FUNCTIONAL SIGNIFICANCE OF SENESCENCE AND AUTOPHAGY IN CANCER-ASSOCIATED FIBROBLASTS FROM ORAL SQUAMOUS CELL CARCINOMA

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2020

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF DENTISTRY
UNIVERSITY OF MALAYA
KUALA LUMPUR

2020

UNIVERSITY OF MALAYA

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is a malignancy that arises from the epithelial cells within the oral cavity. It accounts for approximately 355,000 new cases worldwide and is exceptionally prevalent in particular geographical areas such as Papua New Guinea, the Indian subcontinent and South-East Asian countries. Despite advances in surgical management and therapeutic approaches, the five-year survival rate of OSCC patients has not improved significantly over the past few decades. The mortality associated with OSCC is particularly high often due to late presentation, locoregional recurrence, distant metastases and the development of second primary tumours. A comprehensive understanding of the molecular pathogenesis of OSCC is required to identify new druggable targets and inform innovations in the therapeutic approach. OSCCs are a heterogeneous group of tumours and, whilst the majority of tumours are aggressive and genetically unstable (GU-OSCC), a subset of genetically stable cancers (GS-OSCC) has been identified that have a more favourable prognosis. Intriguingly, cancer-associated fibroblasts (CAFs) from these tumours are phenotypically and functionally distinct. Many of the characteristics ascribed to CAFs are shared by autophagic and senescent fibroblasts, suggesting that these stress responses contribute to the tumour-promoting properties of CAFs. The present study was designed to investigate the possible link between autophagy and senescence in CAFs from OSCCs and normal oral fibroblasts as well as to investigate the functional significance of these CAF phenotypes in terms of promoting tumour growth, migration and invasion. The results showed that autophagic and senescent phenotypes were closely related and CAFs from GU-OSCCs were shown to be more senescent and also displayed impaired autophagic flux than normal oral fibroblasts and CAFs from GS-OSCCs. Next, the contribution of autophagy to the activated and senescent phenotypes of oral fibroblasts was investigated using TGF-β1 as an inducer of myofibroblast differentiation and

senescence, together with inhibitors of autophagy. The results demonstrated that altered autophagy could regulate the activated and senescent phenotypes in oral fibroblasts. This had functional significance because conditioned media collected from oral fibroblasts with altered autophagy significantly enhanced migration and invasion of OSCC cells *in vitro*. These data indicate that the autophagy-regulated secretion by fibroblasts might be responsible, at least in part, for modulating the malignant phenotypes of OSCC cells. Lastly, an *in vitro* model to conditionally induce senescence in normal oral fibroblasts was established. Using this model that allowed synchronous induction of senescence in normal fibroblasts, it was demonstrated that the secretome from senescent fibroblasts enhanced OSCC cell migration and invasion. Taken together, the results of the present study suggest that the physiological states of CAFs within the OSCC tumour microenvironment might reflect different stages of the same sequential pathway and/or be part of a unified biological programme in which autophagy precedes activation and subsequent senescence during the acquisition of pro-tumorigenic CAF phenotypes. These physiological stages of oral CAF transdifferentiation could possibly be targeted therapeutically in the future for the better clinical management of patients with OSCC.

Keywords: OSCC, Autophagy, Senescence, Cancer-associated fibroblasts, fibroblast activation

ABSTRAK

Kanser mulut jenis squamous sel karsinoma (OSCC) adalah sejenis karsinoma yang timbul dari sel-sel epitelium dalam rongga mulut. Ia menyumbang kira-kira 355,000 kes baru di seluruh dunia dan sangat lazim di kawasan geografi tertentu seperti Papua New Guinea, benua kecil India dan Asia Tenggara. Kadar hidup lima tahun pesakit OSCC tidak bertambah baik sejak beberapa dekad yang lalu walaupun terdapat kemajuan dalam pengurusan pembedahan dan terapi. Kematian yang dikaitkan dengan OSCC amat tinggi kerana pengesahan lewat, pengulangan locoregional, metastasis jauh dan ketumbuhan tumor utama kedua. Pengetahuan komprehensif mengenai patogenesis molekular OSCC amat diperlukan untuk mengenalpastikan sasaran pengubatan baru dan memaklumkan inovasi dalam perkembangan terapeutik. OSCC adalah kumpulan tumor heterogen. Walaupun majoriti tumor adalah agresif dan tidak stabil secara genetik (GU-OSCCs), satu subset kanser mulut stabil secara genetik (GS-OSCCs) telah dikenalpasti dan mempunyai prognosis yang lebih baik. Fibroblas berkaitan kanser (CAFs) dari tumor ini adalah fenotipikal dan berbeza secara fungsional. Kebanyakan ciri-ciri CAFs dikongsikan dengan fibroblas autofagi dan senescent, mencadangkan bahawa tindak balas tekanan ini juga menyumbang kepada ciri-ciri CAFs yang mempromosikan tumor. Kajian ini direkakan untuk menyiasatkan kemungkinan hubungan antara autofagi dan senescence dalam CAFs dari OSCCs dan fibroblas mulut biasa, dan untuk menyiasatkan kepentingan fungsi fenotip CAFs ini dari segi peningkatan pertumbuhan tumor, migrasi dan invasi. Keputusan kajian menunjukkan bahawa fenotip autofagik dan senescent berkaitan rapat, dan CAFs dari GU-OSCCs menunjukkan fenotip yang lebih jelas dan juga fluks autofagik yang terjejas. Selanjutnya, sumbangan autofagi kepada fenotip aktif dan senescent di fibroblas mulut telah disiasatkan dengan menggunakan TGF-β1 untuk induksi myofibroblas diferensiasi dan senescence. Hasilnya menunjukkan bahawa autofagi yang diubahsuaikan dapat mengawal fenotip aktif dan senescent dalam

fibroblas mulut. Ini mempunyai kepentingan fungsi kerana media yang dikumpulkan dari fibroblas mulut dengan autofagi yang telah diubahsuaikan boleh meningkatkan migrasi dan invasi sel-sel OSCC dengan ketara *in vitro*. Data ini juga menunjukkan bahawa secresi yang dikawalkan oleh autofagi daripada fibroblas mungkin bertanggungjawab, sekurang-kurangnya sebahagiannya, untuk modulasi fenotip malignan dalam sel-sel OSCC. Akhir sekali, senescence model *in vitro* fibroblas oral biasa yang boleh diaktifkan secara konduktif telah dihasilkan. Model ini membolehkan induksi senescence secara serentak dalam fibroblas mulut biasa dan ia menunjukkan bahawa secresi dari fibroblas senescent meningkatkan migrasi dan invasi sel-sel OSCC. Kajian ini, buat pertama kalinya, menunjukkan bahawa keadaan fisiologi CAF dalam persekitaran mikro tumor OSCC mungkin mewakili peringkat-peringkat yang berbeza daripada laluan berurutan yang sama dan/atau adalah sebahagian daripada program biologi bersatu, di mana autofagi mendahului pengaktifan dan berikutnya senescence semasa pemerolehan fenotip CAF yang pro-tumorigenik. Tahap-tahap fisiologi transdiferensiasi CAF mulut ini berpotensi untuk disasarkan secara terapeutik pada masa akan datang untuk pengurusan klinikal pesakit OSCC yang lebih baik.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and heartfelt thanks to my PhD supervisor, Prof Ian Paterson for his gentle guidance throughout the formulation of this thesis and his expertise during the course of this PhD project. I am sincerely grateful for his patience in meticulously mentoring me, especially with my scientific writing. I would also like to thank my co-supervisors Associate Prof Yap Lee Fah and Prof Ken Parkinson for their generous advice and assistance. I would like to further extend my appreciation to Prof Ian Paterson and Prof Ken Parkinson for giving me the opportunity to expand my horizons during my research attachment in Blizard Institute, Queen Mary University of London. It has not only allowed me to gain valuable experience and develop technical lab skills but has also helped me with my personal growth.

I would like to acknowledge all lab members from both the University of Malaya and Blizard Institute who have helped impact my life both on a personal and professional level. Without their constant encouragement and support, the course of this process would have been insurmountably more difficult.

I could not have been more grateful to my family, partner and friends, especially Joel, Melissa, Lynnette, Chae Yin and Chai San for their constant love and emotional support in motivating me throughout trying times and encouraging me in all of my pursuits. Thank you, Joel, for always believing in me and sharing in my dreams.

Last, but definitely not least, in loving memory of a great friend, Min who has been there for me since the beginning. Thank you for showing me perseverance and that nothing is impossible with passion and dedication.

"For from You are all things, and to You are all things, You deserve the glory."

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LIST OF SYMBOLS AND ABBREVIATIONS

3-MA	:	3-methyladenine
ANE	:	Areca nut extract
AMPK	:	Adenosine monophosphate-activated protein kinase
ATG	:	Autophagy-related gene
ATP	:	Adenosine triphosphate
α-SMA	:	Alpha smooth muscle actin
β-ME	:	β-mercaptoethanol
BECN1	:	Beclin 1 gene
BSA	:	Bovine serum albumin
CAF	:	Cancer-associated fibroblast
CDK	:	Cyclin-dependent kinase
cDNA	:	Complementary DNA
CGH	:	Cyclooxygenase
CNA	:	Copy number alteration
COX	:	Cycle threshold
Ct	:	Computed tomography
CT	:	Adenosine triphosphate
CQ	:	Chloroquine
CXCL	:	CXC motif chemokine ligand
DDR	:	DNA damage response
DMEM	:	Dulbecco's Modified Eagle's Medium
DMSO	:	Dimethyl sulphoxide
DNA	:	Deoxyribonucleic acid
EBV	:	Epstein-Barr virus

ECL	:	Enhanced chemiluminescence
ECM	:	Extracellular matrix
EDTA	:	Ethylenediaminetetraacetic acid
EGF	:	Epidermal growth factor
EGFR	:	Epidermal growth factor receptor
EMT	:	Epithelial-mesenchymal transition
FAP	:	Fibroblast activation protein
FBS	:	Fetal bovine serum
FC-II	:	FetalClone^{TM} II serum
FFPE	:	Formalin-fixed paraffin-embedded
FGF	:	Fibroblast growth factor
FHIT	:	Fragile histidine triad gene
GS-OSCC	:	Genetically unstable OSCC
GU-OSCC	:	Genetically stable OSCC
Gy	:	Gray
HCl	:	Hydrochloric acid
HCQ	:	Hydroxychloroquine
HGF	:	Hepatocyte growth factor
HIAR	:	Hypoxia-induced angiogenesis regulator
HIF	:	Hypoxia-induced factor
HIV	:	Human immunodeficiency virus
HNSCC	:	Head and neck squamous cell carcinoma
HPV	:	Human papillomavirus
hTERT	:	Human telomerase reverse transcriptase
IL	:	Interleukin
IGF	:	Insulin-like growth factor

IHC	:	Immunohistochemistry
IR	:	Ionising radiation
ISH	:	*In situ* hybridization
LC3	:	Microtubule-associated protein light chain 3
LOH	:	Loss of heterozygosity
MCP	:	Monocyte chemoattractant protein
MMP	:	Matrix metallopeptidase
MRI	:	Magnetic resonance of imaging
mRNA	:	Messenger RNA
MSC	:	Mesenchymal stem cell
mTOR	:	Mammalian target of rapamycin
mTORC1	:	mTOR complex 1
NHOF	:	Normal human oral fibroblast
NK	:	Natural killer cell
NF-κB	:	Nuclear factor-κB
OIS	:	Oncogene-induced senescence
OSCC	:	Oral squamous cell carcinoma
PAGE	:	Polyacrylamide gel electrophoresis
PDAC	:	Pancreatic ductal adenocarcinoma
PD-1	:	Programme cell death protein 1
PDGF	:	Platelet-derived growth factor
PDGFRβ	:	Platelet-derived growth factor receptor-β
PE	:	Phosphatidylethanolamine
PET	:	Positron emission tomography
PBS	:	Phosphate buffered saline
PI3K	:	Phosphatidykinositol-3-kinase

PFA	:	Paraformaldehyde
PVDF	:	Polyvinylidene difluoride
p16	:	$p16^{Ink4a}$
p21	:	$p21^{Waf1/Cip1}$
p62	:	p62/SQSTM1, sequestosome 1
pRb	:	Retinoblastoma
qPCR	:	Quantitative polymerase chain reaction
RNA	:	Ribonucleic acid
RNAseq	:	RNA sequencing
ROS	:	Reactive oxygen species
rRNA	:	Ribosomal RNA
SA-β-gal	:	Senescence-associated β-galactosidase
SASP	:	Senescence-associated secretory phenotype
SDF	:	Stromal cell-derived factor
SDS	:	Sodium dodecyl sulphate
SFT	:	Secondary field tumour
SIRT1	:	Sirtuin 1
SNB	:	Sentinel node biopsy
SPT	:	Second primary tumour
TBS	:	Tris buffered saline
TBST	:	Tris buffered saline with 0.1% Tween 20
TCGA	:	The Cancer Genome Atlas
TGF-β	:	Transforming growth factor-β
TEM	:	Transmission electron microscopy
TME	:	Tumour microenvironment
TNM	:	Tumour, node, metastasis

TORS : Transoral robotic resections

VEGF : Vascular endothelial growth factor

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CHAPTER 1: INTRODUCTION

1.1 General introduction

Oral squamous cell carcinoma (OSCC) accounts for almost 355,000 new cases annually worldwide and there is an increasing incidence in many countries, particularly Papua New Guinea, the Indian subcontinent and South-East Asian countries (Bray et al., 2018). Despite advances in cancer therapy, only approximately 50% of patients survive for five years after diagnosis (Warnakulasuriya, 2009). The mortality rate associated with OSCC is particularly high because of late presentation, locoregional invasion and recurrence, the development of second primary tumours and metastatic disease. The prognosis for patients remains very poor (Omar, 2013; Warnakulasuriya, 2014). Unlike many other solid tumours, targeted molecular therapies are not in routine use for the treatment of OSCC and, therefore, there is an urgent need to develop new therapeutic modalities. Future developments are likely to result from a better understanding of the molecular pathogenesis of the disease.

Whilst our current knowledge of genetics and molecular alterations that occur in the malignant epithelial component of OSCCs is quite extensive (Cancer Genome Atlas Network, 2015; Leemans, Snijders, & Brakenhoff, 2018; Veeramachaneni et al., 2019), non-malignant components within the tumour microenvironment (TME) are now known to influence tumour development and progression (Hanahan & Coussens, 2012; Maman & Witz, 2018). Cancer-associated fibroblasts (CAFs) are often the most abundant stromal cell type, and they actively participate in the reciprocal communication between tumour cells and other host cells in the TME, potentially contributing to the establishment of a tumour-permissive microenvironment and disease progression in many different cancers (Kalluri, 2016).

CAFs react to stress within the tumour environment in a variety of ways that not only allow them to adapt to severely stressful conditions but can also enhance pro-tumourigenic behaviour in tumour cells and modulate the response to treatment (Kalluri, 2016). Such stress responses include activation, autophagy and senescence, which demonstrates heterogeneity in CAF phenotypes (Vitale, Manic, Galassi, & Galluzzi, 2019). CAF senescence (permanent proliferative arrest) is a characteristic associated with a more aggressive and genetically unstable subset of OSCCs (GU-OSCCs), compared to their genetically stable counterparts (GS-OSCCs) (Parkinson, James, & Prime, 2015). Despite playing a tumour-suppressive role when induced in epithelia, senescence in CAFs is tumour-promoting, essentially due to a unique secretome that is collectively known as the senescence-associated secretory phenotype (SASP) (Coppé, Desprez, Krtolica, & Campisi, 2010; Kuilman & Peeper, 2009). Interestingly, activated myofibroblasts share overlapping gene expression profiles with senescent fibroblasts (Mellone et al., 2016) and their pro-tumorigenic phenotypes may be regulated through similar signalling pathways (Alspach et al., 2014; Demehri, Turkoz, & Kopan, 2009; Procopio et al., 2015). Further, many of the characteristics ascribed to senescent CAFs are also shared by fibroblasts that have been induced to undergo autophagy, a regulated self-catabolic digestion mechanism that is frequently used by cells to recycle dispensable or dysfunctional cellular components and maintain cellular homeostasis (Capparelli et al., 2012). Accumulating evidence indicates that aberrant autophagy in stromal fibroblasts might contribute to the acquisition of the senescent phenotype (Capparelli et al., 2012; Galluzzi, Bravo-San Pedro, & Kroemer, 2016; Young & Narita, 2010; Young et al., 2009). However, this needs to be rigorously tested experimentally, especially because studies of autophagy in OSCCs are largely confined to epithelial tumour cells but not cells of the TME.

Unlike tumour cells, CAFs are genetically stable and diploid, which creates a potential opportunity to target CAFs therapeutically in OSCCs and other cancer types (Kalluri, 2016; Valkenburg, de Groot, & Pienta, 2018). Therefore, the present study examined autophagy and senescence in CAFs from OSCCs and investigated the relevance of these CAF phenotypes in the pathogenesis of oral cancer. A fuller understanding of how these stress responses in CAFs drive tumour development and progression could lead to the identification of potential novel therapeutic targets.

1.2 General aims

The present study focused on investigating the importance of CAFs in the pathogenesis of OSCC. A previously published microarray gene expression study revealed that fibroblasts from the normal oral mucosa, GS-OSCCs and GU-OSCCs displayed distinctive gene expression profiles that reflected the stages of epithelial tumour progression in OSCC (Lim et al., 2011). Furthermore, CAFs from OSCC were demonstrated to be activated and prematurely senescent, and these features were more marked in CAFs from GU-OSCCs (Hassona et al., 2013; Lim et al., 2011).

Intriguingly, many of the characteristics ascribed to CAFs are shared by autophagic and senescent fibroblasts, suggesting that these stress responses contribute to the tumour-promoting properties of CAFs (Young et al., 2009). Therefore, this study was initiated to explore the possible link between autophagy and senescence in CAFs, with an emphasis on the different subsets of CAFs from OSCC (CAFs from GU-OSCC and CAFs from GS-OSCC) and normal oral fibroblasts (NHOFs), and to investigate the functional significance of these CAF phenotypes in terms of promoting tumour growth, migration and invasion.

The first part of this study focused on characterising the autophagy- and senescence-associated phenotypes in the different subsets of fibroblasts and explored the

relationship between these two stress responses. During the course of this work, autophagy and senescence were found to be closely associated and, therefore, work was undertaken to determine if the autophagy pathway was compromised in CAFs from GU-OSCCs, as these cells were shown to be more senescent.

Having shown that the autophagy and senescence-phenotypes were closely related in oral fibroblasts, the second part of this work investigated the involvement of autophagy in fibroblast activation and senescence. Experiments were performed to examine whether alterations in the autophagy pathway in fibroblasts might indirectly affect the phenotypic characteristics (proliferation, migration and invasion) of OSCC cells. Normal oral fibroblasts were treated with pharmacological agents targeting autophagy (SAR405) and transforming growth factor-β (TGF-β), which was used as an activator of autophagy, and conditioned media from these cells were used in assays of tumour behaviour *in vitro*.

The final part of this study aimed to develop *in vitro* models with which to study the functional significance of fibroblast senescence. Senolytic drugs were used in an attempt to eliminate senescent cells from CAFs and a temperature-sensitive mutant SV40 large T-antigen was used to develop a model in which fibroblast senescence could be induced. These modified fibroblasts then served as a useful homogenous model to examine the biological significance of senescence to the tumour-promoting properties of CAFs in OSCC.

1.3 Objectives

The objectives of the present study were:

1. To characterise the autophagic- and senescent-associated phenotypes in a panel of fibroblast strains and to use these fibroblasts to examine the relationship of autophagy and senescence in CAFs from OSCCs.

2. To establish *in vitro* models to examine the involvement of autophagy in fibroblast activation and senescence, and its influence on tumour behaviour.

3. To establish senescence-eliminated or/and conditionally senescence-inducible fibroblasts strains and to use these modified fibroblasts to examine the contribution of senescence to the tumour-promoting properties of CAFs in OSCCs.

CHAPTER 2: LITERATURE REVIEW

2.1 Oral squamous cell carcinoma

Carcinomas of the mouth (oral cancer), together with squamous cell cancers of the pharynx (nasopharynx, oropharynx and hypopharynx) and larynx, are known collectively as head and neck squamous cell carcinomas (HNSCCs) (Marur & Forastiere, 2016). Oral cancers represent a group of tumours that arise from multiple anatomic subsites within the oral cavity, which can develop on the surface of the tongue, and other intraoral sites such as the buccal cavity, gingivae, palate and floor of mouth (Figure 2.1). The site distribution of cancers of the lip and oral cavity based on the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) is shown in Table 2.1 (World Health Organization, 2016).

Oral squamous cell carcinoma (OSCC), which begins in the squamous epithelial cells that line the oral mucosal surface, is the most common type of malignancy of the oral mucosa and squamous cell carcinomas (SCCs) account for more than 90% of all forms of head and neck cancers (Speight & Farthing, 2018).

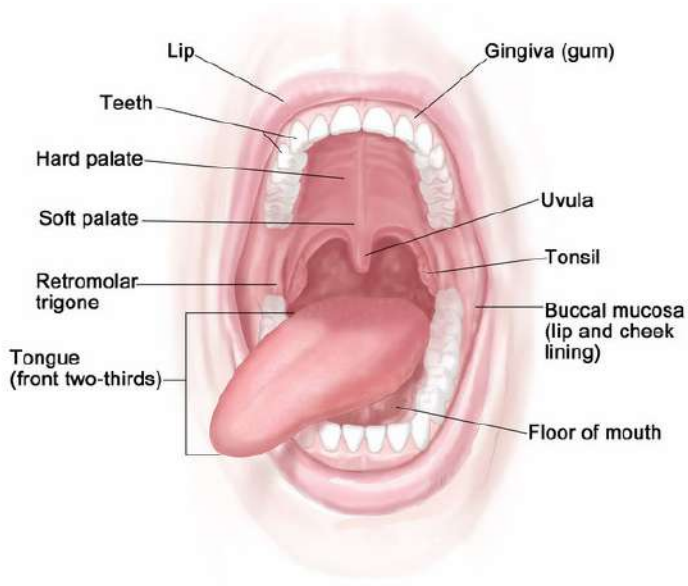


Figure 2.1: Anatomy of the oral cavity

The anatomy sites and subsites of the mouth and oral cavity. Figure reprinted from the National Cancer Institute © 2012 Terese Winslow LLC, U.S. Govt. has certain rights. Retrieved from https://www.teresewinslow.com/head. Reprinted with permission.

Table 2.1: Site distribution of oral cancer

ICD site code	Oral cancer sites
C00	Lips
C02	Other and unspecified parts of tongue
C03	Gingivae
C04	Floor of mouth
C05	Palate
C06	Other and unspecified parts of mouth

2.1.1 Epidemiology, aetiology and risk factors

Based on GLOBOCAN 2008, oral cancer accounted for approximately 263,900 new cases and 128,000 deaths globally (Ferlay et al., 2010). According to the most recent GLOBOCAN estimates in 2018, the number of new cases and deaths from lip and oral cavity cancers increased dramatically over a decade to approximately 354,864 (age-standardised rate, ASR, 4.0 per 100,000) and 177, 384 (ASR 2.0 per 100,000) annually, respectively (Bray et al., 2018). OSCC can be thought of as an Asian disease with an annual estimated incidence of around 227,906 cases in Asia, with the disease being particularly prevalent in Papua New Guinea, the Indian subcontinents and South-East Asian countries (Bray et al., 2018). It is one of the most common cancers in South-Central Asia and Melanesia (Jemal et al., 2011) and has reached epidemic proportions in these particular regions due to specific habits, such as betel quid and tobacco chewing (Omar, 2013; Warnakulasuriya, 2009). However, the incidence of oral cancer is also rising in many Western countries, such as the United States, United Kingdom, Iceland and Finland (Chi, Day, & Neville, 2015; Ferlay et al., 2013). In Malaysia, the incidence of oral cancer is predominant among the Indian ethnic group and the indigenous people of Sabah and Sarawak (Zainal Ariffin & Nor Saleha, 2011).

The occurrence of lip and oral cavity cancer is approximately two-fold higher in males compared to females (Bray et al., 2018). Classically, the risk of developing OSCC increases with age (Neville, Damm, Allen, & Chi, 2015) but recent epidemiologic studies have demonstrated an increasing trend of OSCC in young individuals and also females (Chi et al., 2015; Patel et al., 2011; Schmitd, Tjioe, Assao, & Oliveira, 2015). The aetiology of oral cancer is multifactorial and it has been traditionally attributed to tobacco misuse and excess consumption of alcohol (Warnakulasuriya, 2009). Betel quid with or without tobacco and smokeless tobacco products are also among the major risk factors of oral cancer (International Agency for

Research on Cancer, 2004; Jayalekshmi et al., 2009; Wen et al., 2010). These well-established behavioural risk factors may act synergistically or separately (Blot et al., 1988). The risk of getting OSCC multiplies in the case of combined abuse of tobacco and alcohol, and it could limit treatment efficacy, as well as increase the risk of secondary cancers (National Comprehensive Cancer Network, 2018). The role of human papillomavirus (HPV) in the pathogenesis of HNSCC was controversial for many years but it is now known that HPV accounts for less than 4% of OSCCs and HPV-related disease is found mainly in oropharyngeal squamous cell carcinomas (OPSCCs) (Castellsagué et al., 2016). This has recently led to a separate staging system for $p16^{Ink4a}$ positive (used as a surrogate marker of HPV) OPSCCs (Amin & Edge, 2017; Brierley, Gospodarowicz, & Wittekind, 2017; Huang & O'Sullivan, 2017). Nasopharyngeal carcinoma (NPC), is associated with Epstein-Barr virus (EBV) and is a distinct type of head and neck cancer (Rickinson & Lo, 2019). In addition, human immunodeficiency virus (HIV) infected and immunosuppressed individuals have a higher prevalence of developing oral cancer, with an estimated twofold risk relative to the general population (Neville et al., 2015).

2.1.2 Histological and clinical features

Histologically, 90% of the malignant lesions in the oral cavity appear as SCC and typically arise from the epithelial lining of oral cavity (Speight & Farthing, 2018). The tongue is the most commonly affected site (40-50%) for OSCC in Western populations (American Cancer Society, 2015), while the most common site in the Asian population is the buccal mucosa due to prevalence of betel quid usage (Rao, Mejia, Roberts-Thomson, & Logan, 2013). The pathogenesis of OSCC is multistage and lesions are thought to progress through varying degrees of hyperplasia, dysplasia and carcinoma *in situ* (defined as a localised precancerous lesion without invasion) prior to the development of invasive OSCC (Neville et al., 2015).

Leukoplakia (white patch), erythroplakia (red patch), erythroleukoplakia (combination of red and white patches) and oral submucous fibrosis are among the common potentially malignant lesions in the oral cavity that have increased risk of malignant transformation (Neville et al., 2015; Warnakulasuriya, Johnson, & Van der Waal, 2007). The majority of oral precancers have been documented to be preceded by or associated with leukoplakia (Neville et al., 2015), which has a relatively low malignant transformation rate ranging from 8% to 18% (Chi et al., 2015). In contrast, erythroplakias are less common but have a higher malignant transformation rate and almost all true erythroplakias will demonstrate high-grade epithelial dysplasia, carcinoma *in situ* or develop into invasive SCC (Neville et al., 2015; Reichart & Philipsen, 2005).

Based on WHO classification, OSCCs are graded into three histopathological types according to the tumours' resemblance to normal oral epithelia; approximately 30% of cases being well-differentiated, 60% moderately differentiated and 10% poorly differentiated SCCs (Pindborg, Reichart, Smith, & Van der Waal, 2012; Speight & Farthing, 2018). The histopathological grading considers variables such as degree of keratinisation, cytologic pleomorphism, invasion pattern, tumour thickness, lymphocytic response and mitotic activity (Neville et al., 2015). Well-differentiated OSCC often shows less prominent dysplasia, slower growth rates and metastasise later. It is histologically characterised by features such as evident keratinisation and well-defined tumour islands in the form of keratin pearls. Poorly differentiated OSCC show marked cellular pleomorphism with little or no keratinisation and typically grow rapidly with early metastasis (Neville et al., 2015; Speight & Farthing, 2018). Clinically, the degree of differentiation has high prognostic value (Woolgar, 2006) and is used to predict prognosis and patient survival (Woolgar & Triantafyllou, 2009), although

histological grading can be subjective. This underscores the need for more accurate prognostic tools.

2.1.3 Clinical presentation, assessment and management

The early clinical symptoms of OSCC can be painless and go unnoticed, but the disease usually manifests with indurated ulceration or discoloured patches of tissue in the mouth that could be overlooked as a common ulcer. Other important signs and symptoms of oral cancer include persistent sore throat, lumps in the cheek, and numbness in the mouth and swollen lymph nodes in the neck. Patients might also experience difficulties in swallowing, chewing, tasting and speech.

The diagnosis and treatment of OSCC primarily depend on TNM (tumour-node-metastasis) staging, histopathological grading and the anatomic site of the primary tumour (National Comprehensive Cancer Network, 2018). The diagnosis of oral cancer can only be confirmed by pathological examination of biopsy specimens, while modern imaging modalities such as computed tomography (CT), positron emission tomography (PET), magnetic resonance imaging (MRI) and/or fused CT/PET aid the assessment of tumour extension and staging (Rumboldt, Gordon, Bonsall, & Ackermann, 2006). Other molecular-based methods, such as immunohistochemistry (IHC) to detect epidermal growth factor receptor (EGFR), matrix metalloproteinases (MMPs) and p53 in OSCC (Oliveira & Ribeiro-Silva, 2011) and α-smooth muscle actin (α-SMA) positive cancer-associated fibroblasts (CAFs) in oral stroma (Dourado, Guerra, Salo, Lambert, & Coletta, 2018), have been suggested to have prognostic value, but these are currently not in clinical use.

For early tumours, the main treatment approaches are surgical excision and definitive radiotherapy, in which radiotherapy is also commonly given as adjuvant therapy post-surgery. In addition to surgery as a primary modality, patients with advanced tumours or

recurrence may be treated with radiotherapy, chemotherapy, concurrent chemoradiotherapy and/or targeted therapy. The chemotherapeutic drugs used most often in oral cancers are cisplatin, carboplatin, 5-fluorouracil, paclitaxel and docetaxel (Neville et al., 2015). Other promising molecular-based treatments that are currently being tested in clinical trials include targeted therapies with EGFR inhibitors (e.g. cetuximab), mammalian target of rapamycin (mTOR) inhibitors (e.g. rapamycin) and anti-vascular endothelial growth factor (VEGF) antibodies (H. Lu et al., 2019; Neville et al., 2015; Taberna, Oliva, & Mesía, 2019; Tan, Bai, Saintigny, & Darido, 2019). Importantly, immunotherapy with checkpoint inhibitors, such as programmed cell death protein 1 (PD-1) inhibitors (e.g. pembrolizumab and nivolumab) have shown great promise in a subset of patients in recent trials (Kao & Lou, 2019; National Comprehensive Cancer Network, 2018). Other recent advances in the clinical management of HNSCC include image-based and adaptive radiotherapy, sentinel node biopsy (SNB) and transoral robotic resections (TORS) (Leemans et al., 2018; Schilling et al., 2015).

OSCC is a highly invasive carcinoma with frequent invasion of lymphatic, blood vessels and perineural space (Roh, Muelleman, Tawfik, & Thomas, 2015; Speight & Farthing, 2018). Unfortunately, most OSCCs are HPV-negative and these tumours seem to have a less favourable treatment outcome and a higher risk of developing secondary tumours, compared to HPV-positive HNSCC (Chi et al., 2015). The prognosis and curability rate of OSCC are also typically poor as the majority of the tumours are diagnosed as stage of III or IV with nodal or distant metastases (Speight & Farthing, 2018). The five-year survival for patients with stage III and IV tumours is less than 60% but if detected early (stage I and II), survival may be as high as 80% (Speight & Farthing, 2018). At present, early diagnosis remains a key challenge. The discovery of

specific tumour biomarkers, as an alternative to the classic TNM, would aid early diagnosis and prognosis.

2.1.4 Oral carcinogenesis and field cancerisation

The development of OSCC is a multi-step process and the disease progresses through various grades of premalignant dysplasia prior establishing invasive carcinoma as a result of progressive accumulation of molecular alterations and the effects of carcinogens (Califano et al., 1996; Sapp, Eversole, & Wysocki, 2004). Like most solid tumours, OSCCs are clonal in origin (Bedi, Westra, Gabrielson, Koch, & Sidransky, 1996). It has been concluded that in order for a normal cell to be transformed into a malignant tumour cell, five distinct requisite mutations and alternations are necessary in humans (Hahn & Weinberg, 2002).

OSCC is likely to originate from the stem cells of the oral mucosa as these cells have extensive proliferative capacity and the ability to host the number of necessary mutations, owing to their prolonged existence within the oral epithelial tissue (Owens & Watt, 2003). These genetically altered stem cells can subsequently form a patch or "field" of daughter cells with similar genetic alterations through clonal expansion and selection. This phenomenon, referred to as field cancerisation, describes the progressive replacement of normal oral epithelium by a genetically aberrant mucosal field, and commonly manifests as leukoplakia or alternatively could be invisible clinically (Braakhuis, Tabor, Kummer, Leemans, & Brakenhoff, 2003).

A monoclonal, preneoplastic precursor field does not display metastatic or invasive behaviour. Additional heterogeneous genetic mutations within this field of cells may progressively accelerate the expansion of this cell population and result in premalignant lesions, some of which will eventually transform into cancer (Braakhuis, René Leemans, & Brakenhoff, 2004; Van der Waal, Schepman, Van der Meij, & Smeele,

1997). The heterogeneity of these genetic alterations is thought to explain differences in patient responses and treatment outcomes, even though some tumours are diagnosed at the same clinical stage and localisation.

The concept of field cancerisation has important clinical implications, as there is a high frequency of local recurrence (arising from residual cancerous cells). Recurrences include second field tumours (SFT, second tumour develops from same field of primary tumour) and second primary tumours (SPT, second tumour with independent origin from the initial tumour) after surgical excision of the primary carcinoma (Braakhuis et al., 2003; Braakhuis et al., 2002). Hence, early detection and monitoring of field of cancerisation would have profound consequences to improve cancer prevention and reduce risk of recurrence, SFT or SPT.

2.2 Molecular basis of HNSCC

Whilst the focus of the present study is on OSCC, the molecular basis of the disease is often described in the wider context of HNSCC. Therefore, a detailed description of the common genetic alterations of HNSCC is given below.

As our understanding of HNSCCs has progressed, it is now widely accepted that HSNCCs can be divided into two different subgroups, i.e. HPV-positive and HPV-negative (Seiwert et al., 2015). Comprehensive genomic data from HNSCCs, results from The Cancer Genome Atlas (TCGA) consortium, have supported the concept that HPV-negative HNSCCs are biologically distinct from HPV-positive HNSCCs in terms of their gene expression and DNA methylation profiles, and intriguingly, a distinct subgroup of HPV-negative HNSCCs with very few copy number alterations has also been identified (Cancer Genome Atlas Network, 2015). An integrated genomic progression model of HNSCC carcinogenesis is depicted in Figure 2.2 and further described below.

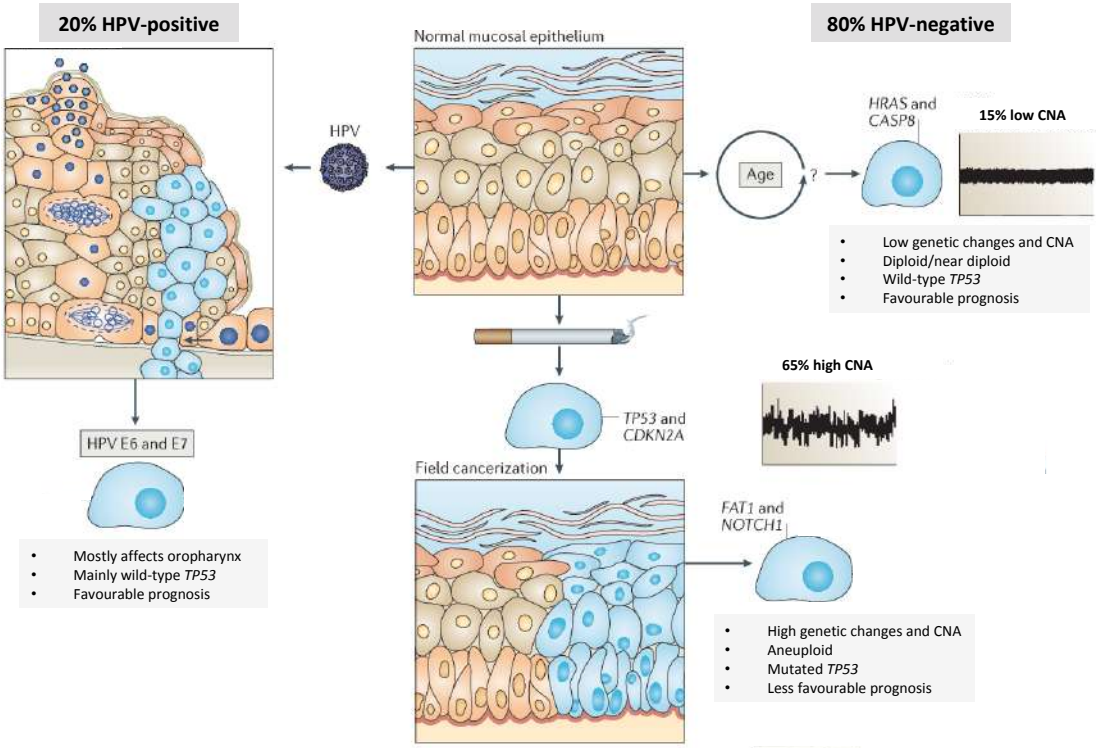


Figure 2.2: Integrated genomic progression model of head and neck squamous cell carcinoma carcinogenesis

HNSCCs can be classified into three genetic subclasses at present: HPV-positive tumours that contain transcriptionally active HPV, HPV-negative tumours that are characterised by either high copy number alterations (CNA) or low CNA. HPV-negative HNSCCs with low chromosomal instability might arise from different molecular routes, independent of the pathways progress by classical HPV-negative high CNA subgroup. At present, the specific aetiology of this subgroup remains vague, but it is hypothesised to be attributed to ageing. The precise clinical impact of this HNSCC subclass is still preliminary and uncertain due to a limited number of studies. The indicated prevalence rates are estimations adopted from Leemans et al., 2011. Figure modified from Leemans et al., 2018.

2.2.1 HPV-negative HNSCCs

HPV-negative HNSCCs, including OSCCs, which are primarily associated with tobacco and alcohol, are typically characterised by field changes, extensive genetic damage and frequent chromosomal gains and losses (Leemans et al., 2018). Comprehensive epigenetic and genomic analyses revealed that these genetic alterations that are frequently detected in HPV-negative HNSCCs are relatively rare in HPV-positive subclasses (Cancer Genome Atlas Network, 2015; Chung et al., 2015). The

histological progression and severity of dysplastic changes that occur during HPV-negative oral carcinogenesis were shown to reflect the number of tumour-associated genetic defects in oral cells (Todd, Donoff, & Wong, 1997). While the chronological order or precise timing of these genetic defects appears to be less of a contributing factor in determining the malignancy of cells, the total number of genetic alterations is the major factor in determining the carcinogenic fate of cells (Califano et al., 1996).

Classical HNSCCs are characterised by extensive chromosomal rearrangement. Loss of heterozygosity (LOH) at chromosomes 3p, 9p and 17p are amongst the early genetic abnormalities that contribute to the malignant transformation of oral dysplasia, while modifications at chromosomal arms 4q, 8p and 11q typically present in late-stage carcinomas (Califano et al., 1996). Importantly, these chromosomal arms harbour genes that are essential for genomic stability, cell cycle regulation and cellular senescence. Chromosome 3p harbours the telomerase repressor genes such as *SETD2* (Cuthbert et al., 1999) and tumour suppressor genes, such as *FHIT* (fragile histidine triad gene; 3p14.2) (González et al., 1998; J. I. Lee et al., 2001). The loss of genes on chromosome 3p can result in replicative stress and DNA damage and is an early event in the pathogenesis of HNSCCs (Califano et al., 1996) even though it was surprisingly not found in the majority of HNSCC in cultures (de Boer et al., 2019). The chromosome 9p21 *INK4A* locus carries two essential tumour suppressor genes, *CDKN2A* and *ARF*, which encode $p16^{Ink4a}$ and $p14^{ARF}$ proteins, respectively (Sharpless & DePinho, 1999). The chromosomal loss of 9p21 occurs in up to 80% of oral precancerous lesions and its gene products are often inactivated by deletion or methylation (Sailasree et al., 2008). The combined effect of loss of *CDKN2A* and gain of *CCND1* allow the premalignant/malignant cells to proceed through G1-S checkpoint without DNA repair and continuously propagate the genetic alterations in the cell cycle (Leemans et al., 2018).

LOH of chromosome 17p and missense mutation of *TP53* gene (resides at 17p13) are also prevalent genetic events observed in early dysplastic fields and OSCCs (Tabor et al., 2001). The p53 protein is a key tumour suppressor and apoptosis inducer in many tumour types. Somatic mutations of p53 are found in 60-80% of HNSCCs and other genes within the p53 pathway are also frequently mutated (Leemans et al., 2018; A. J. Levine & Oren, 2009). Activation of p53 has a multitude of functions in DNA repair, cell cycle arrest, apoptosis, metabolism, autophagy and senescence, which collectively function to prevent tumour formation (A. J. Levine & Oren, 2009; Vousden & Ryan, 2009; White, 2016). Inactivated or mutant p53 provides tumour cells with growth advantages and has been widely implicated with tumour progression and resistance of malignant cells to anticancer therapy (Schulz-Heddergott & Moll, 2018). Besides LOH, other signature chromosomal alterations found in OSCC also include gains on chromosomal arms 3q, 5q, 7p, 8q and 20p, and copy number variation may be associated to a subtype of OSCCs with more aggressive behaviour (Leemans, Braakhuis, & Brakenhoff, 2011; Samman et al., 2015).

Consistent with other solid tumours, genetic alterations in tumour suppressor genes and oncogenes are frequently detected in HPV-negative HNSCCs. Aberrations in tumour suppressor genes, such as *TP53*, *CDKN2A*, *PTEN*, *FAT1*, *AJUBA*, *NOTCH1*, *NOTCH2*, *KMT2D*, *CDH1*, *FHIT*, *SMAD4*, *NSD1* and *CSMD1* have been reported (Agrawal et al., 2011; Cancer Genome Atlas Network, 2015; de Boer et al., 2019; Leemans et al., 2011; Leemans et al., 2018; Neville et al., 2015; Samman et al., 2015; Veeramachaneni et al., 2019). Moreover, several oncogenes include *CCND1*, *EGFR*, *PIK3CA*, *HRAS*, *MET*, *STATS*, *MYC*, *COX-2* and *BCL-2* have been shown to be amplified or displayed gain of function mutations in HNSCCs (J. A. Bauer et al., 2005; Cancer Genome Atlas Network, 2015; Das, Majumder, & DasGupta, 2000; Leeman, Lui, & Grandis, 2006; Leemans et al., 2018; Pandey, Prakash, Santhi, Soumithran, &

Pillai, 2008). Table 2.2 shows a list of frequently observed genetic changes in HPV-negative HNSCCs, including OSCCs.

Table 2.2: Cancer genes and pathways with frequent genetic changes in HPV-negative HNSCCs

Cellular process	Chromosome	Gene	Protein	Type of gene
Cell cycle	3p14	*FHIT*	FHIT, Fragile histidine triad protein	Tumour suppressor
	9p21	*CDKN2A*	$p16^{Ink4a}$	Tumour suppressor
	11q13	*CCND1*	G1-S-specific cyclin D1	Oncogene
	17p13	*TP53*	p53	Tumour suppressor
Growth signal	7p11	*EGFR*	EGFR	Oncogene
Survival	3q26	*PIK3CA*	Catalytic p110α subunit of class PI3Ks	Oncogene
	10q23	*PTEN*	PTEN	Tumour suppressor
TGF-β signalling	18q21	*SMAD4*	SMAD4	Tumour suppressor
HGF/c-Met signalling	7q31	*MET*	MET	Oncogene
WNT signalling	4q35	*FAT1*	Protocadherin FAT1	Tumour suppressor
	14q11	*AJUBA*	LIM domain-containing protein AJUBA	Tumour suppressor
	9q34	*NOTCH1*	NOTCH1	Tumour suppressor

Data summarised from Leemans et.al, 2011 and Leemans et al., 2018.

2.2.2 Subgroups of HPV-negative HNSCCs

In addition to the subgroups of HNSCCs that are distinguishable based on their HPV status, the use of karyotyping, cytogenetic analyses and array comparative genomic hybridisation (CGH) have demonstrated the existence of a distinct subclass of HPV-negative tumours with minimal or silent copy number alterations (CNA) (Cancer Genome Atlas Network, 2015). Differing from most classical HPV-negative oral tumours that are aneuploid, this specific subgroup of HNSCC has a seemingly near-normal genome that is diploid or almost diploid (Hermsen et al., 2001; Jin et al., 2006; Smeets et al., 2009).

The heterogeneity in HNSCCs and dysplasia was reported by Hunter et al. (2006) who showed using *in vivo* in biopsies and *in vitro* in culture that approximately 40% of OSCCs and 60% of dysplasia were mortal in culture. It has been shown that two variants, i.e. genetically unstable (GU-, immortal) and genetically stable (GS-, mortal) both exist in oral dysplasias and OSCCs (Edington, Loughran, Berry, & Parkinson, 1995; O Loughran et al., 1997; Veeramachaneni et al., 2019). These two classes generally have distinguishable molecular backgrounds; keratinocytes of GU-OSCC have p53 and $p16^{Ink4a}$ inactivation, deregulated telomerase, extensive CNA and LOH and poorer prognosis, whilst their stable counterparts typically have wild-type p53 and $p16^{Ink4a}$, regulated telomerase, minimal CNA and LOH and better prognosis in patients (Edington et al., 1995; Hunter et al., 2006; O. Loughran et al., 1996; McGregor et al., 2002). Further, it was shown that both variants of OSCC retained most of the gene expression profiles found in their respective dysplasias but with additional carcinoma-associated transcriptional changes (Hunter et al., 2006). The development of GU- and GS-OSCCs from their respective preceding dysplasias involves distinct transcriptional changes, indicating that immortal and mortal tumours progress along divergent pathways (Hunter et al., 2006).

Later, the findings of Hunter et al. (2006) were verified and extended by independent research groups who showed that this separate CNA-silent subgroup was typically characterised as having wild-type *TP53*, retention of chromosome 3p, activating *HRAS* mutations, inactivation of caspase 8 and a more favourable prognosis (Cancer Genome Atlas Network, 2015; Gross et al., 2014; Smeets, Braakhuis, Leemans, & Brakenhoff, 2007). Furthermore, this phenotype has proficient DNA mismatch repair and was surprisingly observed more frequently in females without a history of alcohol consumption and smoking (Smeets et al., 2007). At present, the specific aetiology of this subgroup remains uncertain, but it is generally hypothesised to be attributed to ageing. The impact of this particular subgroup in clinical trials is still uncertain due to the limited number of studies (Leemans et al., 2018).

2.2.3 HPV-positive HNSCCs

After decades of research, clear evidence now exists to identify HPV as a novel etiological factor for a specific subset of HNSCCs. In particular, HPV infection is specifically associated with SCCs of the oropharynx and HPV-positive-OPSCC is now viewed as a distinct entity to HPV-negative HNSCC (including OSCC and OPSCC) (Leemans et al., 2018; Mirghani, Amen, Moreau, & St Guily, 2015; Speight & Farthing, 2018). OPSCCs usually arise from tonsillar tissues of oropharynx, pharynx, Waldeyer ring, tonsil and base of tongue (World Health Organization, 2016). The high-risk genotype HPV-16 is the most common HPV subtype and specifically accounts for the majority (approximately 83%) of HPV-positive OPSCCs (Castellsagué et al., 2016; Leemans et al., 2018). The prevalence of HPV-positive OPSCC appears to vary substantially by geographical regions (Castellsagué et al., 2016) and has a rising trend particularly in developed countries (Dalianis, 2014; Deschler, Richmon, Khariwala, Ferris, & Wang, 2014). Recent studies have reported that more than 60% of OPSCCs are associated with HPV in the United States (Benson, Li, Eisele, & Fakhry, 2014).

HPV-positive OPSCCs generally have better treatment outcome than HPV-negative HNSCCs despite often exhibiting lymph node metastases clinically (Spector et al., 2014). Hence, HPV status of the tumour is routinely assessed for HNSCC patients (Chi et al., 2015) by performing $p16^{Ink4a}$ IHC staining in combination with reverse transcription PCR for HPV viral oncogenes *E6* and *E7* mRNA or DNA *in situ* hybridisation (ISH) for high-risk HPV sequences (Bishop, Lewis Jr, Rocco, & Faquin, 2015; Holzinger et al., 2017; Prigge, Arbyn, von Knebel Doeberitz, & Reuschenbach, 2017). IHC staining for $p16^{Ink4a}$ is a useful surrogate marker for HPV detection in OPSCCs as the HPV E7 oncoprotein targets retinoblastoma protein (pRb) for degradation, resulting in an overexpression of $p16^{Ink4a}$ (J. S. Lewis, 2012). The use of these reliable diagnostic tests has clearly shown that only a subgroup of HNSCCs is HPV-positive; for example, OSCCs rarely express $p16^{Ink4a}$ and are associated with HPV with only less than 4% of OSCCs being HPV-positive (Castellsagué et al., 2016). The conventional staging system for OSCCs based on prognostic indicators such as grade and invasion depth does not apply to HPV-positive OPSCCs, and HPV-related lesions in the oropharynx are currently graded based on their $p16^{Ink4a}$ status, as a marker of HPV association, together with traditional clinical characteristics (Speight & Farthing, 2018).

It is still unclear why HPV-associated OPSCCs have an overall better prognosis and improved survival compared to HPV-negative HNSCCs, but it could be due to their less extensive genomic alterations and field changes (Chaturvedi, 2012). Comprehensive genomic characterisation revealed that genes which are commonly found aberrant in HPV-negative HNSCCs such as *TP53* and *CDKN2A*, are essentially unaffected in HPV-positive HNSCCs (Cancer Genome Atlas Network, 2015; Chung et al., 2015). In HPV-related HNSCCs, HPV E6 and E7 oncoproteins function through the inactivation of p53 and pRb, which is functionally equivalent to the genetic or epigenetic damages in HPV-

negative HNSCCs that leads to the inactivation of p53 and pRb pathways (Leemans et al., 2018). Genomic profiling and RNA sequencing (RNAseq) studies revealed that HPV-positive OPSCCs have more heterogeneous gene expression profiles, and could be generally classified into two biologically distinct subgroups of tumours, namely one associated with HPV-keratinocyte differentiation and oxidative stress (HPV-KRT) and the other with transcriptional profiles related to HPV-immune responses and mesenchymal cell differentiation (HPV-IMU) (Keck et al., 2015; Y. Zhang et al., 2016). However, this has not been fully validated and is still under extensive research (Leemans et al., 2018).

2.3 The tumour microenvironment (TME)

The hallmarks of cancer first described in 2000 comprise six distinct and complementary biological capabilities namely (i) sustaining proliferative signalling, (ii) evading growth suppressors, (iii) enabling replicative immortality, (iv) resisting cell death, (v) stimulating angiogenesis, and (vi) inducing invasion and metastasis (Hanahan & Weinberg, 2000). Together with the two additional hallmarks – evading immune destruction and reprogramming of energy metabolism, they lay the foundation for our understanding of cancer biology (Hanahan & Weinberg, 2011). For decades, neoplastic cells were the focus of attention for cancer researchers and tumorigenesis was considered as a multistep, cell-autonomous process in which the infinite proliferation of neoplastic cells is predominantly due to consecutive accumulation of genetic alterations in the genome (Kenny, Lee, & Bissell, 2007; Vogelstein & Kinzler, 1993). In recent years, this "epitheliocentric" view of oncogenesis has been gradually replaced by the tumour microenvironment (TME) paradigm (Maman & Witz, 2018). This concept was originally proposed by Stephen Paget in 1889 in his "seed and soil" theory where he postulated that a particular "seed" i.e. neoplasm frequently metastasises to "soil" i.e. sites depending on the similarity of environments between the original and secondary

tumour sites (Jodele, Blavier, Yoon, & DeClerck, 2006; Paget, 1889). We now know that cancer development is not driven solely by transformed epithelial cells, but occurs via an integrated system of neoplastic cells and their surrounding microenvironment (Egeblad, Nakasone, & Werb, 2010). Therefore, cancer is now considered to be a disease which involves interactions and crosstalk between multiple cell types within an active TME (Maley et al., 2017). The TME contains not only the neoplastic epithelial cells but also many other seemingly normal resident and recruited host cells that can be corrupted by the transformed cells (Maman & Witz, 2018), leading to the eventual acquisition of hallmark traits (Hanahan & Weinberg, 2011).

In healthy tissues, the normal cellular microenvironment functions to allow normal tissue development, remodelling and homeostasis, thereby indirectly inhibiting malignant cell growth, (Junttila & de Sauvage, 2013). However, the presence of transformed neoplastic cells may initiate crucial modifications that can shape the surrounding microenvironment to synergistically support cancer progression, allowing the transformed cells to circumvent the body's defences and metastasises to other locations. This occurs because tumours have the capability to recruit stromal cells, enhance matrix remodelling, support vascular networks, enslave the immune system and shape the microenvironment to support both the development of malignant and non-malignant cells (Junttila & de Sauvage, 2013). The influence of the TME was first demonstrated in experiments showing that clonal transformed cells propagated in syngeneic mice *in vivo* were more tumorigenic than the same cells cultured *in vitro* (Halachmi & Witz, 1989; Witz, 2006), which confirmed the significance of factors in the TME in the promotion and maintenance of cancer.

The TME acts as a local store to sequester a plethora of molecules, metabolites and growth factors, which are known to provide contextual cues for cell proliferation,

migration, angiogenesis and tumour metastasis (Hanahan & Weinberg, 2011; Sounni & Noel, 2013). Furthermore, it has also become evident that the TME may facilitate the acquisition of drug resistance, thus affecting the sensitivity of tumour cells towards chemotherapeutic drugs and reducing the accessibility of drugs from reaching their targets (McMillin et al., 2010; McMillin, Negri, & Mitsiades, 2013; Meads, Gatenby, & Dalton, 2009; Straussman et al., 2012).

2.3.1 Cellular and non-cellular complexity of the TME

Much of the complexity within carcinomas, including OSCCs, is found in the stromal compartment and the non-cancerous cells of the TME have dynamic and often tumour-promoting characteristics at all stages of tumorigenesis. The TME consists of numerous non-malignant cell types, including CAFs, myofibroblasts, endothelial cells, adipocytes, smooth muscle cells, pericytes, immune cells such as neutrophils, basophils, eosinophils, mast cells, natural killer (NK) cells, macrophages, dendritic cells, T and B lymphocytes (Balkwill, Capasso, & Hagemann, 2012; Hanahan & Coussens, 2012). Additionally, the non-cellular compartment of the TME, such as extracellular matrix (ECM) molecules, together with other chemical and physical parameters such as pH, hypoxia, interstitial pressure, oxygen tension and fluid flux, are also recognised as important regulators of carcinogenesis (Sounni & Noel, 2013). Figure 2.3 shows the multiple stromal cell types and subtypes of non-cellular constituents in the TME of OSCC (Rivera & Venegas, 2014).

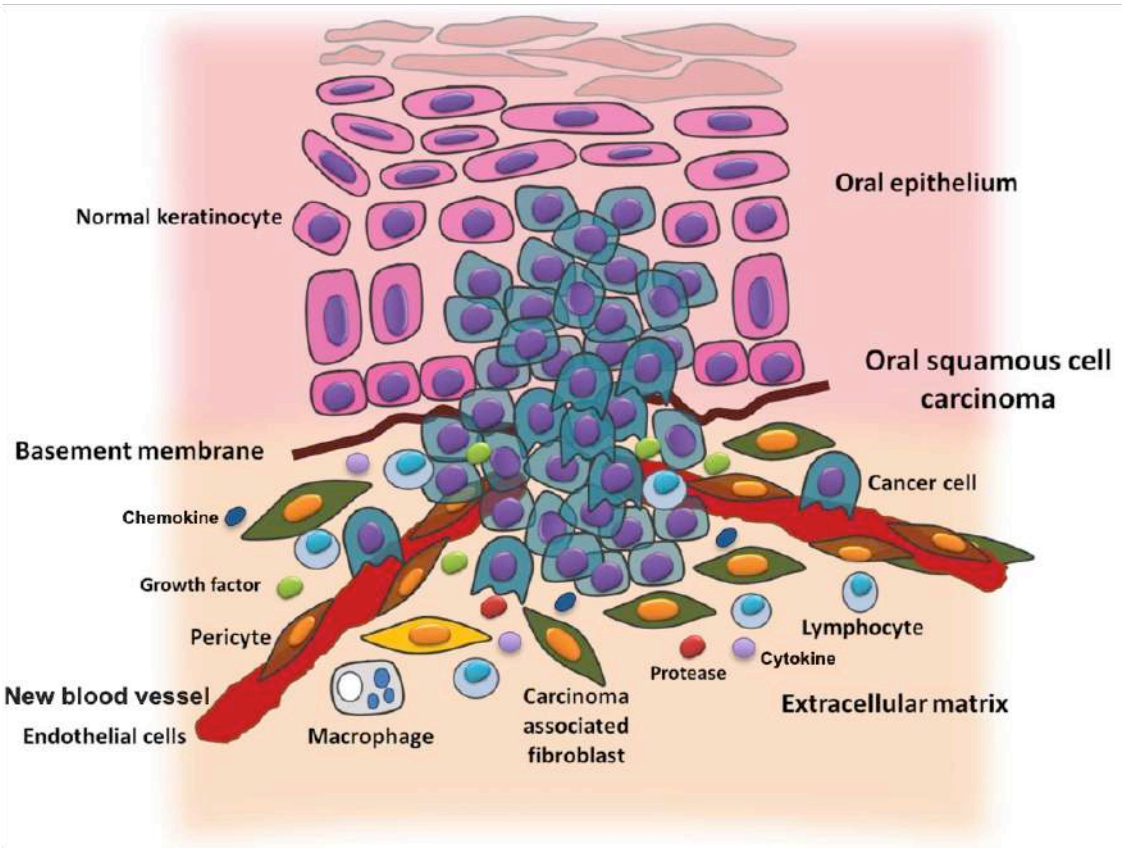


Figure 2.3: Cellular and non-cellular constituents of the tumour microenvironment in OSCC

Apart from the cancer cells, the TME of most solid tumour comprises a heterogeneous mass of non-malignant host cellular and non-cellular constituents that collectively participate in tumour growth and progression. In normal healthy tissues, the stromal compartment is separated from the oral epithelium by the well-delineated basement membrane. During tumourigenesis, both epithelial and stromal compartment co-evolve and undergo modifications to enable primary, invasive and metastatic growth. Tumour cells release chemical signals to recruit and activate host cells in the TME. Fibroblasts become activated and differentiated into cancer-associated fibroblasts (myofibroblasts). The recruited inflammatory cells increase in number. As a result of all these events, expression of growth factors and ECM molecules is promoted. ECM is degraded, facilitating the progression of carcinoma *in situ* to metastasis of invasive tumour cells. New blood vessels are formed to support angiogenesis. The ECM-orchestrated events and reciprocal interactions between tumour cells and host cells in the TME are critical to establishing a favourable microenvironment for tumour development and metastatic dissemination. Figure adapted from Rivera & Venegas, 2014.

2.3.2 Stromal components: fibroblasts and extracellular matrix

In order to metastasise, tumour cells migrate from the primary tumour, invade into the proximate tissue and its ECM, infiltrate into the blood circulation or lymphatic systems via basement membrane and endothelial lining transmigration, and finally extravasate and metastasise to targeted sites (Ungefroren, Sebens, Seidl, Lehnert, & Hass, 2011). As a key element in the TME, the tumour stroma has substantial effects on facilitating the achievement of these processes, as well as therapy resistance (Hanahan & Weinberg, 2011; Valkenburg et al., 2018). These effects and interactions are gained via the intrinsic properties of the tumour stroma and additional pro-tumorigenic properties obtained as part of an adaptive stress response to cancer therapy intervention (Ferrarelli, 2015; Valkenburg et al., 2018).

The constituents of stroma include specialised connective cells, importantly fibroblasts, and the non-cellular ECM (Valkenburg et al., 2018). The ECM is a collection of extracellular molecules and metabolites that provide structural and biochemical support to neighbouring cells and mediate intercellular paracrine signalling (Mammoto & Ingber, 2010). Fibroblasts play multiple roles in shaping and maintaining the ECM in which they reside. The canonical functions of fibroblasts include degradation of ECM by the secretion of proteolytic enzymes, such as collagenase and matrix metalloproteinases (MMPs) and re-deposition of ECM by synthesising collagen and fibronectin (Alexander & Cukierman, 2016). This constant ECM remodelling serves as a reservoir for a continuous supply of growth factors and signalling proteins in the stroma to maintain tissue homeostasis and wound repair (Valkenburg et al., 2018).

As a key source of ECM proteins, fibroblasts are principal mediators of tissue injury, scar formation and fibrosis (Kalluri, 2016). In normal healthy epithelial tissues, fibroblasts are quiescent but they can be activated, transiently increased in abundance

and recruited to sites of wound healing in response to paracrine signals such as transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) (Kalluri, 2016). Activated fibroblasts, also referred to as myofibroblasts due to the induced expression of α-SMA (*ACTA2*), a smooth muscle cell-associated cytoskeletal protein (Micallef et al., 2012). The functions of fibroblast activation include increased secretion of ECM components, cytokines and chemokines, the gain of migratory and proliferative properties, recruitment of immune cells and modification of tissue architecture (Parsonage et al., 2005; Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002).

Myofibroblasts, induced by TGF-β signalling (Rønnov-Jessen & Petersen, 1993), can reverse their acquired activated phenotypes, decrease in number and restore their resting phenotype once the tissue repair stimuli are attenuated (Tomasek et al., 2002). In most healthy non-malignant or premalignant tissues, fibroblasts in the stroma are tumour-suppressive in nature and their ability to influencing tumourigenic hallmarks such as proliferation and motility of epithelial cells is tightly regulated (Trimboli et al., 2009; Valkenburg et al., 2018). During the early stages of carcinogenesis in certain cancer types, an inactive stroma can also confer tumour-suppressing properties, thus preventing tumour progression (Rhim et al., 2014). However, an acidic and hypoxic stroma can be formed when carcinoma *in situ* or secondary tumours progress to a more advanced phenotype (Amend & Pienta, 2015), leading to the establishment of a reactive stroma (Valkenburg et al., 2018). An activated or reactive stroma has extensive stromal cell infiltration into a wound site or cancer lesion, accompanied by enhanced deposition of ECM proteins (Dvorak, 1986). Differing from an acute tissue injury, cancerous lesions are very much similar to chronic inflammation, in which persisting injurious stimuli are often sustained, leading to perpetual activation of fibroblasts in the reactive tumour stroma (Kalluri, 2016; Kalluri & Zeisberg, 2006). Owing to the presence of reactive

stroma, fibroblasts at the tumour sites typically resemble myofibroblasts (Shiga et al., 2015; Tuxhorn et al., 2002). Due to some of these striking similarities shared between tumour and chronic tissue repair responses, tumours are also regarded as "wounds that do not heal" (Dvorak, 1986, 2015). Although beneficial in wound healing and tissue regeneration, the abundance of irreversibly activated myofibroblasts can be detrimental in chronic inflammation and tumour sites (Foster, Jones, Ransom, Longaker, & Norton, 2018).

2.4 Cancer-associated fibroblasts (CAFs)

Fibroblasts found at tumour sites are termed CAFs and these cells represent one of the preponderant populations of stromal cells within the TME. α-SMA, fibroblast activation protein (FAP) and platelet-derived growth factor receptor-β (PDGFRβ) are some of the characteristic markers of CAFs, but they are not restricted to CAFs or their fibroblast lineage (Öhlund, Elyada, & Tuveson, 2014). The molecular definition and terminology of CAFs are debatable due to their high complexity and plasticity (Öhlund et al., 2014). A recent review by Kalluri (2016) also termed CAFs as tumour-associated fibroblast (TAFs), activated fibroblasts or myofibroblasts. Fibroblasts can be recruited from different potential sources and reprogrammed by tumour cells or other factors like hypoxia and ROS, converting these tumour-suppressing fibroblasts to tumour-supportive CAFs as the tumour evolves (Öhlund et al., 2014; Toullec et al., 2010). CAFs can originate from heterogeneous resident precursors: (i) fibroblasts adjacent to neoplasm can become activated, (ii) cells of the vasculature system such as smooth muscle cells and endothelial cells (Rønnov-Jessen, Petersen, Koteliansky, & Bissell, 1995), (iii) local or bone marrow-derived mesenchymal stem cells (MSCs) (Paunescu et al., 2011; Quante et al., 2011) or (iv) fully differentiated epithelial cells can be induced to differentiate into myofibroblasts through epithelial-to-mesenchymal transition (EMT) (Hanahan & Coussens, 2012; Orimo & Weinberg, 2007; Radisky, Kenny, & Bissell,

2007; Willis, duBois, & Borok, 2006). The multiple origins of CAFs may contribute to their phenotypic and functional heterogeneity in the TME (Kalluri, 2016).

These recruited myofibroblasts to the tumour sites have been implicated in stimulating pro-tumourigenic phenotypes in a number of ways and notably by enhancing cancer cell proliferation, angiogenesis, metastasis and invasion (Hanahan & Weinberg, 2011). Early experiments demonstrated that CAFs isolated from various tumours exhibited distinct properties and gene expression profiles compared to normal fibroblasts derived from healthy individuals (M. Bauer et al., 2010; Delinassios, Kottaridis, & Garas, 1983; Ronnov-Jessen, Petersen, & Bissell, 1996). Unlike normal fibroblasts which are bystanders and quiescent within the ECM, CAFs gain proliferative ability and are functionally diverse, adding to the complexity of the dynamic TME (Kalluri, 2016; Östman & Augsten, 2009). They have been demonstrated to play a regulatory role in promoting cancer progression by exhibiting enhanced migratory capability, autocrine signalling and dynamic secretome (Kalluri, 2016).

2.4.1 Tumour-promoting functions of CAFs

It has been clearly demonstrated that CAFs can stimulate and support the hyperproliferation of tumour cells by the secreting high amounts of mitogenic epithelial growth factors such as hepatocyte growth factor (HGF), epidermal growth factor (EGF) family members, insulin-like growth factor 1 (IGF1), fibroblast growth factors (FGFs) and stromal cell-derived factor-1/CXC motif chemokine ligand 12 (SDF1/CXCL12) (Cirri & Chiarugi, 2012; Hanahan & Coussens, 2012; Orimo et al., 2005). However, CAFs do not simply influence the growth of neoplastic cells by sustaining proliferative signalling and we have known for some time that they can enhance various malignant phenotypes in co-culture systems (Dimanche‐Boitrel et al., 1994; Orimo et al., 2005).

Subsequently, it has been shown that CAFs regulate a number of key processes during carcinogenesis and also regulate some therapeutic responses, as outlined below.

CAFs can directly or indirectly orchestrate tumour angiogenesis (Kalluri, 2016). For example, they can produce a variety of potent angiogenic signalling molecules such as VEGF, FGFs, PDGF-C and interleukin IL-8/CXCL8 (Crawford et al., 2009; Hanahan & Coussens, 2012). Secretion of CXCL12 by CAFs can stimulate the recruitment of endothelial progenitor cells, directly increasing vascularisation of tumours (Orimo et al., 2005; Orimo & Weinberg, 2007). Expression of certain MMPs, e.g. MMP9 and MMP13 by CAFs can indirectly influence angiogenesis, mainly by modulating the bioavailability of VEGF (Bergers et al., 2000; Kessenbrock, Plaks, & Werb, 2010; Lederle et al., 2009). CAFs can also respond to hypoxia in regulating pro-angiogenic factors like hypoxia-induced angiogenesis regulator (HIAR) and hypoxia-induced factor (HIF), which in turn mediate the expression of VEGF-A (De Francesco et al., 2013; Kugeratski et al., 2019). Finally, CAFs produce cytokines that recruit pro-angiogenic immune cells, such as macrophages and neutrophils, thereby indirectly supporting capillary sprouting (Corliss, Azimi, Munson, Peirce, & Murfee, 2016; Hanahan & Coussens, 2012).

CAFs are also key players in creating an inflammatory and immunosuppressive microenvironment (Gieniec, Butler, Worthley, & Woods, 2019). They can release a repertoire of pro-inflammatory mediators such as osteopontin, cyclooxygenase 2 (COX2), CXCL1, CXCL2, interleukin (IL)-6 and IL-1β in a nuclear factor (NF-κB)-dependent manner (Erez, Truitt, Olson, & Hanahan, 2010). This pro-inflammatory gene signature can also mediate the tumour-enhancing inflammation in the TME. Notably, certain CAF populations can convert into a senescent state and acquire a unique secretome known as the senescence-associated secretory phenotype (SASP), which

includes a spectrum of cytokines, chemokines and pro-inflammatory mediators that drive malignant transformation (Pazolli et al., 2012; Pazolli et al., 2009). Co-culture systems of CAFs and HNSCC cells revealed that crosstalk between tumour cells and CAFs is very much influenced by these soluble factors secreted by CAFs (Jung et al., 2010; Peltanova, Raudenska, & Masarik, 2019).

CAFs can also contribute to tumour immune evasion by inducing an immunosuppressive TME and supporting tumour vasculature (Gieniec et al., 2019; Hanahan & Coussens, 2012). CAFs are implicated in the deposition of a desmoplastic (dense collagenous) stroma during ECM remodelling that could limit the motility and infiltration of tumour-killing T cells to the tumour site (Neesse, Algül, Tuveson, & Gress, 2015; Salmon et al., 2012). Additionally, an extensive tumour vasculature can also act as a barrier to T cell influx into the tumour mass, thereby facilitating the escape of tumour cells from immune surveillance (Hanahan & Coussens, 2012). For example, in pancreatic carcinoma, it was demonstrated that CXCL12 expressed by CAFs could inhibit T cell extravasation and entry to the tumour mass (Feig et al., 2013). Immunoinhibitory PD-1 ligand (PD-L1) expressing CAFs have also been shown to suppress T cell activation and function in several different cancer models. Collectively, these studies strongly suggest that CAFs play a regulatory role in limiting cancer cell killing by T cells and creating an immunosuppressive TME (Monteran & Erez, 2019).

CAFs can convert an anti-metastatic stroma into a pro-metastatic environment through the modulation of ECM proteins and ECM remodelling (Murata, Mekada, & Hoffman, 2017). The crosstalk between tumour cells and CAFs is regulated by chemical signals released from ECM components and by the organisation of ECM. For example, the expression of MMPs by CAFs enables the degradation and metabolism of ECM, facilitating tumour cell penetration through the ECM, intravasation into the blood or

lymphatic circulation and dissemination to secondary metastatic sites (Kessenbrock et al., 2010). Importantly, TGF-β, which is often highly expressed by CAFs induces EMT in tumour cells, which promotes their motility and invasion (Y. Yu et al., 2014). Furthermore, CAFs were shown to physically remodel ECM and accelerate cancer cell invasion by generating directional tracks in the ECM via which the tumour cells follow during stromal invasion (Gaggioli et al., 2007; Labernadie et al., 2017; Neri et al., 2015).

CAFs have also been implicated in reducing cancer cell drug sensitivity, limiting drug access, driving drug resistance and tumour relapse (Kalluri, 2016; Su et al., 2018; Valkenburg et al., 2018). As a primary source of collagen type I, CAFs contribute to the reduced uptake of chemotherapeutic drugs in tumours (Dangi-Garimella, Sahai, Ebine, Kumar, & Munshi, 2013). Due to their role in building a rigid ECM, CAFs indirectly create a physical barrier that can affect drug diffusion and delivery (Miao et al., 2017; Provenzano et al., 2012). Therapy-induced DNA damage can cause stress responses in CAFs, which in turn secrete many tumour-promoting factors that could contribute to therapy resistance (Valkenburg et al., 2018). In an *in vitro* study of HNSCC, conditioned media collected from CAFs were demonstrated to increase cisplatin resistance (Steinbichler, Metzler, Pritz, Riechelmann, & Dudas, 2016). Similarly, chemotherapy can trigger activation in quiescent fibroblasts to develop CAF phenotypes (Peiris-Pagès, Sotgia, & Lisanti, 2015), which could lead to the development of chemoresistance.

2.4.2 TME and CAFs as targets for cancer therapy

Since many of the cells, molecules, metabolites and signalling intermediates found in the TME are involved in the regulation of some fundamental aspects of tumorigenesis such as EMT, migration, invasion, metastasis, angiogenesis, neovascularisation,

apoptosis and chemoresistance, the TME represents a particularly exciting candidate for targeted therapeutic interventions in addition to the malignant parenchymal cells (Sounni & Noel, 2013; Valkenburg et al., 2018).

Understanding the interactions between tumour cells and CAFs may provide new approaches to chemoprevention and therapeutic strategies by targeting the components of the TME (Salo et al., 2014; Valkenburg et al., 2018). It has been suggested that cancer may be a reversible disease and that malignant cells can be possibly reverted or re-programmed into a "normal" phenotype when in receipt of cues from a "normal" microenvironment (D'Anselmi et al., 2013; Ingber, 2008; Kalluri, 2016; Sherman et al., 2014). Whilst TME components and CAFs can influence the development of drug resistance in cancers; they can also be modified to enhance drug sensitivity and responsiveness of tumour cells (Landry et al., 2018; McMillin et al., 2013).

Numerous therapeutic approaches targeting multiple compartments within TME have been successfully implemented, approved by the US Food and Drug Administration (FDA) or are currently undergoing clinical trials in several advanced cancers (Belli et al., 2018; Gieniec et al., 2019; Junttila & de Sauvage, 2013; Valkenburg et al., 2018). Some of the popular therapeutic targets to promote anti-tumour responses in the TME include chemo-attractants or molecules produced by CAFs, ECM, vasculature, immune cells etc. (Albini & Sporn, 2007; Junttila & de Sauvage, 2013; Valkenburg et al., 2018). For instance, tumour ECM can be targeted to enhance chemotherapy responses by increasing the drug access to the tumour (Provenzano et al., 2012). Transcriptional factors and associated regulatory proteins within signalling pathways, e.g. VEGF and TGF-β signalling are also important molecular targets in the TME for chemoprevention and cancer therapy [reviewed in (Albini & Sporn, 2007; Belli et al., 2018; Carmeliet & Jain, 2011)]. Furthermore, stromal fibroblast-directed therapy or chemoprevention

generally focuses on the possibility to eliminate, to potentially re-programme or 'to normalise' CAFs back to their normal quiescent state. An ideal target molecule for CAFs in cancer would be one which is able to eliminate the tumour-promoting phenotypes but retain the anti-fibrotic properties (Kalluri & Zeisberg, 2006). Even though targeting CAFs is seemingly a promising new tool for anti-cancer treatment, a more comprehensive understanding of CAFs is required due to their functional heterogeneity and dynamic cellular "polarisation" – a term adopted to highlight the multifaceted role of CAFs in tumour development (Augsten, 2014).

2.4.3 CAFs in OSCCs

Distinct tumour-promoting subtypes of OSCC-derived CAFs such as CAFs from GU-OSCCs and GS-OSCCs have been identified (Costea et al., 2013; Mellone et al., 2016; Prime et al., 2017). More specifically, fibroblast strains derived from GU-OSCCs were shown to display a distinctive gene expression profile that distinguished them from CAFs isolated from GS-OSCCs and normal human oral fibroblast (NHOF) (Lim et al., 2011). The transcriptional profiles of the two subsets of CAFs (from GU- and GS-OSCCs) reflected the stages of epithelial tumour progression (Lim et al., 2011) and they have differential tumour-promoting abilities (Costea et al., 2013).

CAFs from GU-OSCC are characterised by enlarged cell size, flattened morphology, slow growth *in vitro*, and a distinct pattern of gene expression associated with cellular senescence (Lim et al., 2011). By contrast, CAFs from GS-OSCC proliferated faster and were morphologically similar to NHOFs in cell culture. Interestingly, CAFs from GU-OSCCs were also able to potentiate the invasion of malignant epithelial cells in three-dimensional (3D) organotypic model relative to other fibroblast subgroups from GS-OSCC and NHOF (Lim et al., 2011). The mechanisms underlying this senescent-associated tumour-promoting phenotypes remain uncertain, but the possibilities include

replicative exhaustion (Hayflick & Moorhead, 1961) and oxidative stress due to the generation of ROS (Parrinello et al., 2003; Rai et al., 2011). These CAFs were also suggested to sustain their pro-tumoral phenotypes by miRNA-mediated gene regulation and/or epigenetic mechanisms (Prime et al., 2017).

TGF-β is a pluripotent cytokine that plays a dual role in carcinogenesis; it is known to be tumour inhibiting during the early stages but later, functions as a tumour promoter and some of these pro-oncogenic effects are mediated by its effects on CAFs (Calon, Tauriello, & Batlle, 2014; Roberts & Wakefield, 2003). The activation and recruitment of myofibroblasts in many cancers are TGF-β dependent (Kalluri, 2016; Löhr et al., 2001). Similarly, in OSCCs, different experimental models have demonstrated that malignant cells regulate myofibroblast transdifferentiation via elevated TGF-β1 secretion (Hassona et al., 2013; Kellermann et al., 2008; M. Lewis et al., 2004; Mellone et al., 2016). For example, CAFs displayed upregulated expression of TGF-β target genes relative to NHOFs (Costea et al., 2013) and secretion of elevated levels of TGF-β1 was capable to induce phenotypic features to CAFs, enhance carcinoma cell proliferation and motility, as well as stimulate invasion and metastasis directly through EMT in OSCCs (Kellermann et al., 2008; M. Lewis et al., 2004; Ramos, Dang, & Sadler, 2009; C. Yu et al., 2011).

Some of the features of CAFs appear to be important clinically and are often associated with worse prognosis in OSCC patients (Bello et al., 2011; Fujii et al., 2012; Inoue et al., 2014; Kawashiri et al., 2009). IHC analyses showed that myofibroblasts are frequently present (≈60%) in the stroma of OSCCs (Kellermann et al., 2008; Rodrigues et al., 2015) and the abundance of CAFs correlated significantly with certain clinicopathological features, such as proliferative potential of tumour cells, lymph node metastasis and disease stage of OSCCs (Kellermann et al., 2007; Kellermann et al.,

2008). Furthermore, two genes that characterise the CAFs from GU-OSCC, namely, *ACTA2* (the gene that encodes α-SMA) and *ITGA6* (integrin subunit alpha6; involved in cell surface-mediated signalling and cell adhesion) were observed to be associated with poor clinical outcome in OSCC patients (Lim et al., 2011). Additionally, high α-SMA expression within the tumour stroma has been reported to be the strongest independent risk factor of early OSCC mortality and is an effective predictor of patient mortality in oral cancer (Marsh et al., 2011). Along with α-SMA, podoplanin-positive CAFs are also suggested to be an indicator of poor prognosis in OSCC patients (Inoue et al., 2014).

2.5 Senescence

Ageing at the cellular level is generally referred as cellular senescence, replicative senescence or cellular ageing (Campisi & di Fagagna, 2007). Cellular senescence can be defined as the stable, permanent and irreversible state of cell cycle arrest wherein cells cease proliferating, despite remaining viable and active metabolically (Campisi, 2013). Replicative senescence can occur following the progressive shortening of telomeres upon extensive cell division, known as the Hayflick limit (Hayflick & Moorhead, 1961). On the other hand, cellular senescence can also occur independently of telomere shortening, following certain insults or a variety of cellular stresses, e.g. exposure to chemotherapy or irradiation, oxidative DNA damaging agents, oncogene activation (termed oncogene-induced senescence, OIS) and oxidative stress from mitochondrial dysfunction (Gorgoulis & Halazonetis, 2010; Halazonetis, Gorgoulis, & Bartek, 2008; Vasileiou et al., 2019). Regardless of its trigger, senescence is a response to cellular stress (Vitale et al., 2019).

Telomere-dependent senescence is an inevitable consequence of progressive shortening of telomeres that occurs after every cell division and it is a major reason why normal cells cannot divide indefinitely when they reached their Hayflick limit

(Hayflick, 1965). Small segments of telomeric DNA cannot be replicated and are lost after each DNA replication cycle, eventually causing dysfunctional and critically short telomeres (Allsopp et al., 1995). This telomere dysfunction is able to initiate a classical DNA damage response (DDR), which in turn drives replicative arrest and the activation of senescence. Only one or a few such dysfunctional telomeres are sufficient to elicit a senescence-associated cell growth arrest (Herbig & Sedivy, 2006) and persistent DDR signalling enforces this growth arrest (Fumagalli et al., 2012). An important consequence of replicative senescence is the aversion of genomic instability (Campisi, 2013). Senescence in response to a variety of intrinsic and extrinsic stimuli that occurs independently of telomere shortening is more acute and is termed stress-induced premature senescence (Gorgoulis & Halazonetis, 2010; Liakou et al., 2016; Munoz-Espin & Serrano, 2014).

Senescence growth arrest is generally established and maintained by either or both the p53/$p21^{Waf1/Cip1}$ and $p16^{Ink4a}$/pRb signalling pathways, which can reinforce senescence in a ROS-dependent positive feedback manner (Childs, Baker, Kirkland, Campisi, & van Deursen, 2014; Passos et al., 2010; Takahashi et al., 2006). Both of these complex pathways are usually cell-type- and species-specific, while involving numerous upstream regulators, downstream effectors and modifying side branches which may cross-regulate each other (Campisi, 2013; Salama, Sadaie, Hoare, & Narita, 2014). DDR signalling is stimulated by diverse stressing triggers of senescence, which in turn initiate a transient p53/$p21^{Waf1/Cip1}$-dependent proliferative arrest for DNA repair. Failure to repair DNA results in a persistent DDR response, which leads to the subsequent activation of the $p16^{Ink4a}$/pRb pathway to ensure permanent cell cycle arrest (Loaiza & Demaria, 2016). Overexpression of p53, $p21^{Waf1/Cip1}$, pRb or $p16^{Ink4a}$ is generally adequate to stimulate a senescent-induced cell cycle arrest (Beauséjour et al., 2003; Campisi, 2013).

2.5.1 Characteristics of senescent cells

Senescent cells undergo distinctive morphological and physiological alterations, including profound changes in chromatin organisation, gene expression and secretome (Campisi & di Fagagna, 2007; López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013). Morphologically, senescent cells are larger in volume with enlarged nucleoli and increased numbers of Golgi apparatus and lysosomes. In culture, senescent cells have a flattened morphology with more diverse morphotypes than younger cells (Cristofalo, Lorenzini, Allen, Torres, & Tresini, 2004). These senescence-associated morphological alterations are not restricted to fibroblasts but were also observed in epithelial cells (Delfarah et al., 2019; Sikora, Mosieniak, & Alicja Sliwinska, 2016).

Senescence-associated β-galactosidase (SA-β-gal) is a commonly used histochemical marker to identify senescent cells. It is a lysosomal enzyme that shows increased activity in senescent cells at pH 6 *in vitro* and *in vivo* and can be visualised microscopically as perinuclear blue staining (Dimri et al., 1995; Kurz, Decary, Hong, & Erusalimsky, 2000). The cell cycle inhibitors, $p21^{Waf1/Cip1}$ and $p16^{Ink4a}$, are two important CDK inhibitors commonly highly expressed by senescent cells in tissues and in culture (Campisi & di Fagagna, 2007). $p16^{Ink4a}$ is also a commonly used senescent marker, as its expression is generally low and undetectable in normal cells but is frequently detected in many, but not all senescent cells. Its expression also increases gradually with biological age (Ben-Porath & Weinberg, 2005; Waaijer et al., 2012). Interestingly, $p16^{Ink4a}$ can also be found in tumour cells, especially those that have dysfunctional pRb (Gil & Peters, 2006). A low level of sirtuin 1 (SIRT1), a NAD-dependent deacetylase, has also been reported to be marker of senescence (James, Lane, Michalek, Karoly, & Parkinson, 2016; Michishita, Park, Burneskis, Barrett, & Horikawa, 2005; T. Sasaki, Maier, Bartke, & Scrable, 2006).

One of the main features of senescence is the inability of cells to initiate DNA replication even in adequate growth conditions. Senescent cells can remain viable and metabolically active for a long period of time but are unresponsive to apoptotic and growth-promoting stimuli (Cristofalo & Pignolo, 1993; Goldstein, Ballantyne, Robson, & Moerman, 1982). This growth arrest is often considered irreversible and permanent, as currently there are no recognised physiological signals that have the ability to rescue senescent cells and stimulate them to re-enter the cell cycle (Campisi, 2013). However, molecular genetic manipulations, such as sequential inactivation of certain tumour suppressor genes or oncogene transformation, can stimulate the growth of senescent cells (Beauséjour et al., 2003). Genetic manipulation of TERT can restore telomerase activity, inhibit telomere shortening and immortalise fibroblasts without alterations in their cell cycle checkpoints or karyotype (Morales et al., 1999). In addition, viral system carrying an oncoprotein such as simian virus (SV40) large tumour antigen (LT) can be manipulated to inhibit the activity of tumour suppressor proteins p53 and pRb, restoring cell cycle progression and immortalising cells (Ali & DeCaprio, 2001; Hardy et al., 2005; O'Hare et al., 2001).

2.5.2 Senescence-associated secretory phenotype (SASP)

Despite having restricted proliferative ability, senescent cells are metabolically active with widespread alterations in protein expression, which results in an altered secretory profile. These changes in protein expression ultimately develop into the SASP or senescence-messaging secretome (SMS) (Kuilman & Peeper, 2009; Loaiza & Demaria, 2016). SASP components are key feature of senescent cells and they can be collectively categorised into the following major groups of soluble signalling factor; cytokines (e.g. IL-6, IL-1), chemokines (e.g. IL-8, monocyte chemoattractant proteins [MCPs], CCL2, CXCL2, CXCL12), growth factors (e.g. TGF-β, insulin-like growth factor binding proteins, IGFBPs), proteases (e.g. MMPs, serine protease) and ECM components (e.g.

fibronectin) (Coppé et al., 2010; Faget, Ren, & Stewart, 2019). By means of the complexity of the SASP, senescent cells can communicate with other cells and influence the surrounding tissue microenvironment via paracrine mechanisms (Di Mitri & Alimonti, 2016).

Persistent DDR signalling is required for the induction and maintenance of some of these SASP factors (Kuilman & Peeper, 2009). DNA damage caused by a rapid robust DDR does not induce a SASP in senescent cells, because the SASP is stimulated by DDR proteins only after the establishment of persistent DDR signalling (Rodier et al., 2009). In cultured cells, a full SASP develops slowly (>5 days) after senescence initiation and often after the initial DDR subsides (Coppé et al., 2010; Coppé, Patil, et al., 2008).

Proinflammatory chemokines and cytokines are among the highly conserved SASP constituents in different cell types and are induced by several senescence-inducing stressors. This suggests that senescent cells generally share common properties to attract immune cells and promote local inflammation (Coppé, Patil, et al., 2008). A significant number of factors are upregulated (e.g. IL-2, IL4, IL-10) or unaltered (e.g. GCP-2, GITR) upon senescence, while no factors were observed to be downregulated significantly in different senescent states (Coppé, Patil, et al., 2008). In spite of this, cells with different senescence statuses appear to display distinct features. Normal cells that senesce owing to telomere disruption, replicative exhaustion, epigenomic perturbation or X-irradiation seem to have similar SASP profiles, whilst RAS-induced or p53 dysfunctional senescent cells produce a higher level of more secretory factors. By contrast, cells that senesce simply due to ectopic overexpression of $p16^{Ink4a}$ and $p21^{Waf1/Cip1}$ but fail to activate DDR signalling do not express a SASP despite displaying other characteristics of senescent cells (Coppé et al., 2011). Although many similar

factors are highly conserved features in all senescent cells, the quality and robustness of the SASP vary depending on senescence-inducing stimuli and cell type (Coppé et al., 2010). Additional genetic and epigenetic alterations may drive cells more to a late senescence state, further driving the changes in transcriptional profiles and SASP heterogeneity (Van Deursen, 2014).

2.5.3 Bimodal roles of senescence in cancers

Senescence-induced growth arrest is proposed to be a potent tumour-suppressive mechanism because cancer development requires relatively unconstrained cell proliferation (Hanahan & Weinberg, 2000); thus any mechanism that limits cell growth stringently may protect against cancer development (Campisi & di Fagagna, 2007). Due to senescent cells' unresponsiveness to mitogenic stimuli, senescence is regarded as a defence mechanism to limit the proliferation of genetically damaged or altered cells. It is widely recognised that senescence is a tumour-suppressive biological response early in cancer development, with p53/ $p21^{Waf1/Cip1}$ and $p16^{Ink4a}$/pRb signalling being the two primary tumour suppressor pathways responsible for the growth arrest of senescent cells (Faget et al., 2019; Loaiza & Demaria, 2016). It has been suggested that the transformation and progression of cells to form a tumour requires the bypass of the senescence (Bartkova et al., 2006; Faget et al., 2019). However, overcoming senescence alone is often insufficient for malignant transformation and not all immortal cells are tumorigenic (Campisi & di Fagagna, 2007). It has also been demonstrated that cellular senescence acts synergistically with the immune system as an alternative approach to apoptosis in eliminating impaired and/or potentially malignant cells (Parkinson, 2010).

Despite its obvious tumour-suppressive functions, senescence might be a double-edged sword that plays opposing roles in tumorigenesis (Loaiza & Demaria, 2016). Studies have indicated that senescent cells might fuel the progression of neighbouring

preneoplastic cells (Campisi & di Fagagna, 2007; Pazolli et al., 2012). Indeed, it has been shown in several studies that tumour initiation is not possible without the support and facilitation of the surrounding stromal compartment (Kuperwasser et al., 2004; Olumi et al., 1999). As mentioned above, senescent cells actively communicate with adjacent cells and the surrounding extracellular matrix through a plethora of secreted factors within the SASP (Faget et al., 2019). This communication might regulate a myriad of biological activities, such as stimulation of cell proliferation (or inhibition), cell motility (invasion, migration and metastasis), angiogenesis (new blood vessel formation), EMT, chemotherapy resistance, inflammation, tissue repair, stem cell renewal and differentiation (Campisi, 2013; Demaria et al., 2017). SASP components may potentially create a permissive tissue microenvironment that facilitates the survival, growth and propagation of cells that may harbour premalignant mutations. This may possibly explain, at least in part, the role of senescence in cancer development of ageing organisms and the marked surge of cancer incidence beyond middle age (Campisi, 2013; Van Deursen, 2014).

Intriguingly, the acquisition of the SASP is reminiscent of some CAF functions, which may be active contributors of carcinogenesis (Campisi, 2005; Vitale et al., 2019). Convincing evidence has been presented to show that senescent CAFs can drive hyperplastic pathology in numerous cancers including breast, prostate cancer and melanoma (Coppé et al., 2010). For example, in xenograft and *in vitro* co-culture studies, senescent fibroblasts significantly stimulated the growth of premalignant cells and neoplastic cells (Coppé, Kauser, Campisi, & Beauséjour, 2006; Krtolica, Parrinello, Lockett, Desprez, & Campisi, 2001; Lawrenson et al., 2010; D. Liu & Hornsby, 2007). MMP3, a prominent component of the SASP, which acts on the extracellular matrix, is known to promote tumorigenesis in adjacent preneoplastic cells in a paracrine manner (Krtolica et al., 2001). Secreted MMP3 can also alter the morphological and functional

differentiation of mammary epithelial cells in three-dimensional cultures, suggesting that senescent stromal cells might contribute to preneoplastic cell growth *in vivo* (Parrinello, Coppé, Krtolica, & Campisi, 2005). In addition, cytokines such as IL-6 and IL-8 promoted tumorigenesis through the induction of EMT and inflammation in premalignant cells and non-aggressive malignant epithelial cells, but this phenomenon was not seen in normal cells (Coppé, Patil, et al., 2008; Laberge, Awad, Campisi, & Desprez, 2012; Ortiz-Montero, Londoño-Vallejo, & Vernot, 2017). VEGF secreted by senescent human lung fibroblasts was shown to fuel the malignant phenotype and stimulate tumour-driven angiogenesis in a mouse model (Coppé et al., 2006). Lastly, several studies have reported that senescent cells, specifically those that senesce in response to chemotherapeutic drugs and DNA-damaging irradiation, secrete chemoprotective secretory proteins such as IL-6, TIMP-1 and WNT16B that can trigger chemoresistance of tumour cells (Gilbert & Hemann, 2010; Sun et al., 2012).

Collectively, data from decades of research has indicated that during earlier stages of carcinogenesis, senescence-induced growth arrest arises in incipient tumour cells tends to be a tumour-suppressive mechanism. However, cellular senescence via the SASP from the stromal compartment more often promotes the acquisition of cancer hallmarks in malignant cells (Faget et al., 2019).

2.5.4 Fibroblast senescence and senescent CAFs in OSCC development

Fibroblast senescence is thought to be an early event in the pathogenesis of OSCC (Hassona et al., 2013; Pitiyage et al., 2011). Primary risk factors of OSCC such as tobacco, alcohol and betel quid alkaloids have been shown to induce senescence in fibroblasts (Coppé, Boysen, et al., 2008; Parkinson et al., 2015; Salem et al., 2013). Senescent fibroblasts are generally characterised by the overexpression $p16^{Ink4a}$, increased SA-β-gal, altered gene expression and a distinctive secretory profile of SASP

(Campisi, 2013). Importantly, these phenotypes are more apparent in CAFs from aggressive GU-OSCCs and it has been suggested that senescence in these cells is due to oxidative DNA damage (Hassona et al., 2013; Lim et al., 2011).

Similar to myofibroblastic CAFs, senescent CAFs have also been shown to possess tumour-promoting properties in various models (Coppé et al., 2010; Krtolica et al., 2001). Likewise, many of the properties ascribed to CAFs are also shared by senescent cells and many of the molecules that mediate CAF-induced phenotypes, such as TGF-β (Coppé, Patil, et al., 2008; Hassona, Cirillo, Heesom, Parkinson, & Prime, 2014), MMPs (Hassona et al., 2014; Parrinello et al., 2005; Pitiyage et al., 2011) and collagen (Lim et al., 2011) are also secreted by senescent oral fibroblasts, provoking the hypothesis that many properties of OSCC-derived CAFs may be due to their prematurely senescent state.

Due to the overlapping functions of the TGF-β/Smad signalling pathway, it has been suggested that myofibroblast activation and senescence in fibroblasts might occur via similar molecular mechanism(s) and they may be part of the same regulatory system of fibroblast responses at different stages of cancer progression (Mellone et al., 2016; Prime et al., 2017). In an experimental model of OSCC, tumour-derived epithelial TGF-β1 induced-fibroblast activation preceded cellular senescence and was associated with elevated ROS production (Hassona et al., 2013). It was demonstrated that epithelial invasion was stimulated by fibroblast activation and becomes further amplified when fibroblast senescence is more marked (Hassona et al., 2013). Further, malignant keratinocytes of GU-OSCCs were shown to reinforce their malignant phenotypes by stimulation of fibroblast activation and senescence in a ROS- and TGF-β-dependent manner (Hassona et al., 2013).

Interestingly, many of these characteristics ascribed to activated and senescent CAFs are also shared by autophagic fibroblasts, suggesting that these fibroblastic stress responses could contribute to the tumour-promoting properties of CAFs. With respect to OSCC, the picture remains unclear, but the fact that fibroblast activation, autophagy and senescence statuses are aberrant in CAFs from OSCC, suggests that these mechanisms may potentially reflect a unified programme in regulating CAF phenotypes.

2.6 Autophagy

The ubiquitin-proteasome and lysosomal pathways are two of the principal protein degradation systems observed in eukaryotic cells to routinely breakdown intracellular proteins (Hansen & Johansen, 2011; Mani & Gelmann, 2005). These two systems are both evolutionarily conserved and have been observed and characterised from yeast to human (Hochstrasser et al., 1999; Reggiori & Klionsky, 2002). Autophagy is one of the major pathways that function through the activation of lysosomal degradation. This self-catabolic digestion system is often referred to as "self-eating" or "self-cannibalism" whereby a cell self-digests its own long-lived cytosolic proteins and organelles (Klionsky, 2004; Yorimitsu & Klionsky, 2005).

Autophagy can either target the degradation of cellular components selectively or non-selectively, depending upon the stimuli or environmental cues (Nair & Klionsky, 2005; Yorimitsu & Klionsky, 2005). Induced autophagy is important during nutrient deprivation and metabolic stress as an adaptive response. In situations when nutrients are scarce, this catabolic pathway and recycling of intracellular molecules can provide the fundamental building blocks to sustain the metabolic pathways and restore nutrient availability. On the other hand, basal autophagy is responsible for the constitutive turnover of cytosolic components in maintaining cell homeostasis (Mizushima, 2007). Autophagy is constitutively active in order to maintain cellular homeostasis by

eliminating dysfunctional proteins and organelles, such as mitochondria, to prevent the accumulation of unfolded proteins and excessive ROS generation by dysfunctional mitochondria (Mizushima & Komatsu, 2011). Inherently, autophagy, therefore, is cytoprotective and facilitates cell survival (Maes, Rubio, Garg, & Agostinis, 2013).

Autophagy is a multi-step, highly dynamic process. It is functionally regulated by a unified set of autophagy-related genes (*ATG*), which are conserved between mammals and yeasts (Noda & Inagaki, 2015). Activation or increased activity of overall autophagic degradation is commonly termed autophagic flux. Conceptually, autophagy proceeds through several sequential steps including induction, cargo packaging and selection, nucleation, vesicle expansion, docking and fusion, degradation and eventually the export of metabolites (Kundu & Thompson, 2008; Melendez & Levine, 2009; Suzuki & Ohsumi, 2007). When induced by a cue, the cytosolic cargos are sequestered by a phagophore (isolation membrane) during the nucleation step. The phagophore expands and encloses all the cargos into a double-membrane vesicle, termed autophagosome. Subsequently, the complete autophagosome fuses with the lysosome, producing an autophagolysosome to internally degrade the sequestered materials (Klionsky, 2005). The breakdown products are then released back to the cytoplasm for metabolic recycling, synthesis of new macromolecules and energy generation (Klionsky, 2005). Figure 2.4 depicts the multi-step autophagy process.

Beyond its roles in adaptive defence and cellular housekeeping, other non-canonical functions of autophagy include roles in antiviral immunity, endocytic trafficking and secretory processes (Green & Levine, 2014; B. Levine, Mizushima, & Virgin, 2011). Defects or deregulation in autophagy are widely implicated in diverse pathologies, including tumorigenesis, neurodegeneration, bacterial and viral infections, ageing, diabetes, myopathies, cardiovascular and pulmonary diseases (Choi, Ryter, & Levine,

2013; Masini et al., 2009; Mizushima, Levine, Cuervo, & Klionsky, 2008) and this is due to the key roles it plays in cell metabolism and development (Kimmelman & White, 2017). Notably, autophagy is emerging as a key biological process in tumorigenesis and in modulating tumour-stroma crosstalk (Mowers, Sharifi, & Macleod, 2018).

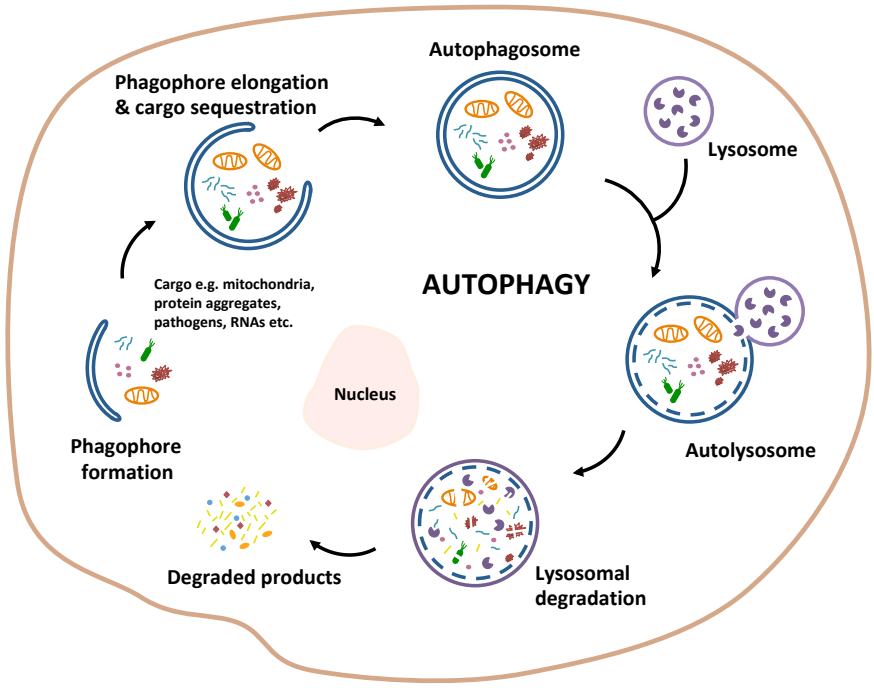


Figure 2.4: Schematic diagram of multi-step autophagy pathway

The process of autophagy starts with the formation of isolation membrane or phagophore in a cell. Cytosolic components, e.g. organelles, pathogens and protein aggregates are sequestered through the expansion of phagophore to form mature autophagosome. Subsequently, the autophagosome fuses with the lysosome and the contents are degraded to be recycled.

2.6.1 Autophagy in tumour suppression

Mounting evidence has shown that autophagy plays important but complex roles in carcinogenesis, as it can promote or inhibit tumourigenesis, depending on the cellular context such as tissue origin and tumour type (Santana-Codina, Mancias, & Kimmelman, 2017). Autophagy was first identified as a tumour-suppressive mechanism based on observations from tumour suppressor and oncogene alterations studies

(Santana-Codina et al., 2017). Beclin 1 (gene: *BECN1*, also known as *ATG6*) was the first identified mammalian autophagy-related protein that plays a prominent role in tumour development. It was shown to possess a haploinsufficient tumour suppressor function (Yue, Jin, Yang, Levine, & Heintz, 2003) and to be monoallelically mutated in 40 to 75 % of human breast, ovarian, prostate and other cancers (Aita et al., 1999; Kondo, Kanzawa, Sawaya, & Kondo, 2005; Liang et al., 1999; Qu et al., 2003). Transgenic mice with haploinsufficient *BECN1* were observed to be more susceptible to the development of spontaneous tumours, e.g. lymphomas, hepatocellular carcinomas, lung carcinomas and precancerous mammary lesions (Qu, Yu, Bhagat, & Furuya, 2003). Besides *BECN1*, similar genetic studies of autophagy-related genes such as *ATG4C*, *ATG5* and *ATG7* also demonstrated a link between autophagy impairment and an increased risk of malignant transformation (Mariño et al., 2007; Takamura et al., 2011). Likewise, mutation of *p53* and *PTEN*, two well-established tumour suppressor genes, can also regulate autophagy (Arico et al., 2001; Feng, Zhang, Levine, & Jin, 2005). The gain of function of *PI3K* or *AKT* can activate mammalian target of rapamycin (mTOR), which in turn inhibits autophagy; they are common targets for oncogenic mutation in cancer, suggesting the potential relevance of autophagy during tumour initiation (Maiuri et al., 2009; Santana-Codina et al., 2017).

In the early stages of carcinogenesis, autophagy plays an anticancer role and functions as a tumour suppressor. It safeguards against metabolic stress in cells, prevents inappropriate cell proliferation and reduces genetic instability by promoting the clearance of specific functionally disabled organelles and ROS, thus limiting potential tumour cell growth (Choi et al., 2013; Hippert, O'Toole, & Thorburn, 2006). Deregulation of mitophagy, which is the selective autophagic clearance of dysfunctional mitochondria, can trigger increased ROS in cells (Frank et al., 2012; Lemasters, 2005). More specifically, cancer cells deficient in autophagy regulators, coincide with

defective mitochondrial accumulation, increased DNA damage and ROS levels (Komatsu et al., 2005; Scherz-Shouval & Elazar, 2007; Stephenson et al., 2009). Basal autophagy involves the continuous clearance of a scaffold protein for ubiquitinated cargo, called p62/SQSTM1 (Lamark, Kirkin, Dikic, & Johansen, 2009). Accumulation of p62 aggregates that occur as a result of defective autophagy can activate the DDR pathway (Duran et al., 2008; Mathew et al., 2009). Such events can all result in oxidative stress and can be considered to be pro-tumorigenic (Liou & Storz, 2010).

In *in vitro* models of OIS and therapy-induced senescence, a positive correlation between autophagy and senescence was demonstrated by the delayed onset of senescence arrest during autophagy inhibition, suggesting a possible effector role for autophagy in senescence (Goehe et al., 2012; Young et al., 2009). Another possible mechanism by which autophagy functions as a tumour suppressor is via its association with cellular senescence, particularly OIS that can be stimulated in response to oncogenic stresses to induce replicative arrest and prevent malignancy even though its relevance in activating senescence is often context-dependent (C. Kang et al., 2015; Perez-Mancera, Young, & Narita, 2014). Alternatively, autophagy plays a prominent role in preventing apoptosis-deficient cancer cells from undergoing necrosis that might stimulate inflammation and exacerbate tumour growth (Degenhardt et al., 2006). Furthermore, the deletion of *ATG* genes and autophagy disruption may foster genomic instability; and eventually cause deregulation in cell proliferation and necrosis (Degenhardt et al., 2006). Consequently, cellular contents are released to adjacent environments and stimulate inflammatory responses that may facilitate tumour growth (Vakkila & Lotze, 2004).

2.6.2 2.6.2 Autophagy in tumour promotion

Autophagy is such a complex and dynamic process that influences cancer development in multiple ways and, paradoxically, it is believed that autophagy plays a dual role in tumourigenesis. Despite its possible pro-death function in restricting early malignant transformation, there is evidence that indicates a pro-survival role for autophagy that facilitates the survival of malignant cells (B. Levine, 2007). These opposing roles of autophagy can be explained, in part, as the same triggers (ROS, inflammation and DDR) that promote cancer initiation during the early stages can be detrimental at later stages of disease progression (Poillet-Perez, Despouy, Delage-Mourroux, & Boyer-Guittaut, 2015; Santana-Codina et al., 2017). In this regard, tumour initiation is associated with downregulation of autophagy activity, which results in the accumulation of ROS and oncogenes. In contrast, autophagy activity is frequently upregulated during tumour maintenance (Folkerts, Hilgendorf, Vellenga, Bremer, & Wiersma, 2019).

In advanced tumours, autophagy enhances the survival advantages of tumour cells when they are subjected to various pressures and metabolic stresses, such as nutrient deprivation, hypoxia and insufficient vascularisation (Degenhardt et al., 2006; Karantza-Wadsworth et al., 2007). Under growth/survival factor-deprived conditions, studies have shown that autophagy acts as a stress response of malignant cells that protects them from apoptotic elimination and by preventing autophagy, the tumour cells undergo apoptosis (Gurkan, Arisan, Yerlikaya, Ilhan, & Unsal, 2018; Tian et al., 2018). Furthermore, autophagy may also contribute to the acquisition of therapy resistance in tumour cells during therapeutic interventions (Fitzwalter et al., 2018) or by contrast, facilitate autophagic cell death (type II programmed cell death) after cancer treatments (Kondo et al., 2005; Trenti et al., 2014). Interestingly, if excessive autophagy persists

due to microenvironment and metabolic stresses, it may also eventually lead to autophagic cell death (Y. Liu & Levine, 2015). Taken together, these data indicate that autophagy can either be cytoprotective or responsible for cell death during cytotoxic therapy, depending on the treatment and cell type (Folkerts et al., 2019).

2.6.3 Autophagy in the tumour microenvironment

Cancer cells have been shown to induce and exploit autophagy in stromal fibroblasts to support their proliferation via mechanisms that can involve a variety of different mediators, including ROS (Martinez-Outschoorn, Balliet, et al., 2010; Pavlides, Tsirigos, Migneco, et al., 2010), metabolic by-products (Capparelli et al., 2012; Martinez-Outschoorn, Lin, Whitaker-Menezes, Howell, Lisanti, et al., 2012; Martinez-Outschoorn, Lin, Whitaker-Menezes, Howell, Sotgia, et al., 2012) and hypoxia (Bellot et al., 2009). The outcome of this epithelial/fibroblast interaction is the induction of glycolysis and the secretion of high-energy metabolites (lactate, pyruvates and/or ketones), together with amino acids and nucleotides in CAFs (Martinez-Outschoorn, Pestell, et al., 2011). This, in turn, stimulates mitochondrial biogenesis and oxidative phosphorylation in carcinoma cells. The process has been termed the “reverse Warburg effect” and the result is a tumour-promoting cycle composed of catabolism in the tumour stroma and anabolic metabolism in tumour cells. This new idea is different from the conventional Warburg effect in that tumour cells obtain energy supply via aerobic glycolysis from within the tumour stroma, and in CAFs specifically, rather than from aerobic glycolysis in the tumour itself (Pavlides et al., 2009).

This enhanced reverse Warburg effect observed in CAFs in the tumour stroma is related to heightened catabolism and autophagy (Chaudhri et al., 2013; Guido et al., 2012; Martinez-Outschoorn, Lisanti, & Sotgia, 2014). Autophagy is compartment and cell type-specific (Chiavarina et al., 2010) and distinct effects may occur in the tumour

stroma, as opposed to the epithelial cancer cells (Capparelli et al., 2012). This suggests that the function of autophagy is different in the tumour and stromal compartments. It has been postulated that the malignant cells and CAFs are metabolically coupled (Martinez-Outschoorn, Pestell, et al., 2011), resulting in a parasitic cancer metabolism in which the autophagic CAFs in the tumour stroma provide recycled nutrients to support the anabolic growth of tumours and to fuel oxidative mitochondrial metabolism in tumour cells (Capparelli et al., 2012; Pavlides et al., 2012). This concept of stromal-epithelial metabolic coupling is termed two-compartment tumour metabolism or autophagic tumour stroma model of cancer (Martinez-Outschoorn, Pestell, et al., 2011; Martinez-Outschoorn, Sotgia, & Lisanti, 2012; Salem et al., 2012; Scherz-Shouval & Elazar, 2007). The role of autophagy in cellular secretion has gained much attention recently (Ponpuak et al., 2015) and the soluble factors secreted by autophagic fibroblasts have been reported to promote tumour progression (New et al., 2017; New & Thomas, 2019; Sousa et al., 2016). In this respect, CAFs may play a role in the catabolic and anabolic balance of malignant epithelial cells in the TME milieu (Kalluri, 2016) and autophagy can increase malignant tumour potential when it is activated in CAFs (Pavlides et al., 2012).

2.6.4 Autophagy in OSCCs

Even though autophagy has gained much of the attention in recent years, the exact role of autophagy in OSCC carcinogenesis remains is not yet fully understood. Autophagy can be either facilitative or inhibitive in oral carcinogenesis, and the expression of ATGs is stage-dependent or specific to cell type (J. L. Liu et al., 2014). Areca nut extract (ANE) was reported to induce autophagic flux in OSCC cells in a ROS-dependent manner. A similar response was also observed in stromal cells treated with ANE, while ANE triggered autophagic cell death in immune cells and contributed to the chronic inflammation in oral cavity (M.-H. Lin et al., 2010).

Several studies have shown that deregulation of ATGs or autophagy activity is associated with unsatisfactory and poorer prognosis in OSCC patients (Kapoor, Paliwal, Singh, Mohanti, & Das, 2012; Tang, Hsi, et al., 2013; Y. Wang et al., 2013). For example, autophagy-related 16-like 1 (ATG16L1) protein is essential for autophagosome formation and was suggested to have prognostic significance in OSCC patients with a more aggressive tumour phenotype (Nomura et al., 2009; Tang et al., 2015). Overexpression of cytoplasmic and plasma membranous ATG16L1 was observed in IHC-stained tissues of OSCC as well as premalignant lesions, suggesting that the altered expression of ATG16L1 could be an early event during oral tumorigenesis (Nomura et al., 2009). Interestingly, overexpression of ATG16L1 in the stromal compartment was found to have a correlation with lymph node metastasis and lymphovascular invasion in OSCC patients, suggesting the relevance of ATG protein subcellular localisation and translocation in malignant transformation. Further, the same report also indicated that the deregulated expression of stromal ATG proteins could influence the metastatic and invasive behaviour of OSCC and might be a potential target for therapy (Nomura et al., 2009). Additionally, Y.-R. Lee et al. (2012) explored the role of autophagy in immune cells of TME and suggested that autophagy actively mobilised immune cells and contributed to increasing tumour antigenicity of OSCC cells. Other ATGs and autophagic components that were reported to have prognostic significance in OSCC include Beclin-1, ATG5, LC3 and p62 (Abd-El Raouf et al., 2019; K. Lai et al., 2018; J. L. Liu et al., 2014; Tang, Fang, et al., 2013; Tang, Hsi, et al., 2013).

The extensive crosstalk between autophagy and apoptosis appears to be important to how OSCC cells respond to cancer therapies (Gordy & He, 2012). ANE-induced autophagy via the upregulation of oxidative stress and HIF-1α (H.-H. Lu et al., 2010), and nutrient-deprivation-induced autophagy (L. C. Jiang et al., 2015) were reported to

be cytoprotective to OSCC cells by inhibiting apoptosis. Xu et al. (2017) reported that ANE-induced autophagy mediated by ROS and AMPK/mTOR signalling could also contribute to the reduced chemosensitivity of OSCC in response to cisplatin, as OSCC patients with a history of areca nut chewing showed higher propensity of cisplatin resistance. In this context, targeting autophagy with autophagic inhibitors seems to have important clinical significance to augment apoptosis in tumour cells. Inhibition of autophagy by pharmacological use of 3-methyladenine (3-MA) and chloroquine (CQ) in an OSCC cell model showed promising results by reducing migration and cisplatin resistance (F. Lin et al., 2018). Autophagy inhibition in CAFs by CQ or ATG knockdown suppressed HNSCC progression through the inhibition of autophagic CAFs-secreted factors (New et al., 2017; New & Thomas, 2019). However, by contrast, other reports described autophagy induction as an advantageous strategy to increase anti-apoptotic effects of OSCC treatment (Ahn, Ahn, & Yoon, 2011; Y.-R. Lee et al., 2012). Induction of autophagy with rapamycin enhanced the response to radiotherapy in OSCC patients (S. Y. Wu et al., 2014), while other chemotherapeutic drugs were demonstrated to exert anti-tumour properties through autophagy and apoptosis (Ahn et al., 2011; Y.-R. Lee et al., 2012).

Although numerous studies have highlighted the pivotal role of autophagy in OSCC development and progression, the precise role of autophagy in these processes remains to be elucidated. Significantly, the majority of these studies to date are primarily confined to epithelial tumour cells, whilst the function of autophagy in the oral TME has received little attention.

2.7 Autophagy and senescence in CAFs

Lisanti et al. (2011) observed that the nurturing relationship between a tumour and its surrounding microenvironment begins with the secretion of hydrogen peroxide by cancer cells that drives paracrine crosstalk between these two compartments in a breast cancer-fibroblasts co-culture model. In this model, hydrogen peroxide was identified as the predominant ROS (Lisanti et al., 2011). High levels of ROS and persistent oxidative stress produced by epithelial cancer cells (Toyokuni, Okamoto, Yodoi, & Hiai, 1995) induce activation and senescence in neighbouring CAFs; CAFs, in turn, produce high levels of ROS to reinforce their own senescence phenotype (Hassona et al., 2013). Consequently, oxidative stress from the fibroblasts could also result in increased DNA damage and genomic instability in tumour cells, leading to more aggressive tumour cells with apoptotic resistance (Martinez-Outschoorn, Balliet, et al., 2010; Martinez-Outschoorn, Trimmer, et al., 2010).

In addition to senescence being a stress response in fibroblasts, accumulating evidence indicates that autophagy is also key for CAF activation and survival (Goruppi, Clocchiatti, & Dotto, 2019). In *in vitro* models, an autophagic response in stressed fibroblasts was demonstrated to support the survival and growth of tumour cells (W. Zhou et al., 2017). Oxidative stress produced by tumour cells can drive the induction of autophagy and senescence in adjacent CAFs (Martinez-Outschoorn, Lin, et al., 2011). Consistent with this idea, the senescent phenotype may be a secondary response to the activation of autophagy and mitophagy, causing mitochondrial dysfunction (Capparelli et al., 2012). In these senescent CAFs, the loss of mitochondrial function enhanced a shift towards aerobic glycolysis, resulting in the generation and release of high energy metabolites, e.g. ketone bodies, L-lactate, glutamine and free fatty acids into the tumour stroma (Pavlides, Tsirigos, Migneco, et al., 2010; Pavlides, Tsirigos, Vera, et al., 2010; Pavlides et al., 2012; Pavlides et al., 2009). These metabolic byproducts can act in a

paracrine manner to fuel oxidative phosphorylation and mitochondrial biogenesis in epithelial tumour cells, leading to a positive loop to drive anabolic growth in tumours (Bonuccelli et al., 2010; Martinez-Outschoorn, Prisco, et al., 2011; Sotgia et al., 2012). These metabolic micro-nutrients would be delivered to adjacent tumour cells, entering the Krebs cycle and results in efficient oxidative phosphorylation and ATP production (Martinez-Outschoorn, Pavlides, et al., 2011). It has also been demonstrated that autophagic CAFs can foster tumour cell metastasis via the release of high-energy mitochondrial fuels, which could be exploited by oxidative phosphorylation in adjacent tumour cells (Capparelli et al., 2012).

The co-culture of cancer cells with fibroblasts can produce an activated microenvironment enriched with growth factors and inflammatory mediators. These secreted molecules are able to induce autophagy in CAFs and this observed autophagic phenotype is associated with an inflammatory response in the tumour microenvironment (Martinez-Outschoorn, Whitaker-Menezes, et al., 2011). Interestingly, the SASP, which includes a plethora of cytokines and growth factors, secreted by CAFs in the tumour stroma, has been estimated to contribute to only 50% of the tumour-promoting potential of senescent CAFs (Krtolica & Campisi, 2002; Krtolica et al., 2001), while the remaining 50% may be attributed to the autophagic phenotype and the shift towards aerobic glycolysis (Capparelli et al., 2012). This hypothesis also suggested that autophagy and senescence may be part of a unified physiological programme in CAFs, termed autophagy-senescence transition (AST) (Capparelli et al., 2012) or reflect different stages of the same sequential pathway.

While senescent CAFs have been shown to influence OSCC cell behaviour (Prime et al., 2017), the role of autophagy in the regulation of the senescent CAFs phenotype has not been fully elucidated and has never been examined in the context of OSCC.

However, it is reasonable to speculate that autophagy may be part of the same physiological process as senescence in OSCC-derived fibroblasts. The availability of NHOFs, together with GS- and GU-OSCCs derived CAFs, provides a unique opportunity to study the possible link between fibroblast autophagy and senescence, and the functional relevance of these stress responses in fibroblasts to the malignant phenotypes of OSCCs.

CHAPTER 3: MATERIALS AND METHODS

3.1 Cell strains and lines

3.1.1 Cancer-associated and normal human oral fibroblast strains

A unique series of fibroblast strains consisting of normal human oral fibroblasts (NHOF, n=3), CAFs from GS-OSCCs (n=4) and CAFs from GU-OSCCs (n=4) were used in this study (Table 3.1). The derivation and molecular characterisation of these fibroblast strains have been published previously (Lim et al., 2011).

3.1.2 Human OSCC-derived cell line

A well-characterised human malignant oral keratinocyte cell line, H376 was used in this study. Details of the way by which the OSCC cell line was established have been previously described (Prime et al., 1990). The cells have wild type Ha-ras (Yeudall et al., 1993), wild type methylated CDKN2A (C. L. Wu et al., 1999) and carry a p53 mutation G to T at codon 266 of exon 8 (Yeudall, Paterson, Patel, & Prime, 1995).

Table 3.1: List of fibroblast strains

Origin	Fibroblast strains
Normal oral mucosa	NHOF1, NHOF2, NHOF7
Genetically stable (GS-) OSCC	BICR59F, BICR66F, BICR69F, BICR73F
Genetically unstable (GU-) OSCC	BICR3F, BICR31F, BICR63F, BICR78F

3.2 Materials

Lyophilised recombinant human TGF-β1 (Miltenyi Biotec, Germany) was reconstituted in 4 mM HCL (Sigma-Aldrich, USA) and 1 mg/mL BSA (Bio Basic Inc., Canada). Chloroquine (CQ; Sigma-Aldrich, USA) was dissolved in ultrapure water and sterilised using 0.2 μm filter syringe. Reconstituted CQ was stored in opaque tube at 4°C for not more than a month. Hydroxychloroquine sulphate (HCQ; Sigma-Aldrich, USA) was reconstituted in sterile water, aliquoted and stored at -20°C in the dark. SAR405 (Calbiochem, Merck, USA) was dissolved in DMSO (Sigma-Aldrich, USA) and stored at -20°C protected from light. ABT263 [4-[4-[[2-(4-Chlorophenyl)-5,5-dimethyl-1-cyclohexen-1-yl]methyl]-1-piperazinyl]-*N*-[[4-[[(1*R*)-3-(4-morpholinyl)-1-[(phenylthio)methyl]propyl]amino]-3[(trifluoromethyl)sulfonyl]phenyl]sulfonyl]benzami-de] and ABT737 [4-[4-[[2-(4-Chlorophenyl)phenyl]methyl]piperazin-1-yl]-*N*-[4-[[(2*R*)-4-(dimethylamino)-1-phenyl-sulfanylbutan-2-yl]amino]-3nitrophenyl]sulfonylbe-nzamide were obtained from Abcam, UK. ABT263 and ABT737 were dissolved in DMSO and stored in -20°C. All compounds were stored in small aliquots.

3.3 Cell culture

3.3.1 Maintenance of cell strains and cell lines

All fibroblast strains were cultured in Dulbecco's Modified Eagle's medium (DMEM) medium (Gibco Life Technologies, USA) supplemented with 10% HyClone™ FetalClone™ II serum (GE Healthcare Life Sciences, USA), 1% penicillin-streptomycin (Gibco Life Technologies, USA), 1% HyClone™ L-glutamine (GE Healthcare Life Sciences, USA) and 25 mM HEPES buffer (BioWest, France). OSCC cells were cultured in Dulbecco's Modified Eagle's medium and Hams F12 (DMEM/F12) containing 10% fetal bovine serum (FBS), 0.5μg/mL sodium hydrocortisone succinate and 1% penicillin-streptomycin. The cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

3.3.2 Sub-culture and cell number determination

All cells were cultured in 25cm^2 or 75cm^2 cell culture flasks (Corning, USA) and sub-cultured when they reached 80-90% confluency. The fibroblasts were briefly rinsed with phosphate-buffered saline (PBS) (Gibco Life Technologies, USA) and trypsinised with 0.05% trypsin-EDTA (Gibco Life Technologies, USA) for 3-5 minutes to dislodge cells. OSCC cells were detached using 0.25% trypsin-EDTA (Gibco Life Technologies, USA) for approximately 10 minutes. After the cells were visibly detached, the trypsin-EDTA solution was neutralised by adding 2x volume of growth medium and the suspension was centrifuged at 800 rpm for 5 minutes. The supernatant was aseptically decanted and the cell pellet resuspended with fresh complete growth medium. The cell density was determined by using a Luna automated cell counter (Logos Biosystems, USA) and the cumulative mean population doubling (CMPD) was calculated using the following formula: n = 3.32 x [log (final cell density) – log (initial cell density)] + mean population doubling (MPD). The cells were seeded into tissue culture flasks accordingly at the required cell density.

3.3.3 Cryopreservation and resuscitation of frozen cells

After trypsinisation and cell counting, cell pellets were resuspended in cold fetal bovine serum containing 10% DMSO (Sigma-Aldrich, USA) and aliquoted into sterile cryogenic storage vials (Nunc, USA). The vials were stored overnight in a MrFrosty^{TM} Cryo 1ºC Freezing Container (Nalgene, USA) at -80ºC and the frozen cells were then transferred to a liquid nitrogen tank for long-term storage.

To revive the cryopreserved cells, the cryovials taken from the liquid nitrogen tank were thawed quickly in a water bath at 37ºC. Cells were then resuspended in 9 mL of pre-warmed medium and centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded and the cell pellet was resuspended before transferring the cell suspension

into a cell culture flask containing pre-warmed fresh medium. The cells were maintained in culture, as previously described (Section 3.3.1).

3.3.4 Establishment of transduced fibroblast strains

3.3.4.1 Generation of amphotropic retroviruses

A stable producer cell line, Phoenix-AMPHO carrying the pBabe-puro-hTERT construct and a TEFLYA packaging cell line carrying temperature-sensitive (ts) mutant (U19tsA58) of simian virus 40 large-tumour (SV40LT) antigen (denoted as tsLT) were used for the transduction experiments and were kindly provided by Professor Parmjit Jat from University College London. The pBabe-puro-hTERT plasmid was constructed by using the pBabe-puro backbone encoding the full-length hTERT cDNA with a puromycin resistance gene. The U19tsA58 LT plasmid confers resistance to geneticin (G418) and has a pZipNeoSV(X)1 backbone with tsA58 encoding a thermolabile LT antigen that is wild type at 33ºC upon tsLT activation but inactive at 39ºC upon tsLT inactivation (Jat & Sharp, 1989; O'Hare et al., 2001). Fresh media were added to the producer cell lines at 80% and they were allowed to grow under standard culture conditions until they reach 100% confluence before harvesting after 12 or 24 hours. The viral supernatants were filtered through a 0.45 μm syringe filter and either used immediately for transduction experiments or stored until use at -80ºC.

3.3.4.2 Retrovirus transduction of fibroblast strains

Mid-confluent proliferating fibroblast cultures were exposed to filtered viral supernatants (1:1 ratio with complete DMEM) for 18 hours with 8 μg/mL polybrene and allowed to grow to confluency for a week. The transduced cells were then selected with 2 μg/mL of puromycin (Sigma-Aldrich, USA) and/or 500 μg/mL of G418 (Gibco Life Technologies, USA). Fibroblasts transduced with tsLT either singly or in combination of hTERT were cultured at 33ºC while untransduced cells were cultured at 37ºC.

3.3.5 Collection of conditioned media

Fibroblasts were grown in 75cm^2 cell culture flasks until they were 80-90% confluent. Cells were washed twice with PBS and serum-free media, and then further incubated with serum-free DMEM for 48 hours. The conditioned media (CM) was centrifuged at 2000 rpm for 5 minutes to remove debris and dead cells. The remaining viable cells in the flasks were trypsinised and counted for normalisation. CM was stored at -80ºC. CM was diluted with fresh supplement-free DMEM at a 1:1 ratio prior to use to replace depleted essential components in the CM collected from fibroblasts.

3.4 Cell behaviour assays

3.4.1 Cell proliferation

Typically, OSCC cells were seeded in triplicate in 12-well culture plates (4 x 10^4/well),) and allowed to adhere at 37ºC for 24 hours. The cells were serum-starved overnight with serum-free DMEM/F12, prior to treatment with CM for 48 hours. The cells were then trypsinised with 400 μL of trypsin-EDTA per well, neutralised with 2x volume of growth medium and centrifuged in microcentrifuge tubes at 10,000 rpm for 2 minutes. The supernatant was removed and the cell pellet was resuspended in PBS. Subsequently, 10 μL of cell suspension was mixed with an equal volume of 0.4% Trypan blue solution (Gibco, Thermo Fisher Scientific, USA) and applied into a haemocytometer. Cell viability was quantified by counting the live unstained cells under a light microscope.

3.4.2 Transwell migration and invasion

OSCC cell migration and invasion were assessed using Transwell Boyden chambers system. Polycarbonate filter inserts (8 μM pore size; Corning, USA) were coated with a layer of 10 μg/mL fibronectin or with 250 μg/mL growth factor-reduced Matrigel (Corning, USA) in DMEM for migration or invasion assays, respectively. The inserts were then placed into triplicate holding wells of 24-well culture plate (Costar, Corning, USA). Prior to cell seeding in the upper chambers, OSCC cells were first serum-starved overnight with serum-free DMEM/F12 and treated with 10 μg/mL mitomycin C (Merck Millipore, Germany) for 2 hours at 37ºC to negate any potential effects of proliferation. Cells were then washed with PBS to remove any trace of mitomycin C before they were detached with TrypLe Express enzyme (Gibco, Thermo Fisher Scientific, USA).

For migration assays, 200 μL of cell suspension (5 x 10^5 OSCC cells in DMEM/F12 containing 1% FBS) was then added dropwise into the upper chambers after the removal of fibronectin in the inserts. 500 μL of chemoattractant was added into the bottom chambers. After incubation for 24 hours, the non-migrated cells in the upper compartment were removed by swabbing the inserts with cotton buds. For invasion assays, the upper chamber contained 500 μL of cell suspension (5 x 10^5 OSCC cells in DMEM/F12 containing 1% FBS), while the lower chamber was filled with 750 μL of chemoattractant and the cells allowed to invade for 48 hours.

The migrated/invaded cells in the lower chamber were briefly washed with PBS and stained with 0.1% crystal violet (in 20% methanol) for an hour on the bench. The stained transwell inserts were allowed to air dry before cell counting was performed in five random fields at 20X magnification under the light microscope. The number of migrating or invading cells was normalised to the number of control cells. All of the

experiments were performed in triplicates and independent experiments were repeated at least three times.

3.5 Transmission electron microscopy (TEM)

Specimens for TEM were prepared by the Centre for Ultrastructural Imaging (CUI), King's College London, UK. The fibroblasts were grown to 70-80% confluence on plastic coverslips (Thermanox, EMS) to ensure that autophagy was not induced by excessive confluence. Cells were fixed for 4 hours at room temperature with 2.5% (v/v) glutaraldehyde in 0.1M cacodylate buffer (pH 7.3) and post-fixed in 1% (w/v) osmium tetroxide in 0.1M cacodylate buffer for 1.5 hour at 4^{o}C. Samples were then dehydrated through a graded ethanol series, before infiltration with TAAB epoxy resin and polymerised at 70°C for 24 hours. Ultrathin sections (70-90 nm) were prepared using a Leica EM UC7 ultramicrotome, mounted on 150 mesh copper grids and contrasted using uranyl acetate and lead citrate. Samples were examined on a FEI Tecnai 12 transmission microscope operated at 120 kV. Images were acquired with an AMT 16000M camera.

3.6 Senescence-associated β-galactosidase (SA-β-gal) staining

A senescence detection kit (BioVision, USA) was used to measure SA-β-gal activity histochemically. Fibroblasts were cultured overnight in 12-well culture plates (SPL Life Sciences, South Korea), the culture media were removed and the fibroblasts rinsed with 1 mL of 1X PBS. The cells were then fixed using 500 µL of the provided fixation solution for 10-15 minutes at room temperature. The fixed cells were rinsed twice with PBS and incubated overnight in 500 µL of staining solution mix (470 µL of staining solution, 5 µL of staining supplement and 1 mg/mL X-gal in DMSO) in a dark environment at 37ºC. The next day, the cells were washed with briefly with PBS before counterstaining with Nuclear Fast Red solution (Sigma-Aldrich, USA) for 20 minutes at

room temperature. The cells were rinsed again with PBS before observing under a microscope. Positively blue-stained senescent cells were counted and the percentage of positive cells s was calculated (relative to the total number of cells). A minimum of 100 cells was counted for each sample at a time-point.

3.7 LC3B immunofluorescence (IF) staining

4.5 – 5 x 10^4 cells were seeded onto a Nunc™ Lab-Tek™ II Chamber Slide™ (Thermo Scientific, USA) and allowed to adhere overnight at 37ºC. The following day, cells were fixed for 10-15 minutes; three different fixatives, namely, 4% paraformaldehyde with 0.1% Triton-X, 100% ice-cold methanol and ice-cold methanol: acetone (1:1) were compared. The fixed cells were rinsed three times with washing buffer (0.1% Tween-20 in 1X PBS) and non-specific binding site was blocked with blocking buffer (10% goat serum diluted in washing buffer) for 1 hour at room temperature. The cells were incubated for 2 hours with anti-LC3B antibody (1:1000, Abcam, UK) diluted in blocking buffer. Following three washes in washing buffer for 10 minutes each, the slides were incubated with AlexaFluor 488-conjugated goat anti-rabbit secondary antibody (1:1000, Thermo Fisher Scientific, USA) diluted in blocking buffer for 1 hour in room temperature. The slides were further washed in washing buffer three times, 10 minutes each in the dark and mounted with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, USA). The glass slides were covered with coverslips and sealed with nail polish at the edges. The slides were observed under a confocal microscope (LSM 510 META, Zeiss, Germany) and the staining was analysed using ImageJ software (https://imagej.nih.gov/ij/).

3.8 Western blotting

3.8.1 Cell lysate preparation

Cells were cultured to 70-80% confluence before lysing. The cells were trypsinised and pelleted by centrifuging at 800 rpm for 5 minutes. The supernatant was decanted and the pellet was resuspended with 1 mL of cold PBS and the cell suspension was transferred into a 1.5 mL microcentrifuge tube. The cells were pelleted by centrifugation at maximum speed for 1 minute. The cell pellets were then homogenised with 50 µL of cold RIPA lysis buffer (Bio Basic, Canada) containing freshly added Phosphatase Inhibitor Cocktail (Thermo Scientific, USA) and Protease Inhibitor Cocktail Set III (Calbiochem, Germany). The samples were allowed to stand on ice for 30 minutes and vortexed every 10 minutes. All the samples were then sonicated in a Whaledent Biosonic water bath (Coltene, Switzerland) for 2 minutes before centrifugation at 10,000 rpm for 30 minutes at 4ºC.

3.8.2 Determination of protein concentrations

Protein concentrations were estimated using a Bradford Protein Assay kit (Bio-Rad, USA) according to the manufacturer's protocol. The protein samples were diluted in PBS (1:10). 250 µL of 1X dye reagent was first added into each well of a 96-well microtiter plate, followed by the diluted protein samples, PBS or bovine serum albumin (BSA) standards (0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 µg/µL). The plate was incubated for 5 minutes at room temperature and the absorbance was read at 595 nm using an Infinite 200 Pro NanoQuant microplate reader (Tecan, Switzerland). The protein concentration of each sample was determined from the standard curve plotted using the BSA standards provided in the kit.

3.8.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein lysates were mixed with 4x Laemmli sample buffer (Bio-Rad, USA) containing 5% β-mercaptoethanol (Bio Basic, Canada) and boiled for 5 minutes at 95ºC to denature the protein. The percentage of gel used was chosen based on the molecular weight of the target protein. Gel preparation and SDS-PAGE were carried out using the Bio-Rad apparatus. The denatured protein samples and 5 μL of Precision Plus Protein All Blue standard (Bio-Rad, USA) were loaded into the wells on the gel in the electrophoresis tank filled with running buffer. Electrophoresis was performed at 70V for 30 minutes in the stacking gel, followed by 100V for 1 hour in the resolving gel.

3.8.4 Transferring and visualisation of proteins

Following SDS-PAGE, the proteins were transferred on to Amersham Hybond ECL nitrocellulose membrane (GE Healthcare, USA) at 25V for 27 minutes using a Trans-Blot Turbo Transfer System (Bio-Rad, USA). The membrane was blocked with either 5% non-fat dry milk or 5% BSA (Bio Basic Inc., Canada) in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 hour in room temperature on a laboratory rocker, followed by the overnight incubation with different primary antibodies with appropriate dilution in blocking buffer (Table 3.2) at 4ºC. The membrane was then washed with TBST (3 x 5 minutes) and incubated with the horseradish peroxidase (HRP)-or fluorophore-conjugated secondary antibodies (Table 3.3) for 1 hour at room temperature. After washing with TBST (3 x 5 minutes), the membrane was incubated with WesternBright^{TM} Sirius enhanced chemiluminescence (ECL) reagent (Advansta, USA) and the target proteins were detected using Odyssey FC Imaging System (LI-COR Biosciences, USA). Densitometric analyses were conducted using ImageJ software.

Table 3.2: List of primary antibodies

Antibody	Species	Dilution	Blocking Buffer	Manufacturer
α-SMA	Mouse monoclonal	1:2500	5%milk/TBST	DAKO
LC3B	Rabbit polyclonal	1:3000	5% milk/TBST	Abcam
p62/SQSMT1	Mouse monoclonal	1:2000	5% milk/TBST	Abcam
$p16^{Ink4a}$	Rabbit monoclonal	1:1000	5% milk/TBST	Abcam
$p21^{Waf1/Cip1}$	Mouse monoclonal	1:2000	5% milk/TBST	Merck
SIRT1	Rabbit polyclonal	1:6000	5% milk/TBST	Abcam
GAPDH	Rabbit polyclonal	1:3000	5% milk/TBST	Abcam

Table 3.3: List of secondary antibodies

Antibody	Dilution	Blocking buffer	Manufacturer
Anti-rabbit IgG-peroxidase	1:5000	5% milk/TBST	Sigma-Aldrich
Anti-mouse IgG-peroxidase	1:5000	5% milk/TBST	Sigma-Aldrich
DyLight 800-conjugated goat anti-rabbit IgG (H+L)	1:15000	5% BSA/TBST	Thermo Fisher Scientific
DyLight 680-conjugated goat anti-mouse IgG (H+L)	1:15000	5% BSA/TBST	Thermo Fisher Scientific

3.9 Gene expression analysis

3.9.1 Total RNA extraction and cDNA synthesis

Total RNA was extracted from cell pellets using an RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's protocol. The isolated RNA was eluted in 30-50 µL of RNase-free water. The RNA quality and concentration were determined using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). 1 µg of total RNA in 10 µL RNase-free water was used for cDNA synthesis using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). The reverse transcription master mix was prepared according to the manufacturer's protocol and added to 10 µL of RNA. The samples were mixed well and briefly centrifuged. cDNA synthesis was performed in a thermal cycler (Applied Biosystems, USA) with the

following conditions: 25ºC for 10 minutes, 37ºC for 120 minutes, 85ºC for 5 minutes and let stand at 4ºC. Gel electrophoresis was performed on a 2.5% denaturing agarose gel at 200V for 10 minutes to assess RNA integrity.

3.9.2 Quantitative polymerase chain reaction (qPCR)

qPCR was performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA) with FastStart Universal Probe Master (Rox; Roche Molecular Diagnostics, Switzerland). Commercially available TaqMan® Gene Expression Assays for ATG5 (Hs00355492_ml), Beclin1/ATG6 (Hs00186838_ml) and ESD (Hs00382667_ml) were purchased from Applied Biosystems, USA. cDNA was diluted using nuclease-free water at a ratio of 1:20 prior to adding into the reaction mixtures (Table 3.4). The experiment was conducted in triplicate for each reaction in PCR strip tubes or 96-well plates. The qPCR results were analysed using 7500 Software v2.0 (Applied Biosystems, USA) and cycle threshold (Ct) values were determined. The target gene expression was normalised against ESD expression as an endogenous control based on the following formula:

$$\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{ESD}}$$

$$\Delta\Delta Ct = \text{Mean of } \Delta Ct_{\text{test sample}} - \text{Mean of } \Delta Ct_{\text{reference sample}}$$

$$\text{Fold change} = 2^{(-\Delta\Delta Ct \text{ of each sample})}$$

Table 3.4: qPCR master mix

Reagent	Volume (µL)
TaqMan® Gene Expression Assays	1
ESD Taqman probe	1
FastStart Universal Probe Master (Rox)	10
Nuclease free water	3
cDNA	5

3.9.3 RNA sequencing (RNAseq)

Total RNA was extracted from the panel of fibroblast strains (n=11). After ribosomal RNA (rRNA) removal and preparation of RNAseq libraries, RNAseq was performed by BGI Tech Solutions (Hong Kong) Co Ltd using an Illumina HiSeq 2000 platform. Sequence reads were aligned to hg19 reference genome file using subread aligner and mapped sequencing reads were assigned to hg19 refGene genes using featureCounts. UCSC table browser was used to obtain the refGene exon coordinates. Gene symbol and description were obtained from the NCBI gene database. The total numbers of mapped human sequence reads were used for the calculation and expression of count per million (cpm) of human genes. The bioinformatics analyses were performed by Dr. Wenbin Wei (University of Birmingham, UK).

3.10 Statistical analysis

GraphPad Prism 6.0 (GraphPad Software, USA) was used for all the statistical analyses. Data are reported as mean ± SD. Mann-Whitney's U test was performed to evaluate the statistical difference between different CAF samples. Student *t*-test was used for comparisons between two groups and one-way ANOVA was used for more than two-group comparisons. Pearson's correlation was performed to study the correlation between autophagic and senescent phenotypes in CAF samples. p values < 0.05 were considered as statistically significant.

CHAPTER 4: SENESCENT AND AUTOPHAGIC PHENOTYPES IN NORMAL ORAL FIBROBLASTS AND CAFS

4.1 Introduction

During tumourigenesis, tumour cells gain the ability to alter the properties of their surroundings to create a supportive and conducive microenvironment for tumour progression (Xing, Saidou, & Watabe, 2010). A variety of non-malignant cells, together with the ECM constitute what is now referred to as the TME. Pre-eminent within the stroma are CAFs, which often display an activated phenotype similar to myofibroblasts (Acosta et al., 2008; Balkwill et al., 2012; Hanahan & Weinberg, 2011).

With respect to OSCC, subpopulations of activated and senescent fibroblasts have been identified in the oral stroma and transcriptional analysis has revealed a common gene expression profile between these two subsets of oral fibroblasts (Mellone et al., 2016). It has been demonstrated that CAFs derived from GU-OSCCs have a distinctive senescent transcriptional profile that differentiates them from CAFs GS-OSCCs and normal oral fibroblasts (Hassona et al., 2013; Lim et al., 2011). Interestingly, CAFs from GU-OSCCs were demonstrated to facilitate the invasion of malignant OSCC cells *in vitro* and the stimulation of fibroblast activation and senescence were shown to contribute to this invasive behaviour of OSCC. It has been proposed that the activated and senescent phenotypes were closely interrelated (Hassona et al., 2013) and the autophagic/catabolic phenotype might contribute to the tumour-promoting activity of senescent CAFs (Capparelli et al., 2012).

In addition to activation and senescence, autophagy is also a common stress response observed in CAFs and autophagic fibroblasts also display a pro-tumourigenic secretory profile (Capparelli et al., 2012). Several findings have shown that autophagy activation in stromal fibroblasts could contribute to the acquisition of senescent phenotypes

(Capparelli et al., 2012), although it has also been reported that disruption in autophagy could induce senescence in fibroblasts (H. T. Kang, Lee, Kim, Choi, & Park, 2011) or was an ageing-related phenomenon (Tashiro et al., 2014). These equivocal observations mean that the precise role of autophagy in fibroblast senescence remains to be determined.

The study of autophagy is largely confined to epithelial cancer cells. The detailed role of fibroblast autophagy in contributing to the development of the senescent phenotype and tumour-promoting properties of CAFs remains largely unknown in the context of OSCC and needs to be examined this experimentally in CAFs of OSCC.

Therefore, the aims of this study were 1) to characterise the senescence- and autophagy-associated phenotypes in a panel of fibroblasts, which included CAFs from GU-OSCCs and GS-OSCCs, together with normal human oral fibroblasts and, 2) to use these fibroblast strains to examine the relationship between senescence and autophagy in CAFs of OSCCs.

4.2 Senescent phenotypes of normal oral fibroblasts and CAFs

4.2.1 Oral fibroblasts and CAFs in 2D cell culture

OSCCs are a heterogeneous group of tumours and it has been shown that two classes of OSCC keratinocytes exist, namely, those that are genetically stable (GS-OSCC) or genetically unstable (GU-OSCC). A unique panel of fibroblast strains consisting of normal human oral fibroblasts (NHOFs), together with CAFs derived from GU-OSCCs and GS-OSCCs were used in this study (Table 1). To begin to characterise these fibroblast strains, the cellular morphology of cells was first examined *in vitro*. In 2D cell culture, fibroblasts from GU-OSCCs displayed characteristics of senescent cells, such as enlarged cell size, hypertrophy (increased cell volume and protein content), vacuolisation, flattened cell morphology with indistinct cell membranes, as observed by light microscopy (Figure 4.1). These morphologies are typical of senescent cells. Compared to CAFs from GU-OSCCs, fibroblasts derived from GS-OSCCs and normal oral mucosa (NHOFs) were smaller in size with a more elongated fibroblastic morphology (Figure 4.1). The growth characteristics of the fibroblasts in cell culture demonstrated that the CAFs from GU-OSCCs divided at a slower rate than CAFs from GS-OSCCs and these, in turn, proliferated slower than NHOFs. For example, BICR3F from a GU-OSCC had a mean population doubling (MPD) of ≈1 over a month, BICR73F from a GS-OSCC had an MPD of ≈1.5 over three days while NHOF1 had MPD of ≈2.5 over three days, respectively. Taken together, these observations suggested that CAFs from GU-OSCCs might be more senescent than CAFs from GS-OSCCs and NHOFs.

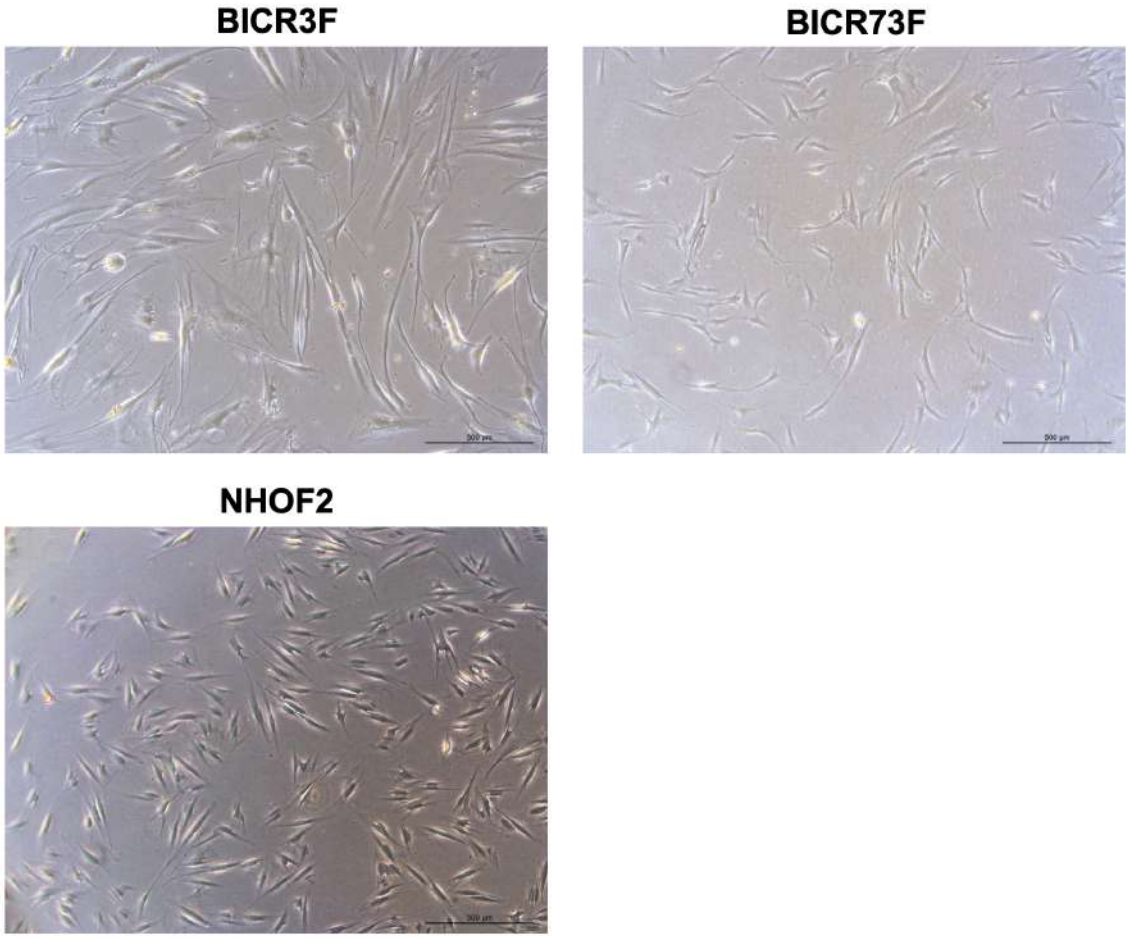


Figure 4.1: Fibroblast morphology in 2D cell culture

Representative images of fibroblasts derived from GU-OSCC (BICR3F) appeared flatter and hypertrophic, while fibroblasts derived from GS-OSCC (BICR73F) and normal oral mucosa (NHOF2) were smaller in size with elongated fibroblastic morphology as seen by light microscopy. Scale bar indicates 500 μm.

4.2.2 SA-β-gal activity in NHOFs and CAFs

In order to confirm that CAFs derived from GU-OSCCs are more senescent than CAFs from GS-OSCCs and NHOFs, I next examined the SA-β-gal activity in these fibroblasts. Staining cells for SA-β-gal exploits a common feature of senescent cells, namely, the increased expression of the enzyme β-galactosidase. This enzyme catalyses the hydrolysis of a chromogenic substrate, X-gal, which produces an insoluble blue precipitate that can be detected by light microscopy. The results showed that there were more positive blue-stained senescent cells in CAFs from GU-OSCCs (57 ± 4.54 %) than CAFs from GS-OSCCs (17.80 ± 3.47 %) and NHOFs (4.83 ± 1.66 %) (Figure 4.2) and this difference was statistically significant (Figure 4.3; $p<0.001$). Taken together, these data confirmed that senescence phenotypes were more apparent in fibroblasts derived from GU-OSCCs.

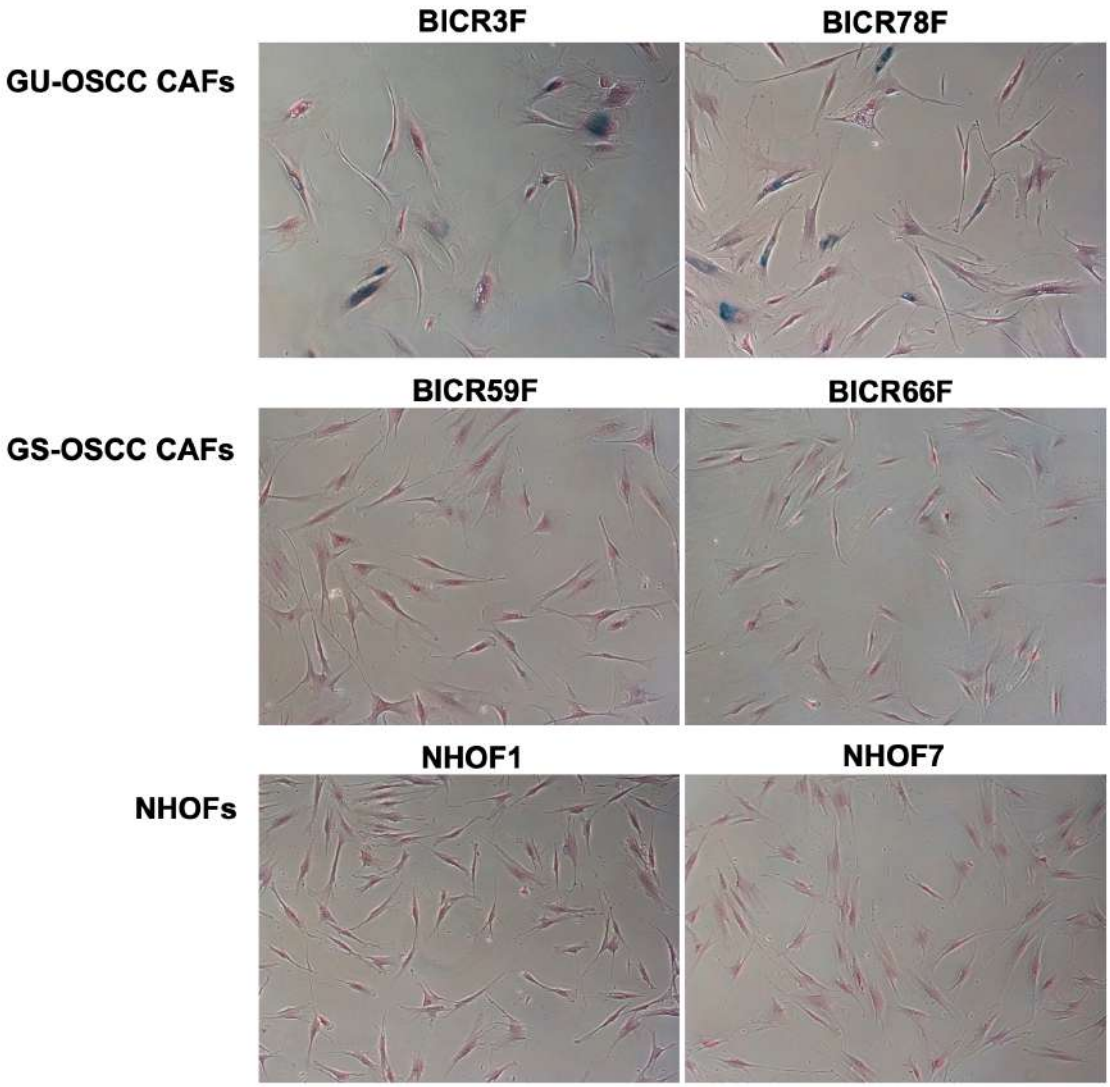


Figure 4.2: SA-β-gal staining was more apparent in CAFs from GU-OSCCs

Representative images of SA-β-gal staining in fibroblasts. Senescent fibroblasts were characterised by dark blue staining of SA-β-gal. Fibroblasts from GU-OSCCs showed more SA-β-gal positive stained cells as compared to fibroblasts from GS-OSCCs and NHOFs.

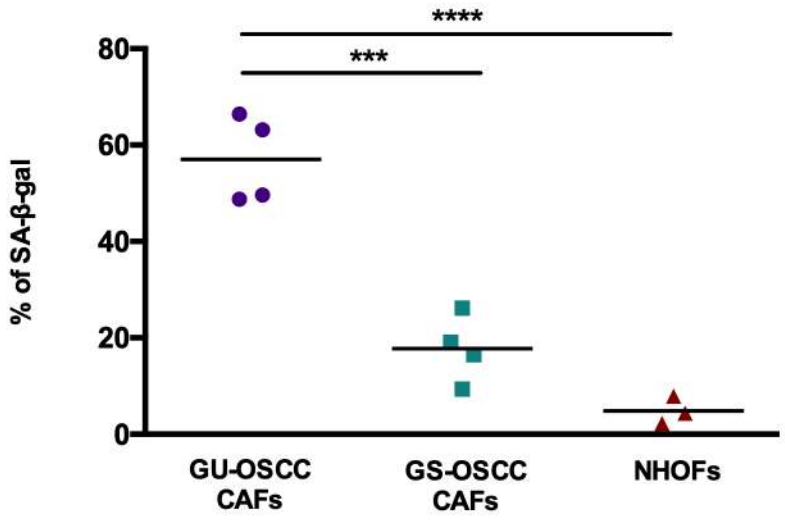


Figure 4.3: CAFs from GU-OSCCs showed a higher percentage of SA-β-gal positive cells

Scatter dot plot showing the percentage of SA-β-gal positive cells in fibroblast cultures from GU-OSCCs (BICR3F, BICR31F, BICR63F and BICR78F), GS-OSCCs (BICR59F, BICR66F, BICR69F and BICR73F) and normal oral mucosa (NHOF1 and NHOF7). The percentage of positive cells in CAFs from GU-OSCCs was significantly higher than both CAFs from GS-OSCCs and normal fibroblasts. *** = $p<0.001$, **** = $p<0.0001$ (ANOVA).

4.2.3 Expression of SIRT1 and $p21^{Waf1/Cip1}$ in oral fibroblasts and CAFs

As CAFs from GU-OSCCs were shown to have more apparent senescent phenotypes, further investigations were undertaken to examine the expression of sirtuin 1 (SIRT1) and $p21^{Waf1/Cip1}$ proteins. SIRT1 is a member of the NAD-dependent deacetylase family that has been linked to the regulation of metabolism, caloric restriction, cell growth, apoptosis and cell senescence (Haigis & Sinclair, 2010). Its expression was shown to be downregulated upon senescence (Rovillain et al., 2011) and reduced levels are considered to be a marker of late senescence (James et al., 2016; Michishita et al., 2005). Consistent with the observation that the senescent phenotype was more apparent in CAFs from GU-OSCCs (Figure 4.2 and 4.3), Western blot analyses demonstrated that the expression level of SIRT1 was generally lower in these

CAFs (Figure 4.4), although the expression was heterogeneous. Although there was variation between lines, generally there was a concomitant increase in the expression of the CDK inhibitor, $p21^{Waf1/Cip1}$ in the GU-OSCC fibroblast group (Figure 4.4), indicating a cell cycle arrest in these cells, as would be expected in senescent cells. The variation could be explained on the grounds that classical senescence is a dynamic process where initial high levels of $p21^{Waf1/Cip1}$ subside as senescence develops to be replaced by high levels of $p16^{Ink4a}$ (Robles & Adami, 1998) and lower levels of SIRT1.

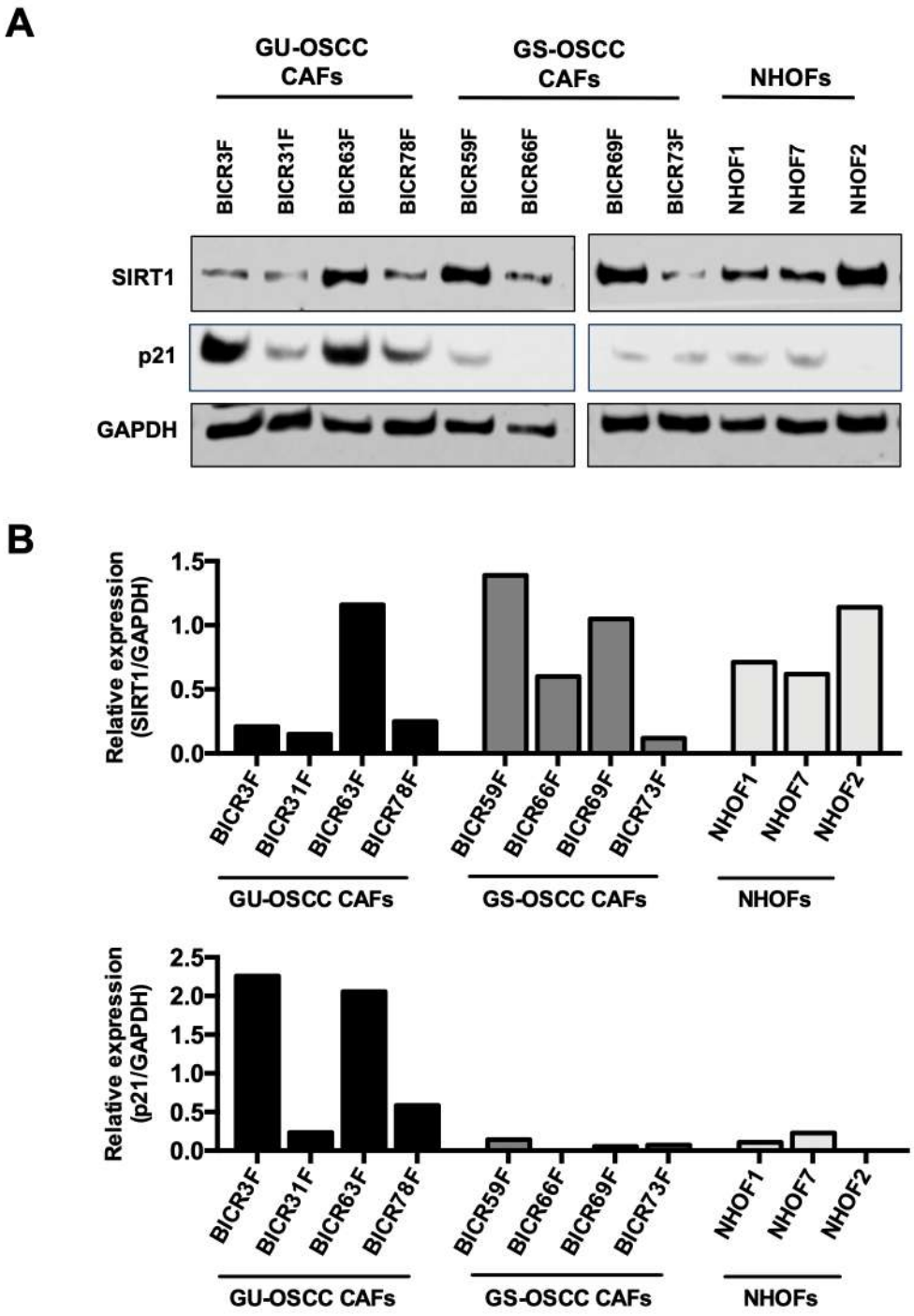


Figure 4.4: Expression of SIRT1 and p21$^{Waf1/Cip1}$ in NHOFs and CAFs

(A) The loss of SIRT1 is a marker for late senescence and its expression level was generally reduced in CAFs from GU-OSCCs. The expression of p21$^{Waf1/Cip1}$ protein was elevated in CAFs from GU-OSCCs. Representative Western blot images of three independent experiments are presented. (B) Densitometric analysis of SIRT1 and p21$^{Waf1/Cip1}$ Western blots was performed using ImageJ software (NIH) and the data were normalised against GAPDH.

4.3 Autophagosomes accumulate in CAFs from GU-OSCCs

4.3.1 Expression of LC3B in fibroblasts of OSCCs

To begin to examine autophagy in fibroblasts from OSCCs, the expression of LC3B-II protein was examined in CAFs and NHOFs. LC3, the mammalian homologue of autophagy-related protein 8 (ATG8), is expressed as three isoforms in mammalian cells, LC3A, LC3B and LC3C. LC3B is the best-studied autophagic marker among the other three isoforms (Koukourakis et al., 2015) and it was used in this study. LC3B-II, which is one of the major components of the autophagosome membrane, can be found on both inner and outer membranes of the autophagosomes. Hence, LC3B-II expression correlates with increased levels of these autophagic vesicles. Endogenous LC3B can be commonly detected as two bands following SDS-PAGE and Western blotting. One band at 16 kDa represents the cytosolic LC3B-I, whereas the other 14 kDa phosphatidylethanolamine-, PE-conjugated LC3B-II is present on autophagosomes and isolation membranes. The amount of membrane-bounded LC3B-II closely correlates with the number of autophagosomes, which is an indicator of autophagic activity. Normal fibroblasts treated with 5 μM of chloroquine (CQ), a lysosomal inhibitor was used as a positive control, as CQ functions as an autophagic inhibitor by inhibiting lysosomes-autophagosomes fusion and protein degradation (Shintani & Klionsky, 2004). Western blot analysis revealed that CAFs from GU-OSCCs expressed more LC3B-II protein than CAFs from GS-OSCCs and NHOFs (Figure 4.5), indicating that there was an accumulation of autophagosomes in CAFs from GU-OSCCs.

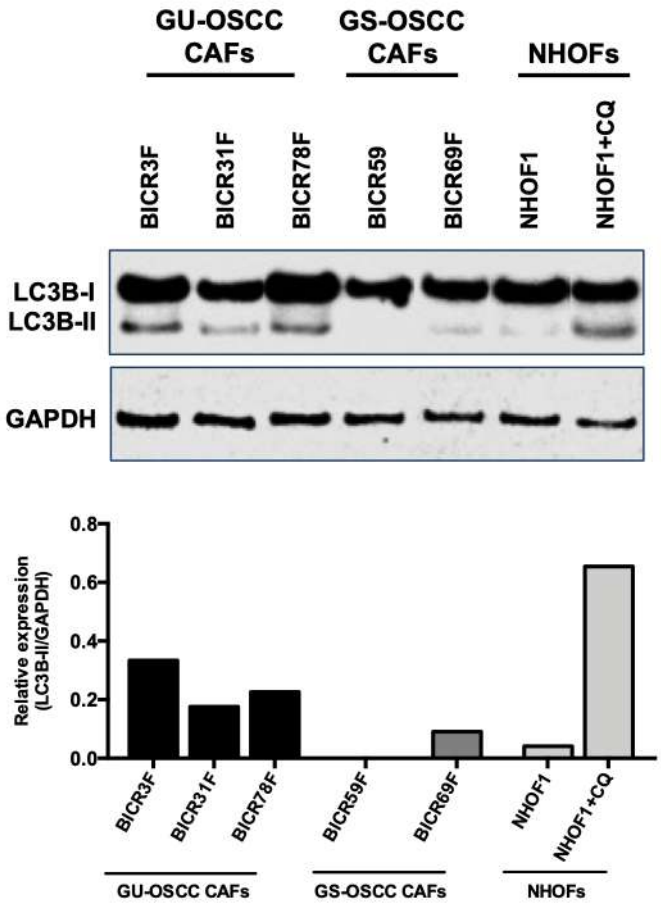


Figure 4.5: Increased level of LC3B-II protein expression in CAFs from GU-OSCCs

The amount of membrane-bounded LC3B-II closely correlates with the number of autophagosomes. Immunoblot analysis revealed higher LC3B-II protein levels in fibroblasts of GU-OSCCs and this data indicated that there was an accumulation of autophagosomes in the CAFs from GU-OSCCs. Normal fibroblasts treated with 5 μM of CQ, a lysosomal inhibitor was used as a positive control. Immunoblotting with GAPDH was used as a control for equal protein loading. Representative Western blot images of three independent experiments are presented.

4.3.2 Optimisation of immunofluorescence staining of LC3B puncta

To date, LC3-II remains the only well-characterised protein that is specifically localised to autophagic structures throughout the autophagy process and only the LC3B-II isoform correlates with the increased levels of autophagic vesicles (Kabeya et al., 2000; Mizushima, 2004). Autophagy is commonly characterised by the redistribution of the LC3B proteins into cytoplasmic puncta/dots, as assessed by fluorescent immunostaining and this was used in the present study to quantify autophagosomes. Firstly, to optimise the staining protocol, three different fixation methods with different fixatives, namely, 4% paraformaldehyde (PFA) with 0.1% Triton-X, 100% ice-cold methanol and ice-cold methanol: acetone (1:1), were compared. Normal fibroblasts treated with 5 μM of CQ were used as controls to assess the three different protocols. The immunofluorescence stained slides were observed under a confocal microscope and the images were compared (Figure 4.6). Fixation with ice-cold methanol and acetone in 1:1 ratio gave the clearest presentation of fluorescent LC3B puncta among the three methods (Figure 4.6C). Hence, this fixation protocol was used for the immunofluorescence staining of oral fibroblasts.

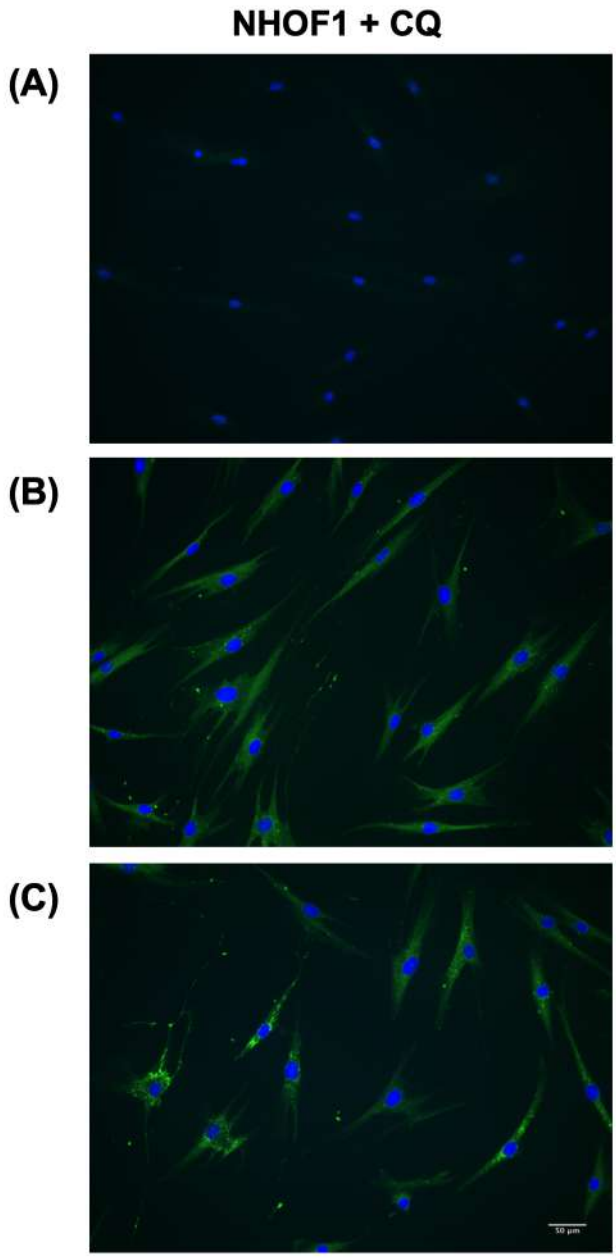


Figure 4.6: Optimisation of immunofluorescence staining

Endogenous LC3 can be visualised by fluorescence microscopy either as diffuse cytoplasmic pool typical of LC3-I or localised distinct punctate structures around the nucleus, which primarily represent LC3-II (autophagosomes). Shown here are representative microscopic images of immunofluorescence staining with an anti-LC3B antibody. NHOF1 treated with 5 μM CQ for 24 hours was used as a positive control. (A) 4 % PFA at room temperature, 15 minutes. (B) Ice cold methanol at -20°C, 10 minutes. (C) Ice-cold methanol and acetone (1:1) at -20°C, 10 minutes. Fixation with ice-cold methanol and acetone (C) gave the clearest presentation of fluorescent LC3B puncta among the three methods. All the images were captured using a confocal microscope at 20x magnification. Scale bar indicates 50 μm.

4.3.3 Immunofluorescence analyses of LC3B puncta in oral fibroblasts

Immunofluorescence staining of LC3B was performed on four fibroblasts strains of GU-OSCCs, four fibroblasts strains of GS-OSCCs and two strains of normal oral fibroblast. A minimum of 350 cells in each fibroblast strain was examined and the number of puncta per cell was calculated. Since lysosomes are unable to degrade the autophagosomes in CQ-treated cells, an accumulation in co-localisation of LC3B puncta on autophagosomes membrane can be observed and these cells were used as a positive control. Immunofluorescence staining showed that punctate LC3B staining was significantly greater in CAFs from GU-OSCCs as compared with GS-OSCCs and NHOFs (Figure 4.7 and 4.8A). The proportion of cells with different numbers of LC3B puncta was also calculated. In general, CAFs from GU-OSCCs contained a higher fraction of autophagic fibroblasts than CAFs from GS-OSCC and normal fibroblasts (Figure 4.8B).

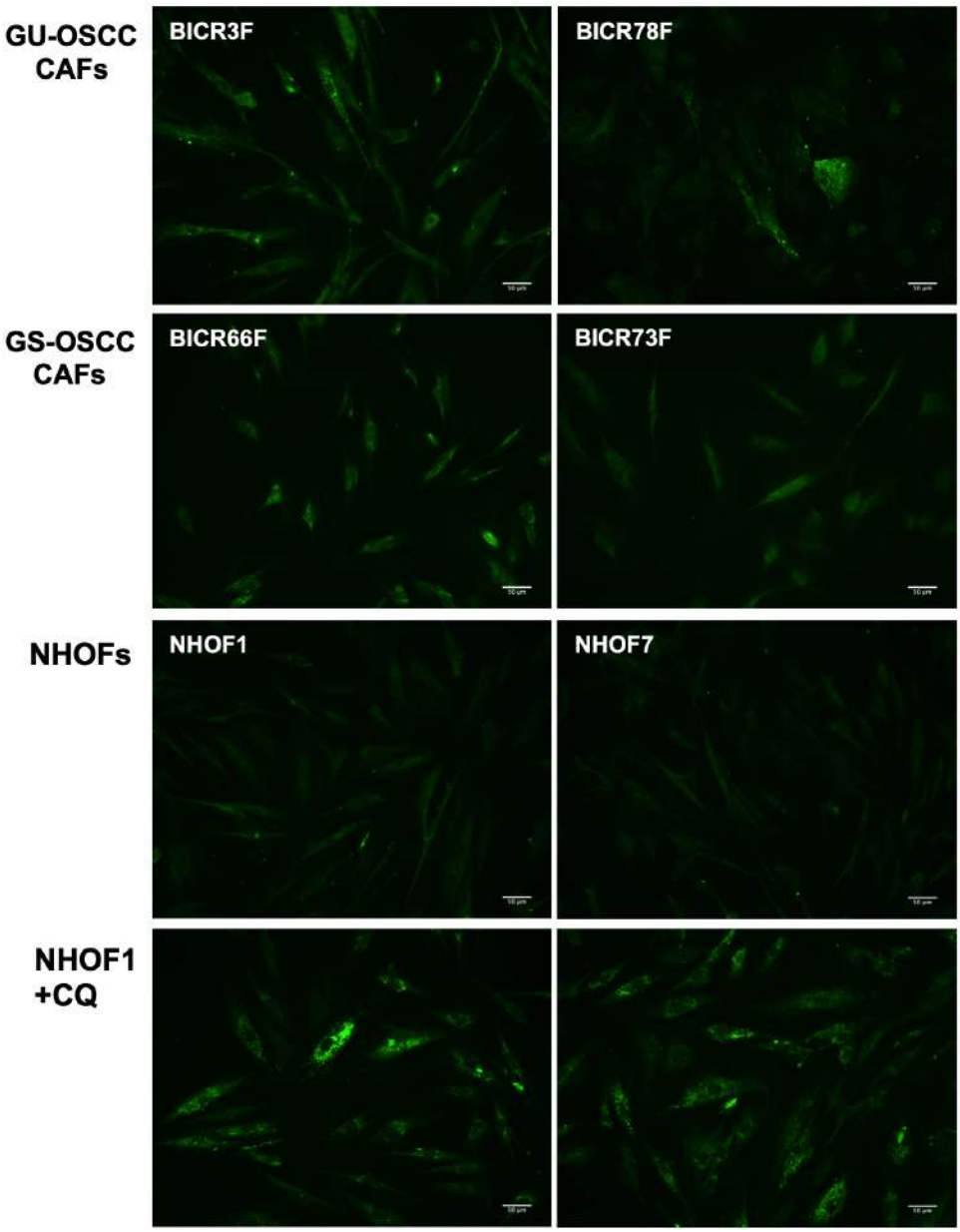


Figure 4.7: Immunofluorescence staining showed more LC3B puncta in CAFs from GU-OSCCs

Cultured oral fibroblasts were fixed and stained with anti-LC3B antibody. LC3B punta were more evidently observed in CAFs from GU-OSCCs as compared to CAFs from GS-OSCCs and normal oral fibroblasts. Normal oral fibroblasts treated with CQ (5 μM, 24 hours) were used as a positive control. Scale bar indicates 50 μm.

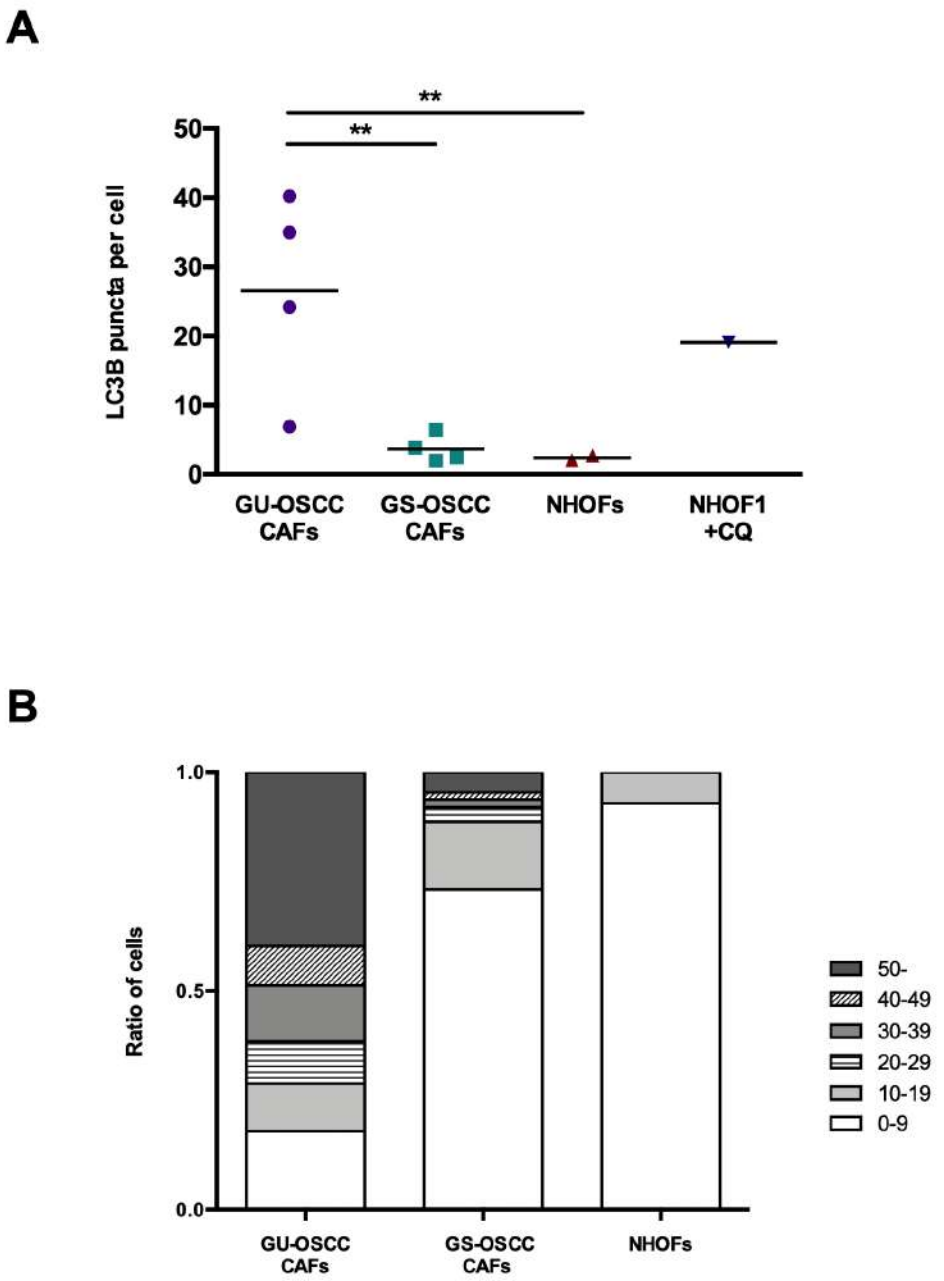


Figure 4.8: Immunofluorescence analyses of endogenous LC3B puncta in oral fibroblasts

(A) A minimum of 350 cells in each fibroblast strain was examined and the number of puncta per cell was analysed using ImageJ software. The number of LC3B puncta was significantly greater ($p<0.01$) in CAFs from GU-OSCCs as compared with GS-OSCCs and NHOFs. (B) LC3B puncta in each stained cell were calculated and the proportion of cells with different numbers of LC3B puncta is presented in a stacked bar chart. ** = $p<0.01$ (Wilcoxon Mann Whitney test).

4.3.4 Visualisation of autophagosomes by TEM

Autophagy was first described by TEM and it remains as one of the most commonly used techniques to detect the presence of autophagic vesicles (Klionsky et al., 2016). In the process of autophagy, cytoplasmic organelles and proteins are engulfed by autophagic vesicles known as autophagosomes. TEM is suitable for qualitative analysis of autophagy, whereby it allows the visualisation of autophagic-specific structures during different stages of autophagy. For example, autophagosomes containing morphologically intact organelles or cytosols can be observed during early autophagy, or during late autophagy, degradative autophagic compartments (autolysosomes) containing partially degraded cytoplasmic materials and/or organelles can be visualised. A late autophagosome typically displays an accumulation of dense multivesicular bodies (a kind of specialised late endosomes) and autophagy-related structures. The presence of autophagic compartments that are electron-dense indicates the accumulation of degraded cytoplasmic materials and/or organelles while partially degraded ribosomes accumulate as dark granular or amorphous clumps inside the autophagosomes. In the present study, TEM was used to confirm the presence of autophagic vesicles in CAFs from GU-OSCCs (BICR3F and BICR78F) and the images provided evidence of advanced autophagosomes in these cells (Figure 4.9). These data support the observations in Figure 4.7 and 4.8 that showed that CAFs from GU-OSCCs contained a high proportion of autophagic cells, as determined by LC3B IF staining.

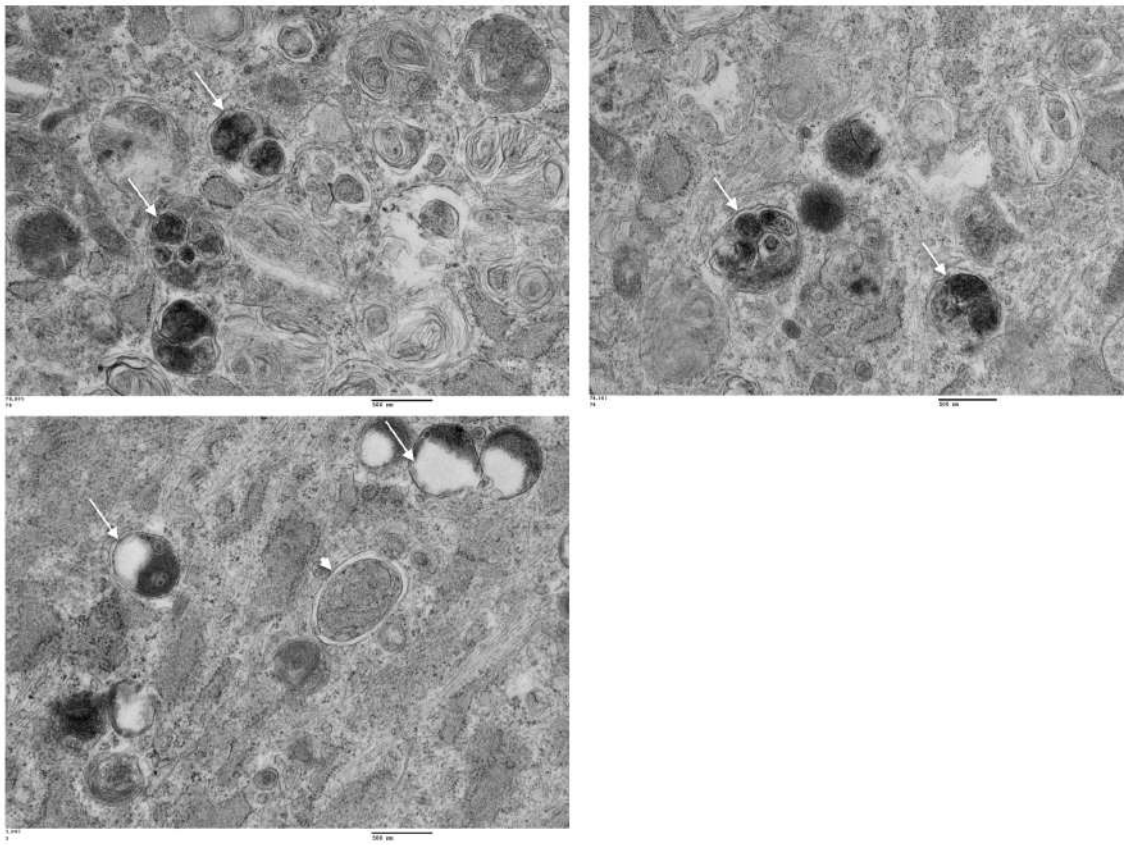


Figure 4.9: Accumulation of autophagosomes in CAFs from GU-OSCCs

Representative transmission electron microscope images of CAFs isolated from GU-OSCCs (BICR3F and BICR78F). The arrows indicate electron-dense late autophagosomes filled with multi-vesicular/lamellated structures while arrowheads point towards early autophagosome. Scale bar indicates 500 nm.

4.4 Senescent and autophagic phenotypes are closely related

To investigate the relationship between fibroblast senescence and autophagy, I cultured CAFs and NHOFs from OSCCs *in vitro* and compared SA-β-gal activity with the number of LC3B puncta. The expression of SA-β-gal correlated with the senescence state while the changes in LC3B puncta reflect the corresponding changes in autophagic structures. In view of the observation that both the percentage of SA-β-gal positive-stained cell (Figure 4.3) and the number of LC3B puncta per cells (Figure 4.8) were significantly higher in fibroblasts from GU-OSCCs, the relationship between senescence and autophagy was examined using Pearson's correlation analysis. Figure 4.10 shows that the percentage of cells staining positively for SA-β-gal significantly correlated (Pearson's $r = 0.9278$, $p<0.0001$) with the number of LC3B puncta in the 10 selected fibroblast strains from normal oral mucosa (n = 2), GS-OSCCs (n = 4) and GU-OSCCs (n = 4). This positive correlation suggests that the senescent and autophagic phenotypes in these cells are closely related.

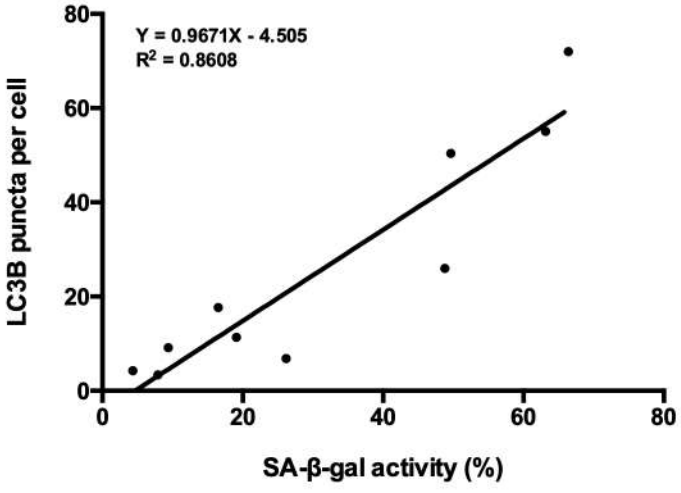


Figure 4.10: Correlation of the number of LC3B puncta and the number of cells staining positively for SA-β-gal activity in 10 fibroblast strains

A panel of fibroblasts at similar culture passage was examined for the number of LC3B puncta and SA-β-gal positive cells. NHOFs (n = 2), GS-OSCC CAFs (n = 4) and GU-OSCC CAFs (n = 4).

4.5 Autophagy was disrupted at the degradation step in CAFs from GU-OSCCs

The completion of the autophagy process involves the fusion of autophagosomes with lysosomes that contain hydrolytic enzymes, following the degradation of sequestered materials together with the inner autophagosomal membrane and release of degraded small molecules or amino acids to the cytoplasm (Mizushima, Yoshimori, & Levine, 2010). The elevation in the number of autophagosomes in CAFs may represent either a true upregulation of autophagic degradation (autophagic flux) or an inhibition in the completion of the autophagic pathway (decrease in autophagic degradation) (Mizushima & Yoshimori, 2007). Hence, the sole determination of the number of autophagosomes is not sufficient to determine overall autophagic activity. To distinguish these two physiologically different scenarios, the expression of autophagy-associated genes was analysed with a recent published RNAseq data (S. L. Lai et al., 2019). The results were validated with qPCR (*ATG5* and *Beclin1/ATG6* mRNAs) and Western blotting (p62 protein).

4.5.1 Expression of autophagy-associated genes in NHOFs and CAFs

Many autophagy-related genes (*ATGs*) have been identified years and now, more than 20 different *ATGs* have been discovered (Reggiori, 2006). The profile of expression of the *ATG* genes that are required for autophagosomal formation and expansion can provide information on the formation of autophagosome during early autophagy. To investigate whether the expression of these *ATG* genes could account for the increased number of autophagosomes in CAFs from GU-OSCCs, the expression of a number of *ATG*s (*ATG5*, *BECN1/ATG6*, *ATG7*, *ATG10*, *ATG12*, *ATG13*, *ATG14* and *ATG16L1*) was examined following an RNAseq experiment to compare gene expression in CAFs and NHOFs. There were no statistically significant differences in the

expression of these genes between CAFs from GU-OSCCs, CAFs from GS-OSCCs and NHOFs by RNAseq (Figure 4.11).

To validate these results, the expression of *ATG5* and *BECN1/ATG6* was examined by qPCR. ATG5 is required during autophagosomal elongation and BECN1/ATG6 regulates the initiation of autophagosome formation. As shown in Figure 4.12, there were no significant differences in the mRNA expression of *ATG5* ($p = 0.073$; Figure 4.12A) and *BECN1/ATG6* ($p = 0.185$; Figure 4.12B) between the fibroblast strains.

Taken together, these results imply that the accumulation of autophagosomes in GU-OSCC CAFs was unlikely to be related to the induction of autophagic flux.

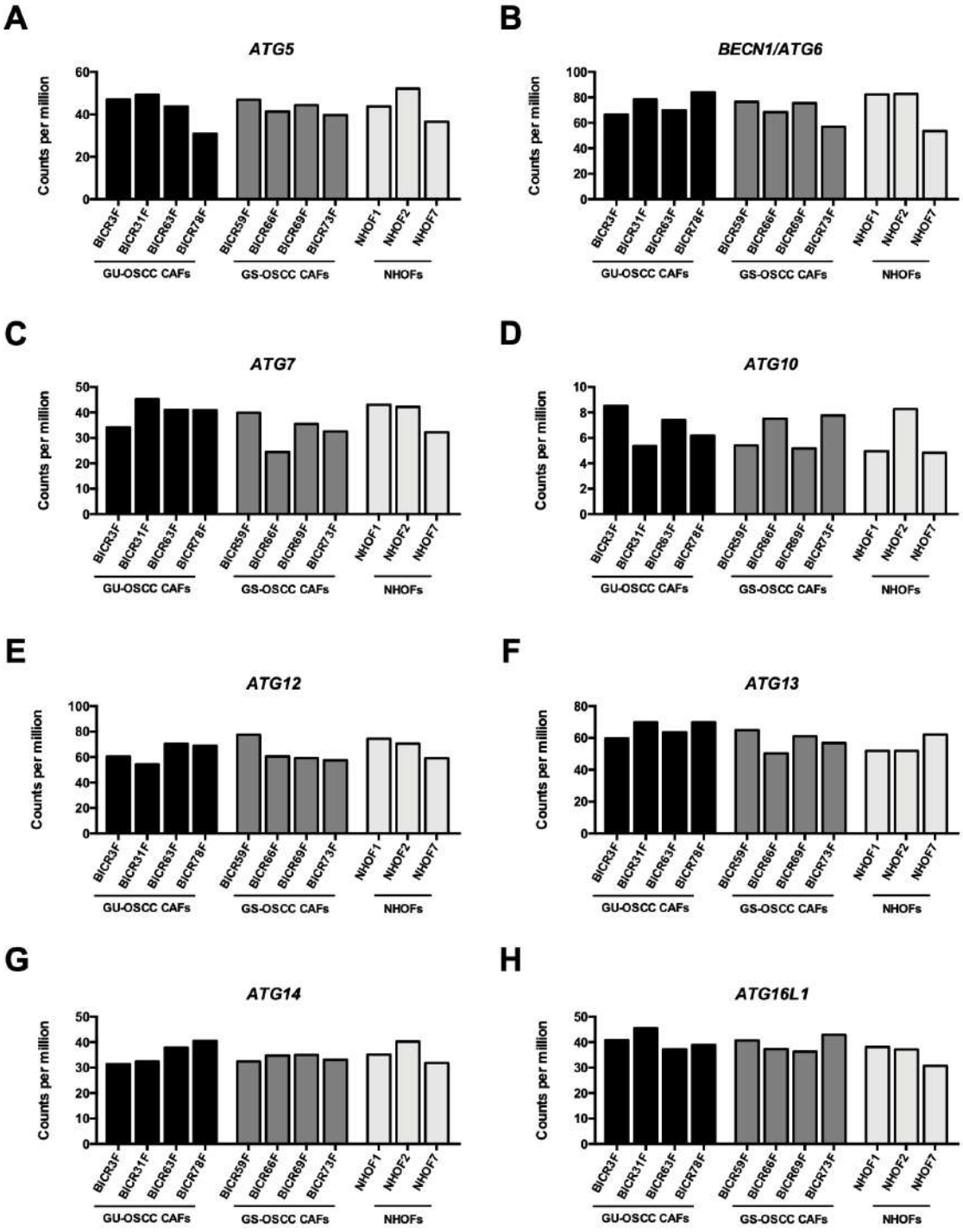


Figure 4.11: Transcript quantification of autophagy-associated genes in CAFs and NHOFs with RNA sequencing

Bar charts displaying RNAseq data from a panel of CAFs from GU-OSCCs (n = 4), GS-OSCCs (n = 4) and normal fibroblasts (n = 3). The transcript abundance of each selected *ATG* gene (A) *ATG5*, (B) *BECN1/ATG6*, (C) *ATG7*, (D) *ATG10*, (E) *ATG12*, (F) *ATG13*, (G) *ATG14* and (H) *ATG16L1* was expressed in counts per million. There were no statistically significant differences (ANOVA) in the expression of these genes between GU-OSCC CAFs, GS-OSCC CAFs and NHOFs by RNAseq.

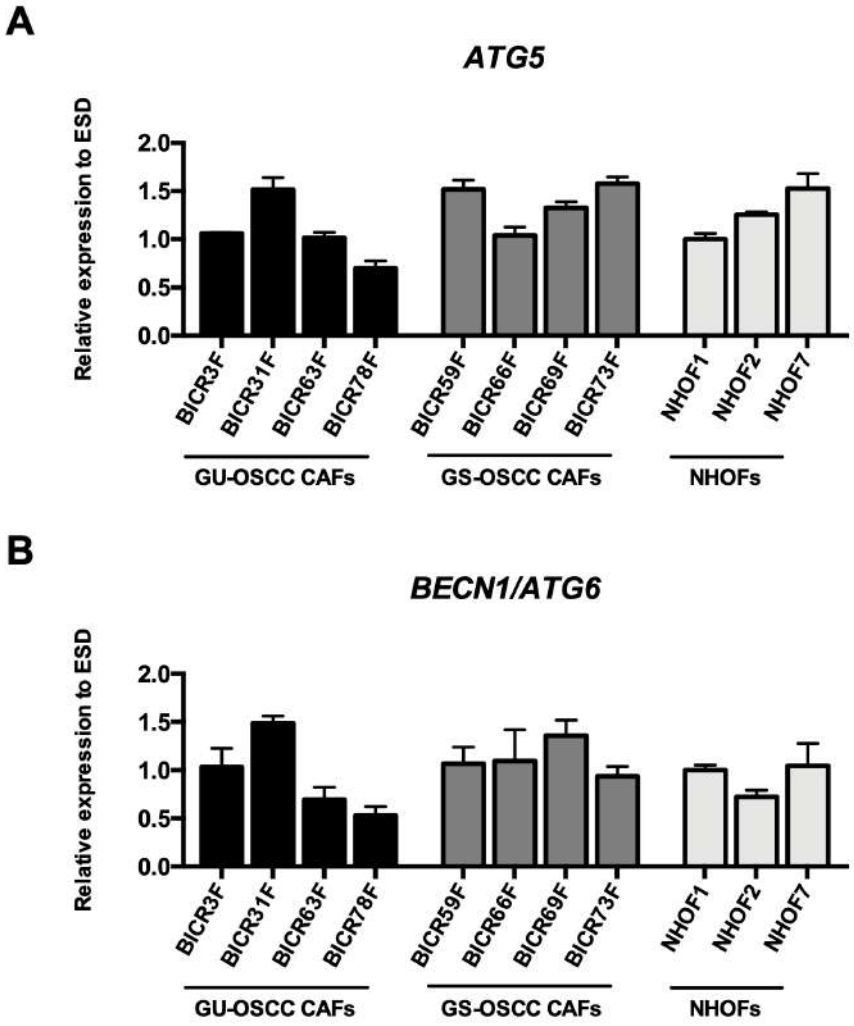


Figure 4.12: Corroboration of RNAseq results by qPCR for *ATG5* and *BECN/ATG6* mRNAs in NHOFs and CAFs

The expression of both (A) *ATG5* and (B) *BECN1/ATG6* mRNAs between all three groups of fibroblasts was not statistically significant (ANOVA). These results indicated that the autophagosome accumulation observed in CAFs was unlikely to be associated with autophagic flux. The expression of *ATG5* and *BECN1/ATG6* was normalised to *ESD* and the data were expressed as mean ± SEM of triplicates. The data presented are representative of two independent experiments.

4.5.2 Concomitant expression of LC3B-II and p62 in oral fibroblasts and CAFs

The cytoplasmic protein, p62, is an autophagic substrate which binds to LC3 selectively and p62 itself is degraded efficiently by autophagy (Bjørkøy et al., 2005; Komatsu & Ichimura, 2010). The changes in the protein levels of p62 can be used to indicate a defect in the autophagic pathway or the turnover of protein aggregates during late autophagy and p62 expression inversely correlates with autophagic flux. The expression of p62 and LC3B-II proteins in fibroblast strains was determined using Western blot analyses. There was a concomitant accumulation of p62 with LC3B-II protein in fibroblasts from GU-OSCCs, and the expression of these proteins was higher in these cells relative to GS-OSCCs and NHOFs (Figure 4.13). Whilst there was heterogeneity in the expression across samples, this result suggested that there was impairment in the later stages of the autophagic pathway in these fibroblast strains and it was in consistence with the mRNA levels of *p62* as determined by RNAseq (Figure 4.14). Taken together with the observation that the expression of *BECN1/ATG6* and *ATG5* mRNA was unchanged in CAFs (Figure 4.12), these data strongly indicate that the accumulation of autophagosomes in CAFs from GU-OSCCs was due to disruption at the degradation stage of the autophagic pathway.

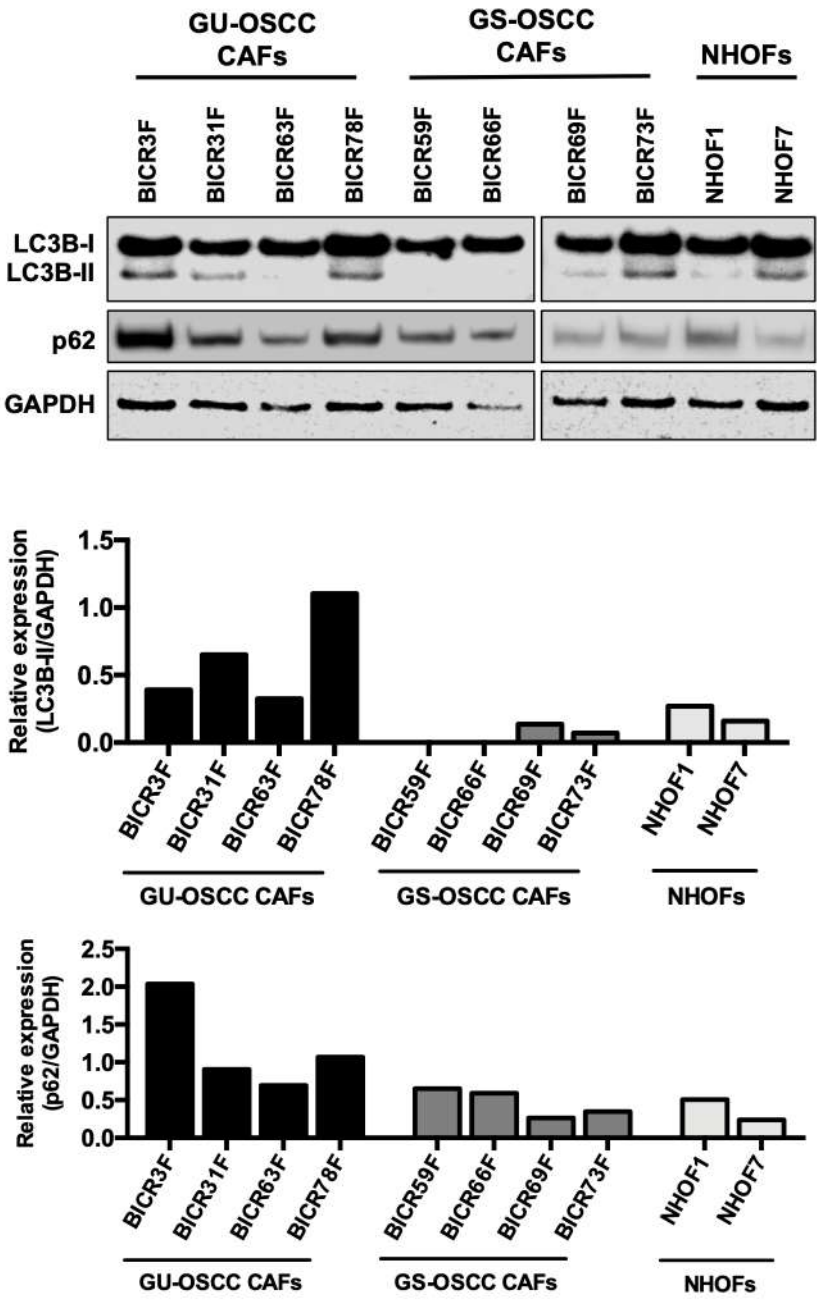


Figure 4.13: Concomitant expression of LC3B-II and p62 proteins in CAFs from GU-OSCCs revealed autophagic impairment in these cells

Western blot analyses demonstrated that there were concomitant accumulations of p62 with LC3B-II at protein levels in CAFs from GU-OSCCs, and the expression of both proteins was relatively higher than of CAFs from GS-OSCCs and normal oral fibroblasts. This observation suggested that autophagosome accumulation in CAFs from GU-OSCCs was most likely to be due to disruption at the autophagic pathway. The densitometric data are expressed as relative expression normalised to GAPDH. Representative Western blot images of three independent experiments are presented.

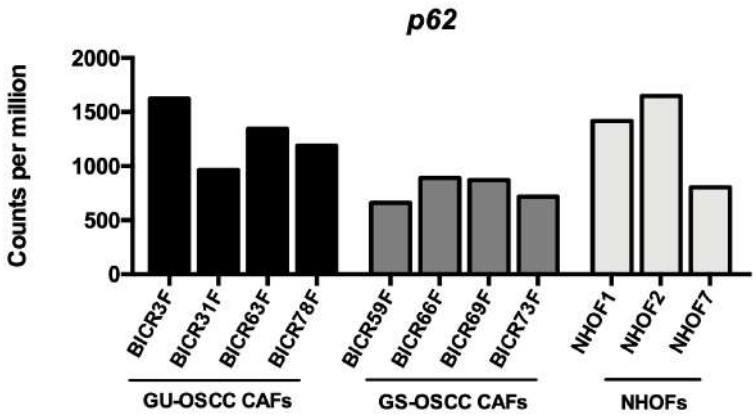


Figure 4.14: Expression of *p62* by RNA sequencing

Bar plots based on counts per million mapped reads across 11 fibroblast samples. The mRNA levels of *p62* is consistent with the p62 protein expression level demonstrated in Western blot.

4.6 Summary

This study examined the relationship of senescence and autophagy by characterising these phenotypes in different subsets of fibroblasts derived from oral carcinomas (CAFs from GU-OSCCs, CAFs from GS-OSCCs) and normal oral mucosa (NHOFs).

The senescence phenotypes were shown to be more apparent in fibroblasts derived from GU-OSCCs. These CAFs were shown to have enlarged cell size, hypertrophy, vacuolisation and flattened cell morphology with indistinct cell membranes. These morphologies are typical of senescent cells in culture. CAFs from GS-OSCCs and NHOFs were smaller in size with elongated fibroblastic morphology as compared to CAFs from GU-OSCCs. CAFs from GU-OSCCs divided at a slower rate than CAFs from GS-OSCCs and NHOFs. Furthermore, SA-β-gal enzyme activity was significantly higher in CAFs from GU-OSCCs than NHOFs and CAFs from GS-OSCCs. The expression level of a late senescent marker, SIRT1, and the CDK inhibitor, $p21^{Waf1/Cip1}$, were generally consistent with the observations that senescence phenotypes were more evident in CAFs from GU-OSCCs.

The expression of autophagy-related LC3B-II and accumulation of LC3B puncta were demonstrated by Western blotting and immunofluorescent staining, respectively, and shown to be more apparent in CAFs from GU-OSCCs. These results revealed that both the senescent phenotype and the accumulation of autophagosomes were more apparent in fibroblasts from GU-OSCCs. These two phenotypes appeared to be closely related because there was a positive correlation between SA-β-gal enzyme activity and autophagosome accumulation. The elevated expression of the autophagic substrate protein p62, together with the unchanged expression of *ATG5* and *BECN1/ATG6* mRNAs in qPCR indicated that autophagy was more likely to be disrupted at the degradation step in CAFs from GU-OSCCs.

Collectively, the results of the present study demonstrate that both senescence and autophagy were deregulated in CAFs from GU-OSCCs. It was also shown that senescence and autophagy were closely associated in fibroblasts, suggesting that these processes may be part of the same biological phenomenon and/or part of a unified programme.

CHAPTER 5: CONTRIBUTION OF AUTOPHAGY TO FIBROBLAST ACTIVATION AND SENESCENCE AND ITS ROLE IN MEDIATING CAF FUNCTIONS

5.1 Introduction

CAFs demonstrate functional heterogeneity with activation, autophagy and senescence being common stress responses. Previous reports have indicated that fibroblast activation (or myofibroblast differentiation) and senescence are closely linked mechanisms and they share common tumour-promoting properties (Hassona et al., 2013; Mellone et al., 2016). The mechanisms underlying the tumour-promoting activities of these two phenotypes are yet to be fully elucidated, but there is evidence to suggest they are regulated through similar signalling pathways (Alspach et al., 2014; Demehri et al., 2009; Procopio et al., 2015). The results of the present study (Chapter 4) showed that senescence is also closely associated with autophagy and both processes are deregulated in CAFs from GU-OSCCs. Collectively, these data suggest that there may be a mechanistic/functional link between activation, autophagy and senescence in fibroblasts. Whilst it has been suggested that these physiological states of CAFs may reflect different stages of the same sequential regulatory pathway in fibroblast differentiation (Hassona et al., 2013; Mellone et al., 2016), this has yet to be tested experimentally in OSCC. Furthermore, the contribution of autophagy to the tumour-promoting properties of senescent CAFs is unclear.

The purpose of the present study, therefore, was to establish an *in vitro* model to examine the involvement of autophagy in fibroblasts activation and senescence. Such a model would also facilitate the investigation of the role of autophagy in mediating CAF phenotypes. To achieve these aims, I used known senescence inducers, e.g. irradiation and TGF-β1 in NHOFs and I took advantage of specific autophagic inhibitors to

examine the functional relevance of autophagy to fibroblast differentiation and how this might indirectly influence OSCC cell proliferation, migration and invasion.

5.2 Autophagy induction

5.2.1 Ionising radiation as an autophagy inducer

High dose of ionising radiation (IR) had been reported to induce cellular senescence and generate a strong DNA damage response (DDR) (Minty, Thurlow, Harrison, & Parkinson, 2008; Rodier et al., 2009). Radiation-induced autophagy has also been demonstrated in various cell types (Gewirtz, 2014; Zois & Koukourakis, 2009) and modulating autophagy in different cellular contexts could have produced different outcomes, as autophagy is known to play a complex role in cancer (Onorati, Dyczynski, Ojha, & Amaravadi, 2018). However, the effect of IR on the induction of autophagy in oral fibroblasts has not been tested. Hence, in this study, the effects of both high and low doses of IR in inducing activation, autophagy and senescence in oral fibroblasts were examined. High doses of IR were used to induce irreparable DNA damage, whilst low levels of DNA damage were induced with low doses of IR.

Normal oral fibroblasts (NHOF1) were irradiated with 0.5 (low dose IR) or 20 Gy (high dose IR) and allowed to recover for 5, 10 and 20 days. Concomitant expression of LC3B-II and p62 proteins indicates autophagic impairment, while p62 expression inversely correlates with autophagic flux. As shown in Figure 5.1, there was an accumulation of LC3B-II when NHOF1 cells were irradiated with 20 Gy, indicating potential accumulation of autophagosomes. However, it was not clear that whether the accumulation of autophagosomes was due to true upregulation of autophagic degradation (autophagic flux) or impairment in the autophagic pathway, as the basal level of p62 expression was relatively high and only varied marginally across the time points.

In addition, the expression of α-SMA was constant across irradiation doses as well as different time points. Nonetheless, there was evidence of deep senescence 10 - 20 days after NHOF1 was irradiated with 20 Gy gamma radiation; SIRT1 was downregulated and this occurred together with an upregulation of $p21^{Waf1/Cip1}$ and $p16^{Ink4a}$ expression. This suggested that the high dose of IR had triggered irreparable DNA damage, resulting in cellular senescence (Minty et al., 2008) by day 10. Low doses of IR, 0.5 Gy, did not result in obvious changes in the expression of α-SMA and SIRT1, most likely because the cells could repair their DNA damage and return to cell cycle after temporary cell cycle arrest (Schäuble et al., 2012), as indicated by slightly elevated $p21^{Waf1/Cip1}$ and $p16^{Ink4a}$ levels.

Taken together, these findings showed that high dose IR enhanced autophagosome (LC3B-II) accumulation and induced senescence, independently of fibroblast activation, although the autophagic status of the cells during these responses was inconclusive due to high and constant basal levels of p62. Consequently, this model was not used for subsequent experiments, as autophagy induction or inhibition could not be confirmed.

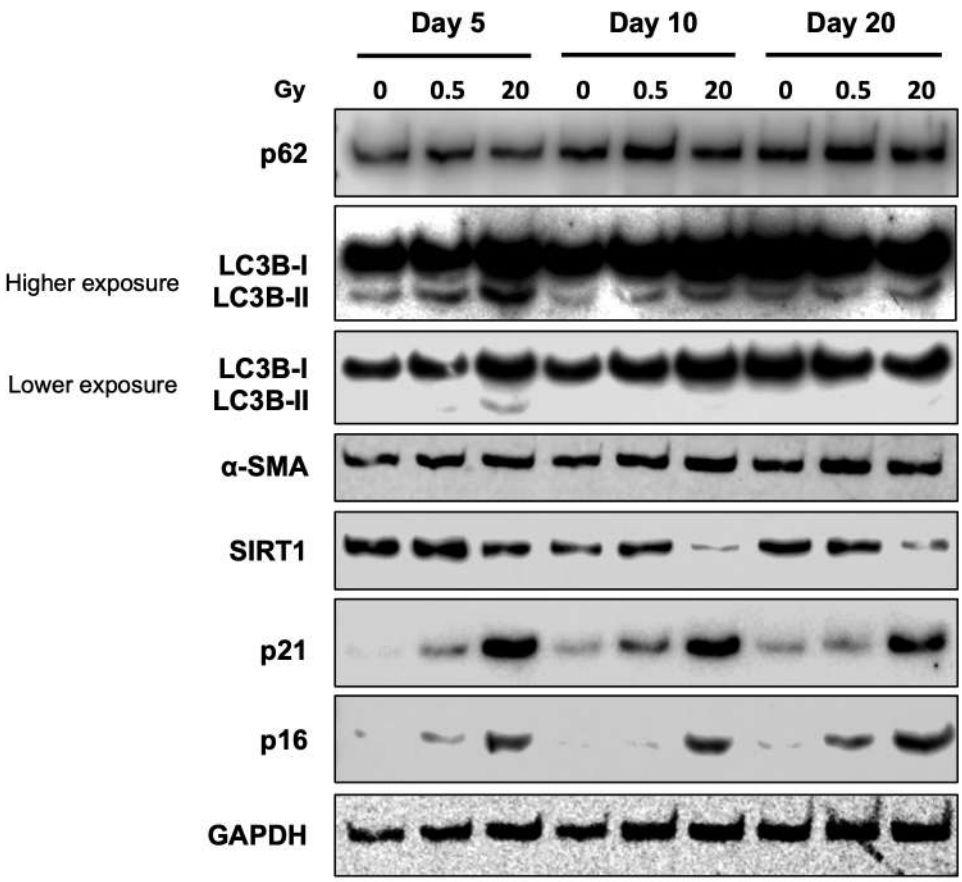


Figure 5.1: High dose of ionising radiation enhanced autophagosome accumulation along with senescence, independently of fibroblast activation

Western blot analysis of markers of autophagy, activation and senescence following irradiation of NHOF1. The blot was exposed to different degrees in order to visualise LC3B-II. Following 20 Gy irradiation, LC3B-II levels increased and this indicated autophagosome accumulation. This occurred concomitantly with SIRT1 downregulation and upregulation expression of $p21^{Waf1/Cip1}$ and $p16^{Ink4a}$ expression. There was evidence of deep senescence when NHOF1 was irradiated with 20 Gy gamma radiation and allowed to settle for 10 and 20 days. Low dose of ionising radiation, 0.5 Gy, did not result in obvious changes in the expression of these markers, as cells could repair their DNA damages and returned to cell cycle after temporary cell cycle arrest.

5.2.2 TGF-β1 as an autophagy inducer

TGF-β1 has been shown to trigger both replicative (Pascal et al., 2005) and oncogene-induced senescence (S. Lin et al., 2012). It was previously reported that CAFs from GU-OSCCs produced high levels of TGF-β relative to normal fibroblasts or CAFs from GS-OSCCs and that CAFs could reinforce their own senescence state by producing increased levels of ROS in a TGF-β1-dependent manner (Hassona et al., 2013). In view of the fact that TGF-β1 is able to induce both activation and senescence in fibroblasts (Hassona et al., 2013; Mellone et al., 2016), the role of autophagy in this process was investigated.

5.2.2.1 Short term treatment of NHOFs with TGF-β1

To develop a model with which to study autophagy in the context of fibroblast activation and senescence, NHOF1 cells were first treated with 4 ng/mL of TGF-β1 for 5 days. The results showed there was a progressive rise of SA-β-gal activity and the TGF-β1-treated normal fibroblasts appeared to be morphologically flatter and hypertrophic (Figure 5.2). Over 5 days, autophagy, fibroblast activation and senescence were induced, with autophagic flux preceding fibroblast activation, which in turn was followed by senescence (Figure 5.3A). The occurrence of real autophagic flux was demonstrated by the increased level of LC3B-II, together with a reduction in expression of p62 as shown by Western blotting (Figure 5.3). Fibroblast activation, as indicated by the elevation of α-SMA expression, started at day 3 and was sustained until day 5. The expression of the cyclin-dependent kinase inhibitors $p21^{Waf1/Cip1}$ and $p16^{Ink4a}$ was generally upregulated following TGF-β1 treatment, as shown in Figure 5.3A. However, even though $p16^{Ink4a}$ was elevated at day 3, the late senescence marker, SIRT1 was not downregulated, suggesting that deep senescence had not occurred during the time course of the experiment. Therefore, I proceeded to perform a similar experiment over a

longer time period in order to examine the relationship between autophagic flux and late senescence.

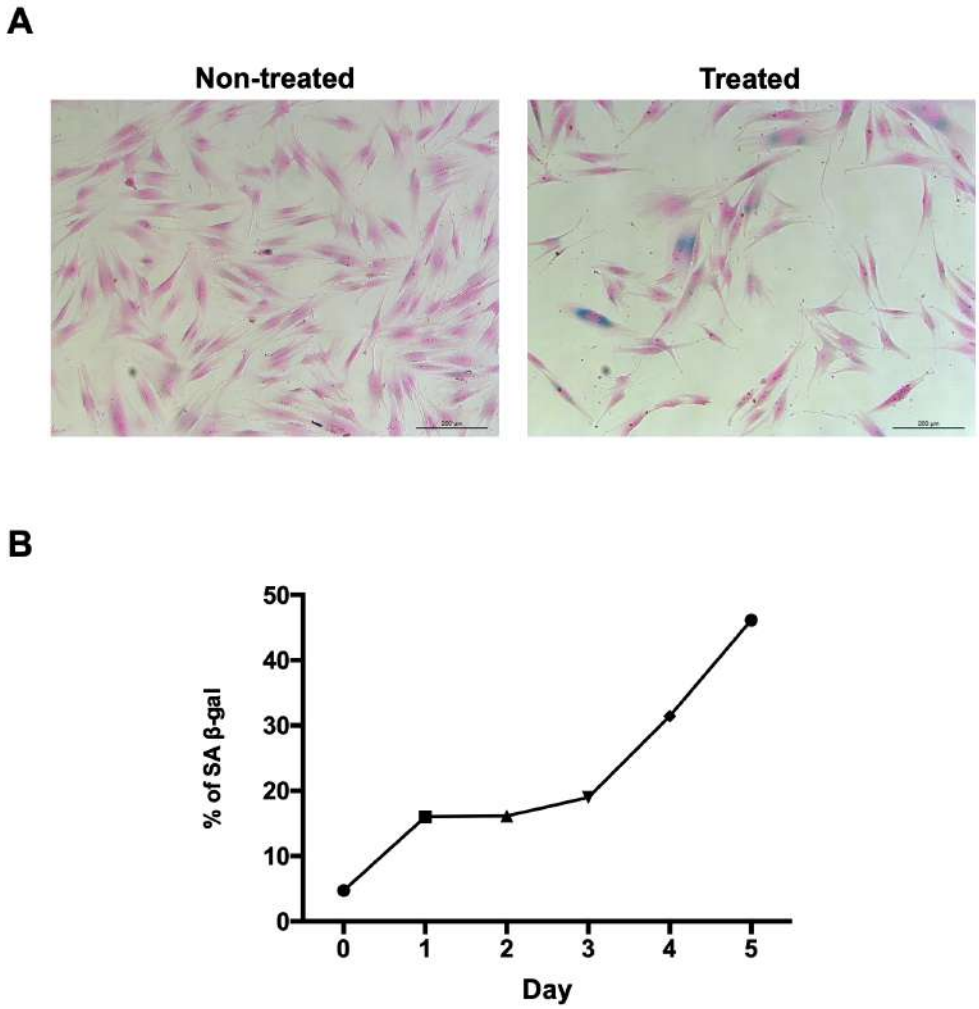


Figure 5.2: TGF-β1 induced progressive elevation of SA-β-gal activity in normal oral fibroblasts

(A) Representative images showing non-treated and TGF-β1-treated NHOF1 at Day 5. Scale bar indicates 200 μm. TGF-β1-treated cells had flattened, enlarged and hypertrophic morphology, which are typical of the senescent phenotype. (B) Percentage of SA-β-gal-positive cells when NHOF1 was treated with TGF-β1. A minimum of 200 cells was counted for each time point. There was a progressive elevation of SA-β-gal activity over 5 days of TGF-β1 treatment.

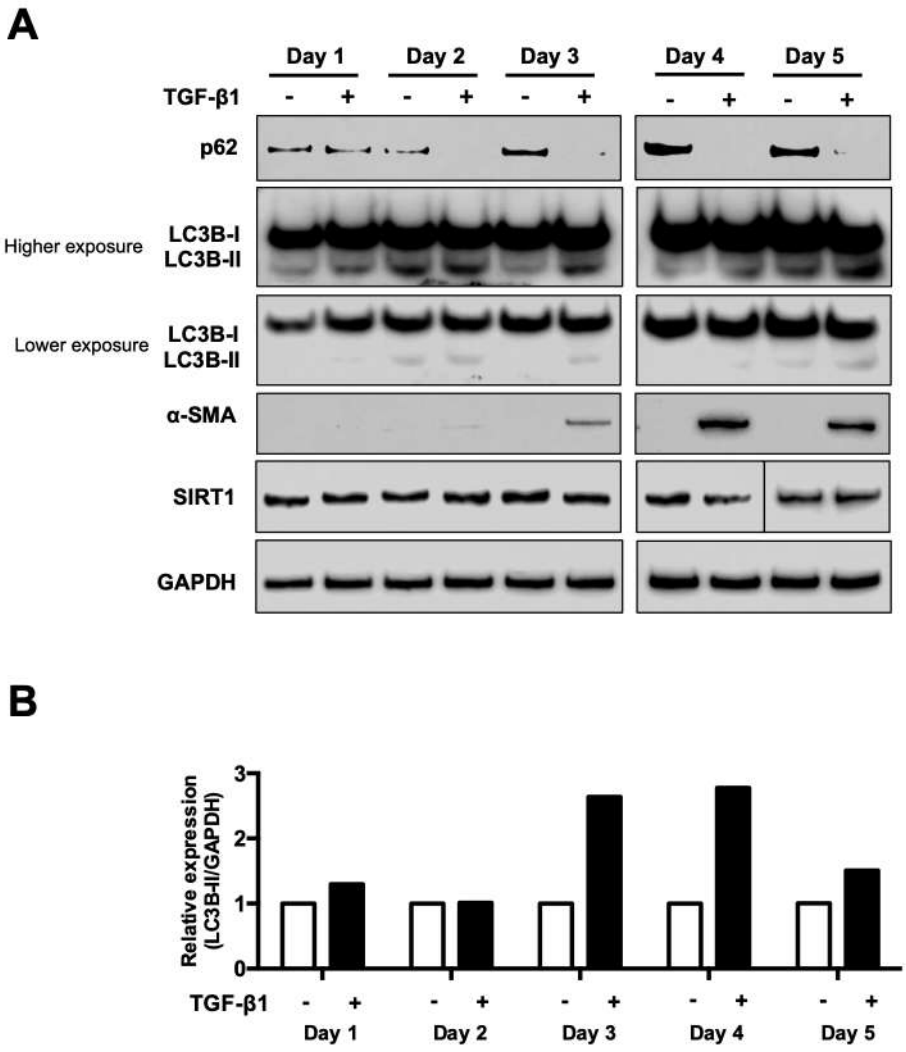


Figure 5.3: TGF-β1 induced fibroblast autophagic flux, activation and senescence

(A) Induction of autophagic flux, α-SMA expression, induction of $p21^{Waf1/Cip1}$ and $p16^{Ink4a}$ in NHOF1 treated with daily doses of 4 ng/mL TGF-β over 5 days. The blot was exposed for different times in order to visualise LC3B-II (lower band). Although $p16^{Ink4a}$ was elevated at day 3, SIRT1 was not downregulated, suggesting that there was no occurrence of late senescence. The results are representative of three independent experiments. (B) Densitometry analysis of LC3B-II expression was performed using ImageJ software (NIH). The data were normalised against GAPDH and expressed relative to the fibroblasts treated with vehicle control (=1). There was an increased level of LC3B-II concomitant with reduction/absence in p62 expression, confirming that there was an induction of autophagic flux.

5.2.2.2 Longer term treatment of NHOFs with TGF-β1

The above results prompted me to examine the relationship between autophagic flux, activation and late senescence. I next examined these phenotypes following TGF-β1-induced senescence over 10 days. The response of normal fibroblasts to 4 ng/mL TGF-β1 treatment was tested in two normal oral fibroblast strains, namely NHOF1 and NHOF2. These parameters (concentration and time) were chosen as they have been shown previously to effectively induce activation and senescence in oral fibroblasts (Hassona et al., 2013).

Over 10 days of treatment with TGF-β1, a striking change in cell morphology was observed. The normal oral fibroblasts gradually changed their spindle-like morphology to more flattened, enlarged and irregularly shaped cells (Figure 5.4A). Both NHOF1 and NHOF2 showed a progressive rise in SA-β-gal activity over 10 days of TGF-β1 treatment (Figure 5.4A and B), indicating that senescence was induced in these cells. Late senescence was confirmed with the loss of SIRT1 protein and fibroblast activation was indicated by the upregulated expression of α-SMA (Figure 5.5). Autophagic flux was demonstrated in Western blot analyses, as there was an increase in the expression of LC3B-II protein accompanied by downregulation of p62 expression following TGF-β1 treatment (Figure 5.5).

Taken together, these results demonstrated that TGF-β1 could induce autophagic flux (activation of autophagy), fibroblast activation (myofibroblast transdifferentiation) and senescence, with autophagic flux preceding fibroblast activation, followed by senescence. Since this effect of TGF-β1 was more evident in NHOF2, this normal fibroblast strain was selected for use in subsequent functional experiments (Section 5.4).

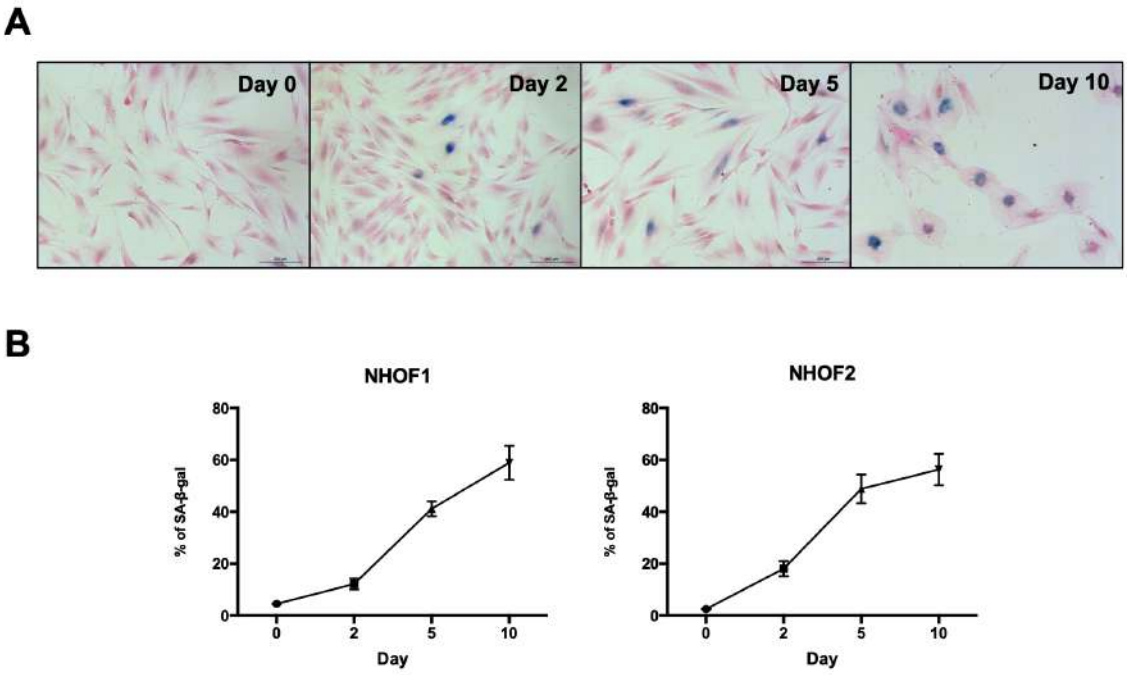


Figure 5.4: TGF-β1 induced senescence over a period of 10 days

(A) Representative images of SA-β-gal staining in 4 ng/mL TGF-β1-treated normal oral fibroblasts over a period of 10 days. Oral fibroblasts underwent a morphological transformation typical of the senescence phenotype with a flat irregular shape. Scale bar indicates 200 μm. (B) Progressive increase of SA-β-gal activity in fibroblasts following TGF-β1 treatment for 10 days. Data presented are of three independent experiments.

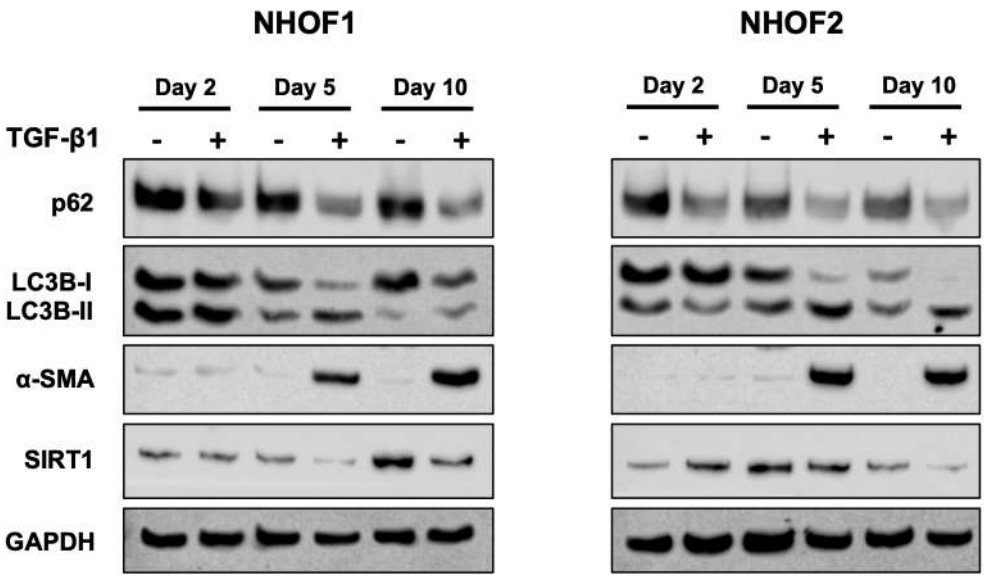


Figure 5.5: TGF-β1 induced autophagic flux, activation and late senescence over a period of 10 days

Western blotting for autophagic (p62 and LC3B-II), activation (α-SMA) and senescent (SIRT1) markers following 10 days of 4 ng/mL TGF-β1 treatment (GAPDH as a loading control) in two different strains of normal fibroblasts, NHOF1 and NHOF2. TGF-β1 could induce autophagic flux, activation and senescence with autophagic flux preceding activation, followed by senescence in normal fibroblasts. The results are representative of three independent experiments.

5.3 Autophagy inhibition

To study the role of autophagy in influencing the processes of activation and senescence in fibroblasts, two pharmacological inhibitors targeting different stages of the autophagic pathway were used.

5.3.1 Hydroxychloroquine (HCQ) as an autophagy inhibitor

HCQ is a derivative of CQ (Manic, Obrist, Kroemer, Vitale, & Galluzzi, 2014). Both of these chemicals are FDA-approved autophagic inhibitors that function by preventing fusion of lysosome and autophagosome, thereby inhibiting the last stage of autophagic degradation (Klionsky et al., 2016). HCQ is widely employed in both *in vitro* as well as *in vivo* experiments to block autophagy and is currently being evaluated in several on-going clinical trials to treat specific cancers (Maes et al., 2013).

In this study, the efficacy of HCQ in inhibiting autophagy was tested and the effect of autophagy inhibition on activation and senescence was examined. Two normal oral fibroblast strains, namely NHOF1 and NHOF2 were treated with increasing doses of HCQ (1 – 10 μM) for 72 hours. The inhibition of autophagy by HCQ in both normal fibroblast strains was confirmed by the concomitant expression of LC3B-II and p62 proteins, as determined by Western blotting (Figure 5.6). The highest dose of HCQ treatment (10 μM) resulted in a moderate reduction of SIRT1 expression, while there was minimal variability in α-SMA expression, suggesting that HCQ treatment has a less pronounced effect in inducing fibroblast activation and senescence at this concentration.

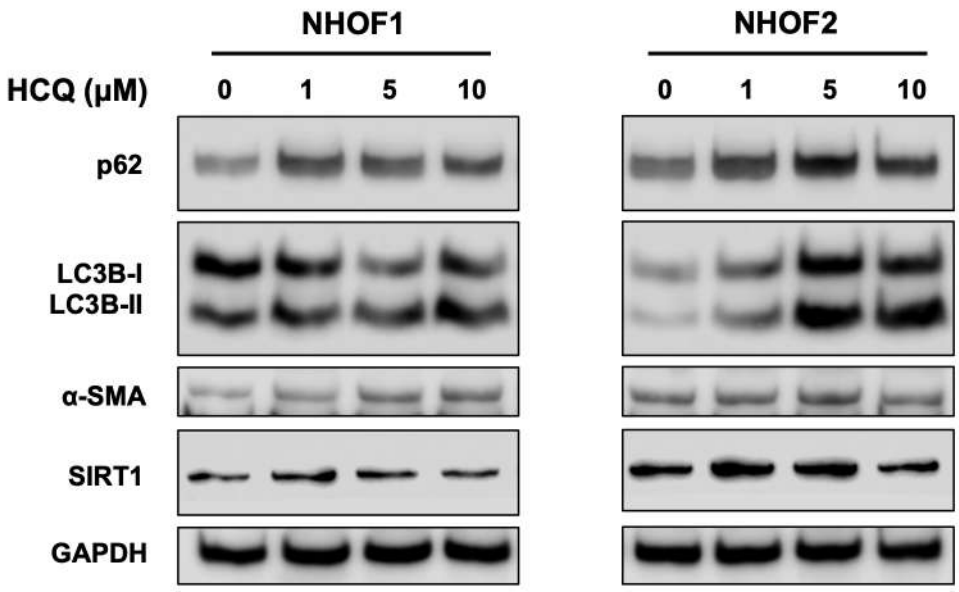


Figure 5.6: HCQ effectively impaired autophagic degradation in NHOFs but had a marginal effect on activation and senescence

NHOF1 and NHOF2 cells were treated for 72 hours with increasing concentrations of HCQ in complete medium, as indicated. Cells were then processed for Western blotting and probed with autophagic (p62 and LC3B-II), senescent (SIRT1), activation (α-SMA) markers and GAPDH was used as a loading control. Autophagic inhibition was observed in fibroblasts after treatment of HCQ (1, 5 and 10 μM) for 72 hours.

5.3.2 SAR405 as an autophagy inhibitor

Having shown that HCQ prevented autophagy degradation in NHOF1 and NHOF2 normal oral fibroblasts but had minimal effect on activation and senescence, the efficacy of autophagic inhibition was further explored using SAR405, a more specific autophagic inhibitor that selectively targets vascular protein sorting 34 (Vps34, also known as PI3K class III) (Ronan et al., 2014). Vps34 is a downstream protein of mTOR and it is commonly found within protein complexes with Beclin1/ATG6 in cells, which is required for autophagy initiation (Backer, 2008; J. Kim et al., 2013).

Western blotting was performed to test the efficacy of SAR405 in inhibiting autophagy in NHOF1 and NHOF2. The results confirmed that SAR405 inhibited autophagy in both normal fibroblast strains, with concomitant expression of LC3B-II

and p62 proteins (10 μM SAR405 treatment in NHOF1, 0.1, 1 and 10 μM SAR405 treatment in NHOF2) (Figure 5.7A). Treatment of fibroblasts with increasing doses of SAR405 (1 – 10 μM) for 72 hours resulted in a dose-dependent accumulation of p62 protein, along with LC3B-II, demonstrating that there was a dose-dependent inhibition of autophagy. In addition, upregulation of α-SMA and downregulation of SIRT1 were also observed in a dose-dependent manner following SAR405 treatment (Figure 5.7B).

Considering that the effect of SAR405 was more prominent in NHOF2 as compared to NHOF1, a time-course experiment was performed using NHOF2 cells with 10 μM of SAR405 to further investigate the effect of SAR405 in inhibiting autophagy over a period of 120 hours. As determined by Western blot analysis (Figure 5.8A), SAR405 inhibited autophagy from 24 hours onwards. At 72 hours, elevation in α-SMA was observed in SAR405-treated cells, suggesting fibroblast activation was initiated at 72 hours post-treatment. It was also shown that SIRT1 expression was gradually lost with a higher concentration of 10 μM SAR405 over the treatment period.

Taken together, autophagic inhibition, senescence and fibroblast activation were more evidently observed in NHOF2 after treatment of SAR405 (10 μM) for 72 hours (Figure 5.7) as compared to HCQ at the same concentration (Figure 5.6) (Section 5.3.1). These results suggested that SAR405 is a more potent inhibitor than HCQ in blocking the autophagy pathway. Inhibition of autophagy with 10 μM SAR405 induced activation and senescence in normal oral fibroblasts, suggesting these two biological responses are regulated by autophagy. Therefore, the treatment of NHOF2 with 10 μM SAR405 for 120 hours was used for subsequent functional studies (Section 5.4).

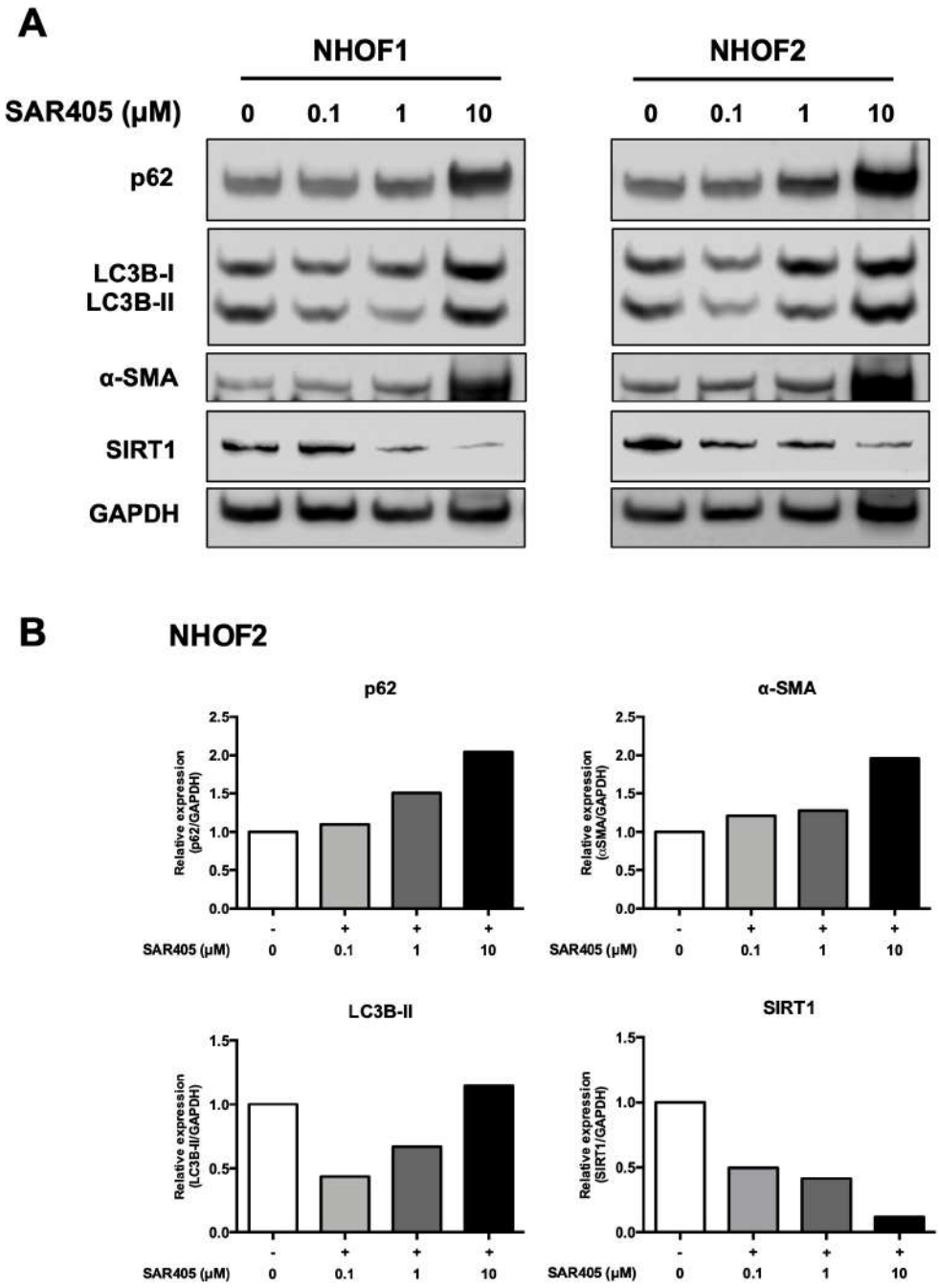


Figure 5.7: SAR405 prevented autophagy in normal oral fibroblasts

(A) Dose-response experiments of SAR405, a specific Vps34 inhibitor were performed using NHOF1 and NHOF2. The cells were treated for 72 hours with increasing concentrations of SAR405, as indicated. Cells were then lysed for Western blotting and probed with autophagic (p62 and LC3B-II), senescent (SIRT1), activation (α-SMA) markers and GAPDH was used as a loading control. (B) Densitometry of protein expression in SAR405-treated NHOF2 fibroblasts was quantified using ImageJ software (NIH). The intensity of bands was normalised to GAPDH and expressed relative to fibroblasts treated with vehicle control (=1). A dose-dependent increase of p62, LC3B-II, α-SMA and reduction of SIRT1 was observed.

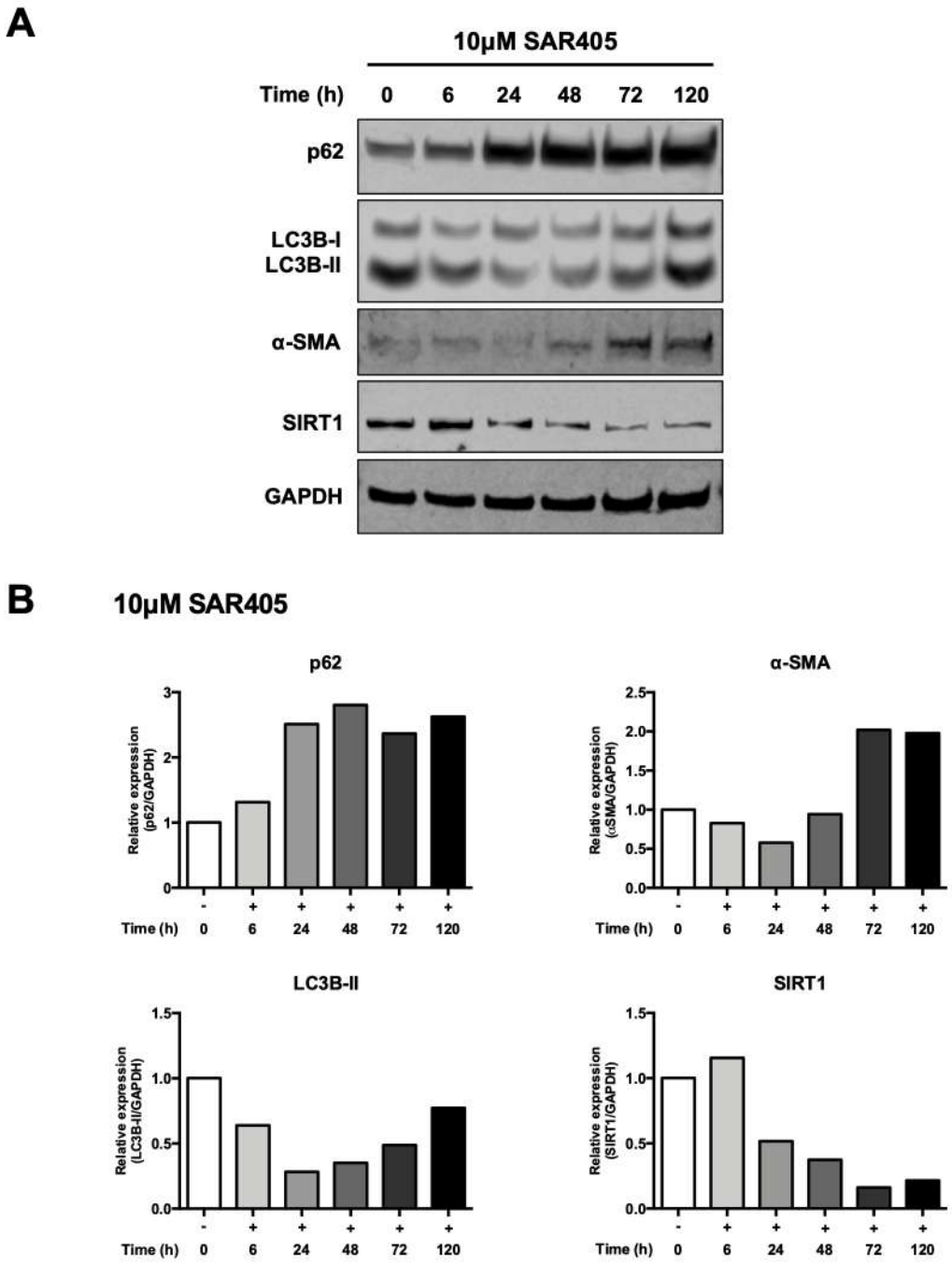


Figure 5.8: Autophagy impairment induced fibroblast activation and senescence in normal oral fibroblasts

(A) Time-course experiments of 10 μM SAR405 in NHOF2 over 120 hours treatment. Cells were prepared for Western blotting and probed with autophagic (p62 and LC3B-II), senescent (SIRT1), activation (α-SMA) markers and GAPDH was used as a loading control. Treatment time and concentration were as indicated. (B) Densitometry of protein expression in 10 μM SAR405-treated NHOF2 was quantified using ImageJ software (NIH). The intensity of bands was normalised to GAPDH and expressed relative to fibroblasts treated with vehicle control (=1). Autophagy inhibition was shown to induce fibroblast activation and senescence.

5.4 Functional significance of fibroblast autophagy in regulating OSCC cell behaviour

To determine if autophagic flux and autophagy impairment in fibroblasts have any biological significance to the malignant behaviour of epithelial cells, the potential of autophagic-inhibited or autophagic-activated fibroblasts to affect OSCC cell proliferation, migration and invasion were assessed. As it has been shown previously that factors secreted by OSCC CAFs could influence OSCC progression (Hassona et al., 2013; Lim et al., 2011; Mellone et al., 2016), conditioned media (CM) were collected from autophagic-inhibited and autophagic-activated fibroblasts to test if these cells show any functional similarity to CAFs.

Normal oral fibroblasts (NHOF2) were treated for 120 hours with 10 μM SAR405 to inhibit autophagy, while 4 ng/mL of TGF-β1 was added to induce autophagic flux. Autophagy inhibition was confirmed by high LC3B-II and high p62 expression, whereas autophagic flux was confirmed by high LC3B-II and low p62 expression on Western blots (Figure 5.9A). Both autophagy inhibition and activation induced an α-SMA-positive phenotype, in addition to suppressing SIRT1 expression in fibroblasts (Figure 5.9A). These findings were consistent with the growth behaviour of NHOF2 as these cells proliferated more slowly after treatment with SAR405 and TGF-β1 (SAR405 = 0.38 ± 0.06 fold, $p<0.0001$; TGF-β1 = 0.68 ± 0.04 fold, $p<0.001$) (Figure 5.9B). In addition, SA-β-gal staining showed that treated fibroblasts exhibited more SA-β-gal positive cells (SAR405 = 2.89 ± 1.13 fold, but this is not statistically significant, $p = 0.31$; TGF-β1 = 5.67 ± 0.21 fold, $p<0.01$) compared to untreated controls (= 1; Figure 5.9C and D). Both SAR405- and TGF-β1-treated NHOF2 displayed features typical of senescent cells (Figure 5.9D). These observations demonstrated that both autophagy inhibition and the induction of autophagic flux could induce senescence in normal oral fibroblasts.

Following treatment of NHOF2 with SAR405 to inhibit autophagy or TGF-β1 to induce autophagic flux, CM was collected after 48 hours and tested for effects on cell proliferation and used as a chemoattractant in transwell migration and invasion assays, using H376 OSCC cells. Compared to vehicle controls, the proliferation of H376 cells was significantly decreased ($p<0.05$) following treatment with CM from both autophagy-inhibited (SAR405 = 0.68 ± 0.07 fold) and autophagy-activated NHOF2 cells (TGF-β1 = 0.69 ± 0.08 fold) (Figure 5.10).

CM from SAR405- or TGF-β1-treated NHOF2 alone increased the migration of H376 cells (Figure 5.11A) relative to CM from untreated cells. However, no additional effect on the migration of H376 cells was observed in the presence of CM from NHOF2 cells treated with both SAR405 and TGF-β1, suggesting that SAR405-induced autophagy inhibition and TGF-β1-induced autophagic flux might influence OSCC cell migration by similar mechanisms. The invasive behaviour of H376 cells was not affected following treatment with CM from SAR405-treated NHOF2 (0.98 ± 0.18 fold) but was greatly enhanced by CM from TGF-β1-treated NHOF2 (5.55 ± 0.61 fold) (Figure 5.11B). In the presence of CM from SAR405+TGF-β1-treated NHOF2 (5.25 ± 0.48 fold), the invasive effect was comparable to CM from TGF-β1-treated NHOF2.

In summary, the migration (Figure 5.11A) and invasion (Figure 5.11B) of H376 cells were both significantly enhanced ($p<0.001$) by CM from TGF-β1-treated normal fibroblasts, in agreement with previous reports (Hassona et al., 2013; Mellone et al., 2016). However, CM from SAR405-treated normal fibroblasts significantly enhanced the migration ($p<0.01$; Figure 5.11A) but not the invasion of H376 cells ($p>0.99$; Figure 5.11B). These data demonstrate that autophagy impairment in normal fibroblasts could enhance the migration of OSCC cells but did not affect tumour cell invasion, whilst

TGF-β1-induced autophagic flux could enhance both the migratory and invasive phenotypes of OSCC cells.

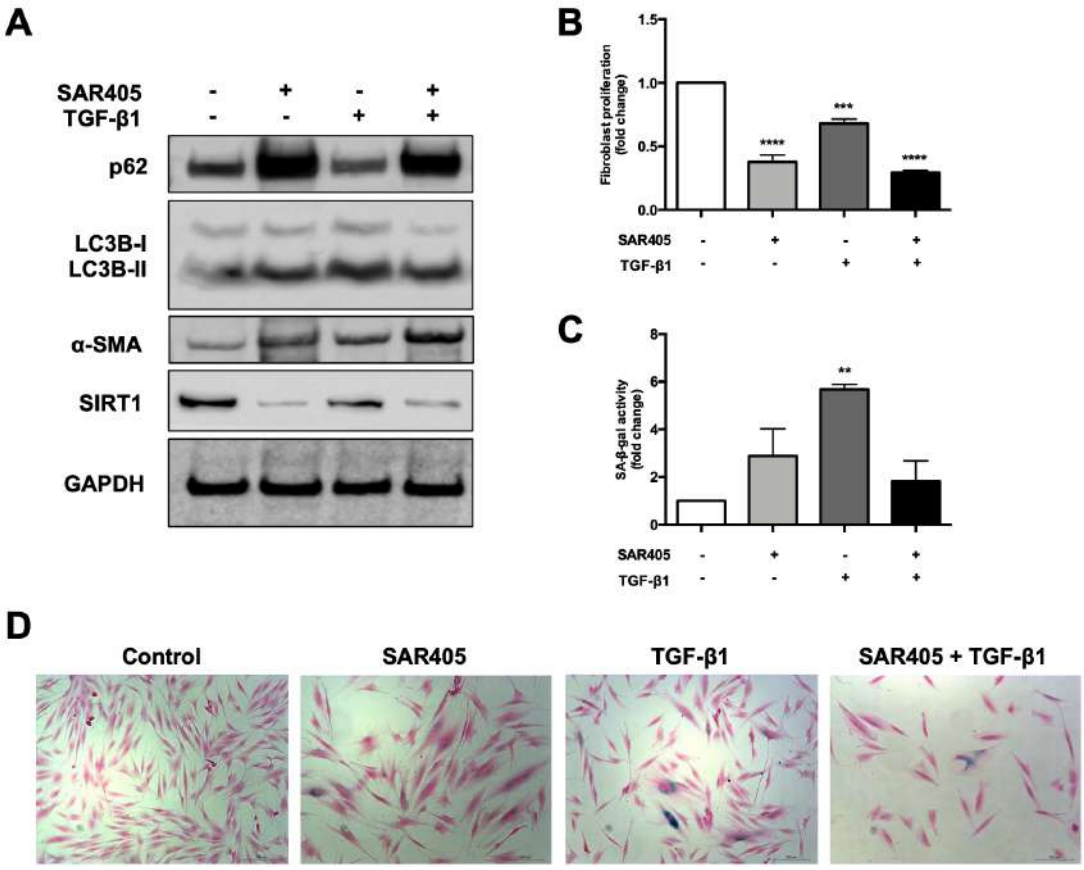


Figure 5.9: Autophagy impairment and autophagic flux induced activation and senescence in normal oral fibroblasts

(A) SAR405 inhibited autophagy (high LC3B-II and high p62) while TGF-β1 induced autophagic flux (high LC3B-II and low p62). Autophagy activity can alter the expression of α-SMA and SIRT1. (B) The proliferation rate of normal fibroblast was significantly lowered after SAR405 or/and TGF-β1 treatment as compared to vehicle control (=1). Bars, mean ± SD. ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$ (ANOVA). (C) These growth-arrested fibroblasts displayed a senescent phenotype of higher SA-β-gal positivity. Bars represent the fold change of SA-β-gal positive cells. Bars, mean ± SEM. * = $p<0.05$, ** = $p<0.01$ (ANOVA). (D) Representative images of SA-β-gal staining of SAR405- or/and TGF-β1-treated NHOF2. Treated fibroblasts exhibited typical characteristics of senescent cells with enlarged cell size, flatten in shape and contractile myofibroblast-like phenotypes. Scale bar indicates 200 μm.

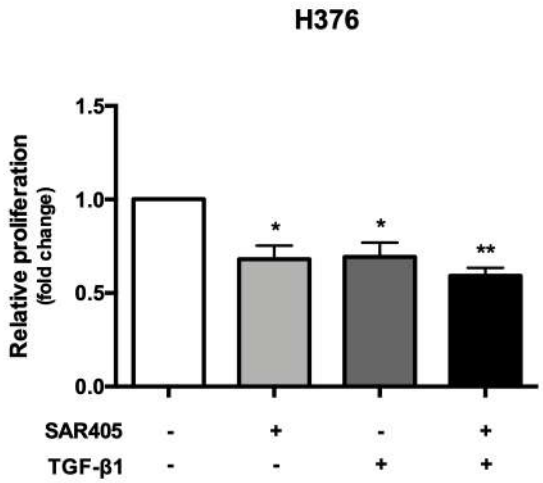


Figure 5.10: Autophagy impairment and autophagic flux in fibroblasts reduced the proliferation of OSCC cells

The proliferation of H376 OSCC cells treated with CM from SAR405-treated normal fibroblasts (autophagy inhibited) and TGF-β1-treated normal fibroblasts (autophagy activated). Negative control included H376 cells treated with CM from normal oral fibroblasts, NHOF2. The data presented are representative of three independent experiments. Bars, mean ± SD; * = $p<0.05$, ** = $p<0.01$ (ANOVA).

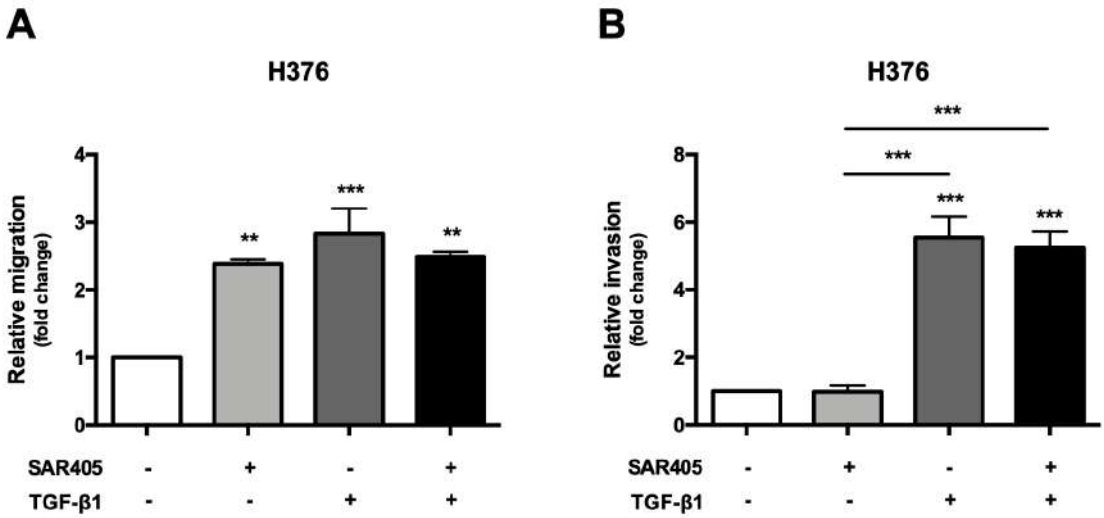


Figure 5.11: Migration and invasion of OSCC cells following SAR405-induced autophagic inhibition and TGF-β1-induced autophagic flux in oral fibroblasts

Transwell migration and invasion assays of H376 OSCC cells were carried in serum-free conditions for 24 and 48 hours, respectively. The lower chamber contained either CM from normal oral fibroblasts NHOF2 (control), SAR405-treated NHOF2, TGF-β-treated NHOF2 or combination of SAR405+TGF-β1-treated NHOF2. The number of migrated (A) and invaded (B) H376 cells are expressed as a relative to control (=1). The data presented are representative of three independent experiments. Bars, mean ± SD; ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.001$ (ANOVA).

5.5 Summary

This study aimed to generate an *in vitro* model with which to examine the involvement of autophagy in fibroblast activation and senescence, as well as its influence on tumour behaviour.

Normal oral fibroblasts exposed to a high dose of IR displayed senescent markers (downregulated SIRT1, upregulated $p21^{Waf1/Cip1}$ and $p16^{Ink4a}$) but did not result in autophagy (unchanged p62) nor fibroblast activation (unchanged α-SMA). This suggested that IR-induced senescence in normal fibroblasts was independent of autophagy and fibroblast activation. There was no obvious correlation between autophagy, fibroblast activation and senescence in this model. Due to these limitations, this model was not selected for subsequent studies.

Senescence in CAFs from GU-OSCCs has been attributed to the production of TGF-β in fibroblasts (Hassona et al., 2013). To mimic this phenomenon *in vitro*, normal fibroblasts were treated with TGF-β1 in order to induce senescence. In the present study, TGF-β1 stimulated fibroblast autophagic flux, activation and senescence, with autophagic flux preceding fibroblast activation, which was subsequently followed by senescence.

Two different autophagy inhibitors that target the autophagic pathway at different stages were then used. It was demonstrated that SAR405 (targeting Vps34 that forms a protein complex with Beclin1/ATG6, responsible for autophagy initiation) is a more potent and specific autophagic inhibitor than HCQ (targeting late stage of autophagy at the degradation step). SAR405 treatment of normal fibroblasts increased the expression of both p62 and LC3B-II proteins, confirming the inhibition of autophagy in a time-and concentration-dependent manner. Inhibition of autophagy with SAR405 was shown to

induce fibroblast activation (elevation of α-SMA expression) and senescence (loss of SIRT1 expression, increased SA-β-gal positive cells).

To study the functional significance of autophagy inhibition and autophagic flux in epithelial OSCC progression, CM was obtained from autophagic-inhibited and autophagic-activated fibroblasts. SAR405 was added to inhibit autophagy in normal oral fibroblasts, while TGF-β1 was used to induce autophagic flux. CM from both autophagy inhibited fibroblasts and cells with enhanced autophagic flux significantly decreased the proliferation of H376 OSCC cells. CM from TGF-β1-induced autophagic fibroblasts increased both the migratory and invasive properties of OSCC cells, whereas CM from SAR405-inhibited autophagic fibroblasts promoted OSCC cell motility but not an invasion.

Collectively, these observations indicate that fibroblast activation and senescence could be regulated by the autophagic pathway. The results of the present study also indicate that both autophagic flux (TGF-β1-induced) and autophagy inhibition (SAR405-induced) displayed overlapping characteristics and they could similarly promote myofibroblast transdifferentiation and senescence in normal oral fibroblasts, as well as the migratory and invasive capabilities of OSCC cells.

CHAPTER 6: THE DEVELOPMENT OF MODEL SYSTEMS TO EXAMINE THE PHENOTYPIC IMPACT OF FIBROBLAST SENESCENCE ON OSCC CELL BEHAVIOUR

6.1 Introduction

We now know that CAFs not only contribute to the growth of primary tumours but also play a significant role in establishing a permissive pre-metastatic niche and in the dissemination of tumour cells to distant sites (Hanahan & Weinberg, 2011). Heterogeneity within CAF populations is becoming more apparent and it has been demonstrated that a subpopulation of senescent CAFs within the TME create a permissive and tumour-promoting environment (Hassona et al., 2014; Hassona et al., 2013; Krtolica et al., 2001), but the precise contribution of cellular senescence to CAF function remains to be determined.

With regards to OSCC, CAFs from GU-OSCCs have been shown to be majorly senescent and slow-growing as compared to CAFs from GS-OSCCs and NHOFs, and these cells were also reported to have altered transcriptional profiles with the expression of cellular senescence-related genes being more predominant (Lim et al., 2011). It has proved difficult to investigate the functional role of senescence in OSCC-derived CAFs because culturing these cells *in vitro* is very challenging due to their highly senescent (i.e. non-proliferative) nature. Therefore, the development of protocols for the elimination of senescent cells from CAF strains and/or the conditional immortalisation of both NHOFs and CAFs would provide an inexhaustible supply of fibroblasts and facilitate further downstream experiments. Specifically, these modified fibroblast strains would be an invaluable resource to examine the contribution of senescence to the tumour-promoting properties of CAFs.

The aims of this study, therefore, were: 1) to establish senescence-eliminated or/and conditionally senescence-inducible fibroblast strains and 2) to use these modified fibroblast strains as homogenous models to examine the contribution of senescence to the tumour-promoting properties of CAFs in OSCC.

6.2 Elimination of senescent cells using senolytic pharmacological agents

The selective elimination of the senescent subset within a heterogeneous CAF population would allow the assessment of the functional importance of cellular senescence in CAFs and might have therapeutic value. Senolytic drugs are a class of pharmacological agents that selectively kill senescent cells by inducing apoptosis (Chang et al., 2016; Yosef et al., 2016). Here, I tested the effect of two senolytic drugs, ABT263 and ABT737, on CAF from GU-OSCC (BICR63F) and normal oral fibroblasts (NHOF1).

Fibroblasts were treated with a single dose (1.25 μM) of ABT263 or ABT737 for 72 hours; this concentration and treatment time were selected as it has previously been shown to be effective in selectively eliminating senescent human lung fibroblasts (Chang et al., 2016). There was an increase in the number of viable cells when BICR63F cells were treated with ABT263 compared to ABT737 treated and controls. Both drugs had a minimal effect on the viability of normal fibroblasts (Figure 6.1A). The percentage of cells staining positive for the senescent marker, SA-β-gal, in both BICR63F and NHOF1 decreased after treatment with both senolytic drugs (Figure 6.1B and C). However, there were no evident changes in the protein expression of markers of autophagy (LC3B and p62), senescence (SIRT1) and activation (α-SMA) in BICR63F following treatment with either drug (Figure 6.1D).

Even though there was a decrease in the percentage of SA-β-gal positivity after treatment with the senolytic drugs, there was no apparent upregulation of the late

senescent marker, SIRT1, suggesting that the senescent status of CAFs from GU-OSCCs was irreversible. Due to this limitation, these drugs were not used to assess the functional importance of cellular senescence in CAFs in this study.

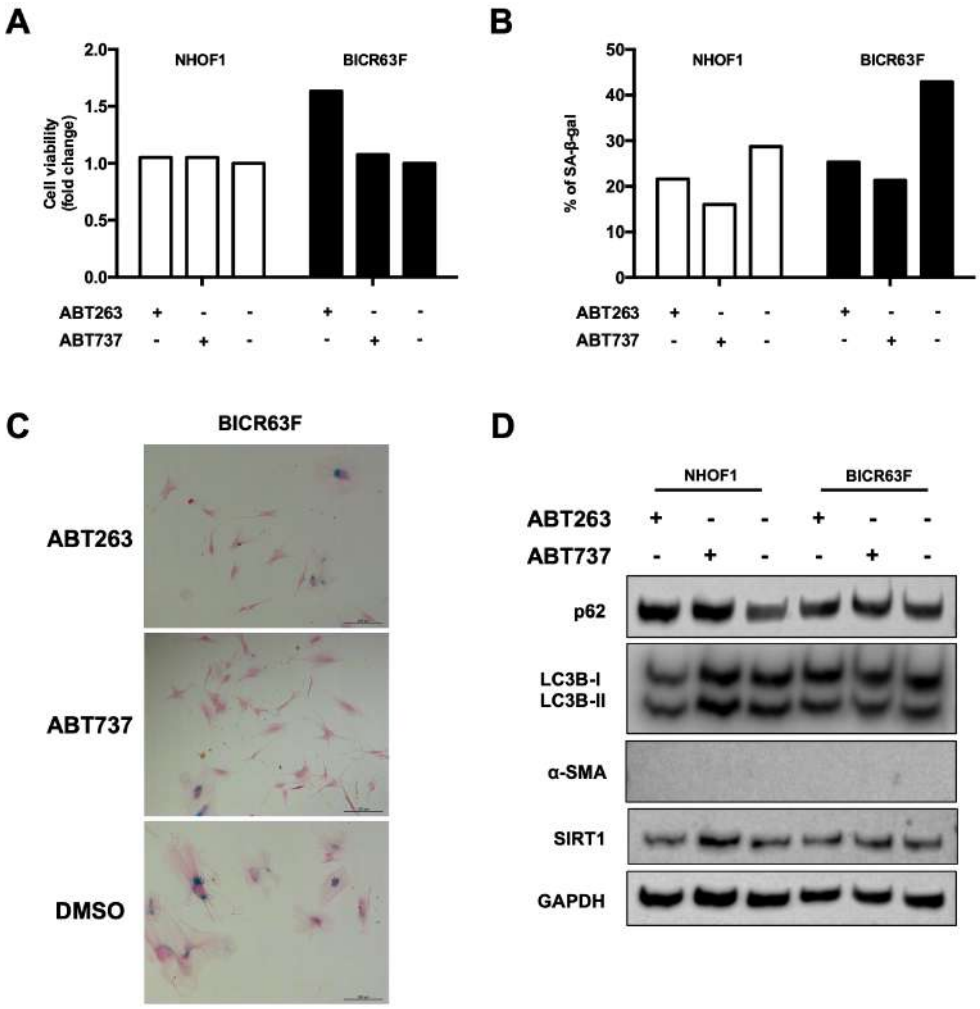


Figure 6.1: Effects of senolytic drugs on GU-OSCC CAF and NHOF

CAFs from GU-OSCCs (BICR63F) and normal fibroblasts (NHOF1) were seeded with equal cell density in 100 mm dishes and treated with vehicle control (DMSO), 1.25 μM of senolytic drugs, ABT263 or ABT737 in complete medium. (A) Following 72 hours of incubation, cell counts were performed. The result represents the fold change of viable cell number relative to control. CAFs from GU-OSCCs treated with ABT263 showed an increased number of viable cells compared to control and ABT737-treated cells. Both the drugs had minimal effect on normal fibroblasts. (B) Results are the percentage of SA-β-gal positive cells. The percentage of SA-β-gal positive cells decreased after treatment with senolytic drugs. (C) Representative images of BICR63F treated with vehicle control, ABT263 or ABT737 (Scale bar indicates 200μm). (D) Western blot analyses showed no apparent differences in protein expression of autophagic (LC3B and p62), senescent (SIRT1), activation (α-SMA) markers in CAFs from GU-OSCCs before and after treatment.

6.3 Establishment of conditionally immortalised CAFs from GU-OSCCs

To immortalise normal cells, hTERT and viral oncogenes are common tools to use. In the present study, an attempt was made to conditionally immortalise four CAF strains from GU-OSCCs, namely BICR3F, BICR31F, BICR63F and BICR78F, by infecting them with amphotropic retroviruses that transduce exogenous hTERT and a temperature-sensitive mutant (U19tsA58) of the SV40 large T antigen (denoted as tsLT). It has been reported that the co-expression of the catalytic subunit of human telomerase and tsLT in fibroblasts allows their immortal expansion at a permissive temperature (33ºC, tsLT activation) but their proliferative potential is lost at non-permissive temperature (39ºC, tsLT inactivation) (Jat & Sharp, 1989). For the purposes of the present study, the same population of cells could be used (by switching temperatures) to examine the contribution of senescence to CAF function.

6.3.1 Characterisation of transduced and untransduced CAFs from GU-OSCCs

The CAF strains were transduced with the hTERT and tsLT genes, either singly or in combination. It was reported previously that the reconstitution of telomerase activity by hTERT transduction alone was not sufficient to immortalise adult mammary fibroblasts (O'Hare et al., 2001). Similarly, in the present study BICR31F and BICR78F cells did not survive puromycin selection following the transduction of either hTERT or tsLT alone (data not shown).

In contrast, the introduction of both genes in the order of hTERT first followed by tsLT into BICR3F and BICR63F resulted in viable cells with an extended lifespan; the expression of the tsLT oncogene alone in these two CAF strains did not produce viable cell colonies. These transduced CAFs (CAFhTERT-tsLT) were designated as BICR3FhTERT-tsLT and BICR63FhTERT-tsLT, respectively, and the growth of the untransduced and transduced CAFs was initially compared at the standard culture

conditions (37ºC). The morphological differences between untransduced and transduced CAFs are shown in Figure 6.2. In 2D cell culture, the untransduced CAFs had characteristics of the senescent cells with less distinctive cell membrane, multi-vacuolated cells, enlarged cell size and a flattened multipolar morphology while the transduced CAFhTERT-tsLT had more distinctive cell membranes, smaller cell size and adopted a bipolar fibroblastic morphology (Figure 6.2A). Cells growing in exponential phase are normally bright and refractile with spherical morphology (Weissleder, 2010). The transduced CAFhTERT-tsLT cells were more round, refractile cells, which were undergoing mitosis, as shown in Figure 6.2A. The average size of a single cell was obtained using a Luna automated cell counter and compared (Figure 6.2B). CAFhTERT-tsLT cells were typically smaller in size comparing to CAFs before transduction.

The untransduced BICR3F had a mean population doubling (MPD) value of ≈ 1 over a month in culture, while untransduced BICR63F had an MPD of ≈ 0.8 over a week in culture. In contrast, both the transduced BICR3FhTERT-tsLT and BICR63FhTERT-tsLT were comparatively faster growing *in vitro* with MPDs of ≈ 2.5-3 over a week. In addition, when subjected to SA-β-gal staining, CAFhTERT-tsLT clearly had decreased levels of positively stained cells, indicating that senescence was overcome by the expression of hTERT and tsLT (Figure 6.3). Figure 6.4 shows the expression of markers of autophagy (p62 and LC3B-II), fibroblast activation (α-SMA), senescence (SIRT1) and cell cycle arrest ($p21^{Waf1/Cip1}$ and $p16^{Ink4a}$) on Western blots of untransduced and transduced CAFs. Transduced CAFs expressed more SIRT1, indicating that the population of cells was relatively less-senescent cells, as compared to the cells before transduction. However, transduction of CAFs also resulted in the induction in expression of the cell cycle inhibitors, $p21^{Waf1/Cip1}$ and $p16^{Ink4a}$, even though they were continuously proliferating. These observations are somewhat paradoxical and the

underlying mechanisms controlling the levels of the CDK inhibitors are uncertain but could be due to the sequestration/perturbation of the Rb and p53 proteins by tsLT (Lane & Crawford, 1979; Serrano, Hannon, & Beach, 1993).

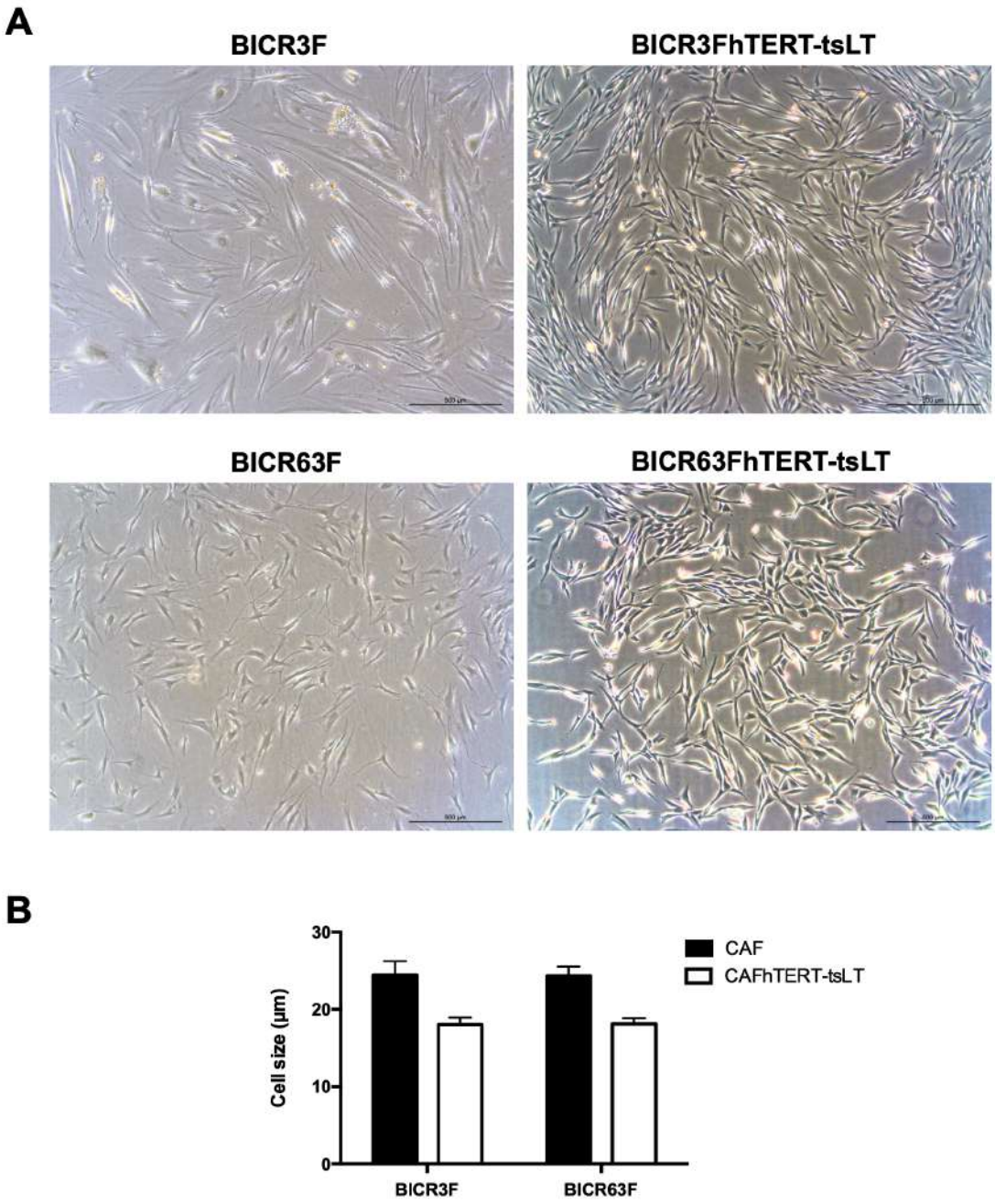


Figure 6.2: Morphological difference between transduced and untransduced CAFs from GU-OSCCs

BICR3F and BICR63F conditionally immortalised with double transduction of hTERT and tsLT are designated as BICR3FhTERT-tsLT and BICR63FhTERT-tsLT, respectively. (A) Representative images of CAFs from GU-OSCCs before and after transduction with hTERT and tsLT under the light microscope. Untransduced CAFs displayed morphological characteristics of senescent cells while transduced CAFhTERT-tsLT resembled normal fibroblasts. Scale bar indicates 500 µm. (B) Comparison of cell size before and after transduction. Cell size was obtained using Luna automated cell counter. CAFhTERT-tsLT were typically smaller in size comparing to CAFs before transduction. Bars, mean ± SD of triplicates.

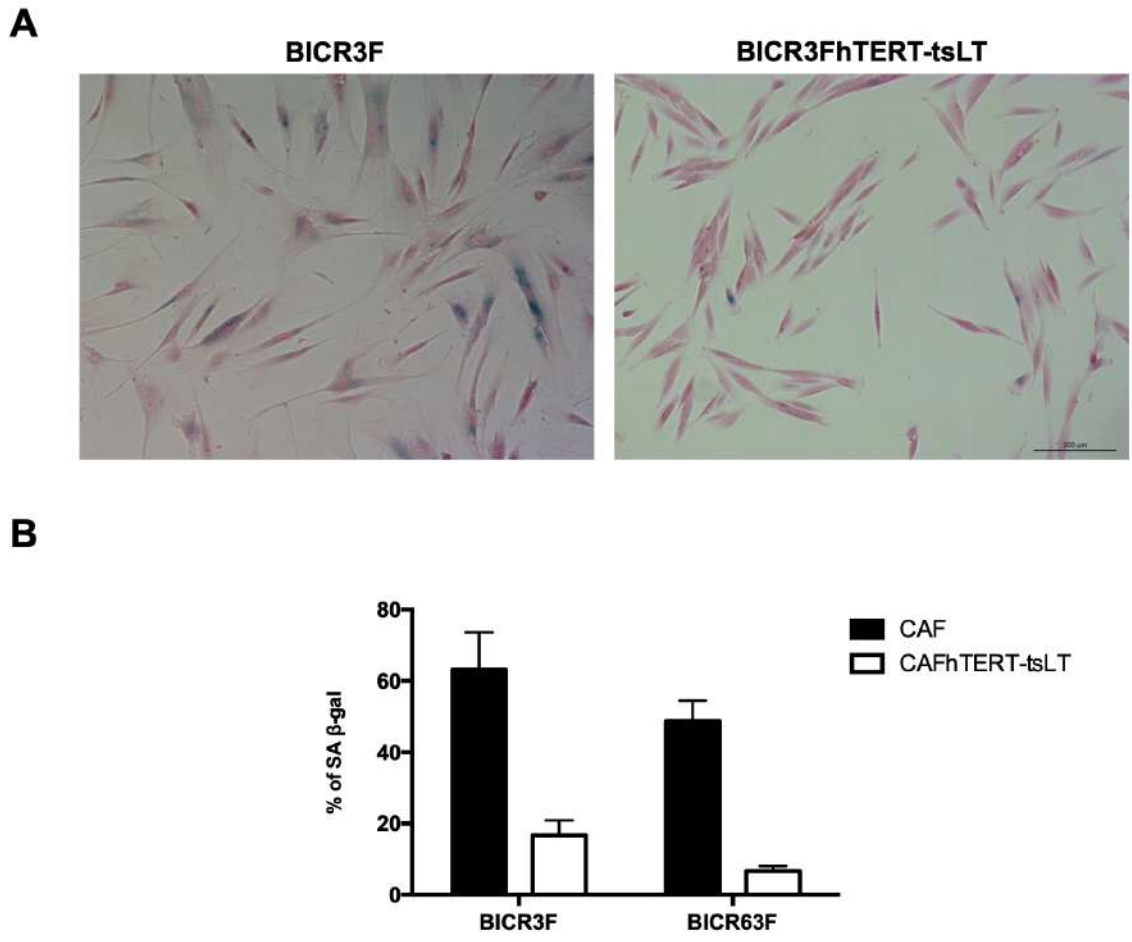


Figure 6.3: Transduced CAFhTERT-tsLT had reduced SA-β-gal positive cells

(A) Representative images of CAF and CAFhTERT-tsLT under bright field microscopy after SA-β-gal staining. Scale bar indicates 200 μm. (B) Percentage of cells positively stained with SA-β-gal. Transduced CAFshTERT-tsLT had a lower percentage of SA-β-gal positive cells, indicating that they were less senescent. Bars, mean ± SEM of triplicates.

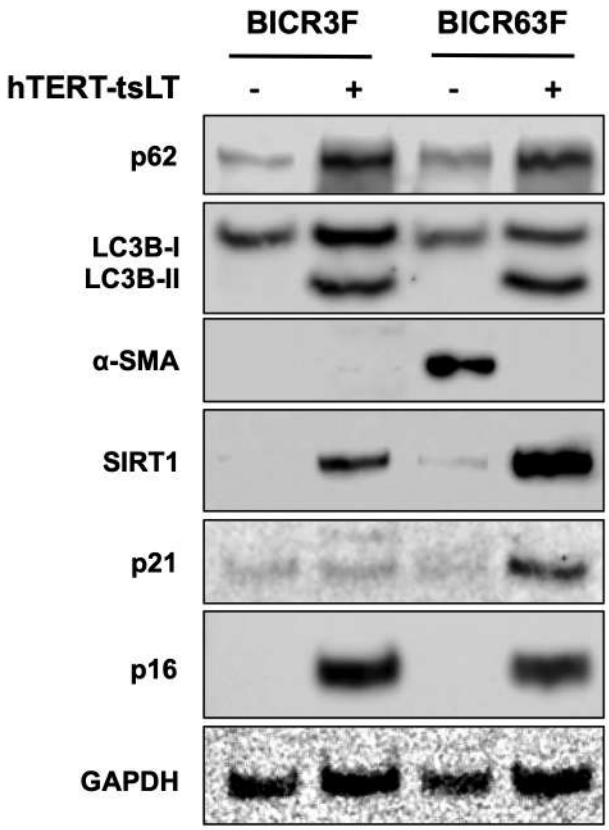


Figure 6.4: Expression of autophagic, activation, senescent and cell cycle arrest markers after double transductions with hTERT and tsLT

Cells lysates were collected from BICR3F, BICR3FhTERT-tsLT, BICR63F and BICR63FhTERT-tsLT, respectively. The samples were subjected to Western blot analysis and probed for autophagic (p62 and LC3B-II), fibroblast activation (α-SMA), senescent (SIRT1), cell cycle arrest ($p21^{Waf1/Cip1}$ and $p16^{Ink4a}$) markers and GAPDH was used as a loading control. Transduced CAFs expressed more SIRT1, indicating that the cells were relatively less senescent compared to cells before transduction. A representative Western blot image of two independent experiments is presented.

6.3.2 Characterisation of transduced CAFhTERT-tsLT at permissive and non-permissive temperatures

The experiments described in Section 6.3.1 represented the establishment and preliminary characterisation of BICR3FhTERT-tsLT and BICR63FhTERT-tsLT. To determine if the tsLT could function in a temperature-dependent manner, BICR3FhTERT-tsLT and BICR63FhTERT-tsLT were cultured at either the permissive (33ºC, tsLT activation) or non-permissive temperature (39ºC, tsLT inactivation).

The cumulative mean population doubling (CMPD) values were compared between the two temperatures (Figure 6.5). These transduced CAFs did not show growth arrest in culture and they grew continuously at 33ºC. However, when shifted to 39ºC, these cell lines continued to divide at a similar rate even though this temperature was thought to be non-permissive and expected to induce growth arrest at G_1 or G_2 phase of the cell cycle (Jat & Sharp, 1989). The doubly transduced cultures grew continuously at both the permissive and non-permissive temperature with no major differences between the growth rate (Figure 6.5), morphology (Figure 6.6A) and average cell size (Figure 6.6B). Somewhat surprisingly, the hTERT/tsLT expressing fibroblast lines grew slightly more rapidly at the higher temperature, compared to 33ºC and they also had a lower percentage of SA-β-gal positive cells (Figure 6.7), even though there was a reduction in SA-β-gal positivity at both temperatures compared with non-transduced cells (Figure 6.3).

The expression of markers of autophagy (p62 and LC3B-II), fibroblast activation (α-SMA), senescent (SIRT1) and cell cycle arrest ($p21^{Waf1/Cip1}$ and $p16^{Ink4a}$) by Western blotting in CAFhTERT-tsLT grown at permissive and non-permissive temperatures is shown in Figure 6.8. The loss of SIRT1 is a marker for late senescence and both transduced CAFhTERT-tsLT had SIRT1 expression at both temperatures, suggesting that they had overcome senescence and progressed through cell cycle arrest. However, all cells expressed the cell cycle arrest markers, $p16^{Ink4a}$ and $p21^{Waf1/Cip1}$, which normally accumulate in late passage senescent cells, at both temperatures even though the transduced cells demonstrated continuous proliferation in culture.

In summary, double transduction of CAFs with hTERT and tsLT generated fibroblast lines with extended growth and no overt crisis. However, these cells did not senesce at 39ºC, suggesting that the effect of hTERT effect may have overridden the effect of the

tsLT antigen. Therefore, this approach could not be used to investigate the functional contribution of senescence in CAFs, as conditionally immortalised cells were not established.

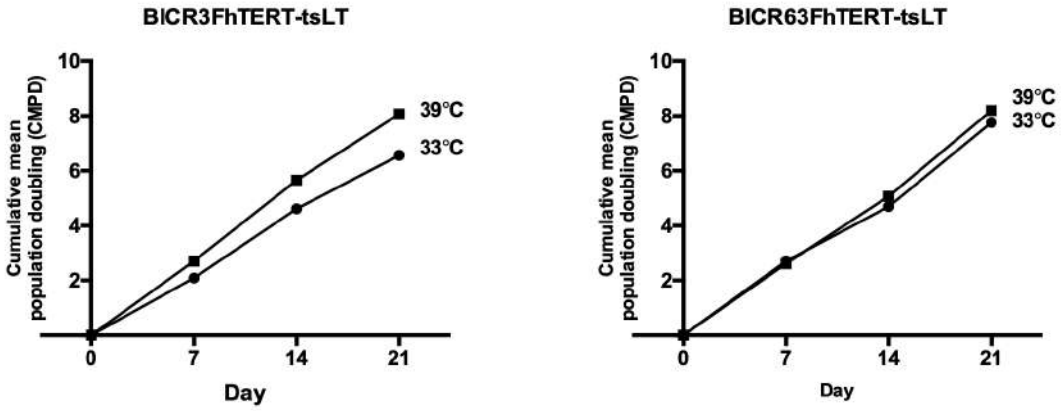


Figure 6.5: Growth of temperature-sensitive CAFhTERT-tsLT culture at 33°C and 39°C

Proliferative potential of CAFhTERT-tsLT (BICR3FhTERT-tsLT and BICR63FhTERT-tsLT) was determined by growing fibroblasts at permissive (33°C) and non-permissive (39°C) temperatures. Tha number of cells was determined by cell count experiment. Line graphs show the cumulative mean population doubling (CMPD) values of cells were compared at indicated days. Both CAFhTERT-tsLT had slightly higher growth rate at 39°C as compared to 33°C.

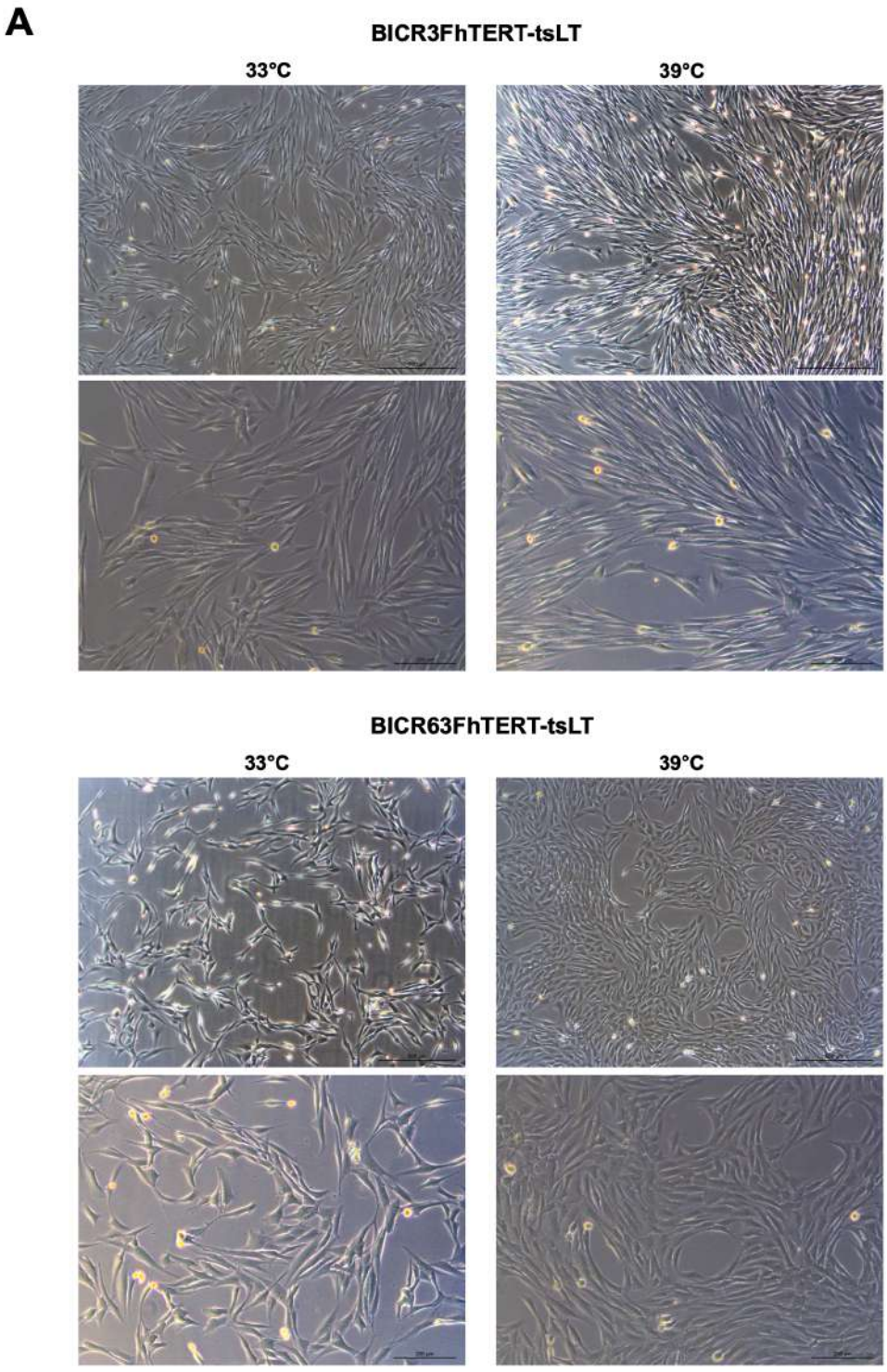
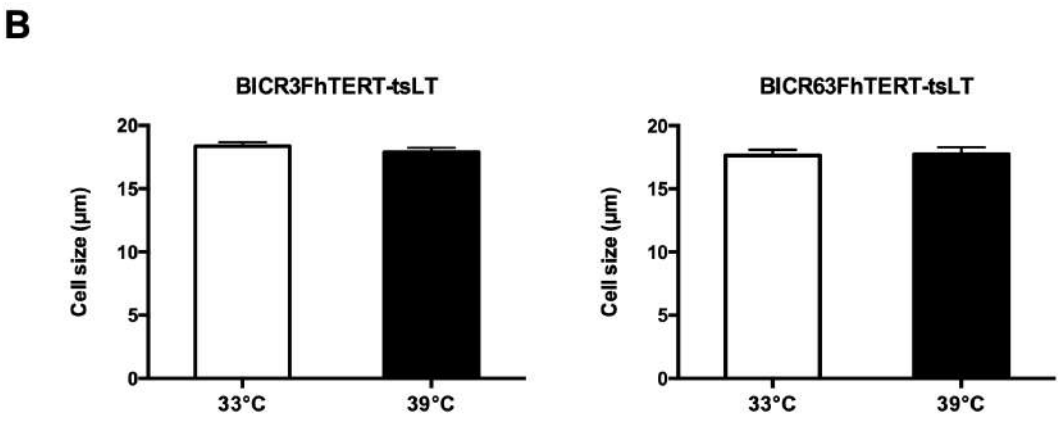


Figure 6.6: Growth and morphology of CAFhTERT-tsLT at 33°C and 39°C

(A) Phase-contrast representative images of BICR3FhTERT-tsLT and BICR63FhTERT-tsLT grown at permissive (33°C) and non-permissive (39°C). No major visible morphological differences were observed under the microscope at two different magnifications. Scale bars indicate 200 and 500µm. (B) Cell size comparison of CAFhTERT-tsLT cultured at 33 and 39°C. Cell size was obtained using Luna automated cell counter. CAFhTERT-tsLT grown at 33 and 39°C were similar in size. Bars, mean ± SEM from more than three sets of samples.



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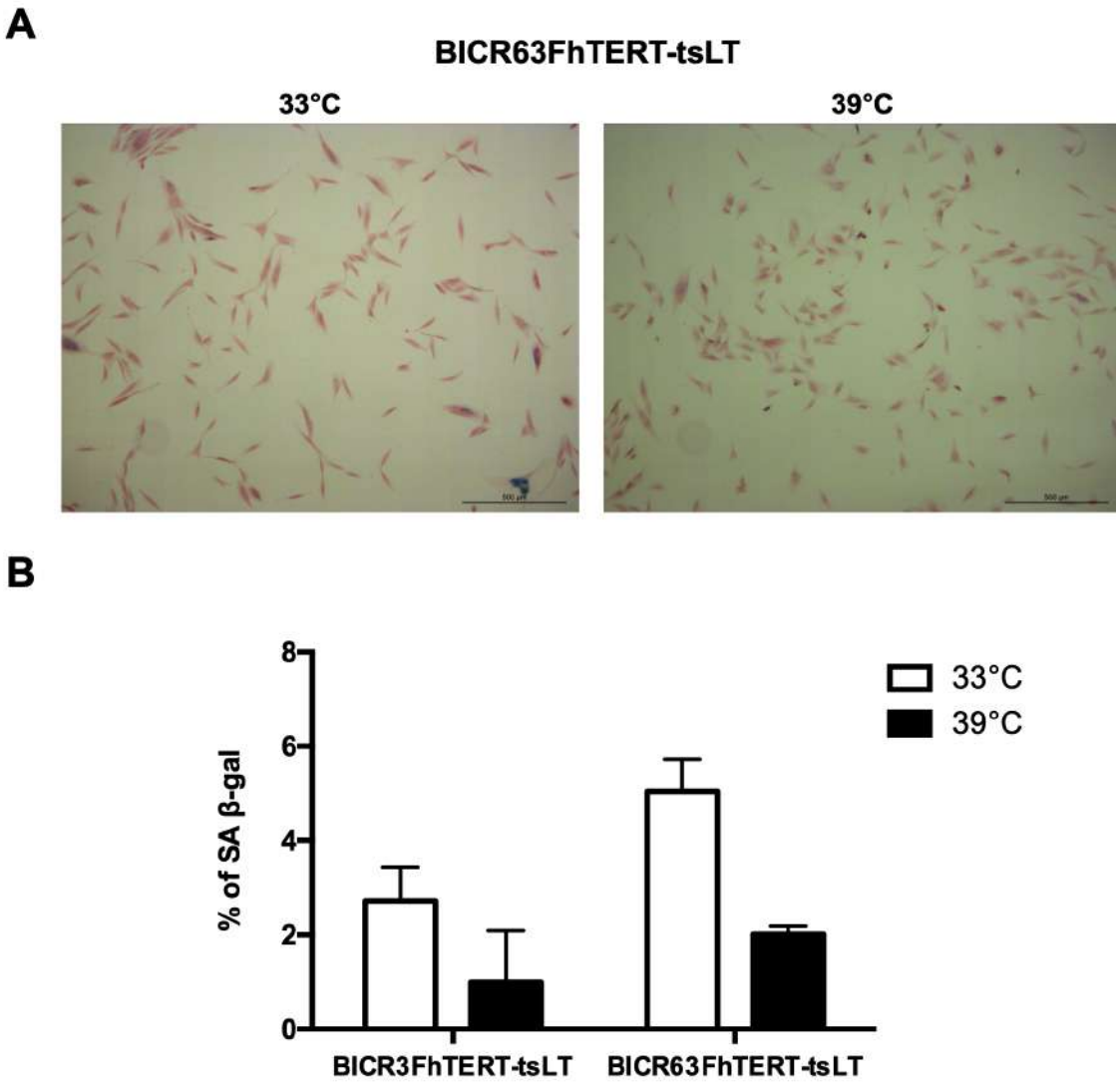

Figure 6.7: SA-β-gal activity in CAFhTERT-tsLT at 33°C and 39°C

(A) Representative images of CAFhTERT-tsLT (BICR63FhTERT-tsLT) under bright field microscopy after SA-β-gal staining. Scale bar indicates 500 μm. (B) Percentage of cells positively stained with SA-β-gal. Transduced CAFhTERT-tsLT had a lower percentage of SA-β-gal positive cells, indicating that they were less senescent. Bars, mean ± SD of triplicates.

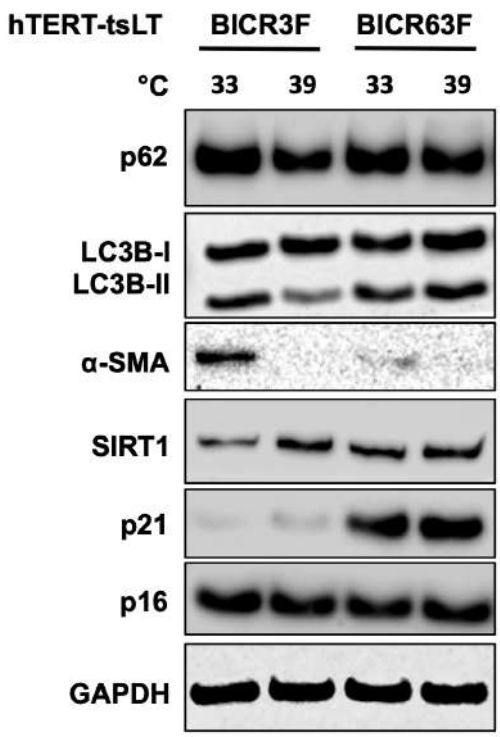

Figure 6.8: Characterisation of protein markers in CAFhTERT-tsLT fibroblasts cultured at 33°C and 39°C

Proteins were harvested from CAFhTERT-tsLT grown at 33 and 39°C, respectively. The expression of autophagic (p62 and LC3B-II), fibroblast activation (α-SMA), senescent (SIRT1) and cell cycle arrest ($p21^{Waf1/Cip1}$ and $p16^{Ink4a}$) markers were examined. GAPDH was used as a loading control. Representative Western blot images of two independent experiments are presented.

6.4 Establishment and characterisation of temperature-sensitive senescence-inducible normal oral fibroblasts

As the transduction of CAFs with hTERT and tsLT failed to produce conditionally immortalised strains, I decided to take an alternative approach to examine the functional significance of fibroblast senescence by attempting to conditionally immortalise normal oral fibroblasts.

NHOF1 and NHOF2 were transduced with tsLT to produce NHOF1-tsLT and NHOF2-tsLT. Unlike CAFs (Section 6.3.1) that did not survive G418 selection after transduction with tsLT alone, NHOFs-tsLT exhibited the properties of an established conditional immortalised cell line of unceasing expansion without crisis at the permissive temperature (33ºC, tsLT activation). Growth arrest was observed (Figure 6.9) when these cells were cultured at 39ºC. The cells ceased proliferating by 24 hours of transfer to the non-permissive temperature of 39ºC upon tsLT inactivation (data not shown). When cell counts were performed at day 7 of culture at 39ºC, approximately 90% of the NHOF1-tsLT remained attached and viable but did not readily expand to colonies when isolated. Approximately 80% of NHOF2-tsLT, however, began to detach from the cell culture flask when cultured at 39ºC and most cells failed to survive. Compared to NHOF2-tsLT, transduced NHOF1-tsLT appeared to be more stable and viable. It also expanded rapidly without any crisis in culture and exhibited temperature-dependent growth at both 33 and 39ºC. Therefore, NHOF1-tsLT was selected for subsequent characterisation and functional experiments.

It was demonstrated previously that tsLT transduced primary mammary fibroblasts cease proliferating and were irreversibly arrested after 7 days of incubation at 39ºC upon tsLT inactivation (Hardy et al., 2005). To determine the effect of tsLT activation on NHOF1-tsLT, the cells were characterised after 2 and 7 days culture at 33ºC and

39ºC. NHOF1-tsLT grew healthily at 33ºC and the morphology of the cells remained constant, but after culturing at 39°C for 7 days the cells displayed a typical senescent morphology (Figure 6.10A). Western blot analyses (Figure 6.10B) showed decreased p62 and LC3B-II levels, and upregulation of α-SMA in cells grown at 39°C for 2 and 7 days, compared to cells cultured at 33°C. SIRT1 was downregulated when cells were shifted to 39°C for 7 days, confirming that late senescence was induced. These data suggested that fibroblast activation precedes senescence in this model, which mimicked the results obtained with TGF-β1-treated NHOF1 (Section 5.2.2).

The average cell size of tsLT activated (33°C) and inactivated NHOF1-tsLT (39°C) at Day 7 is shown in Figure 6.11A. At 39°C, the cells were significantly bigger (27.70 ± 2.55; $p<0.05$) than NHOF1-tsLT at 33°C (19.63 ± 0.45 μm). This observation suggested that hypertrophy, a key characteristic of senescent cells, occurred in cells grown at 39°C. In addition, a marked and significant increase of SA-β-gal activity was observed in NHOF1-tsLT at 39°C (63.30 ± 6.97 %, $p<0.01$) compared to cells grown at 33°C (4.57 ± 1.65 %) (Figure 6.11B). Representative images of SA-β-gal stained NHOF1-tsLT upon tsLT activation and inactivation are shown in Figure 6.11C.

Taken together these data confirmed that NHOF1-tsLT was temperature-sensitive and senescence-inducible. These fibroblasts remained non-senescent at 33ºC and could be readily induced to senesce upon tsLT inactivation at 39°C for 7 days. Since NHOF1-tsLT appeared to be more stable, viable and exhibited temperature-dependent growth compared to NHOF2-tsLT at both 33 and 39ºC, it was selected for subsequent functional studies to study the contribution of senescence to the tumour-promoting ability of OSCC cells.

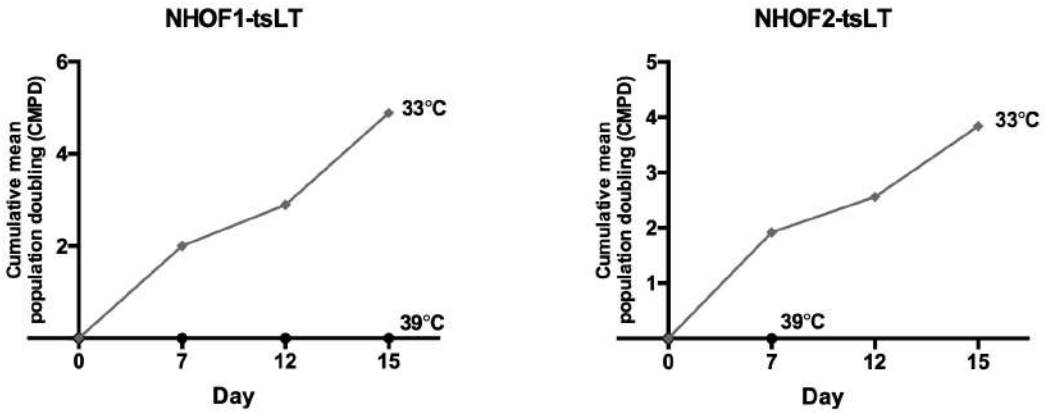

Figure 6.9: Proliferation curves of temperature-sensitive NHOFs-tsLT cultured at permissive (33°C) and non-permissive (39°C) temperatures

Proliferative potential of NHOFs-tsLT (NHOF1-tsLT and NHOF2-tsLT) was determined by growing fibroblasts at 33 (◆) and 39°C (•). Total number of cells was determined by cell count experiment. Data points show the cumulative mean population doubling values (CMPD) of cells at the indicated time points. Continuous growth was observed in NHOF1-tsLT and NHOF2-tsLT at 33°C. NHOF1-tsLT ceased expanding at 39°C in cell number but remained viable throughout the experimental period. The majority of NHOF2-tsLT did not survive at 39°C and no cells could be obtained after day 7.

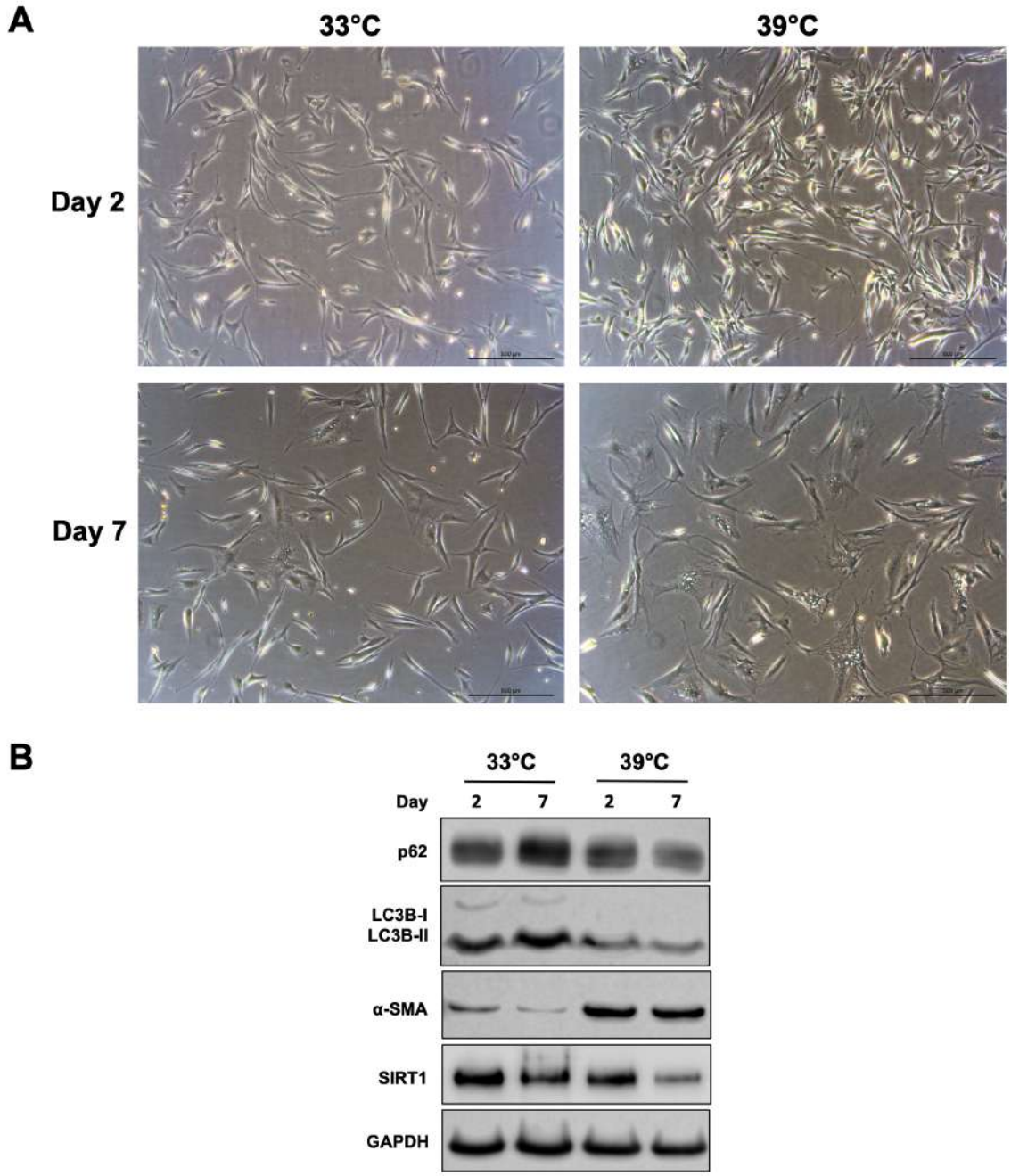

Figure 6.10: Characterisation of NHOF1-tsLT following tsLT activation

(A) Representative images of temperature-sensitive NHOF1-tsLT at day 2 and 7 upon tsLT activation (33°C) and inactivation (39°C). Morphological differences of temperature-sensitive NHOF1-tsLT between tsLT activated and inactivated fibroblasts were more apparent at day 7. The cells remained relatively the same when cultured at 33°C but displayed typical senescent phenotypes after shifted to 39°C for 7 days. Scale bar indicates 500 μm. (B) Western blot analyses showed a decrease in p62 and LC3B-II, and upregulation of α-SMA in cells grown at 39°C. SIRT1 was downregulated when cells were shifted to 39°C for 7 days. This data suggested fibroblast activation precedes senescence in this model. GAPDH was used as a loading control. Representative Western blot images of two independent experiments are presented.

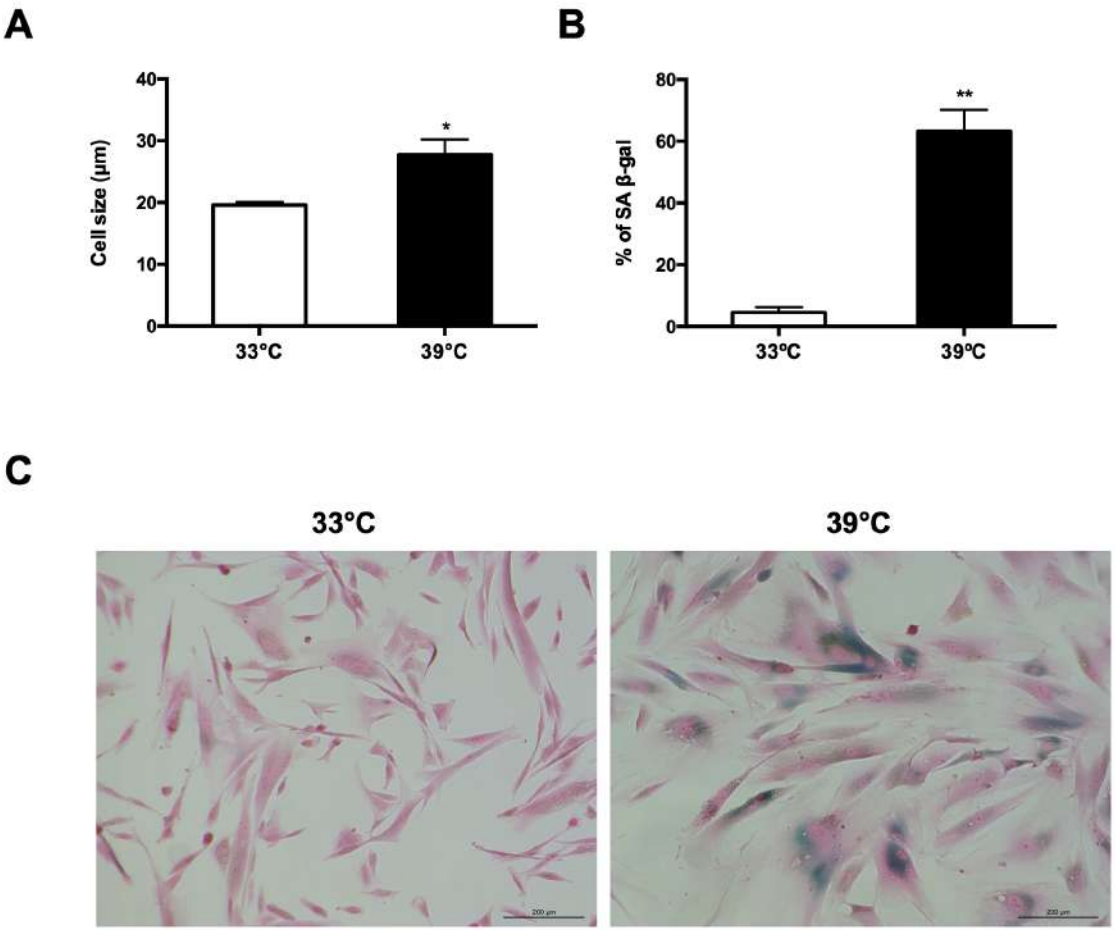

Figure 6.11: tsLT-inactivated NHOF1-tsLT at 39°C was significantly larger in size and had higher SA-β-gal activity

(A) The average cell size of tsLT inactivated NHOF1-tsLT (39°C) was significantly bigger than tsLT activated NHOF1-tsLT (33°C). Bars, mean ± SEM; n=4; * = $p<0.05$ (Student's *t*-test). (B) The SA-β-gal activity of NHOF1/tsLT at 33ºC was compared to NHOF1/tsLT at 39ºC at day 7. NHOF1-tsLT at 39°C a had significantly higher percentage of SA-β-gal positive cells, confirming that they were more senescent than cells at 33°C. Bars, mean ± SEM of triplicates; ** = $p<0.01$ (Student's *t*-test) (C) Representative images NHOF1-tsLT under bright field microscopy after SA-β-gal staining. NHOF1-tsLT had more observable blue stained and flattened cells when shifted upon 39°C. Scale bar indicates 200 µm.

6.5 Biological significance of fibroblast senescence in epithelial OSCC cells

The establishment of temperature-sensitive senescence-inducible NHOF1-tsLT cells described in Section 6.4 enables the exploration of fibroblast senescence on the behaviour of epithelial OSCC cells *in vitro*. In this model, these genetically modified fibroblasts could be induced to undergo senescence synchronously when shifted to 39ºC upon tsLT inactivation, while remaining non-senescent at 33ºC upon tsLT activation. By collecting CM from cells grown at permissive and non-permissive temperatures, the effects of fibroblast senescence on OSCC cell proliferation, migration and invasion were investigated.

6.5.1 Cell proliferation

This study investigated the effect of senescent fibroblasts on OSCC cell proliferation. NHOF1-tsLT fibroblasts were grown until they reached 70-90% confluence at 33 and 39ºC for at least 7 days to ensure senescence was induced in NHOF1-tsLT at 39ºC. The cells were washed with PBS and supplement free DMEM then incubated in supplement free DMEM for a further 48 hours to collect CM from non-senescent (33ºC) and senescent (39ºC) NHOF1-tsLT. H376 OSCC cells were cultured in 1% FC-II supplemented DMEM (control), CM prepared from non-senescent and senescent NHOF1-tsLT for 48 hours. Cell proliferation was examined by cell counting and the results are summarised in Figure 6.12. CM from senescent NHOF1-tsLT (1.24 ± 0.08 fold) significantly ($p<0.05$) promoted proliferation of H376 cells than control (=1). In addition, H376 cells cultured in CM from non-senescent 33ºC NHOF1-tsLT (1.07 ± 0.14 fold) showed similar growth as control cells. There were no significant differences between relative proliferation of CM from non-senescent NHOF1-tsLT versus control ($p = 0.64$) and CM from senescent NHOF1-tsLT ($p = 0.14$). Taken together, these data showed that induction of senescence could promote the proliferation of OSCC cells in the temperature-sensitive senescence-inducible NHOF1-tsLT model.

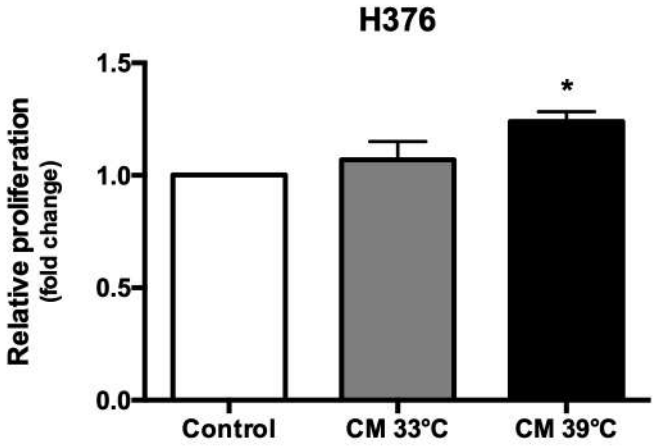

Figure 6.12: CM from senescent NHOF1-tsLT fibroblasts promoted proliferation on H376 OSCC cells

Growth of H376 cells in control medium (1% FC-II supplemented medium), CM from non-senescent NHOF1-tsLT (CM 33°C) or CM from senescent NHOF1-tsLT (CM 39°C). Cell proliferation was measured by performing a cell count assay. H376 cells number is expressed as relative to control (=1). The data presented are representative of three independent experiments. Bars, mean ± SD; * = $p<0.05$ (ANOVA).

6.5.2 Cell migration and invasion

The mobility and ability of tumour cells to disseminate are critical features that dictate the invasive and metastatic growth of cancer cells to secondary sites (Price, Bonovich, Kohn, Welch, & Hershey, 1997). In the present study, the effect of fibroblast senescence on OSCC cell migration was examined by performing transwell migration assays using a Boyden chamber system.

CM from senescent NHOF1-tsLT (39°C), non-senescent NHOF1-tsLT (33°C) or control (supplement free medium) was added into each well. H376 cells were seeded into the upper chamber of fibronectin pre-coated transwell inserts. Following 20 hours of incubation, the numbers of cells that migrated through the transwell membrane were counted and the data are summarised in Figure 6.13. CM from senescent NHOF1-tsLT (2.48 ± 0.21 fold), but not CM from non-senescent NHOF1-tsLT stimulated a marked increase in the migration of H376 cells when compared to control ($p<0.001$). CM from

senescent NHOF1-tsLT also demonstrated a significant increase in cell migration compared CM from non-senescent NHOF1-tsLT ($p<0.01$). H376 cells cultured in CM from non-senescent NHOF1-tsLT cultured at 33°C (1.10 ± 0.12 fold) showed no significant difference ($p = 0.87$) in relative migration compared to controls.

To assess the effect of fibroblast senescence in influencing the invasiveness of epithelial OSCC cells, similar experiments were carried out using Matrigel-coated transwell inserts. Figure 6.14 shows the relative invasion of H376 cells when treated with different conditioned media. The results demonstrated that CM from senescent NHOF1-tsLT stimulated the invasion of H376 cells significantly more than control (1.37 ± 0.10 fold, $p<0.05$). However, the enhancement of H376 cell invasion with CM from senescent NHOF1-tsLT was not statistically significant compared to CM from non-senescent NHOF1-tsLT ($p = 0.07$). H376 cells treated in CM from non-senescent 33ºC NHOF1-tsLT (1.12 ± 0.05 fold) exhibited no significant difference ($p = 0.44$) in relative invasion as control cells.

Taken together, these results demonstrated that the induction of senescence in NHOF1 cells could stimulate OSCC cell migration and invasion.

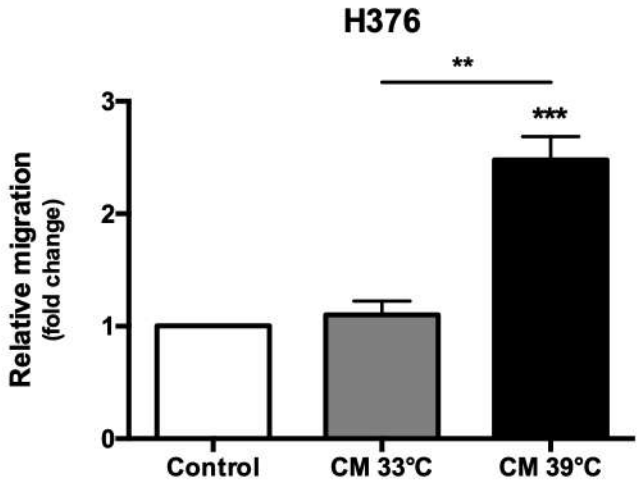

Figure 6.13: CM from senescent NHOF1-tsLT fibroblasts significantly enhanced migration on H376 OSCC cells

Bar chart represents the relative migration of H376 cells in control medium (supplement free medium), CM from non-senescent NHOF1-tsLT (CM 33°C) or CM from senescent NHOF1-tsLT (CM 39°C). Cell migration was measured by performing transwell migration assay with fibronectin-coated transwell inserts. The number of migrated H376 cells was expressed as relative to control (=1). The data presented are representative of three independent experiments. Bars, mean ± SD; ** = $p<0.01$, *** = $p<0.001$ (ANOVA).

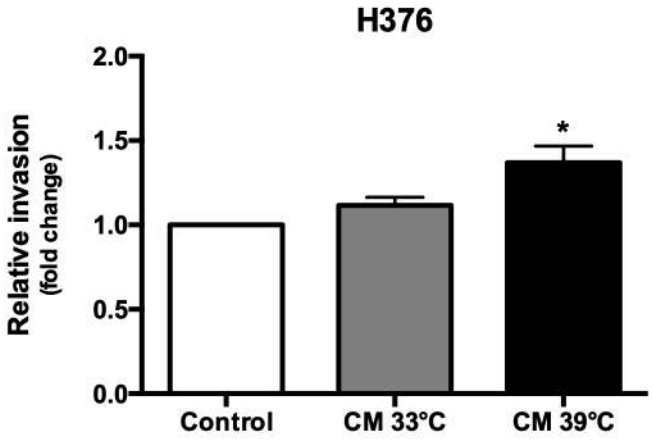

Figure 6.14: CM from senescent NHOF1-tsLT fibroblasts significantly induced invasion of H376 OSCC cells

Bar chart represents the relative invasion of H376 cells in control medium (supplement free medium), CM from non-senescent NHOF1-tsLT (CM 33°C) or conditioned medium from senescent NHOF1-tsLT (CM 39°C). Cell invasion was measured by performing transwell invasion assay with Matrigel-coated transwell inserts. The number of invaded H376 cells was expressed as relative to control (=1). The data presented are representative of three independent experiments. Bars, mean ± SD; * = $p<0.05$ (ANOVA).

6.6 Summary

The present study aimed to establish a sustainable model to evaluate the role and functional significance of fibroblast senescence to the malignant phenotypes of OSCC cells.

CAFs from GU-OSCCs were treated with senolytic drugs, ABT263 and ABT737 in an attempt to eliminate the senescent population. However, despite a reduction in the number of cells staining positive for SA-β-gal, downregulation of the late senescent marker, SIRT1, was not readily observed after the drug treatment, implying that senescence was not reversed. Hence, this model was not used as an *in vitro* model to assess the functional importance of cellular senescence in CAFs.

Next, an attempt was made to conditionally immortalise CAFs from GU-OSCCs by infecting them with amphotropic retroviruses that transduce exogenous hTERT and a temperature-sensitive SV40 large T antigen (tsLT). Double transduction of CAFs with hTERT and tsLT generated two fibroblast lines, namely BICR3FhTERT-tsLT and BICR63FhTERT-tsLT, with extended growth and no overt crisis. However, these cells were not temperature-sensitive as they grew continuously at both permissive (33°C) and non-permissive temperatures (39°C) and senescence was not induced when they were grown at 39°C. Therefore, these cells were not used to investigate the functional contribution of senescence, as conditionally immortalised CAFs were not established.

An alternative approach was taken to generate a temperature-sensitive model of fibroblast senescence, by transducing NHOF1 with tsLT to generate NHOF1-tsLT cells. Characterisation of NHOF1-tsLT demonstrated that these cells could be readily induced to senesce when cultured at non-permissive temperature (39°C), while remaining non-senescent when cultured at permissive temperature (33°C). The establishment of NHOF1-tsLT fibroblasts facilitated the investigation of the effects of fibroblast

senescence on the malignant phenotype of OSCC cells *in vitro*. Using CM collected from NHOF1-tsLT cells grown at permissive and non-permissive temperatures, the results showed that induction of senescence in NHOF1 leads to the secretion of factors that could enhance the proliferation ($p<0.05$), migration ($p<0.001$) and invasion ($p<0.05$) of H376 OSCC cells. Collectively, these results indicate that senescence in fibroblasts influences tumour behaviour by promoting epithelial cell proliferation, motility and invasiveness within the microenvironment of OSCC.

CHAPTER 7: DISCUSSION

7.1 Preface to the discussion

There is compelling evidence to show that CAFs play a pivotal role in all stages of tumour progression in a wide range of cancer types (Kalluri, 2016). CAFs are highly adaptive cell types and their ability to adapt may reside in their intrinsic survival stress responses (Vitale et al., 2019). The mechanisms by which tumour cells adapt to microenvironmental perturbations have been studied extensively; however, there is relatively limited information regarding the role of stress responses of CAFs in OSCC progression, particularly autophagy and senescence. Therefore, this study investigated a possible link between autophagy and senescence in influencing the tumour-promoting phenotypes of CAFs. In particular, different subsets of CAFs (CAFs from GU-OSCCs and GS-OSCCs) and normal oral fibroblasts were compared. The results from the first part of my study revealed that autophagy and senescence were closely associated and CAFs from GU-OSCCs, which were shown to display more senescent phenotypes, also harboured an impaired autophagic pathway. Next, the contribution of autophagy to the activated and senescent phenotypes of CAFs was examined and autophagy in CAFs was shown to promote the migration and invasion of OSCC cells *in vitro*. Lastly, an *in vitro* model of conditionally senescence-inducible fibroblasts was established in order to examine the functional significance of fibroblast senescence to CAF phenotypes in OSCC.

For ease of interpretation, the Discussion chapter has been divided into sub-sections such that consideration is given to the data reported in each of the results chapters. This is followed by a discussion of some of the study limitations and then possible proposals for future work are presented.

7.2 Autophagic and senescent phenotypes in normal oral fibroblasts and CAFs

Various reports have indicated that stromal factors have the capacity to influence tumour behaviour and may be valuable tools in predicting clinical outcome in many cancer types (Eiro et al., 2019; Shan et al., 2014; Wyss et al., 2019), including OSCCs (M. R. Dourado et al., 2018; Marsh et al., 2011). It is well-recognised that OSCCs are a heterogeneous group of tumours (Cancer Genome Atlas Network, 2015; Hunter et al., 2006; Leemans et al., 2018) and likewise, distinct CAF populations and subtypes have also been identified in independent studies (Costea et al., 2013; R. C. Dourado et al., 2018; Lim et al., 2011). These distinct CAF subtypes have been shown to differentially influence tumour behaviour. The phenotypic and functional changes that occur in fibroblasts as a result of cellular stress, such as autophagy and senescence, could mediate the pro-tumourigenic features and heterogeneity of CAFs. In the present study, the autophagic- and senescent-associated phenotypes of CAFs from GU-OSCCs and GS-OSCCs were characterised and these features were compared with normal fibroblasts (NHOFs). CAFs from GU-OSCCs, which are associated with genetically unstable and more aggressive tumours, were shown to contain more senescent cells and also displayed dysfunction at the degradation step of autophagy. Collectively, the data showed that both autophagy and senescence were deregulated in CAFs from GU-OSCCs, and autophagy and senescence are closely associated in fibroblasts. These data suggest that these stress responses of CAFs may be part of the same biological phenomenon and/or part of a unified programme.

7.2.1 Senescent phenotypes were more apparent in CAFs from GU-OSCCs

Senescent fibroblasts can be found in the TME and have been implicated in promoting the progression of a number of solid tumours (E. K. Kim, Moon, Kim, Zhang, & Kim, 2018; T. Wang et al., 2017; Yamao et al., 2019). Fibroblasts undergo striking physiological and morphological changes upon senescence. Senescent

fibroblasts acquire a number of distinctive characteristics that allow their identification *in vitro*, and morphological changes are cardinal features of the senescent phenotype (Munoz-Espin & Serrano, 2014). In the present study, CAFs from GU-OSCCs displayed morphological features typical of senescent cells, such as increased cell size, extensive vacuolisation, and a flattened cell shape with cell surface irregularity. Cellular hypertrophy, which is defined as increased cell volume and protein content, was also observed in CAFs from GU-OSCCs and this feature was more apparent in CAFs from GU-OSCCs compared to CAFs from GS-OSCCs and NHOFs. It can occur as protein synthesis continues in senescence fibroblasts without cell division (Capparelli et al., 2012; Demidenko & Blagosklonny, 2009). Furthermore, it was postulated that cellular hypertrophy observed in senescent CAFs might be a secondary response to compensate for constitutive autophagic protein degradation, resulting in an increase in protein synthesis to avoid cell death (Demidenko & Blagosklonny, 2009; Narita et al., 2011).

During cellular senescence, elongated spindle shape fibroblasts can become enlarged and flattened (Kuilman, Michaloglou, Mooi, & Peeper, 2010). This morphological transformation is often coupled with changes in actin stress fibre formation, focal adhesion and cytoskeleton proteins (Chen et al., 2000; Cho et al., 2004). Proteins that are responsible for the contractile function and actin cytoskeleton of cells, such as α-SMA, are also often profoundly upregulated (Hinz, Dugina, Ballestrem, Wehrle-Haller, & Chaponnier, 2003; Sandbo & Dulin, 2011). This observation is in line with previous findings that activated (characterised by elevated expression of α-SMA) and senescent fibroblast phenotypes are closely related (Hassona et al., 2013). Furthermore, CAFs from GU-OSCCs in the present study grew slowly in cell culture relative to other fibroblast groups, which was also shown by Lim et al. (2011) *in vitro*; this characteristic is most likely due to growth arrest, as would be expected in senescent cells (Kuilman et al., 2010) and was shown by the elevated level of $p21^{Waf1/Cip1}$ in CAFs from GU-OSCCs

(Section 4.2.3). Overexpression of $p21^{Waf1/Cip1}$ inhibits cyclin-dependent protein kinases, whose activity is crucial for G1 cell cycle progression and maintenance of senescent growth arrest.

Even though the induction of SA-β-gal activity is not entirely specific to the senescent state and there is no evidence to support the actual role of this enzyme in the senescence response (Kuilman et al., 2010), it is widely used as biomarker for senescent and ageing cells and can serve as an informative predictor of senescence when tested in combination with other markers *in vitro* or *in vivo* (Collado & Serrano, 2006; Dimri et al., 1995). β-galactosidase is a lysosomal enzyme that is present in all cells to catalyse the hydrolysis of β-galactosides to monosaccharides. In normal cells, this process normally occurs optimally at around pH 4, yet in senescent cells, the activity can be suboptimal at pH 6. By applying an artificial chromogenic substrate such as X-gal, an insoluble blue precipitate will be formed at pH 6 in senescent cells (Dimri et al., 1995). In the present study, the blue staining was visually more apparent and SA-β-gal enzyme activity was significantly higher in CAFs from GU-OSCCs than CAFs from GS-OSCCs and NHOFs, confirming that senescent phenotypes were more evident in these cells.

SIRT1, a NAD-dependent deacetylase, has been increasingly recognised to play important roles in various physiological processes including stress responses, apoptosis, metabolism, autophagy, ageing, inflammation and senescence (Haigis & Sinclair, 2010). In several reports, it has been demonstrated that upregulated expression of SIRT1 can delay cellular senescence, extend cellular life span and thus increase longevity (Langley et al., 2002; Ota et al., 2007; Satoh et al., 2013; N. Zhou et al., 2016). SIRT1 seems to exert a cytoprotective function and protects cells against oxidative stress and multiple cellular stresses (Yao et al., 2014). Loss of SIRT1 occurs with ageing and senescence (Rovillain et al., 2011), and is commonly employed as a senescent marker (James et al.,

2016; Michishita et al., 2005). In the present study, even though SIRT1 protein expression was heterogeneous across the panel of fibroblasts, it was generally downregulated in CAFs from GU-OSCCs relative to the other fibroblast groups. Taken together with the concomitant elevated expression of $p21^{Waf1/Cip1}$, these results indicated that CAFs from GU-OSCCs were more senescent than CAFs from GS-OSCCs and NHOFs. These observations are in agreement with previous reports that CAF senescence is indeed a unique feature of the genetically unstable and more aggressive tumour (GU-OSCCs) in comparison to their more stable counterparts (GS-OSCCs) (Hassona et al., 2013; Lim et al., 2011; Parkinson et al., 2015).

7.2.2 Autophagosome accumulation and autophagic impairment in CAFs from GU-OSCCs

Autophagy is an evolutionarily conserved process crucial for protein degradation, organelle turnover and maintenance of homeostasis. Autophagy is a highly dynamic process that is tightly regulated at multiple sequential steps through the progressive sequestration of cytoplasmic material into double-membrane autophagosomes, delivery and degradation upon fusion with lysosomes (Galluzzi et al., 2017). Several unique molecular features of autophagosome formation and turnover have been exploited for measuring autophagy quantitatively and qualitatively (Klionsky et al., 2016; Mizushima et al., 2010). Central to these approaches is the detection of processed microtubule-associated light chain 3 protein, LC3B-II, on the autophagosome membrane, which serves as a cellular readout of autophagic levels. The formation of autophagosomes involves the conversion of cytosolic LC3B-I to the membrane-bound form of LC3B-II by the conjugation of phosphatidylethanoamine (PE) at its C-terminus and it correlates with the number of autophagosomes (Mizushima & Levine, 2010). LC3B is equally distributed throughout the cytoplasm and localised in the nucleolar regions

(Koukourakis et al., 2015), which can be visualised as fluorescent puncta with immunofluorescent staining.

The results of the present study showed that the number of autophagosomes in CAFs from GU-OSCCs was significantly greater than CAFs from GS-OSCCs and NHOFs. It was confirmed by Western blot, immunofluorescent staining and TEM that autophagosomes accumulated in CAFs from GU-OSCCs, which were also correspondingly found to be more senescent. Similar observations were reported in dermal fibroblasts where autophagosome accumulation was more apparent in aged dermal fibroblasts and by the demonstration that autophagic activity within specific tissues can be affected by age (Tashiro et al., 2014), or possibly cellular ageing, i.e. senescence.

Autophagic flux is a term to describe the overall autophagic degradation and is used to indicate the delivery of sequestered cargo to lysosomes instead of autophagosome formation (Klionsky et al., 2016). LC3B maturation is possibly the most widely used autophagic biomarker due to its correlation with the number of autophagosomes, although it does not accurately monitor the degree of autophagic flux (P. Jiang & Mizushima, 2015). Therefore, it is important to use other biomarkers in combination with LC3B. Another frequently employed marker of autophagic flux is p62, which is useful in monitoring autophagy as its expression decreases upon the completion of autophagy (P. Jiang & Mizushima, 2015; Mizushima & Yoshimori, 2007). p62 is a polyubiquitin-binding protein that binds directly to LC3 and human ATG8 homologue (GABARAP) family proteins and is eventually degraded by autophagy towards the end of the sequential process (Pankiv et al., 2007).

The accumulation of autophagosomes in CAFs from GU-OSCCs observed in the present study may represent either a true upregulation of autophagic degradation

(autophagic flux) or an inhibition in the completion of the autophagic pathway (impairment in autophagic degradation) (Mizushima & Yoshimori, 2007). Western blot analyses showed concomitant elevated expression of LC3B-II and p62 in CAFs from GU-OSCCs indicated defects in the autophagic pathway in these cells. This result was confirmed by the fact that there were no significant differences in mRNA levels of a number of *ATG* genes required for autophagosomes formation and elongation during early autophagy between the fibroblast groups. Therefore, my results showed that the accumulation of autophagosomes in CAFs from GU-OSCCs was due to autophagic impairment at the degradation stage of sequential autophagy, data that are consistent with the observation that p62 accumulates in autophagic-defective cells (Mizushima & Levine, 2010). Similarly, abnormal accumulation of autophagosomes in aged dermal fibroblasts was also due to a deficiency at the degradation step of autophagy and this is thought to contribute to compromised cellular or tissue function (Tashiro et al., 2014).

One of the possible explanations for the impairment of autophagy in CAFs from GU-OSCCs might be due to the lack of SIRT1 in these fibroblasts, as SIRT1 is known to play a pivotal role in the clearance of old and non-functional mitochondria. The lack of SIRT1 could cause accumulation of damaged organelles, especially mitochondria, resulting in the accumulation of p62 (I. H. Lee et al., 2008). This could lead to inefficiency in mitophagy, which is the selective autophagic clearance of damaged mitochondria (Galluzzi et al., 2017). This hypothesis is in accordance with the requirement of SIRT1 in the induction of the autophagy process, as demonstrated in starvation- and oxidative stress-induced autophagy (Hariharan et al., 2010; I. H. Lee et al., 2008; Ou, Lee, Huang, Messina‐Graham, & Broxmeyer, 2014).

7.2.3 Autophagy and senescence are closely related in oral CAFs

In the current study, the autophagic and senescent phenotypes were shown to be closely associated in oral fibroblasts, as demonstrated by the positive correlation between SA-β-gal activity and the number of LC3B puncta per cell. It was also shown that CAFs from GU-OSCCs were more senescent and the abundance of autophagosomes in these fibroblast subsets was due to impairment of their autophagy pathway. The possible link between autophagy and senescence in fibroblasts might be explained in part by the function of SIRT1 in decreasing ROS levels under oxidative stress and the central role of ROS to these phenomena (Hori, Kuno, Hosoda, & Horio, 2013; Salminen, Kaarniranta, & Kauppinen, 2013; W. Zhang et al., 2017).

Paracrine communication has been previously reported between epithelial cells and CAFs to regulate fibroblast activation and OSCC cell proliferation (Kellermann et al., 2008). In addition, it was also shown by Hassona et al. (2013) that ROS produced from GU-OSCC cells induces myofibroblastic phenotypes and senescence temporally in normal fibroblasts in a TGF-β dependent manner. Once the oxidative insults have been induced, fibroblasts can reinforce their senescence in a self-perpetuating manner with further mitochondrial damage and the generation of high ROS levels (Passos et al., 2010; Passos, Saretzki, & von Zglinicki, 2007). SIRT1 regulates mitochondrial function and is required for oxidative stress induced-autophagy, especially the clearance of mitochondrial damage (Ou et al., 2014). The depleted level of SIRT1 in CAFs from GU-OSCCs shown in the present study could possibly result in a loss of mitochondrial membrane potential and accumulation of mitochondrial mass, which eventually leads to impaired autophagic clearance of damaged mitochondria. Several lines of evidence support the notion that failure to eliminate dysfunctional mitochondria could trigger the establishment of senescence in fibroblasts (Correia‐Melo et al., 2016; Moiseeva, Bourdeau, Roux, Deschênes-Simard, & Ferbeyre, 2009; Wiley et al., 2016) and

mitochondrial SIRT proteins SIRT3 and SIRT5 suppress senescent phenotypes (Wiley et al., 2016).

Mitochondrial dysfunction has also been reported in senescent fibroblasts that had irreparable DNA double-strand breaks and were proliferatively exhausted (Dalle Pezze et al., 2014; James et al., 2015) and autophagic impairment due to mitochondrial dysfunction was shown to be an important characteristic of oxidative stress-induced senescence (Tai et al., 2017). This triggering event further allows the maintenance of a persistent DDR via ROS generation, which has been known to be necessary for the establishment of senescence and SASP (Passos et al., 2010; Rodier et al., 2009). In turn, the CAF-mediated SASP would have a bystander effect on neighbouring tumour cells, leading to the promotion of tumorigenesis via paracrine mechanisms. This notion is in accordance with the induction of epithelial invasion by senescent fibroblasts from GU-OSCCs as demonstrated by Hassona et al. (2013) in an *in vitro* model.

7.3 The contribution of autophagy to fibroblast activation and senescence and its role in mediating CAF function

Autophagy is a stress response that has been linked to both fibroblast activation (myofibroblast differentiation) and senescence (Capparelli et al., 2012). These three stress responses share overlapping stimuli such as TGF-β, oxidative stress, DNA damage, oncogene activation and telomere shortening, suggesting an intimate relationship (Ghavami et al., 2015; Prime et al., 2017; Waghray et al., 2005). Previous reports have suggested a close relationship between fibroblast activation and senescence, and that these physiological states of CAFs may reflect sequential stages of the same regulatory pathway during oral fibroblast differentiation, as well as an overlap in their tumour-supporting properties (Hassona et al., 2013; Mellone et al., 2016; Prime et al., 2017).

Autophagy is regulated in a cell type-dependent manner and it can be either a pro- or anti-senescence mechanism (C. Kang & Elledge, 2016). However, the precise role of autophagy in regulating cellular senescence is still debatable. On one hand, the notion of "cellular senescence by autophagy – pro-senescence" is supported by the observation that autophagic flux is activated in senescent cells and that senescence may be a secondary phenotype to autophagic induction (Capparelli et al., 2012; Kwon, Kim, Jeoung, Kim, & Kang, 2017). Autophagy was shown to modulate the SASP during oncogene-induced senescence and was hypothesised to be responsible for providing essential building blocks for SASP protein synthesis (Young et al., 2009). This idea connects the catabolic autophagic process to anabolic SASP synthesis (Narita et al., 2011), whilst also being in accordance with the proposal that the SASP contributes only partially to the tumour-promoting activity of senescent fibroblasts with the remaining activity being a reflection of their autophagic/catabolic phenotype (Capparelli et al., 2012). Conversely, "cellular senescence with autophagy – anti-senescence" can be perceived as a failure of the homeostatic role of basal autophagy in attempting to suppress senescence due to the accumulation of potentially harmful entities (e.g. damaged proteins or increased mitochondrial dysfunction) (C. Kang & Elledge, 2016; Kwon et al., 2017). Several studies have highlighted the critical role of basal autophagy in maintaining homeostasis and preventing cellular senescence. For example, García-Prat et al. (2016) demonstrated that defective autophagy triggers senescence in muscle stem cells and autophagy maintains stemness of cells by suppressing senescence. Under stressful conditions, autophagy has been shown to inhibit oxidative stress-induced senescence, but prolonged oxidative insults could rather impair autophagic activity, resulting in the induction of cellular senescence (Tai et al., 2017).

To test whether autophagy contributes to activation and/or senescence in oral fibroblasts, I took advantage of known senescence inducers and autophagic inhibitors.

The results collectively indicated that the autophagic pathway could regulate fibroblast activation and senescence in mediating the tumour-promoting phenotypes of CAFs in OSCCs and suggest a correlated relationship between autophagy, activation and senescence in oral fibroblasts.

7.3.1 Autophagy induction

A model with which to study autophagy in the context of fibroblast activation and senescence was developed. Previous reports have demonstrated that TGF-β1 secretion was induced by ROS prior to the establishment of fibroblast senescence (Frippiat et al., 2001; Frippiat, Dewelle, Remacle, & Toussaint, 2002) and that TGF-β-induced and senescence-induced myofibroblasts shared similar phenotypic and functional features (Mellone et al., 2016). Activated TGF-β signalling is a consistent feature of the CAF phenotype (Martinez-Outschoorn, Pavlides, et al., 2010). A previous report by Hassona et al. (2013) showed that GU-OSCC cells produce elevated levels of ROS and induce oxidative stress in adjacent fibroblasts in a TGF-β1-dependent manner. The results of the present study have further extended these observations by demonstrating that autophagy could be a regulator in mediating fibroblast activation and senescence.

In this study, normal oral fibroblasts were treated with TGF-β1 over a period of 10 days to induce senescence, which mimics the high expression of TGF-β1 in GU-OSCC CAFs (Hassona et al., 2013), to determine whether autophagic impairment in these cells was TGF-β1-induced. TGF-β1-treated NHOFs showed a progressive rise in SA-β-gal activity and loss of SIRT1 expression, indicating the induction and development of senescence, similar to GU-OSCC CAFs. Morphologically, the cells displayed contractile, flattened features with cellular hypertrophy, which was also consistent with the senescent phenotype in CAFs from GU-OSCCs. Somewhat surprisingly, however, TGF-β1 induced autophagic flux in NHOFs, as opposed to autophagy inhibition as

shown in CAFs from GU-OSCCs. These results extended the previous observation of fibroblast activation preceding senescence in TGF-β1-treated NHOFs by Hassona et al. (2013) and demonstrated the involvement of autophagy. In the current study, TGF-β1 induced autophagic flux (upregulated LC3B-II, downregulated p62), fibroblast activation (elevated α-SMA expression) and senescence (increased SA-β-gal activity, loss of SIRT1) in NHOFs, with autophagic flux preceding fibroblast activation, followed by senescence. The observations of autophagy impairment seen in senescent CAFs from GU-OSCCs and autophagic flux occurs in TGF-β1-treated NHOFs indicate that autophagy plays different roles during the induction of fibroblast senescence depending on the nature of the stimulus. While autophagy impairment could be linked to the senescent CAF phenotype, autophagy could be a pro-senescence response in normal oral fibroblasts following exposure to TGF-β1.

7.3.2 Autophagy inhibition

To further explore the role of autophagy in influencing activation and senescence in fibroblasts, autophagy was inhibited in NHOFs using pharmacological inhibitors to target specific stages of the sequential autophagic pathway.

Inhibitors of autophagy have been gaining much attention in recent years as potential agents for anti-cancer therapy due to their ability to affect various aspects of cancer pathogenesis. CQ and its derivate HCQ are two commonly used FDA-approved autophagic inhibitors and both these drugs are effective antimalarial agents. HCQ contains an additional hydroxyl group compared to CQ, which results in reduced toxicity (Ben-Zvi, Kivity, Langevitz, & Shoenfeld, 2012). They are currently being tested in clinical trials to treat certain cancers and have been shown to function synergistically with conventional chemotherapy drugs, which might result in new combinatorial chemotherapies (Manic et al., 2014; Verbaanderd et al., 2017). However,

it is uncertain whether their efficacy in this regard is solely dependent on the inhibition of the autophagic pathway (Eng et al., 2016; Maycotte et al., 2012). CQ and HCQ were previously assumed to function through the same mechanism as bafilomycin A_1 ($BafA_1$), an endosomal acidification inhibitor that accumulates inside lysosomes and prevents the action of lysosomal enzyme, which requires an acidic pH. $BafA_1$ inhibits autophagy by elevating the lysosomal pH, resulting in the inhibition of lysosome-autophagosome fusion and protein degradation (Klionsky et al., 2016). In contrast to popular belief, it was recently discovered that CQ and HCQ function through blocking the downstream fusion of autophagosomes and lysosomes and not by affecting the degradation capacity of lysosomes (Mauthe et al., 2018).

SAR405 is a more specific autophagic inhibitor than CQ and HCQ that has exquisite selectivity against the upstream lipid kinase Vps34, a PI3K class III isoform that regulates autophagosome formation and vesicle trafficking (Ronan et al., 2014). The Vps34 complex is downstream of the negative regulator of autophagy, mTOR (J. Kim et al., 2013), and has a dual role in autophagosome biogenesis during early autophagy and also between late endosomes to lysosomes during endocytosis (Pasquier, 2015). Lead optimisation and structural analysis of SAR405 identified that SAR405 is a Vps34 inhibitor with high potency and specificity that does not target other protein and lipid kinases (Ronan et al., 2014). Inhibition of Vps34 kinase activity by SAR405 disrupts vesicle trafficking from late endosomes to lysosomes, as well as by the generation of autophagosomes through impairment of lysosomal function (Pasquier, 2015; Ronan et al., 2014).

In the present study, NHOFs were treated with HCQ or SAR405 separately. The differences in how HCQ and SAR405 block autophagic flux could influence different cellular process and have different impacts on cell physiology. Although both HCQ and

SAR405 have clearly been shown to impair autophagic flux (Mauthe et al., 2018; Ronan et al., 2014), these inhibitors appeared to differentially affect activation and senescence in oral fibroblasts in the present study. HCQ treatment effectively blocked autophagic degradation but only had a marginal effect on activation and senescence in two different NHOF strains. Compared to HCQ at the highest treatment concentration (10 μM, 72 hours) used in this study, SAR405 appeared to block autophagy more efficiently (elevated p62 and LC3B-II expression) and also induced fibroblast activation and senescence. Whilst these discrepancies might be due to functional differences between the inhibitors, the results are consistent with the fact that SAR405 is a more potent autophagic inhibitor and possibly highlight the limitations of HCQ, as high concentrations are generally required to effectively modulate autophagy and long duration of HCQ administration at high doses can cause unfavourable side effects (Shi, Yu, Yan, & Xiao, 2017; Verbaanderd et al., 2017; P. Zhou et al., 2018).

Lastly, the inhibition of autophagy by SAR405 in this study also highlights the homeostatic role of autophagy in modulating fibroblast activation and senescence in oral fibroblasts. It was demonstrated that autophagy inhibition could induce fibroblast senescence and this data is in line with the observation of autophagic impairment in senescent CAFs from GU-OSCCs. As described by Hassona et al. (2013), inhibition of TGF-β in conditioned medium of GU-OSCCs only partially inhibited senescence in normal oral fibroblasts, suggesting that other mechanisms or molecules may be involved in the induction of fibroblast senescence. The results of the present study indicate that autophagy impairment in fibroblasts may be required for the full induction of fibroblast senescence.

7.3.3 Autophagy regulates activation, senescence and tumour-promoting functions in oral fibroblasts

In order to examine a possible link between autophagy, activation and senescence, normal oral fibroblasts were first treated for 120 hours with SAR405 to inhibit autophagy and then TGF-β1 was used to induce autophagic flux. The results of these experiments support a molecular link between activation, autophagy and senescence in fibroblasts. It was observed that both the inhibition of autophagy with SAR405 and the induction of autophagic flux with TGF-β1 promoted fibroblast activation and senescence. Therefore, altered autophagic activity induced myofibroblast differentiation and the establishment of senescence. Specifically, SAR405- and TGF-β1-treated oral fibroblasts showed key features of activation and senescent phenotypes such as (1) elevated α-SMA expression, (2) altered cell morphology consistent with cellular hypertrophy, (3) reduced proliferation, (4) downregulation of SIRT1 and (5) increased activity of SA-β-gal. Interestingly, the concept that autophagy and senescence are closely related is supported by the increased lysosomal mass in senescent cells and the function of β-galactosidase as a lysosomal enzyme (Capparelli et al., 2012; Kurz et al., 2000; B. Y. Lee et al., 2006). Together these results demonstrate that deregulated autophagy could induce fibroblast activation and senescence and indicated a direct correlation between autophagy, activation and senescence in oral fibroblasts. These seemingly paradoxical observations that both autophagy inhibition and autophagic flux induced fibroblast activation and senescence in the present study could possibly be due to the balancing act of autophagy and its capacity in counteracting stressful conditions. This emphasizes the critical role of basal autophagy in maintaining cell homeostasis under stressful conditions and indicates that any excessive or impaired autophagic activity could possibly lead to changes in CAF phenotypes.

Tumour cells are proposed to induce oxidative stress in adjacent CAFs in the TME, which then function as a metabolic generator to fuel the co-evolution of the tumour and its surrounding stroma (Martinez-Outschoorn, Balliet, et al., 2010). This metabolic coupling can further induce genomic instability in tumour cells and promote their aggressiveness via a bystander effect. Furthermore, the unique secretome by senescent and/or autophagic CAFs can also drive cancer progression and metastasis through reciprocal communication of tumour cells and TME (Capparelli et al., 2012; New et al., 2017). To further investigate the functional role of fibroblastic autophagy in secreting tumour-promoting factors, CM was collected from NHOFs under conditions of autophagic flux (TGF-β1-induced) and autophagy inhibition (SAR405-treated). Hassona et al. (2013) have shown that epithelial-derived ROS induces activation and senescence in CAFs, and CAFs reinforce senescence by generating an enhanced level of ROS in a TGF-β1-dependent manner. Therefore, the effects of TGF-β1-induced autophagic flux on the ability of fibroblasts to regulate OSCC cell proliferation, motility and invasion were investigated *in vitro*. Furthermore, having shown in the present study that autophagy was impaired in CAFs from GU-OSCCs, the effects of SAR405-inhibited fibroblastic autophagy on OSCC cell behaviour were also investigated *in vitro*.

TGF-β1-induced autophagic flux in fibroblasts resulted in the secretion of factors that enhanced the migration and invasion of OSCC cells in transwell assays. This data extend the results of previous reports by Mellone et al. (2016) and Hassona et al. (2013) and unravel a possible role of TGF-β1-induced autophagic flux in altering secreted factors in the TME. However, in the present study, both TGF-β1-induced autophagic flux and SAR405-inhibited autophagy in fibroblasts resulted in the production of secreted factors that both promoted epithelial cell migration. This paradoxical observation could possibly be explained by the observations in the present study that deregulated autophagy (inhibition and increased autophagic flux) promoted fibroblast

activation and senescence. The inhibition of fibroblastic autophagy by SAR405, however, only enhanced OSCC cell migration but did not affect invasion, suggesting that autophagic impairment in fibroblasts is insufficient to promote tumour cell invasion. These results could possibly be reconciled by the enhanced ROS, and the pro-tumourigenic functions of CAFs from GU-OSCCs reported by Hassona et al. (2013) might occur as a result of the combined effects of autophagic impairment and elevated TGF-β1 expression, especially in facilitating the epithelial invasion of OSCC. These data collectively indicate that altered autophagic activity in fibroblasts could lead to a more motile and aggressive phenotype of OSCC cells through the crosstalk between fibroblasts and epithelial cells.

In summary, autophagy, fibroblast activation and senescence may be part of the same biological phenomenon and/or part of a unified programme and my findings demonstrated that fibroblast activation and senescence are pathways regulated by autophagy. Altered autophagic activity could possibly influence these two stress responses in fibroblasts. CM collected from fibroblasts with altered autophagy enhanced the migratory and invasiveness of OSCC cells. This, in turn, revealed that the autophagy-dependent secretion in fibroblasts might be responsible, at least in part, for regulating the malignant phenotype (tumour cell motility and invasive properties) of OSCC cells.

7.4 The development of model systems to examine the phenotypic impact of fibroblast senescence on OSCC cell behaviour

While temporal induction of senescence can be advantageous in certain settings, long-term accumulation of senescent cells in tissues can be deleterious and may contribute to detrimental age-related pathologies and cancer. These negative effects can often be attributed to the secretion of pro-inflammatory SASP factors. Hence, strategies

to preferentially remove senescent cells from tissues or disrupt the pro-senescent signalling/secretome to revert the senescent-associated phenotype could be beneficial from a therapeutic perspective and are gaining significant attention (Childs et al., 2017).

A major challenge in assessing the functional role of senescence in OSCC-derived CAFs is that these cells are highly senescent (i.e. non-proliferative) and difficult to culture *in vitro* to obtain sufficient biological material for downstream assays. Therefore, I attempted to use senolytic drugs to selectively remove senescent cells from CAF cultures and conditional immortalisation of normal fibroblasts in order to develop sustainable *in vitro* model systems to examine the biological relevance of senescence to the malignant phenotypes of CAFs. A temperature-sensitive *in vitro* model of fibroblast senescence was established and employed to investigate the contribution of fibroblast senescence to the malignant phenotypes of OSCC cells. The results demonstrated that fibroblast senescence plays a key role in facilitating the secretion of tumour-promoting factors and promoting more aggressive OSCC cell behaviour.

7.4.1 Elimination of senescent cells using senolytic drugs

Several compounds with senolytic activity in selectively eliminating senescent cells from mixed cell populations have been reported to exert cancer therapeutic outcomes in model systems, and pre-clinical and clinical trials (Chang et al., 2016; Childs et al., 2017; Yosef et al., 2016). Senescent cells have been shown to be resilient to apoptotic stimuli, mainly through the upregulation of anti-apoptotic proteins of the BCL-2 family (M. Sasaki, Kumazaki, Takano, Nishiyama, & Mitsui, 2001; E. Wang, 1995). ABT263 (Navitoclax) and ABT737 are specific inhibitors targeting the BCL-2 family proteins and are potent senolytic drugs that have been shown to stimulate preferential apoptosis in senescent cells and also to repress the expression of several SASP factors (Chang et al., 2016; Yosef et al., 2016).

To selectively remove the senescent subset from a heterogeneous CAFs population, CAFs from GU-OSCCs were treated with ABT263 and ABT737. Even though these drugs seemed to reduce the number of SA-β-gal positive cells in CAF cultures, there were no marked changes in protein expression of autophagic, activation and senescent markers. The level of the SIRT1 protein in CAFs from GU-OSCCs did not change following treatment with ABT263 and ABT737, implying that these drugs did not reverse the senescent status of the CAF cultures. These results were not totally unprecedented because senolytic drugs target the BCL-2 family members with similar affinities and overexpression of these proteins can sensitise cells (Van Delft et al., 2006). Overexpression of particular proteins of this family can confer resistance to drug-induced apoptosis (Mérino et al., 2012). For example, excess BCL-2 sensitised lymphoma cells following ABT737 treatment but augmented BCL-X_L and BCL-w induced resistance to ABT737 (Mérino et al., 2012). It is notable that the level of expression of specific members of the BCL-2 protein family can have a differential impact on the susceptibility of cells to senolytic drugs. The expression of individual BCL-2 proteins was not fully investigated in the present study and the efficacy of these drugs was not apparent in CAFs of OSCCs. Therefore, this model was not employed for further downstream experiments to examine the biological importance of senescence in CAF phenotypes.

7.4.2 Establishment of temperature-sensitive senescence-inducible oral fibroblasts

Molecular genetic manipulations with the catalytic subunit of hTERT and viral oncogenes have been commonly adopted to establish cell lines/strains in order to overcome cellular senescence and to immortalise normal cells that have limited replicative potential *in vitro* (Ali & DeCaprio, 2001; Morales et al., 1999). In the experiments described in Chapter 6, highly senescent CAFs from GU-OSCCs were

infected with retroviruses that transduce hTERT and SV40 LT antigen in an attempt to conditionally immortalise the cells. Notably, the SV 40 LT antigen used in the present study was temperature-sensitive (tsLT) and has been shown to conditionally immortalise primary fibroblasts or to generate various cell lines from normal tissues of breast, liver, colon, bone marrow, etc. (Jat & Sharp, 1989; Kawata et al., 2006; Obinata, 2001). Cells expressing this tsLT mutant can have extended proliferative potential when grown at the permissive temperature (33°C) upon tsLT activation, but cease growing at the non-permissive temperature (39°C) upon tsLT inactivation.

O'Hare et al. (2001) showed that even though the introduction of both hTERT and tsLT can efficiently generate immortal cell lines regardless of the order in which the genes were transduced, the sequence and timing of sequential transduction could affect the genomic stability of cells. When hTERT is introduced first, followed by tsLT, more stable diploid cells are generated. In the present study, both genes were introduced to CAFs from GU-OSCCs in the aforementioned order and two strains of CAFhTERT-tsLT were generated. Similar to transfection of oncogenes such as EJ c-Ha-*ras*1 gene into rat embryo fibroblasts (Jat & Sharp, 1989), these cells were fast-growing, highly refractile under phase-contrast microscopy and gave rise to morphologically transformed colonies. However, when tested in temperature-shift experiments, these CAFhTERT-tsLT cells did not readily undergo senescence at the non-permissive temperature, even though growth arrest at G_1 or G_2 phase was anticipated (Jat & Sharp, 1989). In theory, growing the cells at 39°C would give rise to CAFhTERT-tsLT that display senescent phenotypes; however, a similar growth rate, morphology and average cell size were observed between cells grown at both temperatures.

The loss of SIRT1 is a marker for late senescence and both transduced CAFhTERT-tsLT strains had detectable SIRT1 expression at both temperatures, suggesting that they

had overcome senescence and progressed through normal cell cycle checkpoints. In contrast, all the samples expressed another senescence marker, $p16^{Ink4a}$, which normally accumulates in late passage senescent cells. Cells may be able to escape senescence through the loss of $p16^{Ink4a}$ and the expression of $p16^{Ink4a}$ in these transduced young cells suggested that they escaped senescence through the loss of pRb by the action of SV40 LT antigen resulting in a relatively high level of $p16^{Ink4a}$ (Hara et al., 1996). However, transduced BICR63FhTERT-tsLT expressed the cell cycle arrest marker, $p21^{Waf1/Cip1}$, at both temperatures even though they demonstrated continuous proliferation in cultures. These results were very perplexing, as the transduced cells did not senesce at 39ºC nor express the relevant markers. It is possible that the effects of hTERT or endogenous mutations may have overridden the effects of the thermolabile tsLT antigen, as this observation was not seen in singly tsLT transduced NHOFs.

As it was not possible to conditionally immortalise CAFs, an alternative approach was taken in which retroviral transduction of tsLT alone was performed using NHOFs to generate NHOFs-tsLT, without the introduction of the hTERT transgene. These cells were cultured in both permissive and non-permissive temperatures in temperature-shift experiments to confirm that tsLT could act in a temperature-dependent manner. Temperature-dependent growth was observed in NHOF1-tsLT, as they bypassed growth arrest and were able to be propagated in culture without evident crisis at 33ºC, while readily differentiating into cells that displayed senescent phenotypes at 39ºC.

Studies with cellular senescence are commonly conducted with primary cells but can be complicated by the proliferative or phenotypic heterogeneity in cultures with subpopulations of growth-arrested and dividing cells (Hardy et al., 2005). Therefore, this genetically modified cell system established in the present study has not only provided an unlimited source of cells that could be induced to senesce synchronously

but also represents a valuable model with which to investigate the mechanism of CAF differentiation and senescence.

7.4.3 Biological significance of fibroblast senescence in epithelial OSCC cell behaviour

Despite undergoing proliferative arrest, senescent cells are metabolically active with significant changes in their protein expression and secretory profile, importantly the SASP (Coppé et al., 2010). A collection of soluble (chemokines, interleukins and growth factors), insoluble (fibronectin, laminin, collagen and osteopontin) signalling factors and proteases (MMPs) constitutes the SASP (Faget et al., 2019; Kuilman & Peeper, 2009). Of note, there is a body of compelling evidence to indicate a pro-tumourigenic role of senescent fibroblasts in the context of the SASP. The SASP confers tumour-promoting paracrine capacities to senescent fibroblasts, which in turn influences the biology of adjacent pre-neoplastic or neoplastic cells and encourages the remodelling of surrounding TME (Acosta et al., 2013; Krtolica et al., 2001; Parrinello et al., 2005).

In the present study, to examine the functional role of fibroblast senescence, CM was collected from the temperature-inducible NHOF1-tsLT cells under permissive (non-senescent) and non-permissive (senescent) temperatures. The senescent state is often associated with metabolic alterations such as mitochondrial function or respiratory chain dysfunction (Malaquin, Tu, & Rodier, 2019; Moiseeva et al., 2009). To avoid confusion of metabolic dysfunction with actual change in cell number, widely applicable cellular metabolic methods to assess cell number, such as MTT assay, were avoided in the current study. Instead, cell counting was performed to evaluate OSCC cell proliferation following co-culture with CM from senescent and non-senescent NHOF1-tsLT. The data showed that CM from senescent NHOF1-tsLT promoted OSCC cell proliferation,

compared to non-senescent NHOF1-tsLT and controls. In similar co-culture systems, CM from senescent fibroblasts was also observed to exert a growth-promoting effect on epithelial tumour cells (Bavik et al., 2006; Coppé, Boysen, et al., 2008), indicating that SASP factors secreted by the senescent fibroblast can amplify tumour cell proliferation in a paracrine manner.

Acquisition of migratory ability and invasiveness are key hallmarks of metastasis and EMT. The effects of senescent fibroblasts on migration and invasion of cancer cells were also explored using CM from the NHOF1-tsLT model. Senescent NHOF1-tsLT demonstrated greater ability to induce both transwell migration and invasion of OSCC cells compared with non-senescent NHOF1-tsLT or blank control. Conversely, non-senescent NHOF1-tsLT failed to stimulate significant migration and invasion of OSCC cells. These results were in agreement with the general notion that secretory factors from senescent fibroblasts can increase tumour cell motility and drive invasion of tumour cells (Malaquin et al., 2013; Ortiz-Montero et al., 2017; Yamao et al., 2019). By using a model that can induce fibroblasts to senesce synchronously, the results of the present study revealed that the contribution of fibroblast senescent secretome in modulating the malignant phenotypes of OSCC cells that warrants further investigation. These senescence-inducible cell lines may be useful in studying the senescent properties of fibroblasts and the reconstruction of the senescent microenvironment or niche *in vitro*. This technique could also be applicable to establishing other types of stromal cell lines, and to investigate the mutual regulatory crosstalk between different stromal cell types or between stromal and tumour cells in the TME.

7.5 Limitations of the study

The present study was carefully designed to address the specific research objectives proposed in Chapter 1. However, there are a number of possible limitations that could influence the interpretation of the findings and the broader concluding remarks.

Firstly, the study was unavoidably limited by the number of fibroblast strains available (Chapter 4). The analysis of such data is subject to the power of statistical analysis and sample bias due to the limited access to more fibroblast samples. The difficulty in culturing fibroblasts, especially CAFs from GU-OSCCs *in vitro* due to their senescence nature, was one of the major factors that contributed to the limited sample size. The power of statistical and preciseness of results can be enhanced when the sample size is increased, but much effort was made to include as many as possible fibroblast samples in the present study.

Secondly, the interpretations of the data deduced from experiments involving the application of pharmacological agents in targeting autophagy are restrained by the assumption that these compounds modulate their target(s) specifically and effectively. Realistically, it would be favourable to also validate such findings using genetic approaches that are more specific such as knockdown or ectopic expression of specific *ATG* genes.

Lastly, although the findings of this present study represent the first *in vitro* study to investigate the relationship of autophagy and senescence in modulating CAF phenotypes in OSCC pathogenesis, the primary limitation to the generalisation of these findings is that these findings have been derived using *in vitro* test systems only. Some of the limitations of this *in vitro* research include the possibility of losing particular cell subpopulation in culture over continuous passaging, limited interaction of tissue with surrounding factors or other stromal cell types in TME and the pharmacokinetics of

drugs which do not necessarily represent the actual situation in the oral cavity. Although it is technically challenging to establish an *in vivo* system, it is not impossible to recapitulate these findings in other relevant models in the future.

7.6 Future work

The work presented in this thesis identified a number of research directions that are likely to have fundamental implications for a more comprehensive understanding of the contribution of autophagy and senescence to the regulation of CAF phenotypes in OSCC pathogenesis. This, in turn, may be beneficial for the development of novel strategies to target these relevant pathways/factors therapeutically in the future.

The results presented in Chapter 4 demonstrated a positive correlation between autophagy and senescence, and that both of these responses were deregulated in CAFs from GU-OSCCs. Further, it was demonstrated that the accumulation of autophagosome observed in CAFs from GU-OSCCs was due to impairment of the autophagic pathway. Considering a role of SIRT1 in regulating mitochondrial function and senescence, it is reasonable to postulate that the autophagic impairment observed in senescent CAFs from GU-OSCCs could be due to mitochondrial dysfunction or failure in eliminating damaged mitochondria. To test this hypothesis, the mitochondrial mass and membrane potential could be further examined in this panel of fibroblasts.

Accumulation of autophagosome in CAFs from GU-OSCCS was demonstrated *in vitro* in the present study by the over-expression of LC3B-II with Western blotting and IF staining. To investigate whether this phenomenon can also be observed in clinical tissue samples, IHC analyses of tumour-associated stroma using formalin-fixed paraffin-embedded (FFPE) can be performed on tissue sections from different OSCC patients. Moreover, it would be beneficial to investigate the prognostic significance of stromal autophagy and its potential as a biomarker by examining the expression of

relevant ATG proteins in OSCC tissues from different histologic grade with known patient outcome.

In the present study (Chapter 5), fibroblast autophagy was demonstrated to have a functional role in modulating fibroblast secretion and enhancing tumour cell behaviour. Accumulating studies have described the role of fibroblast autophagy in regulating tumour metabolism and supporting the high metabolic demand of proliferating tumour cells, but it has not been directly investigated in this study and such studies in OSCCs are very limited. It would be important to extend this study by investigating the metabolic effects of fibroblast autophagy in organotypic and *in vivo* models to mimic the TME of OSCCs as closely as possible. A comprehensive understanding of fibroblast autophagy and tumour metabolism would greatly increase our understanding of the role of fibroblast autophagy in oral carcinogenesis and whether targeting this pathway has therapeutic potential.

The results presented in Chapter 6 showed that an *in vitro* senescence-inducible fibroblast model was established. Since senescence can be induced synchronously under the same culture conditions, this model of "pure" senescence would be a valuable tool in studying the correlation of autophagy and senescence by performing experiments such as ectopic over-expression/knockdown of relevant *ATG* genes or pharmacological intervention. Following such experiments, the autophagy/senescent-regulated secretome can be assessed by cytokine arrays to identify the changes in these fibroblast-secreted factors. The crosstalk between autophagic/senescent fibroblasts and epithelial tumour cells can also be further examined by using this model with *in vitro* transwell or organotypic assays.

CHAPTER 8: CONCLUDING REMARKS

The present study reveals, for the first time, a role for autophagy in modulating the activated and senescent phenotypes of oral fibroblasts, and the contribution of deregulated fibroblast autophagy and senescence to the pathogenesis of OSCC. A working model is proposed to demonstrate the relationship between autophagy and senescence in modulating CAF phenotypes and OSCC cell behaviour. This mechanism is schematically represented in Figure 8.1.

This study demonstrated that autophagy and senescence are closely associated and autophagic impairment was observed in CAFs from GU-OSCCs, which were also shown to display more apparent senescent phenotypes. This process is suggested to begin with the induction of oxidative stress, which occurs in a self-perpetuating manner with further production of ROS and TGF-β, mitochondrial damage and failure to remove such dysfunctional mitochondria due to an impaired autophagic pathway. This ultimately leads to fibroblast senescence.

My results also showed that altered autophagic activity modulated activation and senescence in oral fibroblasts and in doing so promoted the malignant phenotype of OSCC cells. An *in vitro* model of conditionally senescence-inducible oral fibroblasts, that allowed the synchronous induction of senescence, was established for the first time and which could be employed in the future to study fibroblast senescence and the reconstruction of senescent microenvironment *in vitro*. As a proof-of-concept, it was demonstrated that the secretome from senescent fibroblasts induced using this system modulated OSCC cell behaviour by promoting tumour cell migration and invasion.

Taken together, the results of the present study indicate that the physiological states of CAFs within the TME may reflect different stages of the same sequential pathway

and/or be part of a unified biological programme in which autophagy precedes activation and subsequent senescence during the acquisition of pro-tumorigenic CAF phenotypes. Targeting these different stages of oral CAF transdifferentiation within the TME may represent a novel avenue for therapeutic intervention for the better clinical management of OSCC in the future.

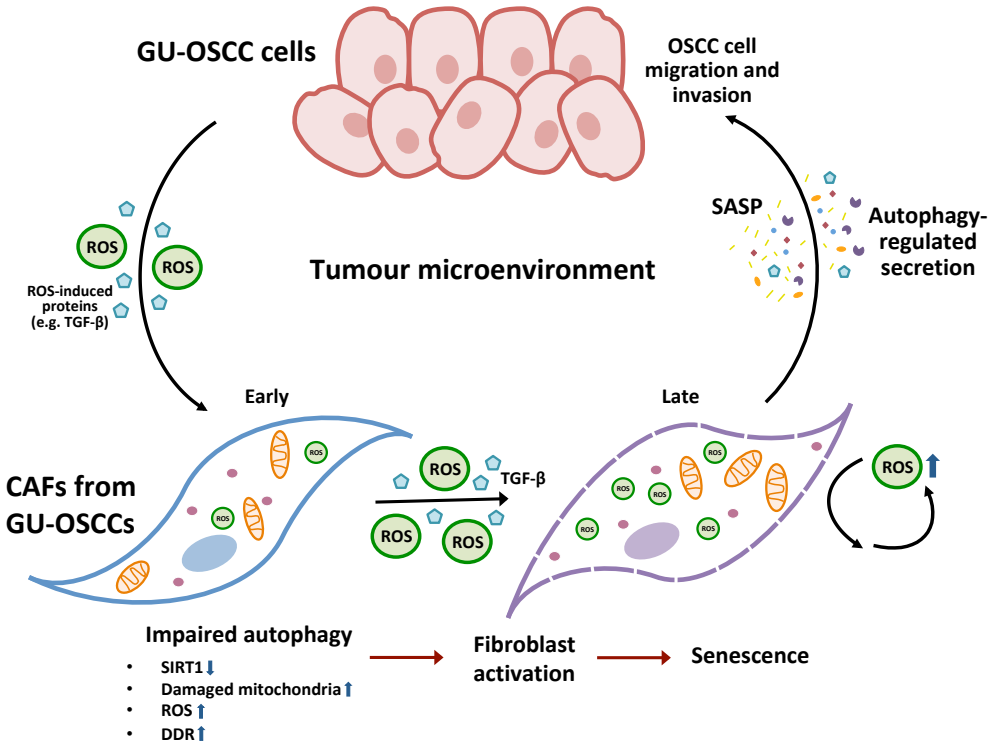

Figure 8.1: A proposed model of the interaction between malignant OSCC cells and CAFs in the oral tumour microenvironment

Keratinocytes from GU-OSCCs produce high levels of ROS and ROS-induced proteins such as TGF-β. Epithelial-derived ROS and TGF-β are transferred to adjacent oral fibroblasts. Depleted level of SIRT1 in CAFs causes the impaired clearance of dysfunctional mitochondria, which further amplifies the generation of ROS in CAFs, resulting in the onset of stromal oxidative stress and a DDR. Autophagy regulates fibroblast activation and senescence; CAFs reinforce and maintain their own senescence phenotypes in a self-perpetuating manner with further mitochondrial damage, DDR and generation of elevated levels of ROS. Autophagy, activation and senescence may reflect sequential stages of the same regulatory pathway during oral fibroblast differentiation. Ultimately, the SASP and autophagy-dependent secretion produced by CAFs enhance the malignant phenotypes of OSCC cells by facilitating the migration and invasion of the tumour cells within the tumour microenvironment.

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LIST OF PUBLICATIONS

1. SL Lai, ML Tan, RJ Hollows, M Robinson, M Ibrahim, S Margielewska, EK Parkinson, A Ramanathan, R Zain, H Mehanna, RJ Spruce, W Wei, I Chung, PG Murray, LF Yap and IC Paterson. (2019). Collagen induces a more proliferative, migratory and chemoresistant phenotype in head and neck cancer via DDR1. *Cancers*, *11*(11), 1766.

2. ML Tan, EK Parkinson, LF Yap and IC Paterson. (2020). Autophagy is impaired in cancer-associated fibroblasts and precedes activation and senescence during the induction of a senescent phenotype by TGF-β. (Manuscript submitted).

3. ML Tan, SS Prime, EK Parkinson, LF Yap and IC Paterson. The role of autophagy in regulating the phenotype and function of cancer-associated fibroblasts. (Manuscript in preparation).

LIST OF PRESENTATIONS

1. ML Tan. Functional significance of senescence and autophagy in cancer-associated fibroblasts from oral squamous cell carcinoma. Presented: PhD Thesis Seminar, University of Malaya, Kuala Lumpur, Malaysia (2018).

2. ML Tan, IC Paterson. Relationship between autophagy, activation and senescence in normal and cancer-associated human oral fibroblasts. Presented: American Association of Cancer Research (AACR) Annual Meeting, South Chicago, Illinois, USA (2018).

3. ML Tan, IC Paterson. Relationship between autophagy, activation and senescence in normal and cancer-associated fibroblasts from head and neck. Presented: Gordon Research Conferences (GRC), Hong Kong (2018).

4. ML Tan. Stress responses of fibroblasts in oral cancer. Presented: University of Malaya Three-Minute Thesis Competition (Faculty Level), Kuala Lumpur, Malaysia (2018). Awarded the best oral presentation.

5. ML Tan. Stress responses of fibroblasts in oral cancer. Presented: University of Malaya Three-Minute Thesis Competition (University Level), Kuala Lumpur, Malaysia (2018).

6. ML Tan. Senescence and autophagy in cancer-associated fibroblasts of oral squamous cell carcinoma. Presented: PhD Candidature Defence, University of Malaya, Kuala Lumpur (2017).

7. ML Tan, EK Parkinson, LF Yap, IC Paterson. Senescence and autophagy in cancer-associated fibroblasts (CAFs) of oral squamous cell carcinoma (OSCC). Presented: International Conference on Molecular Biology & Biotechnology (ICMBB), Kuala Lumpur, Malaysia (2016).

8. ML Tan, EK Parkinson, LF Yap, IC Paterson. Activation, senescence and autophagy in fibroblasts of oral cancers. Presented: Dental Congregation, Kuala Lumpur, Malaysia (2016).

9. ML Tan. Senescence and autophagy in cancer-associated fibroblasts of oral squamous cell carcinoma. Presented: PhD Proposal Seminar, University of Malaya, Kuala Lumpur (2016).