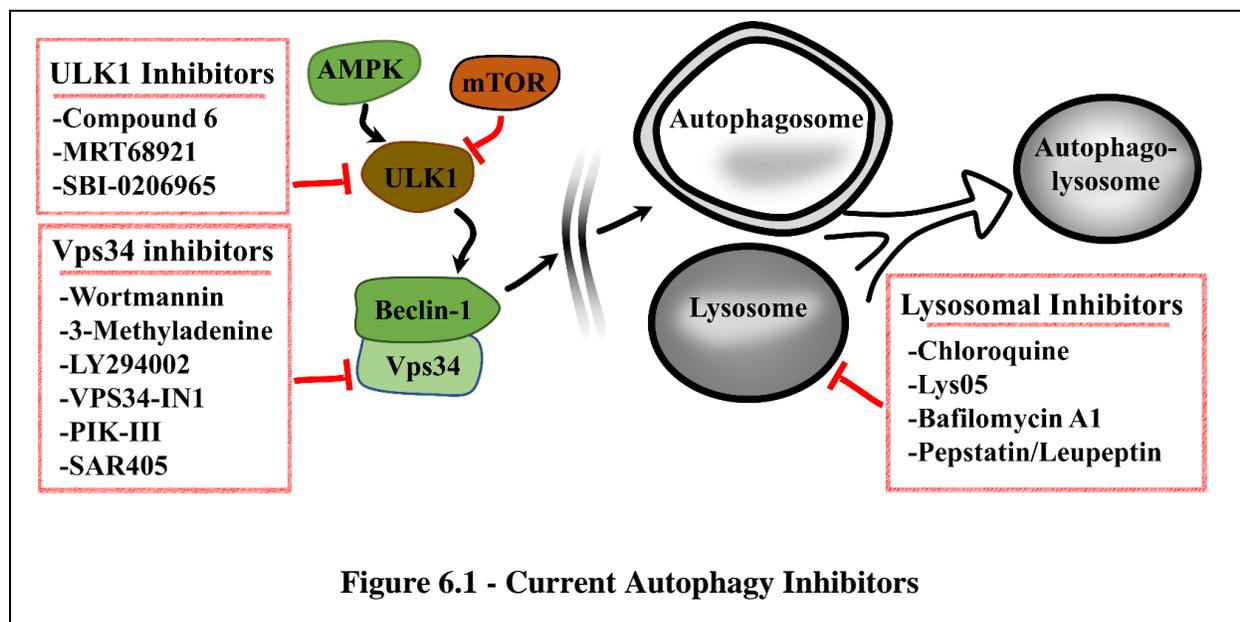
(Rosenfeld et al., 2014). In patients with metastatic pancreatic cancer, HCQ as a single therapy at either 400 or 600 mg qd did not have any significant improvement (Wolpin et al., 2014).

However, also in this trial, patient peripheral lymphocytes had inconsistent inhibition of autophagy (Wolpin et al., 2014). Of patients who demonstrated a greater than 51% increase in LC3-II following treatment, there was a significant difference in overall survival in pancreatic adenocarcinoma (Boone et al., 2015). Although these results suppress the enthusiasm of autophagy inhibition, they also indicate future studies will need greater dosing of HCQ to inhibit autophagy fully.

With this background, 51 studies are currently ongoing to investigate autophagy inhibition for cancer patients (Levy, Towers, & Thorburn, 2017). Yet, all of these studies are focused on CQ or HCQ as methods of inhibiting autophagy. The preclinical and clinical trials aforementioned indicate a response should be expected, albeit limited by the high required doses of CQ needed. There is a need to better delineate autophagy inhibition as a therapy by using new therapeutics.

CQ's mechanism of action limits effectiveness. CQ is a lysosomotropic agent, permeating the lysosome and then neutralizing the acidity. As a base, the process of protonation within the lysosome traps CQ within this organelle. Yet, this mechanism also means that in other acidic environments, like the tumor microenvironment, the acidic conditions limit the permeability of CQ. Additionally, CQ affects other cellular processes that have acidic conditions (Pellegrini et al., 2014). For example, endosomes acidify during intracellular transport. Similarly, the Golgi apparatus has acidic components, as do secretory vesicles, and perhaps even secretory lysosomes (Richard G.W. Anderson, 1988). Although CQ offers a well-known drug that targets

autophagy with an acceptable safety profile, off-target, non-specific effects indicate that claiming CQ as solely an autophagy inhibitor is inaccurate.



Other autophagy inhibitors suffer from this low degree of selectivity. Autophagy inhibitors cluster into two broad categories: proximal inhibitors which target upstream events involved in producing the core autophagic machinery, and late stage inhibitors which target the lysosome (Figure 6.1). Proximal inhibitors target the upstream kinases, ULK1 and Vps34. As Vps34 is a PI3K, most proximal inhibitors are PI3K inhibitors, but have many non-targeted effects as such. 3-methyladenine (3-MA), Wortmannin, and LY294002 compose the three primarily used inhibitors. 3-MA has the dual role in both suppressing autophagy in nutrient starvation conditions, as well as activating autophagy by suppressing Class I PI3K (Y. T. Wu et al., 2010). A high dose is required to obtain physiologic affects, upwards of 10 mM in *in vitro* assays (Y. Wu et al., 2013). This is great enough to affect other kinases, such as MAPK and c-Jun. Wortmannin and LY294002 also suffer from targeting other PI3Ks (Sarkaria et al., 1998) (Vlahos, Matter, Hui, & Brown, 1994). Of these three, only wortmannin has a more specific

IC50 for Vps34 than other PI3K (Benoit Pasquier, 2016). The lack of specificity of these kinase inhibitors has spurred investigation to better targeted agents.

Vps34 is an attractive target for selective autophagy inhibition. Vps34 is necessary for autophagy to occur (Kihara et al., 2001). The only known Vps34 non-autophagy related function is the vesicular transport of endosomal vesicles (E. E. Johnson, Overmeyer, Gunning, & Maltese, 2006). Binding partners of Vps34 seem to dictate its function towards autophagic or endosomal pathways. When Vps34 complexes with UVRAG, it functions as an endosomal mediator; but when it complexes with Beclin-1 and Vps15, it participates in the development of the autophagosome (Vanhaesebroeck, Guillermet-Guibert, Graupera, & Bilanges, 2010). Thus, investigations have sought to inhibit this target

Two primary methods exist to modulate Vps34 activity: 1) hasten the degradation of this kinase, 2) inhibit the catalytic activity. Spautin-1 is the first Vps34 inhibitor, and acts to hasten the degradation. Derived from a parent compound targeted at phosphodiesterase 5 (PDE5) identified in an anti-autophagic screen, spautin-1 was designed to have decreased activity against PDE5, and enhanced anti-autophagic activity (Takase et al., 1994) (L. Zhang et al., 2007). It inhibits USP10 and USP13, two enzymes that deubiquitinate the Beclin-1 complex which leads to the degradation and depletion of autophagy (J. Liu et al., 2011). However, this is an indirect method of inhibiting autophagy, and likely, other functional consequences of inhibiting deubiquitinases exist. Two groups, Sanofi and Novartis, have recently developed inhibitors targeting the catalytic domain of Vps34. Novartis designed VPS34-IN1 and PIK-III, which have biochemical IC50s of 25 nM and 18 nM, respectively, and are highly selective for Vps34 compared to other protein and lipid kinases (Bago et al., 2014; Dowdle et al., 2014). VPS34-IN1 does not have any data concerning its effects on autophagic flux, but PIK-III demonstrated

strong inhibition of autophagic flux at 10 μ M. Sanofi designed SAR405, which is potent and selective for Vps34. No other kinases were affected at 1 μ M, and at the concentration, SAR405 demonstrated excellent inhibition of autophagic flux (Ronan et al., 2014). This lasted even during autophagy induction by mTOR inhibition and starvation (Ronan et al., 2014). After designing SAR405, other compounds were investigated to improve *in vivo* application. Compound 31 demonstrated inhibition of Vps34 *in vivo*, but no autophagic flux inhibition has been demonstrated (B. Pasquier et al., 2015). Thus far, SAR405 is the most potent inhibitor of autophagic flux available, and is the most specific for the autophagy pathway.

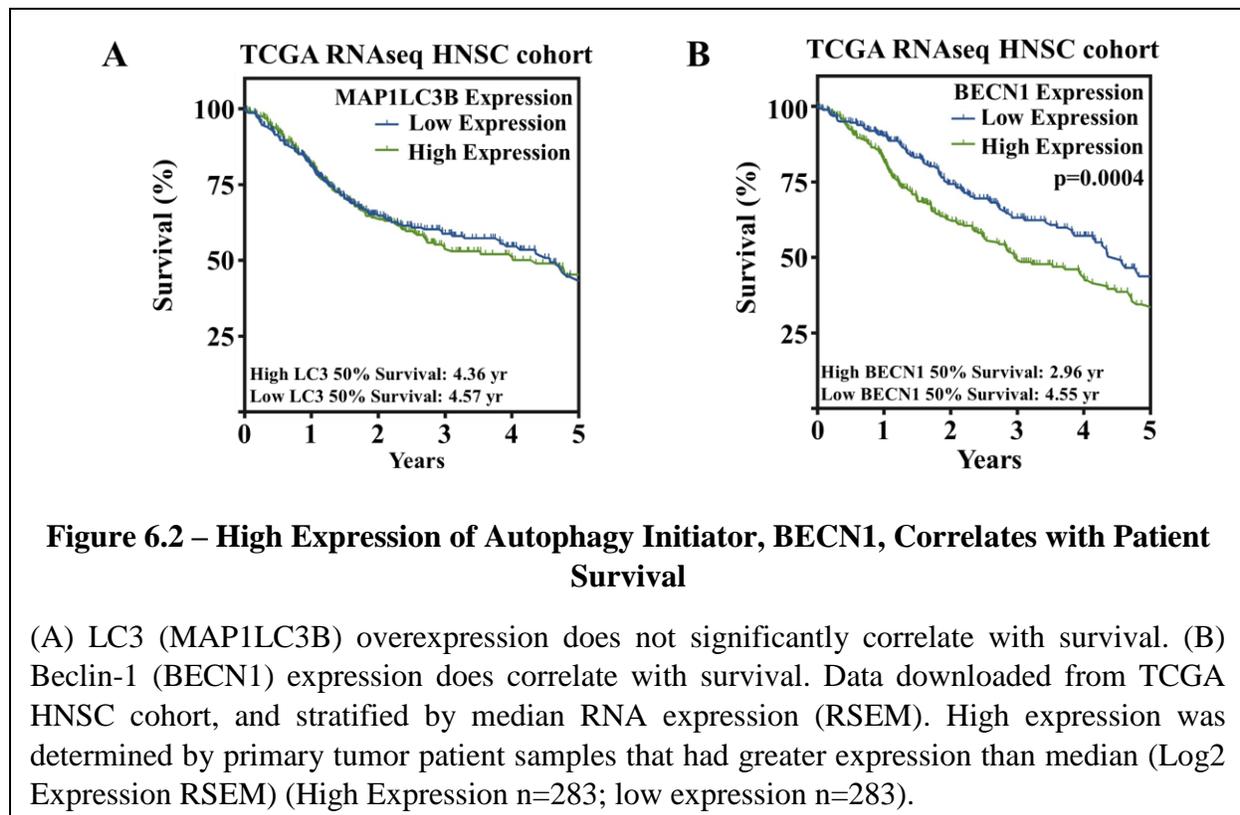
A thorough discussion would be remiss without mentioning ULK1 and late-stage inhibitors. SiRNA screening demonstrates the important role of ULK in mediating autophagy (Chan, Kir, & Tooze, 2007). Three ULK inhibitors exist, Compound 6, MRT68921, and SBI-0206965. However, these either have poor selectivity for ULK1 or have not been assessed in terms of autophagic flux inhibition (Petherick et al., 2015) (Egan et al., 2015). ULK inhibitors are newly made, and poorly selective. However, they may show promise in the future. Yet, current applications are limited by their off-target effects. Late inhibitors of autophagy act upon the lysosomal enzymes to prevent autophagosome degradation (Muller, Denmark, & Reinheckel, 2012). Pepstatin A and E64D act directly on lysosomal cathepsins. Other inhibitors target the acidic pH required for lysosomal enzyme function. Bafilomycin A1 blocks lysosomal proton transport, and therefore inhibits autophagic flux (Klionsky, Elazar, Seglen, & Rubinsztein, 2008). Additionally, the CQ derivative-Lys05 has recently been developed to have a 10-fold greater potency than CQ at raising the lysosomal pH, and demonstrated significant accumulation within tumors (McAfee et al., 2012). However, non-autophagic functions of the

lysosome are still attenuated by Lys05. These ULK1 inhibitors and late stage inhibitors offer a foundation for future drug design, but at the current stage are not specific enough.

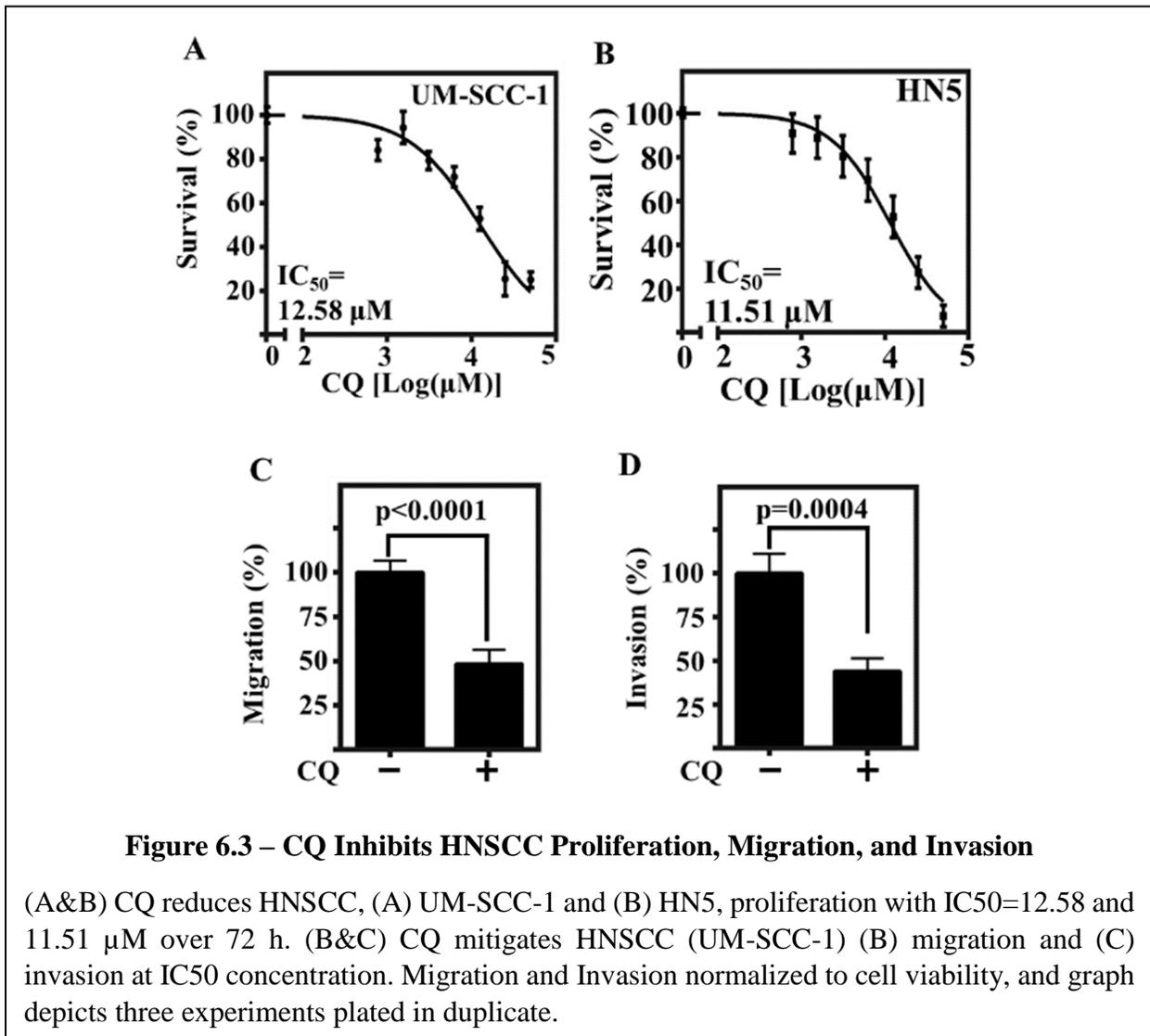
With this in mind, autophagy inhibition as a therapy has not been investigated in HNSCC. Thus, there is clinical utility in understanding the role of the currently available therapeutic, CQ, as a therapy. Additionally, there are no reports of the potent and selective inhibitor of Vps34, SAR405, as a cancer therapeutic in any cancer types. Thus, we undertook investigation to assess the role of autophagy inhibition using both CQ and SAR405 in HNSCC as single agents and in combination with current therapy.

Results

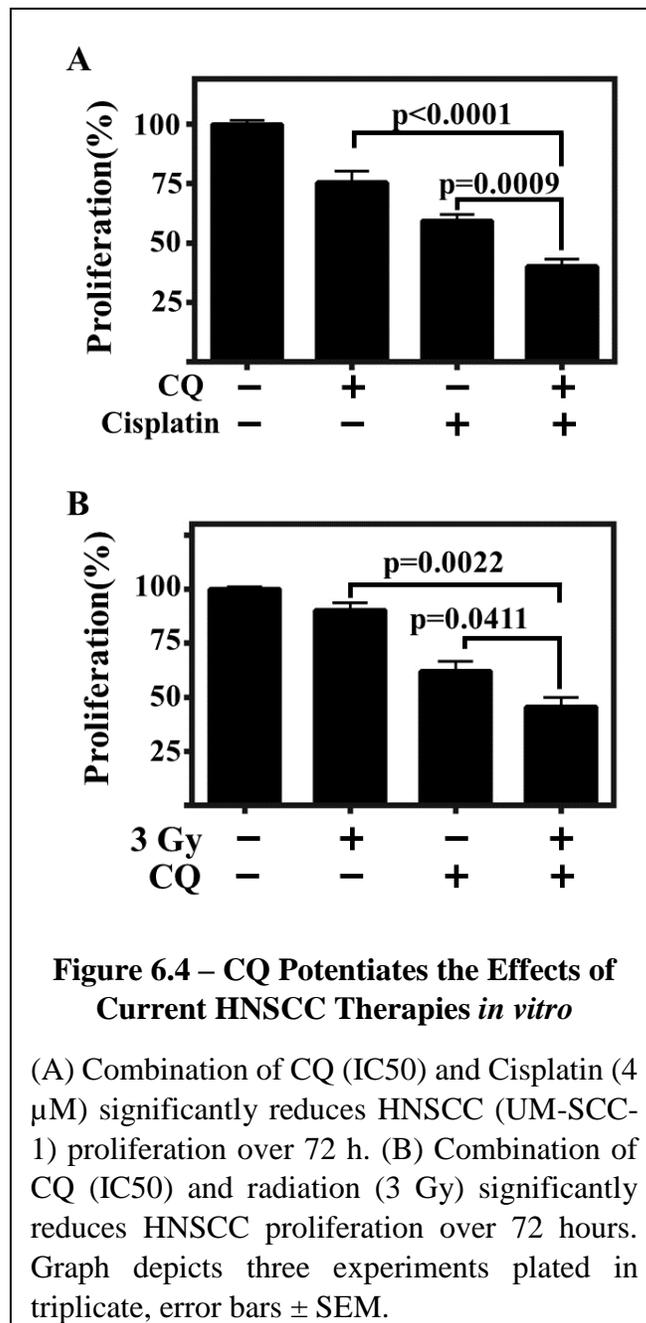
Autophagy associates with poor clinical outcomes in HNSCC. Increased LC3 puncta associates with a poor prognosis, and multiple groups confirm this (J. L. Liu et al., 2014) (J. Y. Tang et al., 2013). However, LC3 studies have consisted of relatively small sample sizes. To



build on this, we assessed the TCGA database for autophagy associated transcripts in the HNSC RNAseq database. Surprisingly, MAP1LC3B, the gene encoding LC3, did not associate with survival. Increased transcripts of LC3 does not by itself increase autophagic flux (Figure 6.2). However, Beclin-1 expression is analogous to autophagic flux (X. H. Liang et al., 1999). In the TCGA cohort, patients with increased Beclin-1 had a significantly worse overall survival (Figure 6.2). This, alongside other reports, indicates the therapeutic potential of targeting autophagy in HNSCC.



With a clear indication for a role of autophagy in HNSCC, we sought to assess the therapeutic potential of autophagy inhibition using CQ. CQ inhibits HNSCC proliferation, albeit with a relatively high IC₅₀ of approximately 12 μM (Figure 6.3). Inhibiting HNSCC with CQ also inhibited HNSCC migration and invasion (Figure 6.3). Therefore, as a stand-alone therapy,



CQ demonstrates potential at limiting HNSCC progression, but reaching effective doses might limit clinical use.

The promising results of CQ used with cytotoxic therapies in other cancers prompted us to investigate the role of CQ in combination with HNSCC current therapies. Cisplatin supplies the therapeutic backbone of HNSCC chemotherapy. By combining IC_{50} doses of cisplatin and CQ, a potentiated antitumor response occurred, greater than either single agent did (Figure 6.4). Radiotherapy also serves as a major therapeutic modality for advanced disease. In combination with 3 Gy of radiation, CQ potentiated the effects of radiotherapy (Figure 6.4). These preclinical data suggest the therapeutic potential of autophagy inhibition in combination with current treatment regimens.

Our previous results indicated a role for autophagy both in the tumor and in the CAFs. To better delineate the effects of autophagy inhibition in the tumor cells compared to autophagy inhibition in CAFs, we established a co-culture assay of both cell types by labeling HNSCC cells with CFSE. Following a 72 h co-culture, HNSCC proliferation was determined by flow cytometry of labeled cells. This allowed for individual assessment of CQ treatment, and/or Beclin-1 knockdown in CAFs. As observed with our previous proliferation study, CQ treatment of cancer cells alone significantly reduced HNSCC proliferation (Figure 6.5). Addition of CAFs significantly increased HNSCC proliferation, which was ameliorated by both knockdown of Beclin-1 in CAFs and the use of CQ. This further established the role of CAF autophagy in promoting HNSCC proliferation, which can be therapeutically mitigated.

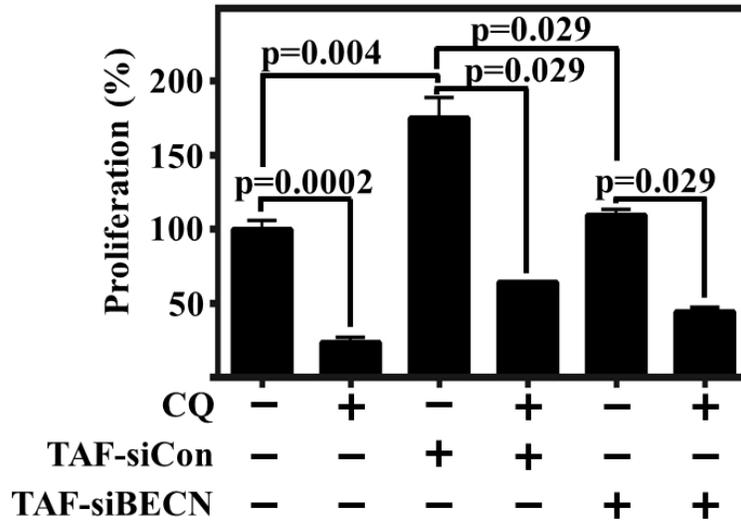


Figure 6.5 – Autophagy Inhibition Reduces CAF-Induced HNSCC Proliferation

CFSE labeled HNSCC (HN5) proliferation over 72 h co-cultured in 1:1 ratio with CAFs with and without pre-treatment of Beclin-1 siRNA knockdown, and with and without CQ (IC50) throughout 72-hour co-culture. Graph depicts results of two separate experiments using two different CAF patient samples, plated in duplicate. Error bars represent SEM.

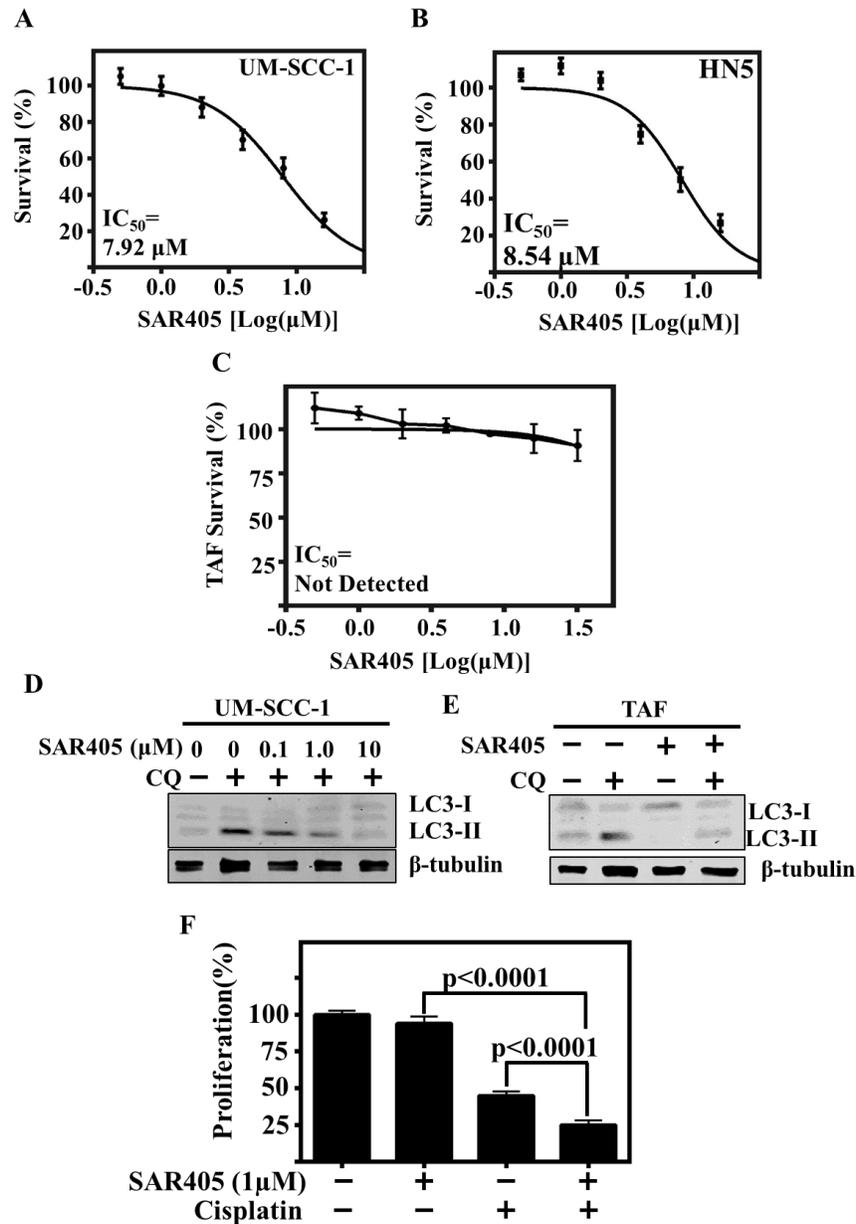
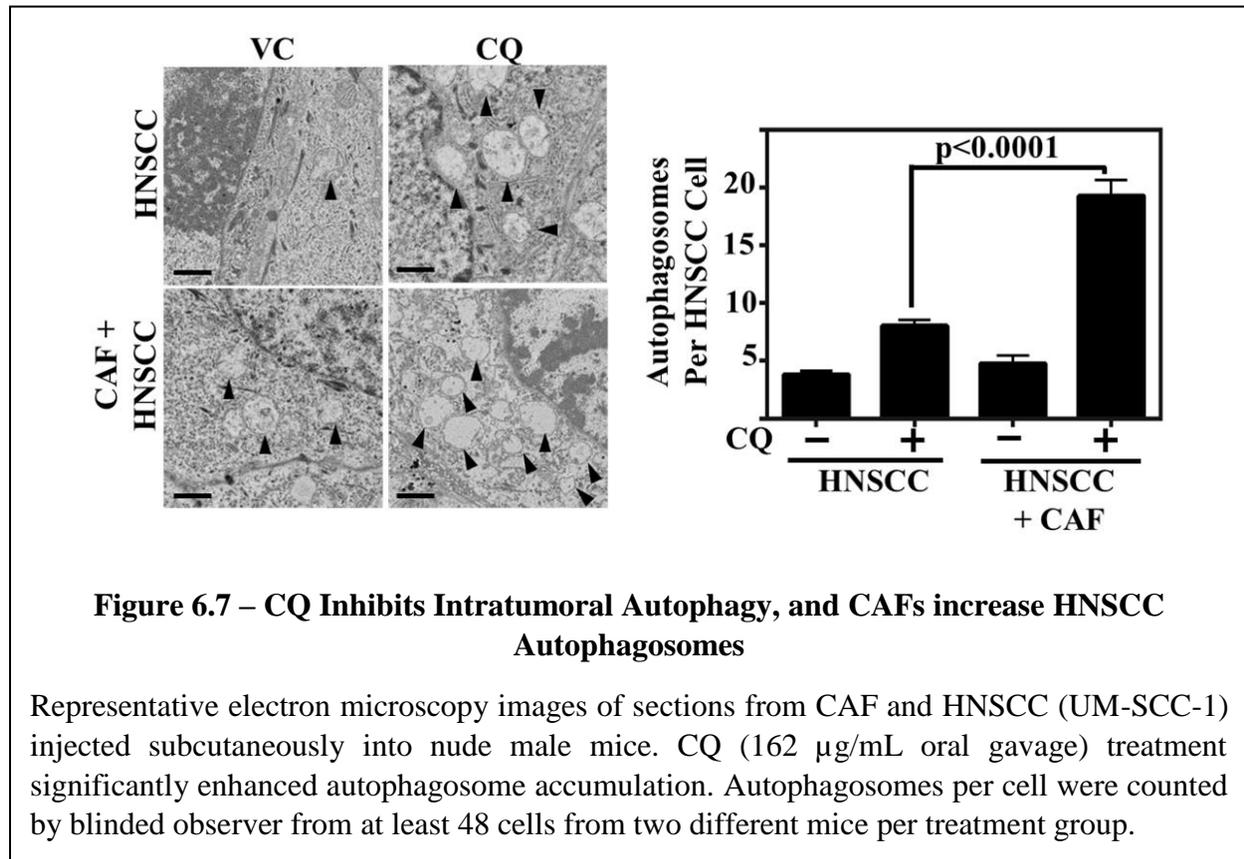


Figure 6.6 – SAR405 Inhibits HNSCC Autophagy, Proliferation, and Potentiates Cisplatin

(A&B) SAR405 reduces HNSCC, (A) UM-SCC-1, (B) HN5, proliferation with IC_{50} =7.92 and 8.54 μ M over 72 h, graph depicts three experiments plated in triplicate. (C) CAF proliferation is not effected by SAR405 inhibition over 72 hour period. (D) Representative immunoblot of increasing doses of SAR405 with and without CQ flux inhibition (20 μ M for 6 h). Experiment repeated twice. (E) Representative immunoblot of 1.0 μ M SAR405 on CAF with and without CQ flux inhibition (20 μ M for 6 h). Experiment repeated twice. (F) SAR405 (1 μ M) potentiates the effects of cisplatin (4 μ M) to inhibit HNSCC (HN5) proliferation over 72 hours, graph depicts three independent experiments plated in triplicate. Error bars represent SEM.

With these studies, promising *in vitro* evidence suggests a role for CQ in HNSCC patients. However, due to the high dosing required, and the non-autophagic effects of inhibiting the lysosome, we sought a more specific autophagy inhibitor. SAR405 is the most selective and potent inhibitor of the autophagy pathway to date. Compared to HNSCC proliferation inhibition by CQ, SAR405 had a slightly improved IC_{50} of approximately 8 μ M (Figure 6.6). However, the dose required to inhibit LC3-II was a significantly lower dose. SAR405 (1 μ M) strongly inhibited autophagic flux in both HNSCC and CAFs (Figure 6.6). Beyond selectively inhibiting autophagy, SAR405 demonstrates pronounced results in combination with HNSCC standard of care (Figure 6.6). SAR405 potentiates the effects of cisplatin. This indicates the therapeutic potential of inhibiting autophagy in combination with HNSCC standard of care.

Given the promising results observed *in vitro*, we sought to assess autophagy inhibition in murine models of HNSCC. The dosing of CQ varied in preclinical studies, so we conducted a



pilot study of HNSCC alone or HNSCC and CAFs inoculated in a 1:1 admixture subcutaneously in athymic mice. The dose we used established robust accumulation of autophagosomes within HNSCC cells, demonstrating the effectiveness of autophagy inhibition in our study (Figure 6.7). Of surprising interest, when CAFs were co-inoculated with HNSCC, more autophagosomes were detected in the HNSCC cells. This demonstrates the role fibroblasts have in inducing increased autophagy in HNSCC.

To assess autophagy inhibition in combination with current standard of care, cisplatin, HNSCC were inoculated in a 1:1 admixture subcutaneously in athymic mice. Mice were treated with autophagy inhibitors CQ or SAR405 and/or cisplatin. With the addition of autophagy inhibitor SAR405 to cisplatin therapy, there was a significant reduction in tumor volume when compared to SAR405 alone, cisplatin alone, or untreated mice (Figure 6.8). Chloroquine and cisplatin combination treatment reduced tumor volume, but the reduction was not as significant as the more specific inhibitor, SAR405. Additionally, by electron microscopy, we were able to observe the significant reduction in autophagosomes per cell due to SAR405 therapy. This indicates the use of targeted autophagy inhibition will potentiate the efficacy of current therapy.

These results support the direction of autophagy inhibition in HNSCC therapy. Both CQ and SAR405 potentiated the effects of cisplatin. Our *in vitro* results demonstrate this potentiation would also occur with radiotherapy. Both radiotherapy and cytotoxic agents increase autophagy in other tumor types, and this likely occurs with our models. Finally, SAR405 demonstrated the most potent effects, indicating the need for clinical studies to focus on specific autophagy inhibitors before affirming or rejecting modulation of this pathway.

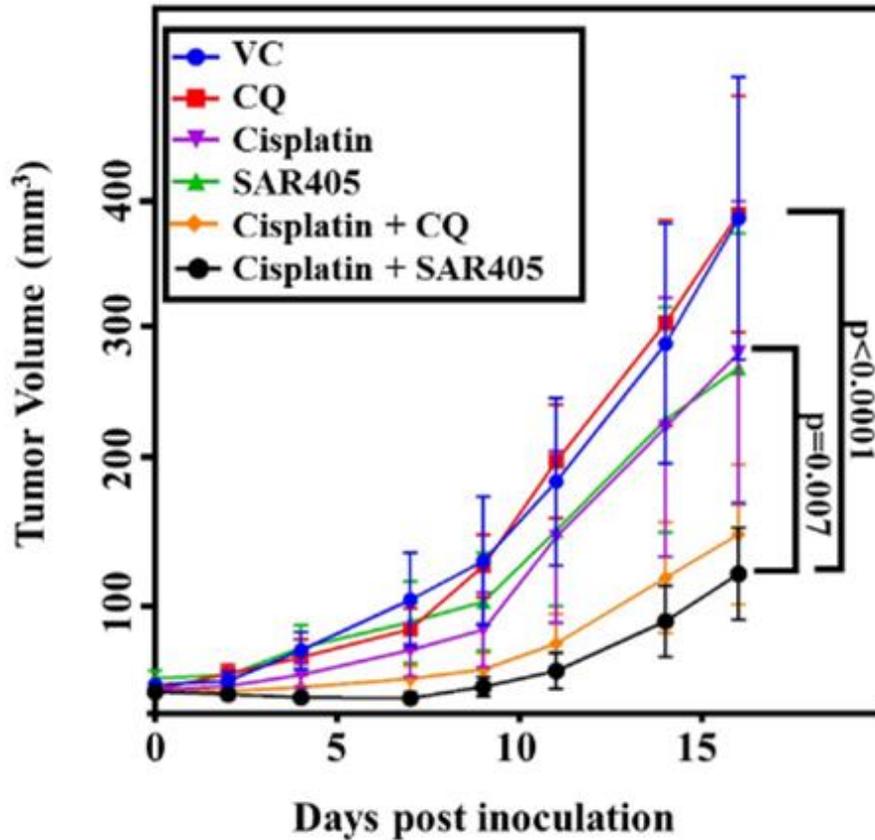


Figure 6.8 – Autophagy Inhibition Potentiates Cisplatin in HNSCC-CAF Xenograft

Autophagy inhibition potentiates standard of care therapy. 1:1 admixture of CAF & HNSCC (UM-SCC-1) were injected subcutaneously in nude female mice. Mice were treated with cisplatin (3 mg/kg i.p. 1x/week), chloroquine (162 mg/kg oral gavage, 5 days/week) or SAR405 (50 μ L intratumoral injection of 10 μ M SAR405 in PBS) (n=9/group). Tumor volumes were assessed by a blinded observer. Error bars represent SEM.

Discussion

Current understanding of the role for autophagy in HNSCC is lacking. Some prior histologic evidence supported the role of high autophagy correlating with worse disease; yet, other reports indicate the opposite. In our studies, we demonstrate a correlation between Beclin-1 expression and worse prognosis. This association is ratified in preclinical models as autophagy inhibition inhibited HNSCC proliferation, migration, invasion, and *in vivo* tumor volume. Autophagy inhibition potentiated the effects of standard of care therapies for HNSCC. All of this supports tumor autophagy as a mechanism that drives tumor progression.

The bulk of current evidence for autophagy inhibition uses the lysosomal inhibitor CQ. Although CQ has late stage effects on autophagic flux, autophagosomes still form and cargo is sequestered following CQ administration. This means autophagic flux is attenuated, but cargo destined for autophagy likely remain sequestered. This limits the effectiveness of this therapy strategy, and is evidenced by the insignificant results of CQ as a single agent in our CAF-HNSCC murine model. However, the early stage inhibitor, SAR405, had a pronounced effect as a single agent, and the greatest effect as a combination component. Thus, future studies targeting autophagy must investigate using more specific inhibitors than CQ.

Despite SAR405 having pronounced results in our hands, this is still a non-selective autophagy inhibitor. SAR405's target, Vps34, assists in trafficking both the autophagosome and the endosome, and the selectivity depends on the associated members of the Vps34 complex. Perhaps future therapeutics will dissect the functionalities of this complex to limit autophagy while still allowing Vps34 to be involved in other processes. Overall, more specific inhibitors to the autophagy pathway would assist therapeutic advancements in this field.

Of interest in our findings, high doses of CQ and SAR405 limited HNSCC growth; however, CAF proliferation was largely unaffected. This indicates that although both the cancer cells and CAFs have significant levels of autophagic flux, autophagy is only required for HNSCC proliferation. Perhaps HNSCC tumors are addicted to autophagy. Autophagic addiction was proposed in breast cancer, where certain subtypes have a predilection for autophagy. Autophagy inhibition in these subtypes leads to an extraordinarily effective strategy (Maycotte et al., 2014). Additionally, Ras mutations, which are commonly associated with HNSCC, are associated with high autophagy, and a dependence on this pathway for survival (Guo et al., 2011; Perera et al., 2015). Whether the tumor is addicted to autophagy or not is overshadowed by autophagy inhibition attenuating tumor growth in multiple cell lines and in multiple models.

One handicap of the current studies is the lack of autophagy inhibition in an immunocompetent host. Results that developed as this project was ongoing indicate the immune system relies on autophagy to mediate its effects. For example, although autophagy increases effectiveness of radiation *in vitro*, in some animal models of other tumor types, autophagy inhibition diminishes the immune response and these tumors grow more following radiotherapy (Ko et al., 2014). Additionally, one group suggests the role for secretory autophagy in mediating the release of ATP from dying tumor cells. Released ATP stimulates the immune system towards a rapid anti-tumor response (Michaud et al., 2011). However, despite these findings, autophagy inhibition limiting the immune response is controversial. No differences in T-cell responses following autophagy inhibition were observed in other tumor models (Starobinets et al., 2016). Additionally, inhibiting autophagy potentiated the effects of natural killer cells and IL-2 therapy (Baginska et al., 2013) (X. Liang et al., 2012). Therefore, future studies should assess the role of autophagy inhibition in an immunocompetent host.

Additionally, there is a need to delineate the specific effects of autophagy in each microenvironment compartment. For example, we have observed both HNSCC autophagy and CAF autophagy contribute to tumor progression, but we do not know which has the greater influence. I attempted three different trials of silencing Beclin-1 using short hairpin inhibition in CAFs, but this had no avail. CAFs did not grow due to technical limitations that prevented direct assessment of autophagy inhibition in a compartment specific manner.

Nevertheless, autophagy within the cancer cells demonstrates a useful therapeutic target in HNSCC. Both single agent and combined therapy demonstrate the therapeutic potential of targeted autophagy inhibition. This builds upon our work of the usefulness in targeting CAF autophagy. Some open questions remain for future work, such as the role of autophagy in the immune response, and the effects of a more targeted autophagy inhibitor. Yet, using the most selective autophagy inhibitor in a robust preclinical model of CAF-HNSCC tumor xenografts, we demonstrate pronounced results in combination therapy with current treatment. Given the dearth of HNSCC therapeutics, this supplies a hopeful avenue for future clinical investigation.

Chapter VII-Discussion

Reciprocal communication between cancer cells and the tumor microenvironment sustains and enables cancer progression. For the past decade, the contribution of stromal cell secreted factors to the progression of cancer has been appreciated (Quail & Joyce, 2013), however the underlying secretory machinery involved remains enigmatic. We observe that CAFs in culture appear phenotypically different from NFs from the same anatomic location, displaying a highly vesicular architecture. This led us to question what fundamental biologic mechanisms account for the cancer-promoting secretory profile. In this study, we observe that primary patient-derived CAFs sustain an increased level of basal autophagy as compared to NFs from cancer-free patients. This is the first observation of enhanced fibroblast autophagy from primary CAFs-derived from patient stroma, and corroborates recent observations of breast cancer cells inducing autophagy in skin fibroblasts, and a *Drosophila melanogaster* tumor model where microenvironmental autophagy was observed (Katherer et al., 2017b; U. E. Martinez-Outschoorn et al., 2010).

We observed an increase in fibroblast autophagy on co-culture with HNSCC cells. The list of known physiologic autophagy inducers is short (Vakifahmetoglu-Norberg, Xia, & Yuan, 2015), with amino acid/glucose starvation and reactive oxygen species being some of the only understood natural inducers. HNSCC metabolically outcompeting CAFs for extracellular nutrients may play a role in inducing CAF autophagy (Chang et al., 2015). However, assessment occurred in a short, 24 h timeframe in high glucose DMEM, which likely indicates this is not a starvation or nutrient depletion phenomenon. We identified IL-6, IL-8, and bFGF, known HNSCC-secreted factors, are all at least in part responsible for CAF autophagy. This corroborates recent findings in a *Drosophila* tumor model that IL-6 secretion from the tumor promotes stromal autophagy (Katherer et al., 2017b). We demonstrate HNSCC secreted bFGF

activates STAT3, induces the transcription of SOX2, which inhibits mTOR transcription (S. Wang et al., 2013). As mTOR represses autophagy (J. Kim et al., 2011), SOX2 inhibition of mTOR conferred increased autophagy in the fibroblasts in our system. The dynamics of CAF autophagy regulation are even more complex when taken into account our observation that autophagy promoting IL-6 and IL-8 are also secreted through autophagic machinery.

There is extensive evidence in the literature demonstrating that both chemotherapeutic drugs and radiation promote cytoprotective autophagy in tumor cells (Sui et al., 2013). Despite multiple studies in a variety of cancer cell types, investigations into the therapeutic potential of autophagy inhibition in HNSCC is lacking (Sannigrahi, Singh, Sharma, Panda, & Khullar, 2015). Our results provide evidence for the first time of autophagy inhibition as having therapeutic potential in HNSCC. The largest limitations to autophagy treatment in clinical trials has been the high doses required of chloroquine, which may fail to achieve intratumoral concentrations sufficient to limit autophagy, and a lack of accepted methodology for monitoring autophagy in patients' tumors to confirm successful inhibition (Poklepovic & Gewirtz, 2014). As such, a large body of research is investigating potential small molecule inhibitors of autophagy, and a few have been discovered (Solitro & MacKeigan, 2016). SAR405 is one such inhibitor that specifically targets PI3K class III, of which the only recognized member is Vps34, an upstream autophagy regulating kinase. We observed that low doses of SAR405 (1.0 μ M or less) were sufficient to limit HNSCC and CAF autophagy *in vitro*. This may provide a feasible therapeutic alternative to chloroquine or hydroxychloroquine for clinical autophagy inhibition. The potentiated effects of HNSCC standard of care, cisplatin, with SAR405 *in vivo* were profound and give hope for future combinational therapy.

Autophagy Inhibition as a Therapeutic Strategy

Our data demonstrate substantial improvement in treatment groups when an autophagy inhibitor is combined with current therapy. Additionally, many clinical trials give evidence to the safety and efficacy of CQ or HCQ in patients. Therefore, should autophagy inhibition progress as a therapy in HNSCC?

Few arguments exist to refute this therapeutic strategy in advanced tumors. In some cases, autophagy can prevent cell death. This occurs when degradative autophagy removes inhibitors of apoptosis, such as Fap-1 (Fas-associated phosphatase 1) (Gump et al., 2014). Inhibiting autophagy leads to increased Fap-1 and decreased Fas induced apoptosis. Additionally, autophagic machinery dictate the cell death mechanism, and inhibition of autophagy can alter the mechanism of cell death (Goodall et al., 2016). Thus, it may be necessary to understand the mechanism of cell-death associated with therapies to determine which therapies would benefit from autophagy inhibition.

Additionally, autophagy inhibition may alter an antitumoral T cell response. Dying cells secrete ATP in an autophagy dependent mechanism, and secreted ATP acts as a mitogen to enhance cytotoxic T cells (Y. Ma, Galluzzi, Zitvogel, & Kroemer, 2013). In some instances, autophagy inhibition in animal models with a fully intact immune system lead to poorer outcomes compared to not inhibiting autophagy (Michaud et al., 2011). Supporting this concept, caloric restriction, a known autophagy inducer, potentiates the effects of chemotherapy (Pietrocola et al., 2016). In addition, vitamin E derivatives, which also induce autophagy, potentiate the effects of chemotherapy (Y. Li et al., 2012). However, this seems to depend on the tumor model used, as autophagy inhibition also potentiates the effects of therapy in other immunocompetent models (Starobinets et al., 2016). Assessing a proposed autophagy inhibition

strategy in an immunocompetent model would likely have better predictive power as to the potential clinical response.

Despite the role of autophagy in mediating different cell death pathways, and altering the immune response, the vast majority of evidence points to autophagy inhibition as a strong candidate for clinical trial. Many cancers depend on autophagy for survival. For example, cancers with mutated Ras require a high basal level of autophagy for their sustained growth (Guo et al., 2011). This stands true for cancers with a high activation of STAT3 (Maycotte et al., 2014). Although direct confirmation of this in HNSCC is still needed, a high proportion of HNSCC patients have mutant Ras and overexpressed STAT3 (Rothenberg & Ellisen, 2012; Sen et al., 2015). Our work demonstrates the role of autophagy inhibition in potentiating the effects of cisplatin. This is supported in other cancer types, such as lung, bladder, and melanoma (Levy et al., 2017). This indicates autophagy inhibition would potentiate the effects of therapy in HNSCC.

Beyond the carcinoma cells, autophagy inhibition would alter the microenvironmental contributions to this disease. Our work demonstrates the role of autophagy dependent secretion in the microenvironment. Other groups also demonstrate how microenvironment autophagy would contribute to disease progression (Sousa et al., 2016) (Endo et al., 2017). Thus, autophagy inhibition would normalize a highly active microenvironment, and likely reduce microenvironmental contributions to disease progression.

All of this points to autophagy inhibition as improving patient outcomes. Yet, although the first report of autophagy as a therapeutic strategy came about more than 10 years ago (Amaravadi et al., 2007), autophagy inhibitors have not reached patients. The lack of a targeted therapeutic explains this dichotomy.

Need for a Selective Autophagy inhibitor

CQ and HCQ offer safe methods of targeting the lysosome. However, high doses are necessary to achieve accumulation of autophagosomes within patient cells (Rangwala et al., 2014). Additionally, CQ targets the lysosome, an important component of degradative autophagy, but a component that may not be necessary for autophagy dependent secretion. Beyond CQ, few other inhibitors targeting the autophagy pathway have minimal adverse effects when given at doses necessary for autophagy inhibition in patients.

There is a need for selective autophagy inhibition. Current inhibitors primarily target the lysosome or upstream kinases. However, few that are widely used are selective. The only one to date that demonstrates potent and selective inhibition of autophagy is SAR405 (Ronan et al., 2014). As such, we used this selective inhibitor in our studies. Our results indicate that SAR405 has a better therapeutic effect compared to CQ as a single agent, and potentiates the effects of cisplatin better than CQ. This indicates that selective and potent inhibition of autophagy would result in better outcomes for patients.

However, our animal trial is the only trial to date that assesses the role of SAR405 in an animal model. Other Vps34 inhibitors by the same group that developed SAR405 demonstrate kinase inhibition *in vivo*, but these have not been assessed in terms of autophagic flux inhibition (B. Pasquier et al., 2015). Additionally, Vps34 has other functions outside of the autophagy pathway, as it also regulates endosomal trafficking (E. E. Johnson et al., 2006). Thus, there is a need for even more selective autophagy inhibition.

Future studies should direct attention towards inhibiting autophagic flux specific events. For example, ULK1 binding to the Beclin-1 complex is known to inhibit autophagy, and might

be a great direction for future therapy. The dearth of therapeutics targeting this pathway creates a molecular frontier in which discovery might occur.

Additionally, there is need for a selective inhibitor that differentiates secretory and degradative autophagy. Current inhibitors inhibit both functions of the autophagic machinery. Inhibition of either secretory or degradative pathways would better define what molecular substrates are chaperoned in an autophagy dependent manner. A selective inhibitor would also allow for targeted inhibition of the secretory functions of fibroblasts that we observed in our data. The main obstacle in developing a selective inhibitor is the lack of mechanistic understanding of the autophagy dependent secretion pathway. Future studies dissecting the mechanisms of these pathways will provide increased selectivity of inhibitors.

Autophagy: An Activated Fibroblast Marker?

Our results from current and past studies demonstrate a role for activated CAFs in supporting tumor progression. CAFs are phenotypically different from NFs, and have a much greater influence on disease. However, despite this understanding, the lexicon describing activated fibroblasts is short. α -SMA is the most widely used marker of activated fibroblasts (Hinz et al., 2007). This is known to be induced by TGF- β (Lewis et al., 2004), and is present in our CAFs (Wheeler et al., 2014). A marker less frequently used is FAP (Fibroblast Activation Protein) (Park et al., 1999). This is widely expressed in CAFs of multiple cancer types (Lee et al., 2011), and high expression of FAP associates with poor prognosis for HNSCC patients (H. Wang et al., 2014). Beyond these two, few other markers distinguish an activated fibroblast from a “normal” fibroblast.

Autophagy might provide an additional descriptor of an activated fibroblast. Our results indicate CAFs have a much greater basal level of autophagy. Upon autophagy inhibition, the role of CAFs in inducing HNSCC proliferation, migration, and invasion diminish. Beyond this,

autophagy seems to be of importance in regulating a stem-like phenotype in fibroblasts (S. Wang et al., 2013). Given these findings, our work sheds light on a previously unrecognized role for autophagy in fibroblast activation.

Conclusions

This work reveals unexpected roles for the supporting stroma and autophagy in HNSCC. The dearth of HNSCC therapeutics that translate from bench to bedside prompted us to investigate microenvironment components that contribute to disease progression. As CAFs are the most abundant microenvironment cell type, and are well known to support tumor progression, we set out to understand the underlying biology of these cells.

To our surprise, CAFs have significantly more autophagic flux compared to NFs. Before these studies, autophagy connoted a mechanism of cell death and degradation to most cancer biologists. Nevertheless, CAFs proliferate quickly, despite a high basal level of autophagy. Autophagy inhibition mitigates CAF secretion of factors important for cancer progression, indicating an autophagy dependent secretome. This reveals that in CAFs, and likely other cell types, autophagy machinery is involved in cellular secretion within the HNSCC microenvironment.

Intriguingly, we found that tumor cells induce autophagy in CAFs, hijacking the microenvironment to promote disease progression. This creates a positive, symbiotic loop between HNSCC and CAFs, which promotes increased autophagy in both cell types. These findings reveal the potential therapeutic strategy of targeting autophagy in HNSCC. Our resulting data from both *in vitro* and *in vivo* studies using combined HNSCC-CAF models indicate that autophagy inhibition would potentiate standard of care therapy in HNSCC. The

studies described offer a new therapeutic strategy in HNSCC, and insights into the biology of autophagy dependent secretion.

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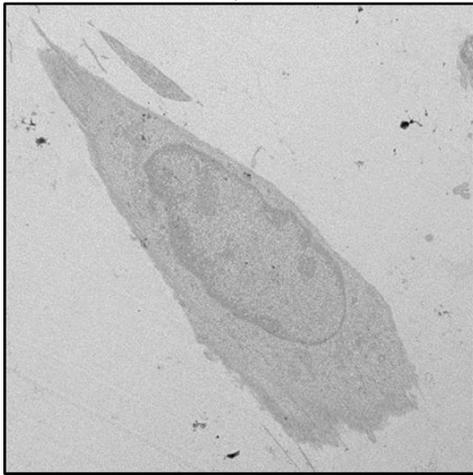
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Appendix
Low Magnification Images

This appendix provides low magnification images of higher powered magnification transmission electron microscopy and immunofluorescent images included in the body of this dissertation.

NF



CAF

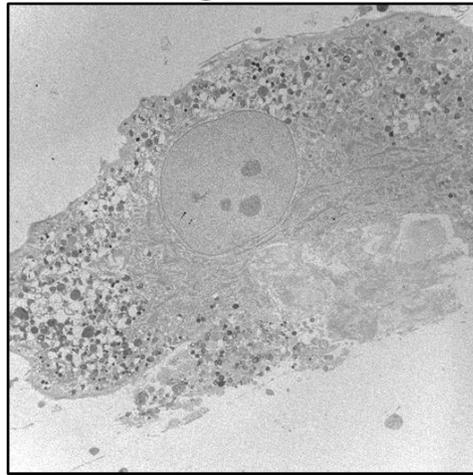


Figure 4.1b-CAFs Demonstrate Increased Autophagosomes than NFs

Representative TEM images of a NF (5000X) or CAF (3000X) cell.

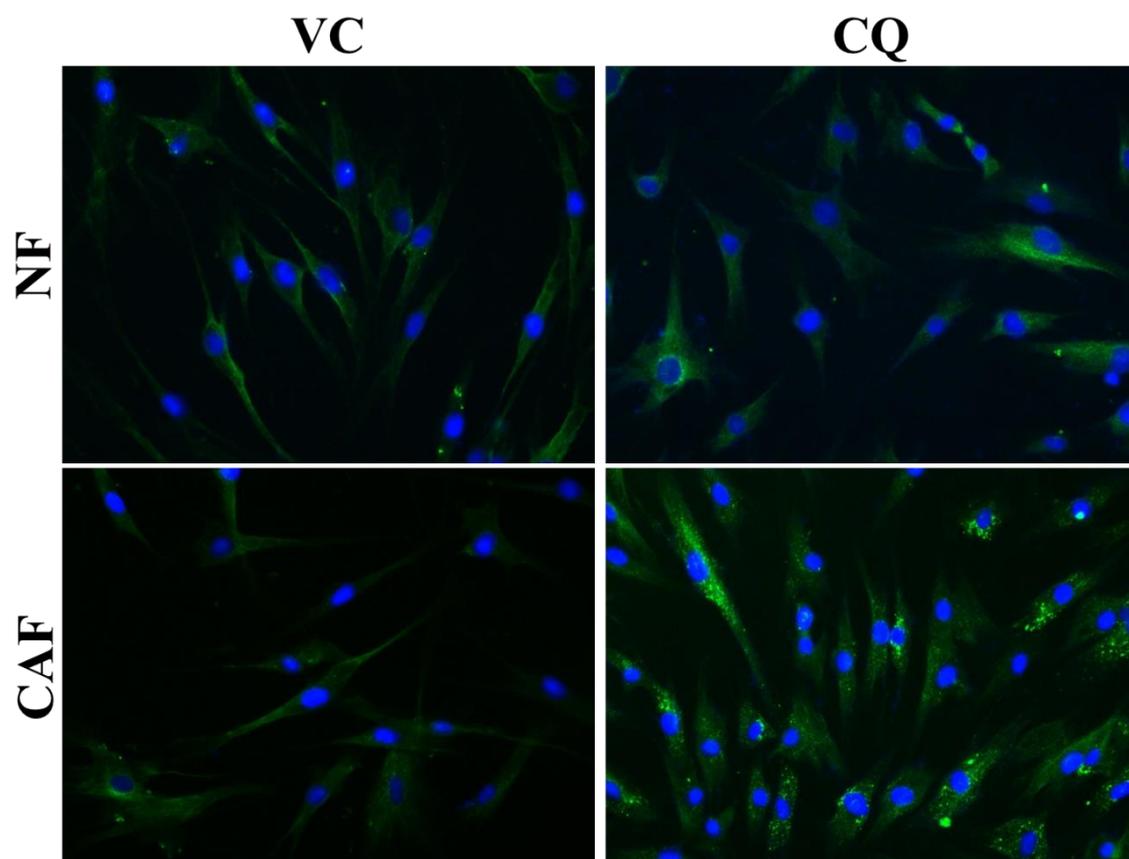


Figure 4.4b - CAFs Demonstrate More LC3 Puncta than NFs

Representative immunofluorescent of LC3 (green) puncta, Hoechst nuclear stain (blue), comparing NF with CAFs with and without CQ (80 μ M for 2 h) (20x magnification).

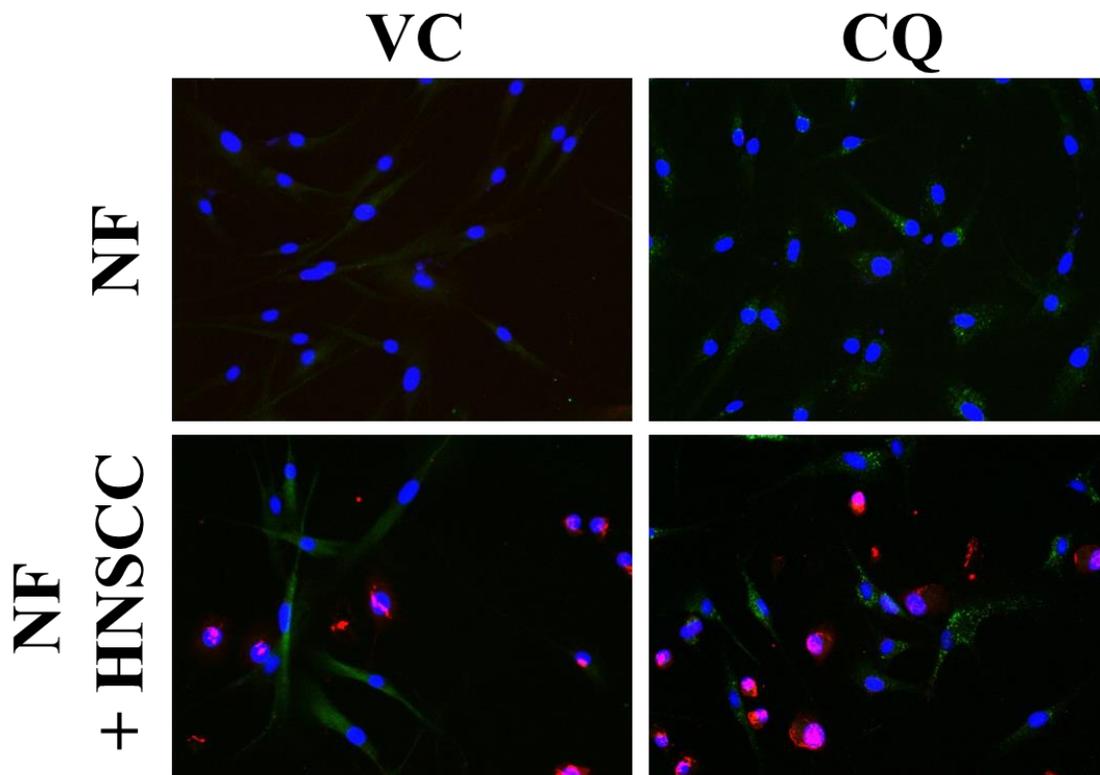


Figure 5.1b – HNSCC Induces LC3 Puncta in Fibroblasts

Representative IF of NFs in a 1:1 co-culture with HNSCC (HN5) with and without CQ (80 μ M for 2 h) with cytokeratin 14 HNSCC label (red), LC3 (green) and Hoescht (blue) nuclear stain (20x magnification).

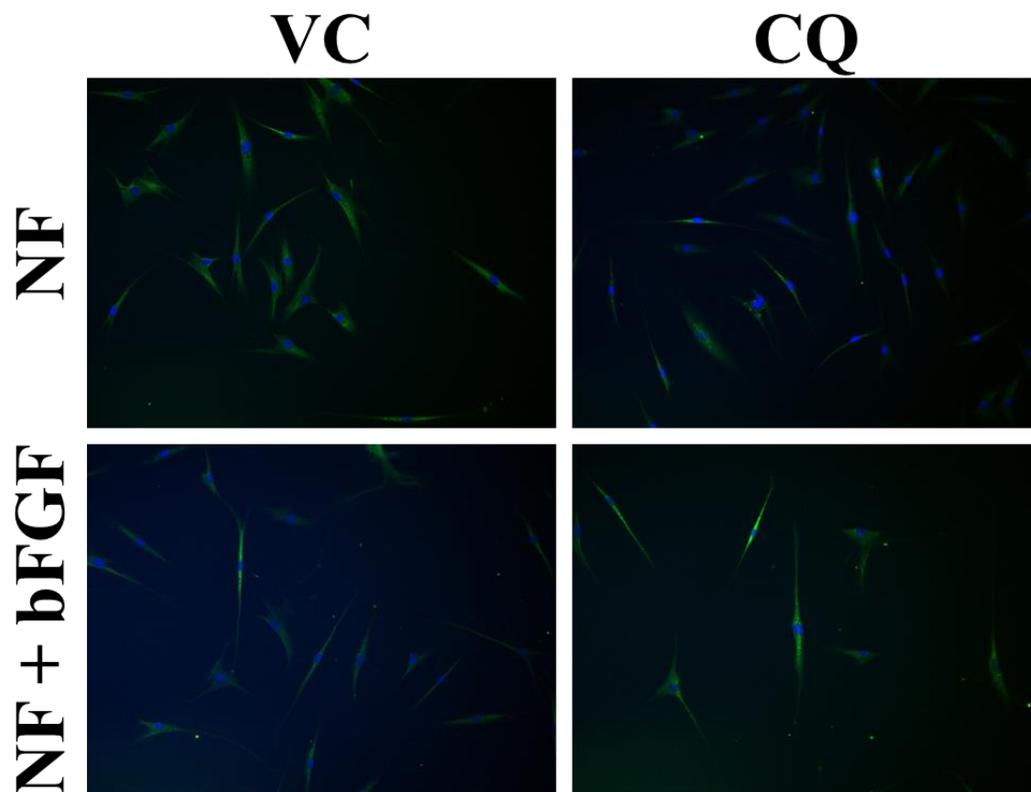


Figure 5.5b – bFGF Induces LC3 Puncta in NFs

Expanded view (10X) of NF with and without bFGF (100ng/mL) and or CQ (80 μ M for last 2 hours), LC3 (green), Hoescht nuclear (blue).

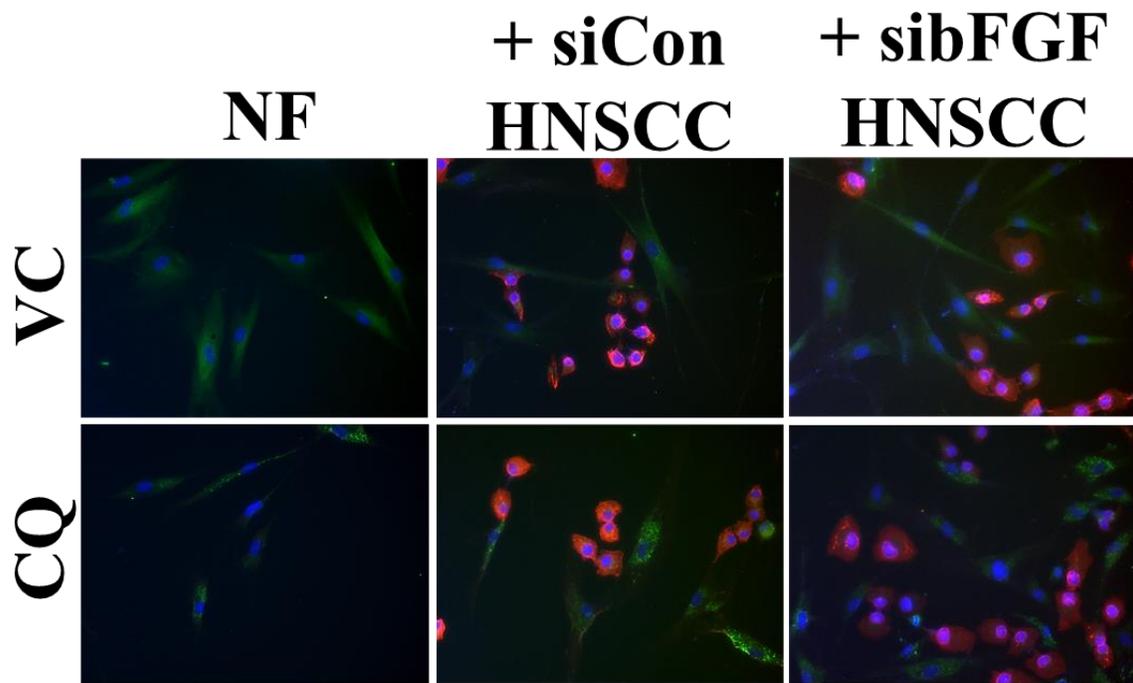


Figure 5.6b – HNSCC bFGF Mediates NF LC3 Puncta Induction

Expanded view (20X) of NF alone or co-cultured with either control siRNA (siCon) or bFGF siRNA (sibFGF) transfected HNSCC (HN5) for 24 h in 1:1 ratio, and CQ (80 μ M for last 2 h); LC3 (green), cytokeratin 14 (red), Hoescht nuclear (blue).