MONASH University

Emergence and Margins in Head and Neck Cancer

A new understanding

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Abstract

Many clinicians are unaware of the philosophies that form the framework of our medical research and accept them as fact. Yet these concepts are only that: concepts. Concepts and frameworks help us to intellectually and logically attempt to understand the complexities of nature and biology, but are not fact. They are simply the best we have at the time.

"Translational Research" has traditionally been defined as taking basic scientific findings and developing new diagnostic tools, drugs, devices and treatment options for patients, "the bench to bedside". It can refer to ensuring that new research is translated in practice, that it reaches the people and populations for whom they are intended and are implemented correctly.[1] The European Society for Translational Medicine (EUSTM) defines translational research as *an interdisciplinary branch of the biomedical field supported by three main pillars: benchside, bedside and community.*[2]

The implication is of a unidirectional flow underpinned by a reductionist philosophy. That is, biology, and specifically cancer, can be explained by breaking it down to chemical or molecular reactions and then by simply tying this knowledge together, we can find all the answers to our clinical questions. As popularized by Richard Dawkins in his book, "The Selfish Gene", biology is all about the DNA. This philosophy has driven the dominance over the last 50 years of the Somatic Mutation Theory of carcinogenesis.

The fall of the gene began with the completion of the Human Genome Project. When it commenced in 1990 it was estimated that humans would require 100,000 plus genes for the proteins required to make up our bodies and at least 20,000 regulatory genes. In 2003, it was discovered humans only have 20,000 genes, the same as rodents. Epigenetics, a field overlooked since Waddington in 1940 started a comeback and an alternative theory of carcinogenesis, Tissue Organization Field Theory, has gone some way to explaining paradoxes and gaps that SMT has been unable to account for.

In this thesis, **"Emergence and Margins in Head and Neck Cancer"**, current philosophies of carcinogenesis are discussed and critiqued, and I propose an alternative ***"Emergence Model of Carcinogenesis".*** The advantage of this model is that it provides a framework that unifies current concepts, models and facilitates bioinformatics integration and provides for the investigation of specific clinical observations from a problem–based approach.

The idea that translational research should include the reverse polarity, "bedside to benchtop" has been argued by a number of pre-eminent scientists, including Nobel laureate, Sydney Brenner.[3] By applying the concepts of "bedside to benchtop" and emergence together, the abundance of scientific knowledge can be reassessed with a view to produce useful translational outcomes.

Max Planck, a German Physicist who is known as the founder of Quantum Physics and recipient of the Nobel Prize for Physics in 1918, is quoted as saying,

"When you change the way you look at things, the things you look at change."

The aim of this thesis is to challenge the reader to do just that.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature: 

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Publications during enrolment

1. Sigston EA, Longano A, Strzelecki AT, Williams BR. Surgical margins in head and neck squamous cell carcinoma: Effect of heat artifact on immunohistochemistry as a future tool for assessment. Head Neck. 2016;38(9):1401-6. DOI: 10.1002/hed.24450

2. Sigston EA, Williams BR. An Emergence Framework of Carcinogenesis. Frontiers of Oncology. 2017. DOI: 10.3389/fonc.2017.00198

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 2 original papers published in peer-reviewed journals. The core theme of the thesis is to consider cancers using a systems biology approach to change the way resection margins are assessed to better predict recurrence. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Hudson Institute of Medical Research under the supervision of Professor Bryan R. G. Williams

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)

In the case of *3* and *4BIII* my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status (published, in press, accepted or returned for revision, submitted)	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
3	*An Emergence Framework for Carcinogenesis*	*Published*	*80%: concept, development of the framework, drafting, revising, and finalizing article*	*Bryan R.G. Williams 20% concept, provided critical feedback with suggested revisions, and was involved in finalizing article*	*No*
4BII	*Surgical margins in head and neck squamous cell carcinoma: Effect of heat artifact on immunohistochemistry as a future tool for assessment.*	*Published*	*55%: Conceived and designed the project, performed the laser resection surgery, analysed and interpreted the data, wrote the paper.*	*Anthony Longano Pathologist 15% assisted with design of interpretation, analysed and interpreted the data, edited the paper.* *Aneta T. Strzelecki 15% Designed and optimized the immunohistochemistry experiments, analysed and interpreted the data, edited the paper.* *Bryan R.G. Williams 15% Supervised the project, provided critical suggestions and discussions, edited the paper.*	*No* *No* *No*

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] **Date:** 4th June 2018

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: [redacted] **Date:** 13th June 2018

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1. Introduction

During my years as a senior registrar and Head & Neck Fellow I was struck by how frequently histological assessment of resection margins seemed to be misleading. Patients who had clear histological margins would return with recurrent disease yet others who had close or positive margins and were anticipated to have disease recurrence, remained disease free. It seemed that there was more to head and neck cancer than morphological properties.

This was further highlighted during my time in France when I pulled together and reviewed a series of laser cordectomies performed for early laryngeal cancer and found that physical margins where in fact not necessarily related to disease recurrence.[4] In my mind I had already developed the concept that there was something about the biological nature of a cancer that was key. A visiting Japanese professor introduced me to laminin γ2 that was the central theme in a paper he had recently authored.[5] His paper not only introduced me to the concept of biomarkers, and specifically laminin γ2, but the heterogeneity of head and neck squamous cell cancer (HNSCC). I felt like I had found the answer.

As it turns out biology and life are never that simple. Not long after returning to Australia I was diagnosed with breast cancer. Whilst not something you would wish on anyone, taking time off for treatment gave me an opportunity to explore my research ideas and to read more broadly. I discovered the fields of epigenetics, systems biology and the concept of emergence. Not that I put it all together then, as I was sold on laminin γ2 being the answer.

Translational research was much harder than I anticipated. At the time of commencement there wasn't a commercial antibody for laminin γ2 available. Progress was made with consenting patients, obtaining specimens and obtaining a clone for laminin γ2, which was successfully amplified. Producing the monoclonal antibody required outsourcing and there were a number of false starts in this area (there are some hybridomas sitting in a lab in China somewhere that took more than 3 years to develop waiting to be verified as I didn't realize that they needed to be cared for on an ongoing basis!). In the interim Dako produced a commercially available and verified antibody which allowed the momentum forward to continue. Test staining was

consistent with predicted results and the protocol advised by Dako was verified on our test samples of tumour slides.

My aim from the outset was to find a way to assess biological margins, using an easily and widely employed technique that was cost effective, with the purpose of improving margin assessment beyond what is currently achieved with traditional H&E assessment, to better predict disease recurrence, enabling those patients to have better directed management. Despite moving forward, though at a pace much slower than a surgeon is typically comfortable with, something was not really gelling. It felt like a part of the puzzle was missing.

It wasn't until I took sabbatical leave and created the space to think that I realized three things. Firstly, the traditional approach to research was to become an expert in a particular 'thing', usually a protein a transcription factor or in my case, laminin γ2. The second was that translational research was generally considered a one-way street, from bench to bedside, with not a lot of the 'bench' making it to the bedside, and my question was a clinical one that required working in the opposite direction. The final, and most profound, thing was medical research in cancer was based predominantly on the concept that cancer is a genetic disease. Like many clinicians, I was unaware of the impact an underlying concept, which is really an assumption, had on where research funds were directed and impacted on what I delivered at the bedside.

So I took a step back, put aside the need for my 'thing' to be 'THE thing', and chose to follow Max Planck's advice, and changed the way I looked at cancer. The result of this approach is contained in this thesis.

Chapter 2 provides the background of what head and neck cancer is, how the epidemiology is changing, how it is staged, treated and the dilemmas around margin assessment and the general lack of significant progress in treating the disease. This Chapter presents the two main current paradigms underlying cancer research: the long standing Somatic Mutation Theory, which has genes as the central cause of cancer and the newer Tissue Organization Field Theory, which sees cancer occurring as a disruption of normal tissue architecture akin to the process of organogenesis during development, or development gone awry. There is discussion how the two theories have been made incompatible and the strong call for a 'systems approach' to cancer.

Chapter 3 presents “An Emergence Framework of Carcinogenesis”. This provides a framework in which cancer is viewed not as a disorganized collection of cells but as an emergent complex system at the level of the ‘functional tissue unit’. This framework is able to unify the diametrically opposed current theories and facilitate bi-directional translational research. A discussion of how to practically apply the framework to cancer research and the subsequent possible implications of employing a methodical systems approach are presented.

Chapter 4 and Chapter 5 provide an example of the practical application of “An Emergence Framework of Carcinogenesis” in addressing the clinical question of assessment of margins using oral squamous cell carcinoma as the study cohort. Chapter 4 walks through the process of defining the ‘functional tissue unit’ system, in this thesis, the oral mucosal membrane. Chapter 5 demonstrates the application of the framework to design the investigative study, create the hypothesis and carry out the research. Results and conclusions are presented.

Chapter 6 provides a summary, conclusion, and discussion of future directions. It highlights how the creation and application of a systems based framework can change the thought process applied in translational cancer research and generate significant outcomes that may not otherwise be produced. By changing the way we look at things, the things we look at change and new understandings can open up.

2. Background

2.1 Head and Neck Squamous Cell Carcinoma

Head and Neck Squamous Cell Carcinoma (HNSCC) is a highly invasive malignant neoplasm associated with a high risk of metastasis and morbidity and is the 6th most common cancer worldwide.[6-12] It is of epithelial origin and can affect any portion of the upper aerodigestive tract. Identified risk factors include smoking, alcohol, dental trauma, and various dietary habits, such as chewing beetle nuts or dried fish, and anaemias.[7, 13-15] Human papilloma virus (HPV) has increasingly been associated with HNSCC of the oropharynx and appears to be associated with an increase in the incidence of HNSCC in non-smokers.[9, 13, 15-18] There seems to be a trend of increasing HNSCC of the oral cavity in non-smokers which is not HPV related.[15, 19-22]

Both the cancer itself and the treatment for it can impact significantly upon the functions of breathing, speech and swallowing. The cosmetic effects can be great. Functional and cosmetic outcomes play a significant role in restricting resection.[23]

Despite numerous advances in our understanding of HNSCC, new surgical and radiotherapy techniques and newer chemotherapeutic agents, overall HNSCC 5-year survival remains at around 60% worldwide. [11, 24, 25]

2.1.1. Sites and Staging

Head and neck squamous cell carcinoma describes malignancies arising from the epithelium in the upper respiratory and digestive tract.[6] Staging is much more complex than in many other cancers as HNSCC comprises many site-specific cancers grouped together.[26] It has long been recognized that different sites have different biological behaviors and this is reflected in differing etiologies, response to different modalities of treatment and varying outcomes.[24, 26, 27]

The most widely accepted staging system for HNSCC is the American Joint Committee on Cancer (AJCC) that has developed into a collegial collaboration between the AJCC and the Union for International Cancer Control (UICC). The staging system is updated periodicity in an effort to continually improve and be reflective of growing knowledge in this field.[26]

The AJCC staging system divides HNSCC into different anatomical and prognostic groups. The major sites include the oral cavity, the oropharynx, the hypopharynx, the larynx, the nasopharynx, and the nose and paranasal sinuses. Each site is further divided into several subsites (*Fig. 1*).

2.1.1.1. Sites

The anterior aspect of the oral cavity is the contact point of the skin, with the vermilion of the lips extending posteriorly to the junction of the hard and soft palates, and with the anterior tonsillar pillars and the circumvallate papillae forming the posterior limits. For the oral cavity the subsites are the lips, anterior tongue, floor of mouth, buccal mucosa, upper and lower alveolar ridges, hard palate, and retromolar trigone. The trigone consists of the mucosa overlying the anterior aspect of the ascending ramus of the mandible.

The oropharynx begins where the oral cavity ends at the junction of the hard and soft palates superiorly and the circumvallate papillae inferiorly, and extends from the level of the soft palate superiorly, which separates it from the nasopharynx, and to the level of the hyoid bone inferiorly. The subsites of the oropharynx are the tonsil, base of tongue, soft palate, and pharyngeal walls.

The hypopharynx begins superiorly at the level of the hyoid bone, where it is contiguous with the oropharynx, and it extends inferiorly to the cricopharyngeus muscle, as it transitions to the cervical esophagus. The major subsites of the hypopharynx are the pyriform sinuses, the postcricoid region, and the pharyngeal wall.

The nasopharynx is a cuboidal structure bounded anteriorly by the choanae at the back of the nose, where pseudostratified ciliated columnar cells are found. The roof and posterior walls of the nasopharynx are made up of the sphenoid bone and the upper cervical vertebrae, covered with a stratified squamous epithelial lining. Inferiorly, at the level of the soft palate, the nasopharynx meets the superior oropharynx.

The larynx is bordered by the oropharynx superiorly, the trachea inferiorly, and the hypopharynx laterally and posteriorly. The larynx is comprised of a

cartilaginous framework, and is subdivided vertically by the vocal folds into the supraglottic, glottic, and subglottic subsites. The supraglottic larynx includes the epiglottis, which has both lingual and laryngeal surfaces, the false vocal cords, the arytenoids cartilages, and the aryepiglottic folds. Anterior to the supraglottis is the pre-epiglottic space. The subsites of the supraglottis are suprahyoid epiglottis, the infrahyoid epiglottis, the laryngeal aspect of the aryepiglottic folds, the arytenoids and the false cords. The glottic larynx describes the true vocal folds, where they come together anteriorly at the anterior commissure, as well as where they meet the mobile laryngeal cartilages at the posterior commissure. The glottic larynx extends from the ventricle to 1 centimeter (cm) below the level of the true folds. Between the thyroid cartilage and the vocal fold lies the paraglottic space, which is continuous with the pre-epiglottic space. The subglottic larynx starts 1 cm below the vocal folds and continues to the inferior aspect of the cricoid cartilage.

The nasal cavity and paranasal sinuses make up the final major site. The paranasal sinuses consist of the paired maxillary sinuses, the superior frontal sinuses, the bilateral ethmoid system, and the central sphenoids. This region includes the lining of the nasal cavity (medial maxillary walls), as well as the nasal septum.

The nasal cavity includes the nasal antrum and the olfactory region. The subsites within the nasal cavity include the septum; superior, middle, and inferior turbinates; and olfactory region of the cribriform plate.

The ethmoid sinus is made up of several thin-walled air cells. Laterally, a thin bone called the lamina papyracea, which separates it from the medial orbit, binds the ethmoid sinus. The posterior border of the ethmoid sinus is close to the optic canal. The anterosuperior border or roof of the ethmoid is formed by the fovea ethmoidalis, which separates it from the anterior cranial fossa. The perpendicular plate of the ethmoid bone separates the ethmoid cavity into left and right sides.

The maxillary sinus is a pyramid-shaped cavity within the maxillary bone. The medial border is the lateral nasal wall. Superiorly, the sinus abuts the orbital

floor and contains the infraorbital canal. The posterolateral wall is anterior to the infratemporal fossa and pterygopalatine fossa. The anterior wall is posterior to the facial skin and soft tissue. The floor of the maxillary antrum extends below the nasal cavity floor and is in close proximity to the hard palate and maxillary tooth roots.

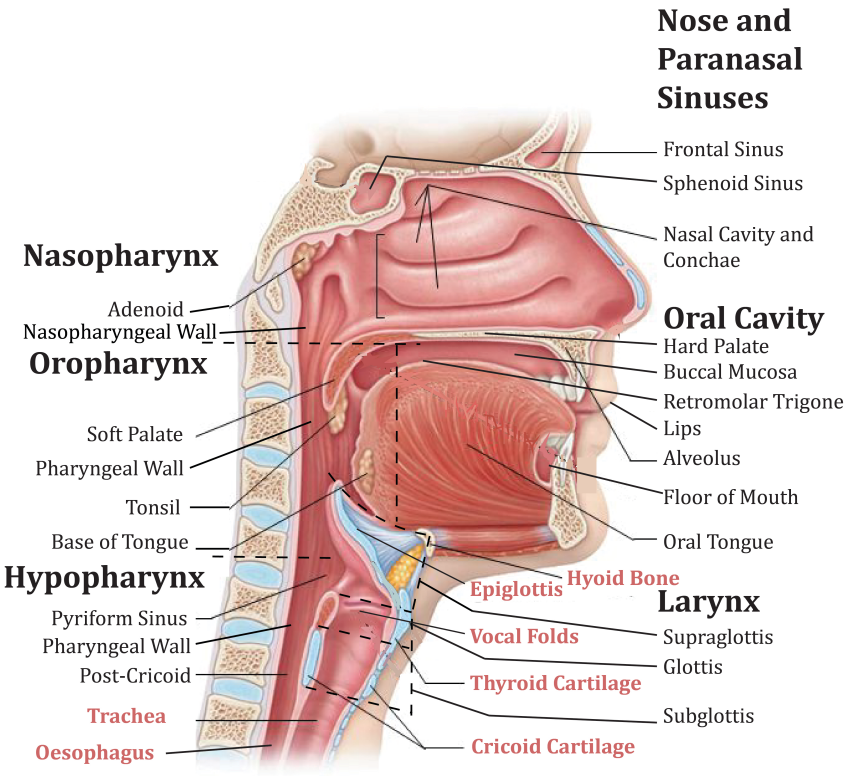

Figure 1 Sites and subsites of the upper aerodigestive tract

Picture from http://human-anatomy101.com/ Labelling modified to demonstrate the surgical sites and subsites used in managing head & neck squamous cell carcinoma

2.1.1.2. Staging

At the start of the 20th century, Halsted and others put forward the concept that most solid tumours spread in a step-wise fashion in stages from the primary site of origin to regional lymphatics and then distant organs. This theory was based on the observation that outcomes worsened as the tumour progressed from the primary site (T) to regional lymph nodes (N) and then to distant organs (M). Subsequently, the French surgeon, Denoix developed the TNM

system at the Institut Gustave-Roussy in Paris between 1943 and 1952. In 1953 he proposed to the Union Internationale Centre le Cancer (UICC) that these three parameters be formally integrated into a prognostic TNM staging system that would be applicable for staging solid tumours. In the United States, the American Joint Committee on Cancer (AJCC) was established in1959 with the mission of formulating and publishing systems of classification of cancer that would be useful not only for selection of treatment and determining prognosis, but for continuing evaluation of cancer control measures. The AJCC and UICC versions of the TNM system were unified in 1987 and these organizations have since maintained a liaison to ensure compatibility of revised staging classifications through continuous collaboration.[28]

HNSCC staging uses the TNM system: primary tumour site, size and other certain features, extent of regional nodal disease and the presence or absence of metastatic disease. Each site has its own tumour classification (*Tables 1-6*). Nodal disease uses a common staging for all sites (*Table 7*) except the nasopharynx (*Table 8*), which due to the behaviour of tumours from this site has its own separate staging for nodal disease (*Table 9*). Metastatic disease is simply staged as present, not present or unknown.[29] There are some minor differences between the AJCCC and the UICC staging systems which impact on database creation but not on clinical application or practice.[30]

Oral Cavity	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour 2 cm or less in greatest dimension
T2	Tumour more than 2 cm but not greater than 4 cm in greatest dimension
T3	Tumour more than 4 cm in greatest dimension
T4a	Moderately advanced local disease* Tumour invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin of face—that is, chin or nose (oral cavity). Tumour invades adjacent structures (e.g., through cortical bone, into deep (extrinsic) muscle of tongue (genioglossus, hypoglossus, palataglossus,and styloglossus), maxillary sinus, skin of face
T4b	Very advanced local disease Tumour invades masticator space, pterygoid plates, or skull base and/or encases internal carotid artery

Table 1 Staging of primary tumours in the oral cavity

Oropharynx	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour 2 cm or less in greatest dimension
T2	Tumour more than 2 cm but not greater than 4 cm in greatest dimension
T3	Tumour more than 4 cm in greatest dimension or extension to lingual surface of epiglottis
T4a	Moderately advanced local disease.Tumour invades the larynx, deep/extrinsic muscle of the tongue, medial pterygoid, hard palate, or mandible*
T4b	Very advanced local disease. Tumour invades the lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base, or encases the carotid artery

Table 2 Staging of primary tumours of the oropharynx

*Will be updated in January 2018 The updated version does not have a material effect on the focus of this thesis.

Hypopharynx	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour limited to one subsite of the hypopharynx and is 2 cm or less in greatest dimension.
T2	Tumour invades more than one subsite of the hypopharynx or an adjacent site, or measures more than 2 cm but not more than 4 cm in greatest dimension without fixation of the hemilarynx or extension to the esophagus.
T3	Tumour more than 4 cm in greatest dimension or with fixation of the hemilarynx or extension to the esophagus.
T4a	Moderately advanced local disease.Tumour invades thyroid/cricoid cartilage, hyoid bone, thyroid gland, esophagus, or central compartment soft tissue
T4b	Very advanced local disease. Tumour invades prevertebral fascia, encases carotid artery, or involves mediastinal structures

Table 3 Staging of primary tumours of the hypopharynx

Larynx	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
Supraglottis	
T1	Tumour limited to one subsite of the supraglottis with normal vocal fold mobility
T2	Tumour invades mucosa of more than one adjacent subsite of the supraglottis or glottis or region outside the supraglottis (e.g., mucosa of base of tongue, vallecula, medial wall of pyriform sinus) without fixation of the larynx
T3	Tumour limited to the larynx with vocal fold fixation and/or invades any of the following: postcricoid area, pre-epiglottic tissues, paraglottic space, and/or inner cortex of thyroid cartilage
T4a	Moderately advanced local diseas. Tumour invades through the thyroid cartilage and/or invades tissues beyond the larynx (e.g., trachea, soft tissues of neck including deep extrinsic muscle of the tongue, strap muscles, thyroid, or esophagus)
T4b	Very advanced local disease. Tumour invades prevertebral space, encases carotid artery, or invades mediastinal structures.
Glottis	
T1	Tumour limited to the vocal fold(s) (may involve anterior or posterior commissure) with normal mobility
T1a	Tumour limited to one vocal fold
T1b	Tumour involves both vocal folds
T2	Tumour extends to the supraglottis and/or subglottis, and/or with impaired vocal cord mobility
T3	Tumour limited to the larynx with vocal fold fixation and/or invades any of the following: postcricoid area, pre-epiglottic tissues, paraglottic space, and/or inner cortex of thyroid cartilage
T4a	Moderately advanced local diseas. Tumour invades the outer cortex of the thyroid cartilage and/or invades tissues beyond the larynx (e.g., trachea, soft tissues of the neck, including deep extrinsic muscle of the tongue, strap muscles, thyroid, or esophagus)
T4b	Very advanced local disease. Tumour invades prevertebral space, encases carotid artery, or invades mediastinal structures.
Subglottis	
T1	Tumour limited to the subglottis
T2	Tumour extends to the vocal cord(s) with normal or impaired mobility.
T3	Tumour limited to the larynx with vocal fold fixation.
T4a	Moderately advanced local disease. Tumour invades cricoid or thyroid cartilage and/or invades tissues beyond the larynx (e.g., trachea, soft tissues of the neck including deep extrinsic muscles of the tongue, strap muscles, thyroid, or esophagus)
T4b	Very advanced local disease.Tumour invades prevertebral space, encases carotid artery, or invades mediastinal structures.

Table 4 Staging of primary tumours of the subsites of the larynx

Maxillary Sinus	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour limited to the maxillary sinus mucosa with no erosion or destruction of bone.
T2	Tumour causing bone erosion or destruction, including extension into the hard palate and/or middle nasal meatus, except extension to the posterior wall of the maxillary sinus and pterygoid plate.
T3	Tumour invades any of the following: bone of the posterior wall of the maxillary sinus, subcutaneous tissues, floor or medial wall of the orbit, pterygoid fossa, or ethmoid sinuses
T4a	Moderately advanced local disease.Tumour invades anterior orbital contents, skin of cheek, pterygoid plates, infratemporal fossa, cribriform plate, sphenoid or frontal sinuses.
T4b	Very advanced local disease. Tumour invades any of the following: orbital apex, dura, brain, middle cranial fossa, cranial nerves other than maxillary division of trigeminal nerve (V2), nasopharynx, or clivus.

Table 5 Staging of primary tumours of the maxillary sinuses

Nasal Cavity and Ethmoid sinuses	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour restricted to any one subsite, with or without bony invasion.
T2	Tumour invades two subsites in a single region or extending to involve an adjacent region within the nasoethmoidal complex, with or without bony invasion.
T3	Tumour extends to invade the medial wall or floor of the orbit, maxillary sinus, palate, or cribriform plate.
T4a	Moderately advanced local disease.Tumour invades any of the following: anterior orbital contents, skin of nose or cheek, minimal extension to anterior cranial fossa, pterygoid plates, sphenoid or frontal sinuses.
T4b	Very advanced local disease. Tumour invades any of the following: orbital apex, dura, brain, middle cranial fossa, cranial nerves other than V2, nasopharynx, or clivus.

Table 6 Staging of primary tumours of the nasal cavity and ethmoid sinuses

Lymph Nodes	
Nx	Regional lymph nodes cannot be assessed
N0	No regional nodes metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension
N2a	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none more that 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N3	Metastasis in a lymph node more than 6 cm in greatest dimension.

Table 7 Staging of regional lymph nodes for head & neck squamous cell carcinoma excluding nasopharynx

Nasopharynx	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour confined to the nasopharynx or tumour extends to the oropharynx and/or nasal cavity without parapharyngeal extension
T2	Tumour with parapharygeal extension
T3	Tumour involves bony structures of skull base and/or paranasal sinuses
T4	Tumour with intracranial extension and/or involvement of cranial nerves, hypopharynx, orbit, or with extension to the infratemporal fossa/ masticator space

Table 8 Staging of primary tumours of the nasopharynx

Lymph Nodes (nasopharynx)	
Nx	Regional lymph nodes cannot be assessed
N0	No regional nodes metastasis
N1	Unilateral metastasis in cervical lymph node(s), 6 cm or less in greatest dimension, above the supraclavicular fossa, and/or unilateral or bilateral retropharyngeal lymph nodes, 6 cm or less in greatest dimension
N2	Bilateral metastasis in cervical lymph node(s), 6 cm or less in greatest dimension, above the supraclavicular fossa
N3a	Metastasis in lymph node)* >6 cm and/or to supraclavicular fossa
N3b	Greater than 6 cm in dimension
N3c	Extension to the supraclavicular fossa

Table 9 Staging of regional nodes for tumours of the nasopharynx

Tumours are assessed according to the TNM and then placed into a numbered staging system with 5 main groups designed to assist with determining treatment and indicating prognosis (*Table 10*).[29]

Stage	T	N	M
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
Stage IVb	Any T	N3	M0
	T4b	Any N	M0
Stage IVc	Any T	Any N	M1

Table 10 Tumour stage calculated from the TNM status.

Stage 0, Stage I and Stage II are considered early disease. Stage III and Stage IV are considered advanced disease.

The system is based anatomically upon the size and extent of tumour and whilst this system has served well, increasing knowledge and observation over time have highlighted number limitations. These include lack of predictive power of stages partly to inhomogeneity in the groups, failure to account for biological tumour factors that are increasingly being recognized as important, and failure to take into account host characteristics.[28]

Whilst a number of tumour factors have been identified as prognostic indicators, such as depth of invasion, pattern or biomarkers at the invasive front [31-33] in patients managed with primary surgical resection, these histological features are not available for patients treated with primary radiotherapy or chemoradiotherapy.[28] Aetiology, particularly HPV[16, 34] and the level of lymph node metastases[35] has been demonstrated to impact prognosis.

Increased use of whole body FDG-PET scans, has contributed to the earlier detection of metastatic disease, improving pre-treatment staging accuracy.[36]

The issue becomes one of detail versus usability.[28] The upcoming version of the AJCCC (8th Edition) attempts to address some of the issues. HPV-related (p16+ve) oropharyngeal disease will have independent TNM criteria, extra capsular spread in regional lymph nodes has been recognized as an important factor in staging non -p16, non-EBER1 cancers of the head and neck, and depth of invasion in oral cavity lesions has been incorporated.[26]

2.1.2. Epidemiology

Head and Neck Squamous Cell Carcinoma (HNSCC) is the 6th most common cancer in worldwide.[6-12] There are approximately 630,000 new cases of HNSCC per year and it is responsible for approximately 350,000 deaths.[7, 8] Incident trends are complex with wide geographical variation in incidence and anatomical sub sites. Developed countries have experienced a decrease in smoking related HNSCC but are seeing increased numbers related to other aetiologies, in particular human papilloma virus (HPV), whilst the incidence in developing countries continues to rise.[7, 8, 11, 37]

HNSCC is more common in men than women in a ratio of 2.2-2.4:1, which is a consistent pattern worldwide.[27, 37, 38] Traditionally HNSCC has been seen in an older population but there is a trend in developed countries of an increasing incidence in younger age groups with 40-60 years being the most common age of presentation.[8] Of particular concern is the increase in the incidence of oral tongue patients under 45 years of age.[21, 22, 37, 39, 40]

In Australia, it is anticipated that there will be an estimated 4,956 new cases of HNSCC diagnosed in 2017, accounting for 3.7% of all cancers and with just over 1,000 deaths attributable to HNSCC, accounting for 2.2% of cancer related deaths.[41] There is a following of trends as seen in other developed countries with decreased incidence of laryngeal and hypopharyngeal cancers but increase in oropharyngeal, particularly HPV related, and oral tongue cancers.[27, 41] The highest rate of oral tongue cancers in females worldwide is found in aboriginal women in the Northern Territory.[27]

Cigarette smoking and alcohol are the most important risk factors for HNSCC and have a synergistic effect.[7, 37] Cigar and pipe smoking are associated with increased oral and lip cancer and chewing of betel leaf and other smokeless forms of tobacco have been linked with oral SCC.[7] Alcohol alone has predominantly been associated with hypopharyngeal cancers.[8]

In developed countries where rates of smoking have been decreasing, there has been a corresponding subsequent decrease in laryngeal, oral cavity and hypopharyngeal cancers.[9, 11, 42] This has however been offset by increased numbers of oropharyngeal and oral tongue SCC, particularly in non-smokers. HPV, oral hygiene and dental issues are now emerging as significant risk factors for HNSCC in these sites.[9,11, 19, 21]

HPV is now recognized as a major risk factor in oropharyngeal SCC, a specific subset of HNSCC. About 60% of oropharyngeal cancers diagnosed in developed countries are positive for HPV. These tumours show genetic alterations specifically related to HPV oncoproteins E6 and E7. This led to a significant increase in oropharyngeal SCC in these countries occurring in a younger, non-smoking age group.[8, 9, 11, 15, 17, 18, 34, 40, 42, 43]

Developed countries have seen a trend of increasing oral tongue cancers in non-smokers. Risk factors identified include repeated trauma to tongue from teeth or ill-fitting dental prosthesis, poor oral hygiene, and an inverse relationship to teeth brushing.[21, 44] A number of studies have not found an identifiable cause for oral tongue SCC in young non-smoking patients.[22, 39, 40] Maxillary and mandibular ridge oral SCCs in non smokers without any specific risk factors has been observed.[45]

Oral lichen planus and submucous fibrosis are known pre-cancerous conditions.[7] Lichen planus is the most common chronic autoimmune inflammatory disorder of oral mucosa that affects 1-2% of adults in middle age. The origin is unknown. It is more common among females and tends to have multifocal lesions, often bilateral and symmetric in distribution. It frequently involves buccal mucosa, gingiva and tongue and tends to show a chronic course with little spontaneous regression. It has an estimated malignant transformation rate of 1% over a 5 year period.[7, 46, 47]

Oral submucous fibrosis is a chronic, progressive, pre-cancerous condition of the oral mucosa, which is associated with betel quid (BQ) chewing habit widely prevalent in South- east Asia and some places in China. Several co-factors such as malnutrition, immunological alterations, and genetic predisposition are implicated. It is characterized by diffuse mucosal rigidity due to dense fibrosis within the lamina propria that might extend into the underlying skeletal muscle. Histopathology shows varying degrees of epithelial dysplasia with up to 25% characterized by pronounced cellular and nuclear pleomorphism. The malignant transformation rate is estimated to be 8-12 % over the period of 10-15 years.[7, 48, 49]

An increased risk of HNSCC is associated with a number of systemic diseases including Plummer Vinson Syndrome, Fanconi Anaemia and dyskeratosis congenita.[7, 50-52]

Survival and mortality in HNSCC are complex to analyse due to increasing heterogeneity in incidence and mortality. [27, 37] Overall there is increasing incidence of HNSCC and increasing mortality worldwide.[27] These statistics are impacted by geographic region and changing aetiologies identified between developed and developing countries. Increased mortality rates in younger people in developed countries including Europe, UK and USA, from oral and oropharyngeal SCC has been observed, but is a reflection of the doubling of the incidence in this group.[27, 37] Overall, mortality from HNSCC has decreased marginally in developed countries. This follows the trend of reduction in incidence related to smoking and better prognosis for HPV related oropharyngeal SCC.[11] In contrast SCC of the larynx has the highest incidence in developing countries and has increased in incidence, likely to be related to increased smoking in these countries. India and Japan have seen significant increases, up to four times from 1965 to 1999, in oral and oropharyngeal carcinomas.[27]

Overall 5-year survival rates worldwide for HNSCC are about 50%. Cancer of the lip has the highest 5-year survival rates, being 90%.[37] Average overall 5-year survival in developed countries, including USA, UK, Europe, Scandinavia and Australia, are about 62%.[24] Australia has an estimated overall 5-year survival rate of 69%.[41]

Deeper analysis of these figures suggests improved overall 5-year survival rates in developed countries are greatly impacted by HPV positive oropharyngeal SCC that has a greater sensitivity to chemo-radiotherapy than HPV negative or unrelated SCC. HPV is now estimated to account for 70% of oropharyngeal SCCs in USA[27], 82% in Denmark[53], 60% in Australia[43] and similar in Canada.[9] Survival in these cancers is statistically much higher.[54, 55]

5-year survivals for oral cavity SCC have not changed in the UK.[37] Europe has had a modest improvement in 5-year survival for oral SCC from 48.5% in 1995-99 to 55% in 2015.[24] USA had a large improvement in 5-year survivals for oral SCC with an increase for late stage disease improving 13.3% and early disease by 4.2% between 1973 and 2005.[56] Improvements in early stage disease may reflect improved surgical technique including less positive margins, elective neck dissections and move to cases being undertaken at higher volume centres.[25] Adjunct chemotherapy may have had a role in later stage disease however the impact on HPV may be underestimated as tongue cancer numbers likely contains a mix of base of tongue lesions and oral tongue lesions.[38, 56] Overall, 5-year survival is still only 53.2%.[25, 38, 56] Australia has one of the highest 5-year survival rates for oral cavity SCC at 75%.[41]

Hypopharyngeal SCC survival rates have improved but still remain poor, about 40%. There has been little change in 5-year survival of laryngeal cancer.[24, 37, 56]

2.1.3. Current Management

Management of HNSCC is dependent largely upon the primarily site and stage of the cancer. Patient factors, such as age, preference of treatment, and co-morbidities, aetiology and access play a role in determining modality or modalities of treatment.[57-60] Treatment regimes may involve surgery alone, surgery and post operative radiotherapy, surgery and post operative chemo-radiotherapy, radiotherapy or chemo-radiotherapy. Rarely, chemotherapy may be used a single modality.[57, 60, 61] The complexity of HNSCC associated with the impacts of treatment mean determination of management is usually undertaken in a multidisciplinary team setting.[58, 60, 61]

In early stage (I–II), single modality treatment is indicated with either conservative surgery or radiotherapy as treatment of choice. Both give similar

loco-regional control, however, this is based only on retrospective studies, as there are no randomized trials available for reference. Later stage tumors are treated with multimodal therapy involving proper combinations of surgery and radiation and/or chemotherapy as the standard of care for these patients. In patients with unresectable disease, oropharyngeal tumours of HPV aetiology or disease that may be clinically resectable but prognosis or functional outcome is so poor, combined concomitant chemoradiation is the standard.[58, 60]

Modern radiotherapy treatment uses 3D conformal radiation therapy or intensity-modulated radiation therapy (IMRT).[56, 60, 62] IMRT typically creates sharp dose gradients between the target volume and any surrounding organ-at-risk (OAR) considered relevant for the development of acute and late radiation-induced adverse effect. Imaging-based dose painting that allows prescription and delivery of nonuniform dose to clinical target volume with in field boosting. This technique aims to conteract to some degree the three main causes of radiotherapy failure in the clinic: tumour burden, tumour cell proliferation, and tumour hypoxia. The typical dosage delivered is in the order of 60-70 Gy delivered in various fractions.[63]

Chemotherapy has been used in HNSCC in neo-adjuvant, concomitant with radiotherapy (CRT) and adjuvant regimes.[58, 60, 64-66] Cisplatin and 5- fluorouracil (5-FU) are the standard chemotherapy agents for HNSCC. Single agent cisplatin is the cytotoxic agent of choice for CRT. Cisplatin acts by forming intra and inter strand DNA adducts, resulting in inhibition of DNA synthesis. Cisplatin potentiates the effect of radiation by inhibiting the repair of the sub-lethal damage, by homologous and non-homologous DNA repair mechanism.[66] Despite improved outcomes with CRT, disease recurrence and treatment toxicity continue to be challenges with this treatment paradigm.[65] Increasing evidence suggests induction chemotherapy followed by CRT reduces distant metastes rates and may offer a survival advantage.[65]

HNSCC is one of the most immunosuppressive cancers resulting in decreased absolute lymphocyte counts, impaired natural killer cell function, reduced antigen-presenting cell function, and a tumour-permissive cytokine profile. This has led to immunotherapy approaches being explored for use in HNSCC.[67, 68] These can be grouped as vaccine therapies, oncolytic viruses, immunomodulators and

monoclonal antibodies.[68]

Anticancer vaccine therapies involve generating an antitumor immune response by presenting a tumour-associated antigen (TAA) plus an immunostimulatory adjuvant, resulting in immune sensitization to tumour antigens.[67, 68] DNA vaccines, peptide vaccines and biological vaccines have been generated.[67, 68] A number of therapeutic HPV vaccines that target E6 and E7 proteins have been developed.[67-69] To date though, none of these have moved passed Phase II trials.[67, 68]

Oncolytic viruses represent a novel approach to cancer therapy that uses recombinant or engineered viruses to selectively kill tumour cells while sparing normal tissues. In addition, tumour cell lysis releases TAAs into the surrounding environment along with viral antigens, which may facilitate the production of an immune antitumor response.[68] Two Phase I trials in HNSCC are nearing completion or have recently been completed, one with a recombinant vaccinia virus (family Poxviridae) that is deleted for the viral thymidine kinase (vTK) gene and contains an exogenous Granulocyte Macrophage – Colony Stimulating Factor (GM-CSF) gene and one a recombinant avian fowlpox virus that expresses three co-stimulatory transgenes, B7.1 (CD80), Intercellular Adhesion Molecule 1 (ICAM-1), and lymphocyte function-associated antigen 3 (LFA-3), plus one or more tumour-associated antigens, such as Carcinoembryonic antigen (CEA) and MUC-1, the results are not yet available.[68]

Immunomodulators include a diverse group of small molecule agonists and compounds designed to increase the host antitumor immune response by generating a generalized increase in immune function rather than a tumour directed specific response.[68] Early approaches with cytokines such as peri-tumoral interleukin 2 (IL2) and perilymphatic injection of IRX-2, a mixture of low-dose IL2, IL1b, IL6, IL8, IFNγ, TNFα, G-CSF, and GM-CSF, showed some promise in randomized phase III and phase II trials respectively. Systemic administration demonstrated limited efficacy with high toxicity.[67, 68] Toll-like receptor (TLR) agonists have been another strategy. TLRs are a family of transmembrane signalling proteins that form an important component of the innate inflammatory response by detecting conserved microbe-associated molecular patterns (MAMPs) and endogenous products known as damage-associated molecular patterns (DAMPs). TLR activation may contribute to clinically relevant antitumor

inflammatory responses. Some Phase I studies are in progress.[68]

A diverse group of monoclonal antibodies have been applied to cancer therapy, and these represent where most advances in HNSCC cancer treatment are being made.[67, 68, 70] Monoclonal antibodies mediate their antitumor effects through similar mechanisms including the targeting of tumour cells for antibody-dependent cell-mediated cytotoxicity (ADCC), the direct inhibition of tumour growth signals, and inhibiting signalling pathways involved in maintaining immune self-tolerance.[67, 68] Monoclonal antibodies against Epidermal Growth Factor Receptor (EGFR) have been approved for use in HNSCC. Cetuximab, a chimeric humanized monoclonal antibody against EGFR, targets the extracellular domain of EGFR and binds with it to prevent receptor dimerization and activation by its natural ligands, induce receptor degradation, and activate antitumoral immune responses.[58, 65, 67-69] Tyrosine kinase inhibitors, such as erlotinib and gefitnib, are small molecules that target the intracellular domain of EGFR and inhibit its phosphorylation activity.[68, 69] Benefit as a monotherapy has been be limited, but used in combination with radiotherapy, with or without platinum based therapy, has shown some benefit.[64, 66-69]

More recently monoclonal antibodies targeting T-cell–inhibitory receptors, such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death/ligand-1 (PD-1/ PD-L1) signalling, that function as immune checkpoints responsible for maintaining the balance between activation and inhibition of immune responses are showing promise in treating advanced HNSCC. Nivolumab and pembrolizumab, monoclonal antibodies against PD-1, have recently been approved as standard of care options for second line treatment in recurrent and/or metastatic HNSCC.[67, 68, 70, 71]

Despite advances in radiotherapy, chemotherapy, immunotherapy and other novel treatments, surgery still remains the predominant modality of treatment in HNSCC and understanding the importance of resection margins is essential.

2.1.4. Margins in HNSCC

Surgical resection remains an effective primary treatment for the majority of head and neck squamous cell carcinomas (HNSCC).[57, 62, 72] The aim of surgery is to completely remove disease and enable further information to be garnered from

pathological assessment to assist with staging and decisions regarding the use of adjunct treatments.[61] An adequate resection margin is important, as failure to eradicate tumour at the primary site is considered the single largest cause leading to death for patients with HNSCC.[57, 73, 74] What defines an adequate resection margin, how it is assessed and what margins mean in terms of patient outcomes remains one of the major dilemmas in HNSCC surgery today.[57, 61, 75]

Cancer resection in the head and neck region is a lot more complex when compared to cancer resection in other regions of the body. The three-dimensional nature of the structures involved and of the cancer itself can make access to, and judgement of, the extent of tumour a challenge. Multiple tissue types may be involved including not just the mucous membrane, but muscle, skin, salivary glands, bone and cartilage. Surgical resection must be balanced with preservation of uninvolved tissue regions critical to orofacial function, social interactions, and quality of life.[61, 76]

Haematoxylin and eosin (H&E) staining of tissue, a technique that has been in use for at least a century, and the use of light microscopy remain the primary tools for recognizing various tissue types and the morphological changes that form the basis of contemporary cancer diagnosis. [77-79] Assessment of tumour margins is performed using H&E stained slides of tissue cut perpendicular to the invasive tumour edge extending to the nearest surgical resection edge. The distance between the tumour cells and the cut edge of tissue can then be measured.[57]

A lack of consensus on what constitutes 'an adequate margin' in HNSCC persists.[57, 61] This is partly due to the heterogeneity of the disease, the impact of the site and/ or subsite involved, varying aetiologies, and even the immune status of the patient.[57, 61, 74] The National Comprehensive Cancer Network defines a 'clear margin' as equal to or greater than 5mm[61], whereas the UK Royal College of Pathologists defines 'clear' as greater than 5mm, with 5mm falling into the category of "close".[75] The agreed exception is early vocal cord cancers where there is consensus that much smaller margins are acceptable due to the need to balance voice preservation with local disease control.[4, 57] There is evidence to suggest that an adequate inferior margin for hypopharyngeal tumours should be much larger than other HNSCC.[80, 81]

There is even more variation in what is considered a 'close margin' and a 'positive margin'. The National Comprehensive Cancer Network defines a close margin as less than 5mm but not at the cut edge.[61] The UK Royal College of Pathologists defines 'close' as between, and including, 1mm to 5mm.[75] A systematic review by Alicandri- Ciufelli et al found that the definition of a 'close margin' depended on the site with a margin of 1mm or less being a 'close margin' for vocal cord cancers, 4mm or less being 'close' for oral cavity cancers, and 5mm or less was considered a 'close margin' for both larger laryngeal tumours and oropharyngeal tumours.[74] A 'positive margin' has been defined as less than 1mm, tumour at the cut edge or tumour or tumour within one high power filed of a cut edge.[57, 61, 74, 75]

The same complexities that make HNSCC resection challenging contribute to the challenges of histological assessment. In particular the three-dimensional nature of resection specimens with multiple air-tissue interfaces requires an excellent understanding of gross anatomy to appreciate what is a true surgical margin and not simply an interface.[57, 61] In addition assessment can be impacted by tissue shrinkage and artefacts. Tongue tissue has been shown to shrink by 20-25% after resection.[57] Resection methods with diathermy, laser or coblation cause shrinkage of elastic fibres in response to heat.[4, 82] Another 10% reduction in tissue can occur with formalin fixation and paraffin embedding required to enable assessment.[57] Resection methods and processing of tissue can both create artefact that may interfere with histological assessment.[4, 57, 82, 83]

Correlation between the status of resection margins determined by H&E and recurrence varies. Positive margins have generally been associated with a higher rate of local recurrence and poorer prognosis.[57, 61] A meta analysis by Anderson et al in the UK found that recurrence rates in oral SCC with clear margins reduced the risk of recurrence by 21%.[75] Binahmed et al in analysis of a historical cohort of patients from a Canadian cancer registry found positive margins to be associated with a much lower 5 year survival rate of 38% compared with a 69% 5 year survival of patients with reported clear margins.[84] Slootweg et al[85] correlated the histologically determined margin status of 394 resected HNSCCs from various sites and found that clear margins had a local recurrence rate of 3.9% whereas involved or close margins had a 21.9% recurrence rate. Of note the rate of regional recurrence was similar in both groups at 10.8% and 11.8%. No comment was made regarding postoperative adjunct treatments. In contrast close or

positive margins in early glottic cancer have been found to be less significant. Sigston et al[4] found that the recurrence rate in 18 patients from a series of 52 early glottic SCCs who had close, suspicious or positive margins, as determined by H&E assessment, was only 16% (three patients). Peretti et al[86] found that reported positive margins with H&E staining for early glottis cancers had no statistical impact on 5-year survival: 23 of 45 patients with positive margins had further treatment in the form of re-resection or radiotherapy, with no difference in survival between those who received additional treatment and those who did not.

The biggest concern is the persistent recurrence rate with clear margins. Iseli et al[72] found that while positive margins in oral tongue SCC increased the likelihood of recurrence, 33% of tumours that had been deemed to be clear of margins recurred. Binahmed's historical cohort reveals a 24.7% recurrence rate with clear margins[84] and Anderson's meta analysis showed a 20% recurrence for clear margins.[75] Other authors have found no direct correlation between margins and recurrence rates and prognosis. McMahon et al[87] assessed the recurrence rate of oral and oropharyngeal carcinomas across two institutions, one in UK and one in Australia and found that margins didn't predict recurrence but a number of histological features, in particular perineural invasion did. Brandwein-Gensler et al [88] found that histological risk assessment was more accurate than margin status in predicting both recurrence and overall survival. Pattern of invasion, perineural invasion and lack of lymphocytic response were significantly associated with locoregional recurrence.

It is now widely accepted that any specific HNSCC tumour is heterogeneous and that the invasive front is the most clinically relevant area to consider with regards to biological activity and prognosis.[5, 31, 32, 89-106] Physiological and functional changes may occur in cancer cells, particularly in early phases of epithelial mesenchymal transition, resulting in altered biological behaviour that is not reflected in morphological changes detected by H&E staining, enabling these cells to appear as unaffected or disguise their aggressiveness.[77, 107, 108] Undetected changes in biological nature may explain why positive margins are not always associated with local recurrence and a clear margin does not guarantee a complete resection and cure.[4, 57, 62, 72, 109] Fischer et al.[77] articulated the need to incorporate an evolutionary framework into pathological tumour classification,

with a focus on tissue level assessment. They highlighted that assessment needed to include metabolic as well as morphological criteria. Consequently there is a growing interest in the use of biological and molecular markers in the assessment of surgical margins.[57, 73, 107, 109-114]

Two major types of techniques have been proposed to assess biological margins in HNSCC by various authors: immunohistochemistry[109, 113, 115-121] and DNA extraction techniques.[73, 107, 109, 111, 115, 116, 122] Each technique has its advantages and disadvantages. DNA techniques have a higher sensitivity, but are associated with a high false positive rate.[109, 116, 122] Concern has been raised that genetic changes do not necessarily relate to expression or biological activity. The techniques used are laborious, require a high level of expertise, and are more costly than immunohistochemistry. Immunohistochemistry is more rapid, more widely available and less expensive, can be performed manually, and is potentially applicable to frozen section specimens. The limitations are the need to identify the appropriate biological markers and for an antibody to the selected marker to be available or developed.

The complex and heterogeneous nature of HNSCC biology makes it unlikely that the information provided by analysing a single biomarker will be adequate to consistently enable comment on biological margins and prognosis.[62, 93, 103, 121, 123-131]

To better understand the biology of HNSCC, and how to better assess and apply biological margins to clinical practice, it is necessary to understand the theories of carcinogenesis that provide the framework for interpreting our clinical and scientific observations.

2.2. Theories of Carcinogenesis – Setting the Framework

Little consideration is given by physicians, particularly surgeons, in their every-day practice to the theories that provide the framework of our apparent understanding of carcinogenesis. Yet it is these thought frameworks that drive cancer research, drug development and ultimately treatment options.

There has been a general acceptance that the predominant model of cancer being a genetic disease is factual, rather than theory. The impact is that this limits directions

of new research and determines the way in which data is analysed.[132] There are in reality numerous models of carcinogenesis that have evolved through the interaction of several disciplines.[133] These can be grouped into two main models: those that consider cancer to be a genetic disease, and those that consider cancer to be an issue of tissue organisation[133]. Most evolutionary models of cancer, which consider the development of cancer to be due to temporal changes related to variation and selection, still place genetic modifications with alteration in phenotype as the key driver of carcinogenesis, are discussed with this group.[20, 134, 135] These two main theories of carcinogenesis are discussed below.

2.2.1. Cancer as a Genetic Disease – Somatic Mutation Theory

Somatic Mutation theory (SMT), at its core defines cancer as a genetic disease, has been the predominant framework employed for cancer research over the last fifty years. This theory is based on the assumptions that cancer is derived from a single somatic cell that has accumulated multiple DNA mutations, the default state of cell proliferation in quiescence, and that cancer is a disease of cell proliferation caused by mutated genes that control proliferation and the cell cycle.[132, 136-139]

The concept of cancer as a genetic disease started with Theodor Boveri in 1914 postulating that a combination of chromosomal defects could cause cancer.[20, 132, 137] In the 1950's models of carcinogenesis were developed based on the hypothesis that multiple mutations accumulated in the same cell. Much of this work was based on cancer incidence curves and formed the basis for the multistage theory of carcinogenesis.[132, 134, 137] In an attempt to provide simplicity and unification, the multistage model reduced carcinogenesis to a serial acquisition of genetic mutations over time. Mathematical representations of cancer initiation and progression are analogous to Newtonian mechanics, both reducing complex phenomena to simple mathematical models, hypothesising common cause as driving observed patterns. In addition, causes exogenous to the system are ignored. An assumption is made that all cancer is of the same disease process. [140]

Between the 1950's and 2000, various new concepts evolved including oncogenes and tumour suppressor genes, viral transfection, genetic instability and epigenetic events.[133, 141] This research forms the basis for the more modern Somatic Mutation Theory (SMT) and was summarized by Hanahan and Weinberg[142] in 2000. Their

paper presented an inclusive reductionism paradigm to guide cancer research into the current century.[143] They presented the idea of heterotypic cell biology, in which tumour cells recruit normal cells to form a mature tumour. Cancer is defined by six hallmarks: self-sufficiency in growth signals, insensitivity to anti growth signals, evading apoptosis, limitless replication, sustained angiogenesis and tissue invasion.[142] Included in their paper is the acknowledgment of Nowell's paper in 1976[144] and subsequent studies that support the notion of tumour development proceeding via a process formally analogous to Darwinian evolution, in which a succession of genetic changes, each conferring one or another type of growth advantage, leads to the progressive conversion of normal human cells into cancer cells.

A genetic multistage model of carcinogenesis for colorectal carcinoma published by Fearon and Vogelstein in 1990[145] provided strongly supportive evidence for SMT. However, findings from ongoing research started to raise questions. The number of mutations being associated with tumours didn't fit the model, only 6 % of tumour mutations related to the six hallmarks of cancer as proposed by Hanahan and Weinberg[142] and some tumours have been found to be characterized by sudden catastrophic genetic changes, not slow accumulation of mutations.[132]

Various extensions have been added into the theory in an attempt to explain experimental paradoxes and recognition of increased complexity including evasion of immune surveillance, deregulating of cellular energetics[146], separation of the "driver" from "passenger" mutations[147], and an increased interest in evolutionary modelling.[20, 133-135, 148, 149]

Despite modifications to explain increasing complexity, the main philosophical assumptions of SMT remain:

- Cancer is a disease of genetic mutation
- Cancer is derived from a single somatic cell
- The initiation process, and therefore the process of carcinogenesis, is irreversible
- The default state of cell is quiescence
- Adjacent tissue has only a supporting role in carcinogenesis

- A reductionism philosophy: the properties of the whole can be inferred, deduced, calculated and predicted from the properties of the parts. Phenomena occurring at one level (cancer at tissue level) can be explained by understanding the properties at a lower level (molecular and chemical properties at the cellular level)
- There is unidirectional upward causation

2.2.2. Cancer as a Tissue Disruption Disease – Tissue Organisation Field Theory

Tissue Organization Field Theory of carcinogenesis, which has been strongly advocated for by Sonnenschein and Soto[136, 150-152] says that cancer results from a disorder of the microenvironment of the cell that represents the physico-chemical support by the morphogenetic field that drives epithelial cells (in particular) towards differentiation and phenotype transformation according to the systems biology rules that govern non-linear dynamic self-organizing structures. This involves complex and reciprocal biophysical, and biochemical communication between mesenchymal (stromal or connective tissue) and parenchymal (epithelial) cells.[153] Cancer is a problem of tissue organization akin to the process of organogenesis during development (development gone awry).[132, 150]

A key premise is that the default state of cells is proliferative with variation and motility. Adopting proliferation and motility as the default state of all cells makes it unnecessary to search for stimulators of both proliferation (i.e., the so-called growth factors, and oncogenes) and of cellular movement and migration.[150] In this theory common genetic or chromosomal lesions do not primarily cause cancers. Cancers are due to sustained failure to communicate between interacting cell lineages living in the complex society of the organism induced by many different primary agents—bioelectric, chemical, imbalances of hormones and other signalling molecules, ROS, implanted foreign bodies, bacteria and viruses, and mutations. These failures of communication initiate the formation of local disorderly, or rogue, cell groups. These groups wax and wane driven by the non-linear dynamics of the group as well as external factors. A sustained local disturbance of such interactions between different cell populations composing a tissue or organ can lead to disorderly arrangement and proliferation of a subpopulation of cells and to disturbances in the microstructure of the region. If one these corrupted groups, by chance, triggers a mechanism that attracts,

recruits and incorporates into the group adjacent non-neoplastic cells, this may start the formation of a cancer.[153, 154]

The main philosophical assumptions in TOFT:

- Cancer is a disease of tissue organization comparable to organogenesis: carcinogenic agents destroy normal tissue architecture interfering with normal cell-cell communication
- The default state of the cell is proliferative with variation and motility
- Carcinogenesis is reversible
- A holistic or antireductionist philosophy: phenomena occurring at one level cannot be explained by understanding the properties at a lower level
- There is unidirectional downward causation: genetic mutations and altered biochemistry are a result of disrupted tissue organisation

Experimental evidence can be found to support and dispute most theories, but there is no single unified theory to bring it all together. The principle underlying postulates of SMT and TOFT render them incompatible. [132, 138, 139, 143] numerous authors have identified a need for a 'systems' approach to cancer. [143, 155-161]

3. An Emergence Framework of Carcinogenesis

3.1. Systems biology and Development of the Framework

Systems biology is not a new concept. Mihajlo Mesarovic, at the Third International Systems Symposium at Case Institute of Technology, Cleveland, Ohio, originally coined "Systems biology" in 1968; to describe a new field designed to explore how systems theory and biology could benefit from one another. The aim was to develop methods for better understanding complex systems with a focus on relations between biological entities forming a system rather than on the properties of the related entities in isolation.[162, 163] Biological and medical research however followed the path of molecular biology with the central concept that the gene is the fundamental unit of biological information and that chemistry provides effective mechanistic explanation of biological processes.[163, 164] Whilst this approach has led to significant improvement in understanding of human disease, translation to clinical impact has not been as fast as anticipated. Increasingly gaps and paradoxes are being uncovered[136, 141, 165-167] together with the acknowledgement that complexity has been overlooked.[164] Cancer epitomizes complex disease.[143, 157, 162, 167-177]

A 'systems' approach requires that cancer not be viewed simply as a disorganized group of cells but as a complex system. A complex system arises when various elements come together in a manner that sees the development, or 'emergence' of new properties that define the system. Using this as a starting point and with considerable review of existing knowledge on complex systems, a new, unified theory of carcinogenesis is proposed. [1, 3, 20, 77, 132-143, 146, 150-152, 155-161, 165, 169, 170, 172, 173, 175-239]

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An Emergence Framework of Carcinogenesis

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Experimental paradigms provide the framework for the understanding of cancer, and drive research and treatment, but are rarely considered by clinicians. The somatic mutation theory (SMT), in which cancer is considered a genetic disease, has been the predominant traditional model of cancer for over 50 years. More recently, alternative theories have been proposed, such as tissue organization field theory (TOFT), evolutionary models, and inflammatory models. Key concepts within the various models have led to them being difficult to reconcile. Progressively, it has been recognized that biological systems cannot be fully explained by the physicochemical properties of their constituent parts. There is an increasing call for a 'systems' approach. Incorporating the concepts of 'emergence', 'systems', 'thermodynamics', and 'chaos', a single integrated framework for carcinogenesis has been developed, enabling existing theories to become compatible as alternative mechanisms, facilitating the integration of bioinformatics and providing a structure in which translational research can flow from both 'benchtop to bedside' and 'bedside to benchtop'. In this review, a basic understanding of the key concepts of 'emergence', 'systems', 'system levels', 'complexity', 'thermodynamics', 'entropy', 'chaos', and 'fractals' is provided. Non-linear mathematical equations are included where possible to demonstrate compatibility with bioinformatics. Twelve principles that define the 'emergence framework of carcinogenesis' are developed, with principles 1–10 encapsulating the key concepts upon which the framework is built and their application to carcinogenesis. Principle 11 relates the framework to cancer progression. Principle 12 relates to the application of the framework to translational research. The 'emergence framework of carcinogenesis' collates current paradigms, concepts, and evidence around carcinogenesis into a single framework that incorporates previously incompatible viewpoints and ideas. Any researcher, scientist, or clinician involved in research, treatment, or prevention of cancer can employ this framework.

Keywords: emergence, systems biology, carcinogenesis, thermodynamics, chaos, entropy, fractals, translational research

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INTRODUCTION

Consideration given to paradigms that provide the framework for the understanding of carcinogenesis by physicians, particularly surgeons, in every day practice of managing and treating cancer is minimal. Yet it is these frameworks that drive cancer research, translational research, drug development and ultimately treatment options, and impact on where research dollars are allocated (1, 2).

The predominant traditional model of cancer, the somatic mutation theory (SMT), in which cancer is considered a genetic disease (3–7), has been widely accepted as factual, rather than theory (1, 4, 5). This narrows avenues of new research and directs the way in which data is analyzed (6). While there are a number of models of carcinogenesis, these have evolved through the interaction of several disciplines and can be grouped into two main theories: those that consider cancer to be a genetic disease, including SMT, multistage models, and evolutionary models that consider the development of cancer to be due to temporal changes related to variation and selection and place genetic modifications with alteration in phenotype as the key driver of carcinogenesis (8–11), and those that consider cancer to be an issue of tissue organization, such as tissue organization field theory (TOFT) and inflammatory models (11–14). Experimental evidence can be found to support and dispute most theories, but there is no single unified theory to bring it all together. The principle underlying postulates of SMT and TOFT, as outlined in **Tables 1** and **2**, render them incompatible (5–7, 15).

Numerous authors have identified a need for a 'systems' approach to cancer (15–22).

This paper presents a comprehensive framework for carcinogenesis. Through incorporating the concepts of 'emergence', 'systems', 'thermodynamics', and 'chaos', a single integrated framework for carcinogenesis has been developed, enabling existing theories to become compatible as alternative mechanisms, facilitating the integration of bioinformatics and providing a structure in which translational research can flow from both 'benchtop to bedside' and 'bedside to benchtop' (5, 7, 15, 20, 23, 24).

'An emergence framework of carcinogenesis' is based on extensive study of the works of numerous philosophers, researchers, scientists, and writers, including Mario Bunge (20) (physicist, philosopher, and philosopher of science) and Denis Noble (25) (biologist, physiologist, Emeritus Professor University of Oxford). The central proposition is a change in the way cancer is investigated, managed, and treated, by considering 'cancer' as an 'emergent system'. This clear and well-defined framework allows integration of progress and discoveries made to date regarding carcinogenesis and cancer, and provides alternative ways to view that and new knowledge, driving the progress of research toward making an impact at the place that truly matters, the bedside.

TABLE 1 | Somatic mutation theory.

- Cancer is a disease of genetic mutation.
- Cancer is derived from a single somatic cell.
- The initiation process, and therefore, the process of carcinogenesis, is irreversible.
- The default state of a cell is quiescence.
- Adjacent tissue has only a supporting role in carcinogenesis.
- A reductionism philosophy: the properties of the whole can be inferred, deduced, calculated, and predicted from the properties of the parts. Phenomena occurring at one level (cancer at tissue level) can be explained by understanding the properties at a lower level (molecular and chemical properties at the cellular level).
- There is unidirectional upward causation.

TABLE 2 | Tissue organization field theory.

- Cancer is a disease of tissue organization comparable to organogenesis: carcinogenic agents destroy normal tissue architecture, interfering with normal cell–cell communication.
- The default state of the cell is proliferative with variation and motility.
- Carcinogenesis is reversible.
- A holistic or antireductionist philosophy: phenomena occurring at one level cannot be explained by understanding the properties at a lower level.
- There is unidirectional downward causation: genetic mutations and altered biochemistry are a result of disrupted tissue organization.

AN EMERGENCE FRAMEWORK

Background

'Emergence' is about the properties of wholes compared to those of their parts. It refers to complex systems having properties (components, patterns, or processes) that their constituents or precursors in isolation do not have. The new property is more than simply a combination of the properties of its pieces, meaning there is no simple mathematical model that explains this new property. It is a qualitative, not quantitative measure. An emergent property may (ontological) or may not (epistemological) be predictable through understanding the properties of its components. Emergent phenomena are found across all areas of study, including physics, chemistry, biology, sociology, psychology, economics, and IT, and led to the development of the field of quantum physics (20, 26–29) and the concept of the chemical reaction network theory in the study of proteins (30).

Thermodynamics is a physical theory that describes a system in terms of the thermodynamic properties (heat and temperature in relation to energy and work) of the system or its parts. Thermodynamics makes no assumption about the microscopic nature of the system; it describes the macroscopic properties and remains correct even if the microscopic assumptions about a system are proved wrong (29, 31).

Biological, or living, organisms are open thermodynamic systems that have acquired complexity through non-linear self-organizational processes and defy the second law of thermodynamics by mechanisms of metabolism. These properties cannot be deduced from molecular biological and genetic knowledge alone (20, 25, 29, 31).

Somatic mutation theory is based on the classic form in which biological systems have been described assuming that it should be possible to reconstruct complex living systems from the bottom up, starting with raw DNA code (22, 32). However, complexity in biological systems, as demonstrated by self-organizational studies and the Human Genome Project, does not require complexity at the level of the genome (32). Complexity is achieved by the repeated application of simple rules by large units (25, 29). An attempt to apply the non-linear mathematics of complexity to understand the combination of gene interactions to generate a single function in a genome of 30,000 genes, as an example, would yield 2×10^{72403} possible combinations (33). Therefore, to understand cancer only as a genetic disease is to underestimate its complexity (32–34).

Somatic mutation theory reasoning leads to the concept that the ultimate causative level is the most microscopic one, the molecular level of genes. This has caused attention to be focused on a level that does not enable an understanding of cancer as an emergent complex system (17). This does not mean that genetics, molecular biology, and immunology have not contributed enormously to advances in the understanding of the biology of cancer (1, 17). This has led to the identification of therapeutic targets, development of small molecule cancer drugs, and application of immunotherapeutics. However, these advances at the same time have both colored and limited the way in which data has been interpreted. Shortcomings and paradoxes uncovered by research findings have been attempted to be addressed through modifying the model (35), rather than questioning the fundamentals (1).

Tissue organization field theory addresses many of the shortcomings of SMT (1). It moves focus away from genes as the centric cause of cancer and instead directs it to disruption of patterns of tissue organization (3, 12, 13, 36). This framework, however, implies that genetic mutations are always the result of disruption of tissue organization and play no causative role in carcinogenesis (7, 15). Brücher's proposed paradigm places chronic inflammation as an essential key in carcinogenesis and is supported by human and animal studies (4). However, it too discounts entirely a causal role of genetic changes.

Somatic mutation theory and TOFT both assign causation to a specific level. The causation of cancer, however, involves numerous processes of a multistage nature from molecular to environmental levels (17). There is no single privileged level of causation: causation flows in both upward and downward directions (17, 22, 25, 37, 38).

A common flaw in both SMT and TOFT is the importance attached to assuming the default state of an individual cell as either quiescent or proliferative. There is ample evidence to suggest a 'default state' of individual cells *in vivo* does not exist. The 'state' of cells (quiescent or replicating) is largely shaped by their roles and positions within their community, or, as discussed later, their 'system' (7). As pointed out by several authors, SMT and TOFT have been made artificially incompatible; yet experimental evidence supports that both models have value (5, 7, 15).

A systems approach enables the absorption of SMT, TOFT, and other theories into a unified concept, allowing them to be compatible as contributors to a 'cancer system' within an emergence framework (5, 7, 15, 20, 23).

Key Concepts

In generating an emergence framework of carcinogenesis, a basic understanding of the concepts of 'emergence', 'levels', 'system', 'complexity', 'thermodynamics', 'entropy', 'chaos', and 'fractals' is required.

Emergence, Levels, Systems and Complexity

An 'emergent property' typically refers to a property or properties possessed by a whole that its parts lack. Some philosophers argue that 'emergence' can only be used when the property is unpredictable or unexplainable by contemporary theories (20). Mario Bunge, a preeminent physicist and philosopher of science, identified that this is not the sense in which 'emergence' is used in biology or science. He provided a clear and simple definition, suitable for science, in his 1977 Treatise as follows (39):

> *P* is a global (or collective or non-distributive)
> property of a system of kind *K*, none
> of whose components or precursors possesses *P*
> where '*P*' is the 'emergent property' of the system

This definition is not limited by the need to be 'unpredictable'.

A 'level' is a collection of things that have a certain property in common. Combinations of lower-level things assemble to constitute a new level, or higher-level thing. Every higher level is characterized by an emergent property, not possessed by any of the lower-level things. This implies a level hierarchy (7, 23, 32). This is demonstrated below in **Figure 1**, using the formation of a water drop as an example.

Level ascription is not a one-off process for any specific entity, but depends on the way one chooses to decompose the parts. This will be determined by the 'system' that is the subject of investigation (37).

For a complex 'system' to come into being, it requires (20):

- The combination of two or more precursors that combine to form a new object that is *characterized by properties its precursors do not have*. Combination, as opposed to association, requires that the original items alter in the process, the system is more stable and cohesive than a simple association, it requires energy and/or time to form, and/or is rarer than an association. A proton and electron coming together to form an atom of hydrogen, as above, is an example.
- A *bonding structure* that *enables self-organization* of the collection of relations among its components generated by the combination.

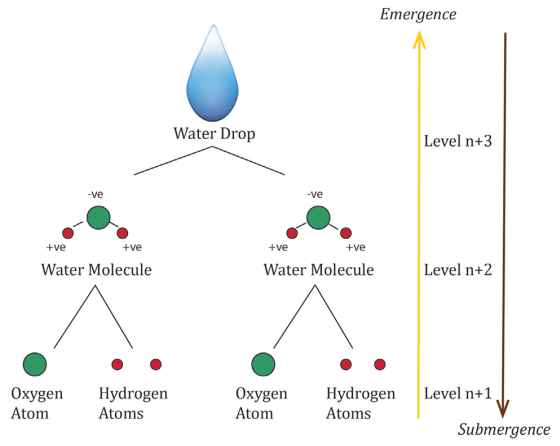

FIGURE 1 | Formation of a water drop as an example of emergent hierarchical levels, with each level possessing a property or properties that is more than simply the sum of the properties of its parts.

- A *mechanism*, which is a process or set of processes that *bring the emergence of a property or other process in the system as a whole*.

A 'system', in particular a concrete system as biological systems are, is not an isolated entity. It comes into being in some sort of surrounds or environment (20, 37). To factor in the impact of the environment on a system, structure needs to be considered in two parts: the *endostructure*, which is the collection of bonds among the system components to enable self-organization as described above, and the *exostructure*, which is the collection of bonds between the system and the environment. The exostructure then determines how the environment acts upon the system (input) and how the system acts upon the environment (output). The subset of the system members that hold direct relationship with the environment are considered the system boundary (17, 20).

A 'system' is dynamic in nature. Components may alter over time due to changes in external influences or internal influences. The 'system' itself may induce feedback loops, generating downward causation or upward causation. The ability to change is the only consistent property across all systems (20, 25).

A 'system' has a point of self-organized criticality. At such a point, there is a system-wide transformation, which moves a system rapidly into a new state. The 'system' either collapses and there is submergence of properties, or the transformation results in the emergence of a new 'system'. The transition may be triggered by a very minor event with little significance on its own. This is a qualitative leap (7).

A system, μ, called 's' at a single point in time can be defined as (20):

$$\mu(s) = \{C(s),\ E(s),\ S(s),\ M(s)\}$$

where C(s) = Composition: collection of all the parts of 's'; E(s) = Environment: collection of items, other than those in 's', that act on or are acted upon by some or all of the components of 's'; S(s) = Structure: collection of relations, in particular bonds, among components of 's' or among these and items in its environment 'E(s)'; M(s) = Mechanism: collection of processes in 's' that make it behave the way it does.

As this would require an understanding of all parts of all systems at all levels, it is not practically useful. This can be limited by focusing on the *given level* that is being studied by using the intersection or logical product:

$$C(s) \cap a = C_a(s)$$
$$E(s) \cap b = E_b(s)$$
$$S(s) \cap c = S_c(s)$$
$$M(s) \cap d = M_d(s)$$

So that

$$\mu_{abcd}(s) = \{C_a(s),\ E_b(s),\ S_c(s),\ M_d(s)\}$$

Using the water drop from above in **Figure 1** as an example: $\mu_{abcd}(s)$ = water drop with the emergent properties of '*P*' = surface tension; $C_a(s)$ = molecules of H_2O; $E_b(s)$ = atmospheric temperature and pressure, gravity, air, or other surface the water drop is in contact with; $S_c(s)$ = endogenous: the arrangement of water molecules with each other *via* hydrogen bonds that pull each molecule equally in every direction resulting in a net force of zero, =exogenous: interaction of the molecules at the surface that do not have the same molecules on each side causing them to be pulled inwards and contract to the minimal area; $M_d(s)$ = cohesion between water molecules being stronger than adhesion with molecules in air.

To capture the dynamics of a 'system', or in other words the qualitative and quantitative changes of a system over time, a state-space approach needs to also be included. The quantitative properties of a system can be combined into a single function, '*F*', of the system. In a simple system that has two quantitative properties, called X and Y, which are considered to be the attributes of the system, then

$$F = \{X,\ Y\}$$

A snapshot at a specific point in time, 't', can then be represented by *F*(t) as follows:

$$F(t) = \{X(t),\ Y(t)\}$$

This is called the 'state function' of the system.

A vector that describes a trajectory from this point in the 'state space' can then represent changes over time. The trajectory represents the *history,* '*H*', of the system over a period of time, 'T':

$$H = < F(t) \mid t \in T >$$

The history is confined within a box, the 'state space' that represents all of the really possible states of the system as determined by the law that governs the system.

This can be demonstrated by considering the movement of a pendulum, which is a linear oscillator. The *function (F)* is swinging. It has two salient properties: *momentum (q)* and *position (p)*. The law that governs the system is that 'energy is constant'. The potential energy related to position and the kinetic energy related to momentum must always equal the total energy of the system.

At any point in time, '*t*' the state of the system can be analyzed in terms of its position and momentum. This changes over time, creating a trajectory that reflects the history, '*H*'. If the energy is added from an environmental source, the trajectory will alter.

An 'event' is represented by an ordered couple of points in the 'state space'.

If a new property arises (emergence), the tangent of the trajectory acquires a new axis. If a property is lost (submergence), an axis is lost.

Thermodynamics, Entropy, Chaos, and Fractals

While thermodynamics is a physical theory that describes a system in terms of the thermodynamic properties of the system or its parts, it makes no assumption about the microscopic nature of the system; it describes the macroscopic properties and remains correct, even if the microscopic assumptions about a system are proved wrong (29). Moreover, it should be recalled that total energy in a closed system is constant and energy of any system can be ordered and available for use, or disordered and unable to be used by the system (40).

Living systems are open systems and defy the second law of thermodynamics (the universe is constantly aiming toward a state of maximal entropy or thermodynamic equilibrium), by interacting with and acquiring energy from their environment. This enables the development of complexity. If their ability to acquire energy from the environment is reduced, energy is gradually dissipated as heat to surroundings and complexity is lost, as this requires energy to be maintained, and the system will degenerate. This is the process that occurs in aging and death (18, 29, 40).

New disciplines, such as non-linear dynamics ('chaos theory') and fractal geometry, have brought new tools and perspectives into pathophysiology. Many physiological systems are highly complex networks, with numerous recursive feed-back and feed-forward circuits, and thus they may be especially prone to develop chaotic behaviors and display fractal structures. Non-linear dynamics and fractal models have been increasingly applied in physiology and medicine (41).

Fractals are spatial structures and have the properties of self-similarity (consists of miniature copies of itself at different levels of magnification) and/or fractal dimensions (the parameter of an object that displays how much space it occupies which is, unlike Euclidean geometry, not a whole dimension but a non-integer, the value of which is a measure of complexity), and/or rough outlines and infinite length (41–46). Fractals enable the creation of complex shapes. Nature and biological systems have mastered fractals to achieve complexity, adaptability, and efficiency. Fractal patterns can be seen in mountains, coast lines, cloud formation, branching trees, and in the human body in the branching of blood vessels, neural synapses, and lung bronchioles (41–43). One of the most well-known fractal patterns is the Mandelbrot set (**Figure 2**) (18, 40, 46). Fractal geometry has been noted to emerge on the surface of human cervical epithelial cells during progression to cancer (47) and observed in lymphoma and leukemia cells correlating with their biological features (48).

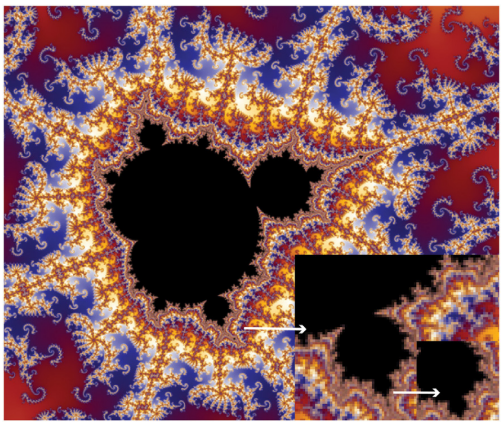

FIGURE 2 | A pattern created by the Mandelbrot set exhibits an elaborate and infinitely complicated boundary that reveals progressively ever-finer recurring detail at increasing magnifications, indicated by the white arrows. The 'style' of this repeating detail depends on the region of the set being examined. The set's boundary also incorporates smaller versions of the main shape, so the fractal property of self-similarity applies to the entire set and not just to its parts.

Chaotic dynamics in a system is a function of time (41). Chaos and complexity share the property of non-linear dynamics (18, 46, 49). A complex system keeps its non-linear processes under control through oscillations, which provide the ability to restore and maintain its steady state within its environment. There is increasing evidence that biological oscillations or 'clocks' and molecular motors that cycle ATP can contribute to the rise of complexity and affect morphogenesis (29, 50–53). Periodic reactions allow periodic signal transmitting between individual cells and regulate cell differentiation within an organism. Such clocks are known to be extremely stable. The predictability of their behavior implies linearity. Under certain circumstances, however, they may exhibit transition into chaotic behavior, implying unpredictability and non-linearity. As decreased usable energy moves a living system toward entropy, increased available energy pushes the system toward chaos with the possibility of creating a new initial state and the emergence of a new system (29).

Ultimately, cancer research and treatment can be progressed by shifting our focus from causal relationships that are non-linear and difficult to predict, to interpreting the patterns of cancer as an emergent system. To do this, a framework that can be employed anywhere from benchtop to bedside is likely to be more impactful.

Applying Emergence to Carcinogenesis

The emergence framework has been established through the creation of 12 principles. Each principle is presented in turn, followed by supporting evidence. Principles 1–10 encapsulate the key concepts upon which the framework is built and their application to carcinogenesis. Principle 11 relates the framework to cancer progression. The 12th principle relates to the application of the framework to translation research.

Principle 1

Cancer is a dynamic complex system emerging at the level of the 'functional tissue unit'.

The first principle in creating an emergence framework of carcinogenesis is defining the 'level' at which 'cancer' as an emergent system arises. Cancer is characterized by alterations in cell and tissue structure, namely excessive accumulation of cells and disruption of normal tissue architecture (36, 54, 55). For some cancers, cell-level criteria are also of value, such as diagnostic nuclear envelope irregularity and chromatin clearing in early-stage thyroid papillary carcinoma (54). Hematological malignancies are diagnosed by assessing the proportion of immature precursor cells in bone marrow. Diagnosis is made using light microscopy and molecular analyses.

Analysis of genes alone does not make the diagnosis of cancer (25, 34). The presence or absence of genetic abnormality does not determine the presence of cancer: a 'cancer' gene may be present but not be expressed; not all malignancies have identifiable mutations; and the number of mutations is highly variable even within the same cancer type. Many normal cells can contain aberrations associated with cancer cells (1, 4, 36, 54).

Tissue-level patterns are diagnostic and tissue-level patterns may indicate mechanism (54, 55). It is this level at which cancer is diagnosed and it follows that this is the level that cancer emerges as a 'system'. This level is not a single tissue type but a combination of tissues in a specific arrangement that have developed to form a system with specific properties and functions. These tissue level systems can be considered 'functional tissue units'. Cancer, therefore, emerges at the level of 'functional tissue units' (56) (**Figure 3**) and can be defined as 'disordered growth occurring at the level of functional tissue units causing changes of both morphology and physiology resulting in loss of normal function'.

This concept is supported by the work undertaken by Bissell (57, 58). Bissell and collaborators, based on experimental results of modeling both normal development of mammary glands and breast tumor formation, determined that the functional unit of the mammary gland is the mammary acinus, not simply the mammary epithelial cell and its extracellular matrix. Using a three-dimensional (3-D) culture system, they demonstrated the importance of the stroma in developing mammary acini and in augmenting function. This did not occur in 2-D models or cell lines. In contrast to normal breast cells, malignant breast cells did not form acini, but formed cell aggregates with large diameters and large number of cells, and did not produce casein (59).

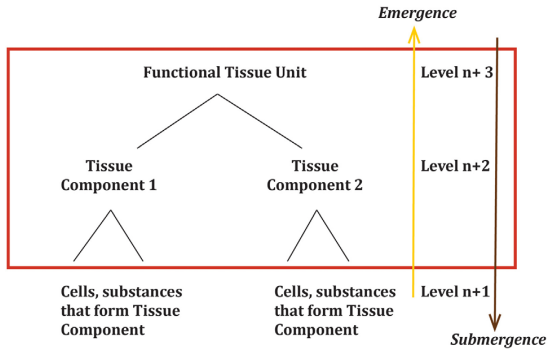

FIGURE 3 | Cancer emerges as a dynamic system at the level of the 'functional tissue unit'. The red box area highlights the levels of importance in an emergence framework. μ(s) or the 'system' is the 'functional tissue unit'. The logical components $C_a(s)$ of the 'functional tissue unit' are the tissue components at the lower level.

Identifying the level at which cancer is diagnosed allows the construct of a normal 'functional tissue unit' system using a systems formula. The components of the system, $C_a(s)$, are the lower level 'tissue components' that combine in a specific self-organized structure, $S_c(s)$, to form the 'functional tissue unit'.

Placing 'mammary acinus' into an emergence model framework (**Figure 4**):

$$\mu_{abcd}(s) = \{C_a(s),\ E_b(s),\ S_c(s),\ M_d(s)\}$$

then $\mu_{abcd}(s)$ = functional tissue unit (mammary acinus) with the unique emergent functional property (to form glands capable of producing and releasing milk); $C_a(s)$ = the parts of the lower hierarchical level of tissue that combine to form the functional tissue unit (bi-layered epithelium, basement membrane, connective tissue stroma); $E_b(s)$ = the environment that interacts with the functional tissue unit or components of the functional unit (systemic hormones and factors in blood, external environmental factors *via* ducts); $S_c(s)$ = the specific arrangement and self-organization of the components of the functional tissue unit to form the functional tissue unit (bi-layered epithelium, basement membrane, connective stroma self-organize into a mammary acinus), the structure that enables interaction with the environment (blood vessels, epithelium); $M_d(s)$ = morphogenesis [collection of processes that enable formation of the functional tissue unit, including dynamic reciprocity (58)] and physiology (collection of processes that enable the functional tissue unit to perform its biological functions).

This principle enables research from both laboratory and clinical studies to be brought to a unified point through understanding the impact of findings on the various components of a normal 'functional tissue unit' that leads to its loss and replacement by an emergent cancer system.

Principle 2

Cancer is not a single disease entity, but an emergence phenomenon that can occur across numerous functional tissue units by multiple processes to generate

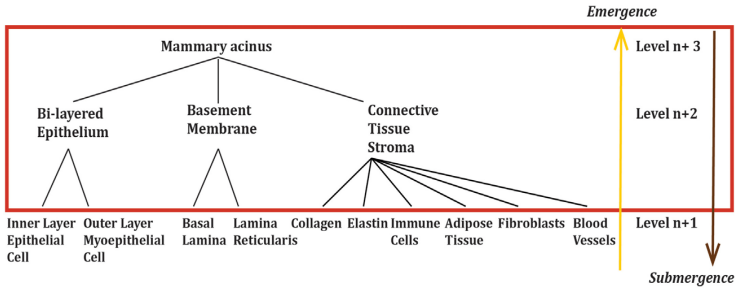

FIGURE 4 | Mammary acinus as an example of a 'functional tissue unit', μ(s), the level at which ductal carcinoma as a system emerges. Again, the red box area highlights the levels of importance in an emergence framework. The logical components, $C_a(s)$, of the 'functional tissue unit' are the tissue components at the level below, Level $n + 2$. Components at lower levels are important only through the impact they have on the components, $C_a(s)$, by changing the components themselves and/or changing the structure, $S_c(s)$. Physiological changes alter the environment, $E_b(s)$, or mechanism, $M_d(s)$.

> a mechanism of carcinogenesis that is specific to that functional unit, and may be specific to an individual tumor; the common properties of cancer can be accomplished via different systems utilizing different mechanisms.

Different organs have different functional units and any one organ may have more than one functional unit. The emergence framework allows for each functional tissue unit to be considered as a unique 'system'. The functional tissue unit of breast tissue is the mammary acinus. The pancreas has both an endocrine functional tissue unit and an exocrine tissue functional unit. The blood system has a functional tissue unit in the bone marrow, but the lymph glands could also be a functional tissue unit.

An example is 'ductal breast cancer'. In an emergence framework, it would be considered to arise from the 'components' that form a 'mammary acinus'. 'Components', 'environment', 'structure', and 'mechanism' enable the incorporation of the major theories of carcinogenesis into a unified concept. The "Hallmarks of Cancer", as described by Hanahan and Weinberg (35, 60) are captured in 'environment' or 'mechanism'. 'Structure' includes the key concept of TOFT as described by Sonnenschein and Soto (3, 13). Chronic inflammation (14) and evolutionary models are covered in 'environment' and 'mechanism', genetic modifications may alter 'components', 'structure', or be included in 'mechanism'. The equation would be as follows.

$\mu_{abcd}(s)$, the 'mammary acinus' system is replaced by $\mu_{abcd}(sc)$, the cancer system emerging from the 'mammary acinus' system, 'ductal carcinoma'

$$\mu_{abcd}(sc) = \{C_a(sc),\ E_b(sc),\ S_c(sc),\ M_d(sc)\}$$

then $\mu_{abcd}(sc)$ = 'ductal carcinoma' with loss of the property to form glands capable of producing and releasing milk and the new emergent properties of limitless replication, tissue invasion, and metastatic potential; $C_a(sc)$ = the parts of the lower hierarchical level of tissue that formerly combined to form the functional tissue unit (bi-layered epithelium, basement membrane, connective tissue stroma) in their altered form; $E_b(sc)$ = the environment that interacts with the 'ductal carcinoma' or components of the 'ductal carcinoma', which still include not only systemic hormones and factors in blood, external environmental factors *via* ducts, but also local tumor microenvironment and tumor-promoting inflammation; $S_c(sc)$ = the specific change in arrangement and self-organization of the components of the functional tissue unit to now form the 'ductal carcinoma', including altered polarity of epithelium, loss of normal tissue architecture, various stromal alterations, including angiogenesis, and loss of contact inhibition, breakdown of basement membrane, and invasion; $M_d(sc)$ = physiological processes that enable the formation of 'ductal carcinoma', including self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, sustained angiogenesis, deregulating cellular energetics, and avoiding immune destruction.

Comparing the systems equation for the relevant functional tissue unit to the systems equation of the cancer that has emerged from that specific functional tissue unit helps to identify the factors relevant for both the loss of the functional tissue unit system and emergence of the cancer system. For each functional tissue unit, the components, environment, structure, and mechanisms will vary. So too, will the specifics of the cancer that emerges from it.

This variation is seen at many levels, including genes. The functional tissue unit from which leukemia emerges is the bone marrow. In leukemia, *Notch1* is considered an oncogene. By contrast, the functional tissue unit from which oral squamous cell carcinoma emerges is the mucous membrane, and *Notch 1* is considered a tumor suppressor gene (61). Squamous cell carcinoma arising from skin and mucous membrane will have a similar phenotype but there can be a wide variety in mechanisms (62).

The overwhelming evidence supports that cancer is not just one aberrant cell or one disease, but many (1, 4). Identifying from which functional tissue unit a cancer has emerged is key.

Principle 3

> Causation of cancer is a property of the system and is not contributable at any single hierarchical level: multiscale causality associates causation at different levels concomitantly.

As can be deduced from **Figure 3**, significant changes in even lower levels could impact the emergence of one or more of the tissue components, which would in turn impact the emergence of the 'functional tissue unit'. This shows the path of upward causation. However, as the relationship between levels is not linear, a single change in a single cell is highly unlikely to generate a change at the level of the 'functional tissue unit'. For a system property to emerge or submerge, a point of criticality must be reached. This is discussed further below.

As opposed to evolutionary models, an emergence framework is not required to start at the genetic level, nor does the process need to be gradual or stepwise. An emergence framework allows for discontinuity and major rapid transformation in state.

Changes in $S_c(s)$ that impact the exostructure or the way the system interacts with the environment, $E_b(s)$, can occur as a direct response to changes in $E_b(s)$, as a result of changes to $C_a(s)$ impacting $S_c(s)$, and/or the output of the system, $\mu_{abcd}(s)$, directly causing changes in $E_b(s)$.

The output of the system, $\mu_{abcd}(s)$, may also create feedback affecting lower levels. This is downward causation.

The mechanism, $M_d(s)$, or group of processes by which the 'functional tissue unit' achieves its emergent property or function, can also be impacted by upward causes, downward causes, and environmental causes. Physiological changes are as important as morphological ones.

Causation can flow in many directions simultaneously, there is no single privileged level of causation, and causation is not necessarily linear (15, 17, 22, 37, 38, 63).

Principle 4

> The 'state' of a cell is determined by its position within the functional tissue unit and the state of the functional tissue unit as a system. All living systems metabolize and are therefore dynamic over time. Defining a 'default'

state of a cell as quiescent or proliferative is not relevant in an emergence framework of carcinogenesis.

All living tissues metabolize and, therefore, create dynamic open systems experiencing various iterations of pendulum-like swings in their morphology and physiology, which are controlled by resetting mechanisms. These are the oscillations or 'clocks', and molecular motors referred to in the preamble (29, 50–53). For each cycle, there is a defined beginning and an end. Cycles express relatedness, a key characteristic of systems, implying the interconnectedness and dependability of all components (18, 39).

The 'state' of cells (quiescent or proliferative) is largely shaped by their roles and positions within their community (7). Mapped in a state-space over time, changes would be seen in the variables that compose the system in response to inputs from its environment and the outputs it puts back and other internal changes. Fluctuations occur in order to maintain the function of the 'functional tissue unit' in accordance with the natural law of that state. Therefore, there can be no 'default state' of a cell in a system, as it is never free of the system it is part of (7). If a cell became a closed system, that is it has ceased to communicate with its environment, it would move into a state of entropy and cease to exist (18).

Evidence that cells *in vivo* function as a community can be found in the science laboratory. Placing cells in culture disrupts tissue architecture and cell–cell relationships, allowing the emergence of cells with malignant potential, that is, those that can grow and replicate autonomously (55). Conversely, growing cells in Matrigel, the extracellular matrix produced by Engelbreth–Holm–Swarm sarcoma cells allows the growth of 3-D structures that resemble normal tissue, and supports, for example, the branching, morphogenesis in mammary tissue that produces duct-like structures and responds to lactogenic hormones (55, 57, 59).

Investigation into morphogenesis, morphogens, and morphostats provides additional evidence for the position within functional tissue units determining the 'state' of any given cell (55, 56). Morphogens are fundamental organizers of tissue morphology and play a critical role in embryogenesis. Morphastats are believed to have a central role in maintaining normal cellular behavior and microarchitecture in adult tissues. Both are substances to which cells respond directly, but there are two or more qualitatively different responses depending on the concentration and, therefore, the distance from, and by extrapolation, the relative position to the source (55).

An example is the regular controlled migration of basal layer to surface layer in adult epithelial tissues. Each epithelial cell passes through several stages: a reproductive transit cell located basally; an intermittent cell with functional capacity located centrally; and a quiescent/senescent cell or cell remnant located at the surface (55). Position determines the state of the cell.

Principle 5

A healthy functional tissue unit is a metastable system oscillating between maintaining optimum function, maximal adaptability in response to inputs and outputs with its environment and self-maintenance through repair, differentiation and apoptosis in accordance with the natural law of a functional tissue unit as defined by

$$F = \frac{k}{M}$$

where F = functional status of the functional tissue unit; M = repair/growth rate; k = non-zero constant.

A functional tissue unit is a dynamic system that is constantly re-setting mechanisms to allow for complex adaptability in balancing inputs and outputs in response to internal and environmental changes, to maintain stable boundaries, and to maintain its function. A healthy functional tissue unit sits in a metastable state oscillating between order and the inner edge of chaos, undergoing cellular renewals, cellular differentiation, and cellular apoptosis as required by the functional tissue unit, maintaining immunosurveillance and controlling angiogenesis (18, 29, 46). The natural law of a functional tissue unit is a reciprocal relationship between functionality and repair/multiplication and can be represented graphically as in **Figure 5**.

Various stimuli can alter the dynamic state of a functional tissue unit moving it toward a higher rate of growth and/or repair, such as in wound healing, with changes in motility, polarization,

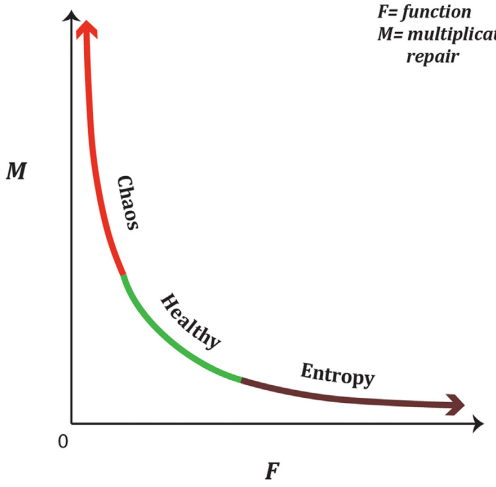

FIGURE 5 | A functional tissue unit is a metastable dynamic system oscillating between maintaining optimum function, maximal adaptability in response to inputs and outputs with its environment, and self-maintenance through repair, differentiation, and apoptosis. The natural law of a functional tissue unit is an inverse non-linear relationship between function, 'F', and growth/repair, 'M'. This is represented graphically, by the equation $F = \frac{k}{M}$ with 'k' being a non-zero constant. If the functional tissue unit swings too far toward optimal function, it reduces its ability to adapt to environmental changes and will ultimately become a closed system with inability to gain energy from its surrounds and in accordance with the second law of thermodynamics will move toward entropy (aging and death). A functional tissue unit that undergoes excessive growth or repair is redirecting energy away from normal function and increases the need to generate additional usable energy from its environment, pushing it into the hyper-energized state.

matrix formation, and expression of various proteins and cytokines (64–67) shifting the oscillation of the functional tissue unit more toward chaos. Alternatively, a shift toward increased function results in a functional tissue unit losing its complexity and ability for renewal (41, 68). This may manifest in the functional tissue unit becoming non-responsive to its environment and functioning autonomously, such as parathyroid glands in primary parathyroidism (69) or involution due to entropy such as in Type 2 diabetes (41, 68).

In carcinogenesis, precancerous states represent a shift of the oscillating metastable state of the functional tissue unit toward one of these two extremes.

Principle 6

> A functional tissue unit will have points of self-organized criticality in both the directions of entropy and chaos, beyond which a critical collapse occurs, resulting in loss of the morphological and physiological properties of the functional tissue unit setting the initial state from which cancer can emerge.

In line with Principle 5, if a functional tissue unit becomes too stable, adaptability and, therefore, resilience to environmental changes is lost, leading to entropy, or degeneration (18, 70). A system that becomes too chaotic will lose its structure and function and be pushed toward the development of a new state. At each extreme of the 'healthy' metastable state, there is a point of self-organizational criticality that when surpassed results in the collapse of the next hierarchical level. At or near this point, a change may occur that in itself is insignificant, but in the system can lead to a massive, rapid, and seemingly disrupted transformational occurrence (7, 18).

Therefore, in this framework, a change or combination of changes in $C_a(s)$, $E_b(s)$, $S_c(s)$, or $M_d(s)$, beyond a point of self-organized criticality in either direction, alters the system causing disruption of the bonding structure that enables self-organization of the collection of relations among the biological tissue components, and/or their environment and/or the group of processes producing the mechanism of morphogenesis. This results in loss of the morphological and physiological properties of the functional tissue unit. Once this occurs, there is an opportunity for a new system, namely 'cancer' to arise.

The work of Nordemar et al. (71) in which they assessed progression of cancer *in situ* (CIS) in the larynx to invasive carcinoma can be used as an example of how this principle may be applied. While not all CIS lesions staining positive for the laminin γ2 chain of laminin 332 (formally laminin 5) progressed to invasive cancer, *only* lesions expressing laminin 332 progressed. In this framework, these results could be interpreted that the expression of laminin 332 was an indicator of the point of self-organized criticality.

Principle 7

> Reduced redundancy of healthy functional tissue units through either entropy (degeneration) or excessive repair in response to tissue trauma is associated with an increased risk of cancer.

'Redundancy' is another key feature of system resilience or robustness (18, 25). Redundancy of functional tissue units and redundancy within the tissue components of the functional tissue units are important considerations in an emergence framework of carcinogenesis.

If the metastable state passes too far toward entropy, there is an increasing inability to adapt to the environment, and the functional tissue unit moves from being an open system toward an increasingly closed one. Increased entropy results in inadequate useable energy within the functional tissue unit to maintain function and structure (18, 70, 72, 73). At a higher hierarchical level, the organ may shut down and rid itself of an entropic functional tissue unit that has passed the point of self-organized criticality toward entropy, in the same manner that a functional tissue unit may trigger apoptosis of a cell to maintain the health of the system (18, 63, 72–74).

Aging has been shown to be associated with breakdown of non-linear dynamics and fractal patterns with subsequent loss of complexity in numerous areas, including liver metabolism, cardiac physiology, and gait (75–78). Degeneration that occurs with aging leads to loss of redundancy through reduction of healthy functional tissue units available to maintain the overall function of the organ (63, 70, 74, 79). This has a number of important consequences. Numerous functional tissue units having such reduced interaction with their environment may become perceived as 'foreign tissue', triggering an immune response, manifesting in an autoimmune disease (18). There are a lower number of healthy functional tissue units to respond to requirements of repair, and less capacity to remove functional tissue units that have swung too far in the other direction into the chaos zone. Finally, the entropic functional tissue units may not be able to respond to the requirements of repair in a healthy manner due to decreased interaction from the environment, and die or attempt to undergo repair and growth that is disordered. Resilience protecting against the emergence of cancer is, therefore, increasingly lost with age (70, 72–74). The same age incidence curves used to propose and support SMT are consistent with an emergence framework of carcinogenesis.

Moving to the other extreme, if a functional tissue unit is required to undergo excessive repair or growth, this could push it past the self-criticality point into chaos. 'Trauma' in all its manifestations, including inflammation, mechanical trauma, carcinogens, and infective causes, would push a functional tissue unit toward repair. As the natural law of the functional tissue unit is that function is inversely proportional to repair/multiplication, this will be the point where the law can no longer be maintained and the functional tissue unit as a system collapses. Loss of tissue organization architecture would also result. An emergence framework is, therefore, supported by the same evidence that supports TOFT as proposed by Sonnenschein and Soto (12), and the evidence that supports the inflammatory paradigm proposed by Brücher and Jamall (14).

Principle 8

> Risk factors for cancer act to reduce redundancy of functional tissue units via a number of mechanisms at a faster rate than in the natural aging process, generating an increased risk for cancer.

Individuals who are exposed to various risk factors such as smoking, excessive alcohol intake, stress, exposure to radiation, and other life style insults, prematurely reduce the redundancy of the functional tissue units of various organs, reducing resilience to aging processes (73). Consequently, they have higher risk of various diseases as they become older, including cancer. Environmental insults that produce repeated or persistent tissue 'trauma' reduce healthy functional tissue units by pushing the system into a state of repair, and beyond, also impact on functional tissue unit redundancy. Hereditary factors or genetic profiles associated with increased cancer rates alter baseline redundancy through increased entropy or excessive repair. Precise mechanisms are wide and varied (72, 73, 80–82).

An emergence framework of carcinogenesis provides a common system end-point at the level of the functional tissue unit, reduced redundancy, and hence resilience and is consistent with the multivariate findings for risk factors linked to the majority of cancers.

Principle 9

> Loss of the properties that make a functional tissue unit a 'system' is a prerequisite to create the 'initial state' for the emergence of cancer.

Prior to a new system emerging, a pre-system or 'initial state' exists. This is a point where potential components become randomly associated rather than being combined in a self-organized structure. Potential exists for the emergence of new systems (18). In carcinogenesis, this is the point where functional tissue units move so far along entropy or chaos vectors that there is a breakdown in structure and loss of function of functional tissue units, leaving only an association of tissue components with the potential to combine into a new form. At the chaos extreme, it is the excessive energy being acquired to drive repair or growth that tips the scale. At the entropy extreme, there is insufficient available energy to maintain structure and function, which causes the system to break down, releasing its tissue components to the state of chaos of the lower level. It is from these points that cancer has the opportunity to arise as an emergence phenomenon. Cancer cannot arise from healthy functional tissue units.

Principle 10

> 'Cancer' is the emergence of a new 'system' arising from the tissue components of a functional tissue unit that has lost its normal self-organization arrangement and function, identifiable by changes in morphology and physiology.

The events that occur in this 'initial state' are critical in determining the potential outcome, including apoptosis or involution, clearing by the immune system, formation of scar or fibrosis, stabilization and reformation of functional tissue units, or the formation of cancer.

Cancer has commonly been described as a state of disorder and 'chaos'. However, using the definition of 'chaos' as previously described, and the exquisite sensitivity to 'initial condition', this would imply that the likelihood of ever seeing two cancers at the same time would be extremely small (18, 40, 46). In reality, this is not the case. Over time, a specific type of cancer exhibits repetitive and largely predictable patterns in numerous patients. For example, breast cancer will most commonly spread to the axillary nodes, before spreading to other tissues, including bone and liver. Head and neck squamous cell cancer will spread to regional nodes before metastasizing to lungs. Papillary thyroid cancer spreads to the local neck nodes first, whereas follicular thyroid cancer may spread elsewhere in the body. This supports that cancer is not simply random disorganized cell multiplication, but a complex, dynamic, and evolving system. Further evidence to support this theory is the identification of fractal patterns appearing in both cancer cells and tumors (42, 83–86).

The importance of considering cancer as a system is that the components will include *all* the components that previously formed a healthy functional tissue unit. This means, for example, in solid epithelial tumors, such as head and neck squamous cell carcinomas, the 'system' includes not only the abnormal epithelial cells but the stroma as well. The importance of stroma and microenvironment is well supported by the work of many researchers (57, 59, 87–93). An emergence framework of carcinogenesis incorporates these findings.

Principle 11

> Cancer progression is the deterministic development of a sequence of rapidly adapting emergent systems, each with identifiable patterns of morphology, physiology and behavior, the dynamics of which can be studied via a state-space approach.

Defining cancer as a system in an emergence framework also enables cancer progression to be seen as a sequence of rapidly adapting emergent systems, each of which will have identifiable patterns of morphology, physiology, and behavior. Despite the likely multiple mechanisms that initiate cancer, there is a surprisingly stereotypical and deterministic pattern of cancer. Epithelial cancers follow a general pattern of growth, dysplasia, invasion, local metastases, distant metastases, and ultimately destruction of the host. Patterns of progression are seen too in sarcomas and hemopoetic cancers (29, 44, 48, 86).

The primary driver of functional tissue units toward chaos is the need for growth and repair, either in an attempt to self-salvage from entropy or in response to environmental factors. These processes require an increase in energy. This energy needs to be brought into the system from the environment (29). A persistent requirement to repair or grow will result in the need for an ongoing increased level of energy within the system. In line with Principle 6, there is a point of self-organizing criticality where the attempt of the functional tissue unit to persistently maintain higher energy levels will push it into chaos and enable the emergence of a new system with new complexity.

Evidence for this is multifaceted. In cancer cells, there is an increase in glycolysis (the Warburg effect) (29, 94–96). This phenomenon has been utilized clinically with the development of PET scans that use radiolabeled glucose analog 18-fluorodeoxyglucose to detect radiologically the higher rate of glucose metabolism found in malignancy (94–96). Originally proposed

to be due to mitochondrial respiration defects, this has now been linked to oncogenic driver mutations, such as activation of K-rags, c-Myc, and phosphatidylinositol-3 kinase, or loss of phosphatase and tensin homolog (Pten) and p53, further increasing energy production through glycolysis in addition to, rather than instead of, mitochondrial respiration (95).

Mitochondria are responsible for about 80% of normal cellular energy (29). In cancer cells, mutations have been noted in mitochondrial DNA; however, importantly, there is a lack of accumulation of mitochondrial genome errors. The errors noted do not inactivate energy metabolism, but appear to alter the bioenergetic and biosynthetic state of the cell (95, 96). Moreover, there is a strong selective pressure to retain and accumulate respiration functional mitochondria in malignant tumors, with active quality control of mitochondria through mitophagy, preventing accumulation of defective mitochondria and release of the substrates for reuse.

1–5% of oxygen consumed will produce reactive oxygen species (ROS), or free radicals (29). In cancer cells, increased demand on mitochondria to produce energy is reflected in increased ROS (29, 95, 96). High ROS production can be toxic to a cell (94–96). Increased ROS production when apoptosis is inhibited, however, changes the cell's redox status, altering activities of transcription factors, such as hypoxia-induced factor (HIF-1α) and FOS–JUN heterodimer, which constitute an AP–1 transcription factor, and lead to histone and DNA methylation, stimulating cancer proliferation (95, 96). Cancer cell ROS production inactivates caveolin 1 in adjacent stromal fibroblasts. This increases mitophagy, reduces mitochondrial function and, increases lactate production in these fibroblasts. Secreted stromal cell lactate then fuels cancer cell oxidative metabolism, which drives tumor growth and proliferation (96).

Angiogenesis is an identified hallmark of cancer. Recent data indicates that angiogenesis contributes to both the microscopic premalignant phase and ongoing phases of neoplastic progression (35). During tumor progression, an 'angiogenic switch' is almost always activated and remains on, causing normally quiescent vasculature to continually sprout new vessels, increasing nutrition and oxygen supply to tumors, and fueling energy needs that help sustain expanding neoplastic growths (35, 97). HIF-1α is a key regulator of hypoxia-induced angiogenesis in tumors (97).

Cachexia, which is characterized by weight loss and inflammation, is present in 40–80% of cancer patients, depending on tumor type, and is associated with metabolic changes involving carbohydrate, lipid, and nitrogen metabolism. These changes are linked with the need to maintain glycemia and to sustain tumor growth (98).

The observation of fractals emerging at various stages of tumor progression is also supportive of viewing cancer progression as a sequence of emergent systems. As described in the preliminary description of chaos, fractals occur in space-chaos and their appearance is reflective of deterministic development with emergence of a new system with new complexity (42, 45, 47, 85, 86).

Fractal patterns have been observed in the neo-angiogenesis patterns associated with tumors (43, 45). Cancer-specific fractal geometry has been found at the tissue level when analyzing tumor perimeters (48, 84). Recently, fractal patterns were observed with atomic force microscopy to appear on the cell surface as premalignant cells transformed into malignant cells in cervical epithelial cells (47).

Applying the systems formula, an emergence framework enables each 'stage' in the progress of cancer (invasion, local

TABLE 3 | An emergence framework of carcinogenesis: the 12 Principles.

Principle 1	Cancer is a dynamic complex system emerging at the level of the 'functional tissue unit'
Principle 2	Cancer is not a single disease entity, but an emergence phenomenon that can occur across numerous functional tissue units by multiple processes to generate a mechanism of carcinogenesis that is specific to that functional unit and may be specific to an individual tumor; the common properties of cancer can be accomplished *via* different systems utilizing different mechanisms
Principle 3	Causation of cancer is a property of the system and is not contributable at any single hierarchical level: multiscale causality associates causation at different levels concomitantly
Principle 4	The 'state' of a cell is determined by its position within the functional tissue unit and the state of the functional tissue unit as a system. All living systems metabolize and are, therefore, dynamic over time. Defining a 'default' state of a cell as quiescent or proliferative is not relevant in an emergence framework of carcinogenesis
Principle 5	A healthy functional tissue unit is a metastable system oscillating between maintaining optimum function, maximal adaptability in response to inputs and outputs with its environment, and self-maintenance through repair, differentiation and apoptosis in accordance with the natural law of a functional tissue unit as defined by: $F = \frac{k}{M}$ where F = functional status of the functional tissue unit, M = repair/growth rate, k = non-zero constant
Principle 6	A functional tissue unit will have points of self-organized criticality in both the directions of entropy and chaos, beyond which a critical collapse occurs, resulting in loss of the morphological and physiological properties of the functional tissue unit setting the initial state from which cancer can emerge
Principle 7	Reduced redundancy of healthy functional tissue units through either entropy (degeneration) or excessive repair in response to tissue trauma is associated with an increased risk of cancer
Principle 8	Risk factors for cancer act to reduce redundancy of functional tissue units *via* a number of mechanisms at a faster rate than in the natural aging process, generating an increased risk for cancer
Principle 9	Loss of the properties that make a functional tissue unit a 'system' is a prerequisite to create the 'initial state' for the emergence of cancer
Principle 10	'Cancer' is the emergence of a new 'system' arising from the tissue components of a functional tissue unit that has lost its normal self-organization arrangement and function identifiable by changes in morphology and physiology
Principle 11	Cancer progression is the deterministic development of a sequence of rapidly adapting emergent systems, each with identifiable patterns of morphology, physiology and behavior, the dynamics of which can be studied *via* a state-space approach
Principle 12	An emergence framework of carcinogenesis provides a common united framework for facilitating and integrating cancer research across all areas of basic, clinical, and translational research by directing focus on a common level at which the diagnosis of cancer is made, the functional tissue unit

metastases, distant metastases, and destruction of the host) to be considered as a new emergent property. Using the state-space methodology, ($F(t) = \{X(t), Y(t)\}$) the dynamics of each stage can be studied using data and information relevant to that stage and to the level at which investigation is being focused. Patterns can be observed, investigated, and ultimately provide guidance in management decisions.

Principle 12

> An emergence framework of carcinogenesis provides a common united framework for facilitating and integrating cancer research across all areas of basic, clinical and translational research by directing focus to a common level at which the diagnosis of cancer is made, the functional tissue unit.

An emergence framework of carcinogenesis provides a framework for translational research that is consistent and workable with the definitions of the European Society of Translational Research, the National Institutes of Health (USA), Australia's National Health and Medical Research Council, and the UK's Medical Research Council. It enables the starting point to be anywhere in the system as it directs all starting points to a common level, that of the functional tissue unit. The interplay of laboratory observations, clinical outcomes, genetic studies, community patterns, or therapeutic trials can be assessed within this framework by considering how they impact on the systems equation of the functional tissue unit and/or the emergent cancer.

Having a common framework provides a consistent format for investigating, allows for cross-referencing, enables integration, and facilitates the use of biosystematics and computational modeling.

CONCLUSION

Living systems are dynamic, non-linear, and complex. Increasingly, biology is recognized as its own discrete discipline that cannot be adequately explained by reducing living processes to their physicochemical properties.

Viewing cancer as a complex emergent system creates a new perspective that moves away from the gene-centric reductionist approach that has been the predominant theory of carcinogenesis for more than half a century. Through the integration of concepts from systems biology, physics, chemistry, and various theories of carcinogenesis, a new practical emergence framework of carcinogenesis has been proposed, based on 12 key principles. These are summarized in **Table 3**.

The motivation in developing an emergence framework of carcinogenesis was to create a common, comprehensive, and integrative framework for researchers, clinicians, philosophers, thought leaders, institutions, and funding bodies involved in researching and treating cancer. The authors have aimed to collate and synthesize current concepts and evidence around carcinogenesis into a single framework that incorporates previously incompatible viewpoints and ideas.

It would not have been possible to construct this framework without the work of the significant body of researchers, scientists, clinicians, and philosophers who have already contributed to this field. Accordingly, the reference list is limited to key publications.

Max Planck, a German Physicist known as the founder of Quantum Physics and recipient of the Nobel Prize for Physics in 1918, is quoted as saying:

> "When you change the way you look at things, the things you look at change".

We hope that this 'emergence framework of carcinogenesis' challenges the reader to look at cancer and carcinogenesis differently.

AUTHOR CONTRIBUTIONS

ES, the primary author, was responsible for the concept, development of the framework, drafting, revising, and finalizing of the article. BW contributed to the development of the concept, provided critical feedback with suggested revisions, and was involved in finalizing the article. Both ES and BW agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.2. Application and Implications

Applying a different framework to understand aetiology, progression and treatment of cancer does not mean ignoring or discounting the large body of work or advances achieved to date. Nor does it obviate the role of basic bench top science to acquire detailed understanding of molecular biology. An emergence framework of carcinogenesis employs the philosophy of systems biology, complexity and emergence to provide a *practical* systems approach enabling a new perspective on how this knowledge can be synthesized and translated into clinical application.

Using a systems approach, cancer becomes viewed as a 'process', carcinogenesis, rather than a 'thing' and its emergence is due to the disruption of the inherent coordination and harmony of healthy state of the system in which it arises.[162, 167, 182] Central to this is clearly defining the healthy system from which cancer arises.[240] **Principle 1**, which states "Cancer is a dynamic complex system emerging at the level of the 'functional tissue unit'." provides this definition in both a qualitative and quantitative form, applicable to any tissue and any cancer. There is no tissue in the human body that does not have a definable property or function. Whether that property or function is essential to whole organism survival is another discussion. Tonsils and an appendix can be removed without impact, but it is not possible to survive without a liver. This does not mean that tonsils or appendices do not have a FTU; rather it implies that the function or property of that FTU is not essential in the higher system level of the whole organism.

In accordance with **Principle 12**, defining the FTU is always the key to integrating any cancer research into the bigger picture whether starting in the lab or with a clinical observation. Having a common definition of the FTU enables a mechanism for connecting lab based research and clinical research, facilitating bidirectional translational research. It helps to identify which lab based discoveries are more likely to have clinical relevance and which clinical questions are important for lab based researchers to be focusing on. A common FTU provides a point for integration across disciplines, research institutions and countries. As per **Principle 2**, "Cancer is not a single disease entity, but an emergence phenomenon that can occur across numerous functional tissue units by multiple processes to generate a mechanism of carcinogenesis that is specific to that functional unit, and may be specific to an individual tumour; the common properties of cancer can be accomplished via

different systems utilizing different mechanisms." Whilst this thesis is focused on carcinogenesis, the concept of FTUs could be applied to other disease processes and to aging. One could imagine the value of an international library of defined FTUs built upon and available to any researcher in the world.

Principle 1 provides a systems formula to define a FTU. A shift from linear thinking is required in applying a systems approach in contrast to considering the role of a single gene, peptide, cytokine, growth factor, transcriptional factor, risk factor or predictive factor. A four square summary map (*Fig. 2*) is a good tool for brainstorming and providing the overall summary before developing the detail. This also enables comparison of different FTUs which may be useful in understanding why some tissues are more prone to developing cancers than others, such as carcinomas arising from epithelial tissues being much more common that sarcomas arising from connective tissues.

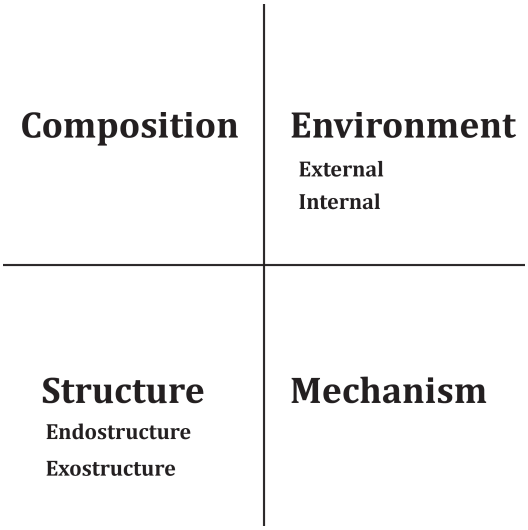


Figure 2 Four-square CESM summary map

A visual tool to enable brainstorming, provide a start point and identify where detail needs to be filled in.
Original Diagram designed from text in reference.[159]

Once the relevant FTU has been defined, the next step is to apply **Principle 2** and define the 'cancer system' relevant to the FTU and the hypothesis being proposed. This involves analysing what has changed in the FTU system to produce the specific 'cancer system'. (*Fig. 3*) **Principle 9** is applied to identify what system property has been lost and what new system property has been gained. **Principle 10** applies here

to ensure that all the components of a normal FTU are accounted for and identify how they have been altered or re-organized, and how the physiology, which encompasses the mechanism(s) have been modified. Research that focuses on carcinogenesis, which may include gene mutations, chronic inflammation or lifestyle risk factors, can then be investigated in terms of the impact they have on the FTU components and/or environment and/or structure and/or mechanism.

Depending on the nature of the investigation, **Principle 3, Principle 4, Principle 5, Principle 6, Principle 7** or **Principle 8** may be of relevance. Research that focuses on cancer progression, which may include prognostic markers, treatment strategies or drug development, will employ **Principle 11,** which considers how the specific cancer continues to evolve. The hypothesis or clinical question determines which further principles are employed and the details (*Fig. 3*).

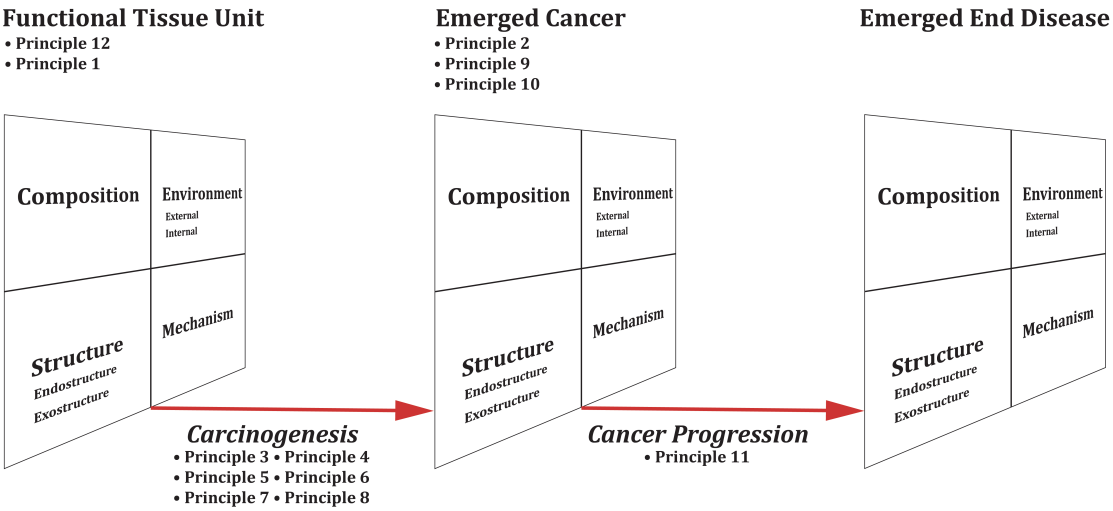


Figure 3 Cancer and cancer progression as emerged complex systems

Starting from the Functional Tissue Unit both the Emerged Cancer and the ultimate Emerged end Disease can be mapped. The various Principles applied depend on the nature of the investigation being undertaken. The starting point for any investigation is always the Functional Tissue Unit.
Original drawing.

The use of a common framework for different cancers offers a mechanism for comparing cancer systems. This could help build an understanding as to why some factors in one cancer system may be an indicator of poor prognosis but an indicator of a good prognosis in another. As an example, regulator T-cells are have been associated with a poor prognosis in HNSCC but a positive correlation has been

reported in other cancers such epithelial ovarian cancer, colorectal cancer and lymphoma.[241] The same process could be utilized to assess why various chemotherapy or immunotherapy treatments have different response rates in different cancers, or alternatively identify potential roles for use of existing agents in different settings.

Finally, an emergence framework provides an integrated framework for carcinogenesis, enabling existing theories to become compatible as alternative mechanisms. The inclusion of a mathematical basis facilitates the integration of bioinformatics and the structure provides a pathway for translational research to flow from both 'benchtop to bedside' and 'bedside to benchtop'.

4. Defining a Functional Tissue Unit: The Oral Mucous Membrane

In this Chapter, OSCC is used as an example of how to apply "An Emergence Framework of Carcinogenesis", utilizing **Principle 12**, and **Principle 1**, to identify and define the relevant FTU. HNSCC is described as arising from the epithelium of the upper aerodigestive tract. [7, 13-15] The relevant FTU of the upper aerodigestive tract that is affected is, however, not the surface squamous cell epithelium, but the mucous membrane. Mucous membranes throughout the body line internal organs and passages that come into contact with the exterior environment. The primary functions of the mucous membrane of the upper digestive tract is as a barrier function to protect underlying tissues from mechanical and chemical damage, prevent the entry of outside pathogens and debris and provide lubrication for the passage of ingested and inhaled substances.[242-244]

In an emergence framework, these functions represent the emergent properties of the mucosal membrane system. There are variations of the mucous membrane across the different sites of the upper aero digestive tract, reflective of differing embryological origins and functions. This in part explains the differences in biological behaviour of HNSCC based on site of origin. For this thesis, the focus will be on the oral cavity and the FTU will be 'Oral Mucous Membrane' (OMM).

To understand the system within this framework the qualitative equation,

$$\mu_{abcd}(s) = \{C_a(s), E_b(s), S_c(s), M_d(s)\}$$

is used.

$\mu_{abcd}(s)$ becomes the system, μ_{abcd} (omm), where 'omm' is abbreviation for 'oral mucous membrane', $C_a(s)$ is the components of the system, $C_a(omm)$, $E_b(s)$ is the environment that the OMM interacts with, $E_b(omm)$,$S_c(s)$ represents the endostructure and exostructure of the mucous membrane, $S_c(omm)$ and $M_d(s)$ are the mechanism(s) by which the system forms and produces it emergent properties, $M_d(omm)$, so that the qualitative equation becomes:

$$\mu_{abcd}(omm) = \{C_a(omm), E_b(omm), S_c(omm), M_d(omm)\}$$

To be workable, the challenge is to stay focused on the level of interest, the FTU. This is achieved by using the concept of logical intercept to limit the discussion to what is relevant at tissue level. The four-square CESM summary map appears as in *Fig. 4.*

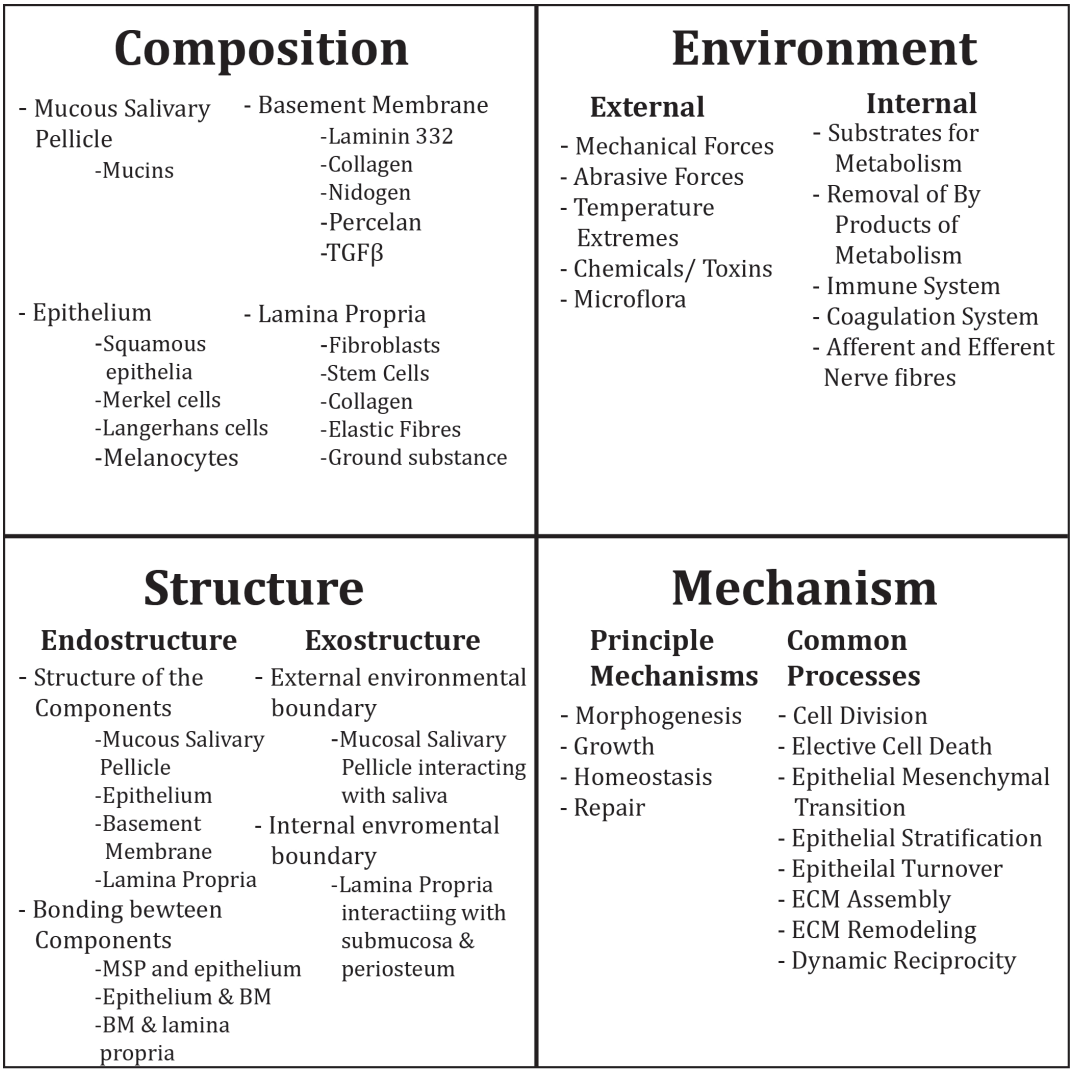


Figure 4 The oral mucous membrane represented in a four-square CESM map

Each of the elements of a complex system can be placed into a CESM summary map. This provides an overview of the system and common starting point for investigations. Note that some elements may appear in multiple parts of the system.
Original drawing.

Each of the system parameters is considered in turn.

4.1. Composition

Determining the tissue level composition, or components, of the OMM requires identifying the components necessary for the μ_{abcd}(omm) to perform its' function.

The components of the OMM are the mucous salivary pellicle, the stratified squamous epithelium, the basement membrane and the lamina propria (*Fig.5*). If any of these components are missing or significantly altered, function is lost.

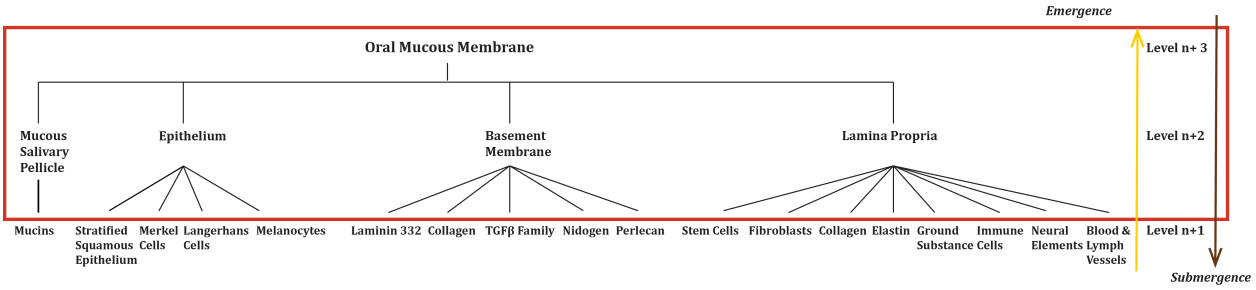


Figure 5 The oral mucous membrane functional tissue unit

The logical components, Ca(s), of the 'functional tissue unit' are the tissue components at the level below, Level *n* + 2. Components at lower levels are important only through the impact they have on the components, Ca(s), by changing the components themselves and/or changing the structure, Sc(s). The logical components of the functional tissue unit of the oral mucous membrane are the mucous salivary pellicle, epithelium, basement membrane and lamina propria. Original diagram.

4.1.1. Mucous Salivary Pellicle

The mucous salivary pellicle (MSP) is a highly hydrated and viscous gel formed by large salivary glycoproteins, mucins. [242-244] These are grouped into 3 sub families: secreted gel-forming mucins, secreted non-gel forming mucins and membrane-bound mucin. Secreted gel-forming mucins, the major constitute of mucous, appear as networks through the arrangement of monomers in homo-oligomeric structures. [242, 245] The secreted non-gel forming mucins can self-aggregate but do not form gels and do not contribute significantly to the properties of mucous.[245] The membrane-associated mucins are typical monomeric, membrane-anchored glycoproteins, which do not form gels.[242, 246]

In the oral cavity, the salivary glands, submucosal glands, and goblet cells secrete mucins.[242, 243, 245-247] MUC5B is primarily produced by goblet cells in the submandibular and sublingual glands.[246, 248] It is composed of approximately 80% carbohydrate in the form of O-linked glycan chains and 20% protein, forming the peptide backbone. MUC5B's complex structure allows it to interact with an array of different salivary proteins and microbes to maintain a healthy oral cavity.[245, 248] MUC19 is another gel forming secreted mucin, and MUC7 is a non-gel forming secreted mucin, both of which are found in saliva.[242, 245, 247]

MUC1 is the primary membrane bound mucin in the OMM, [242, 245, 247] synthesized by both salivary glands and epithelial cells.[242, 249] It is anchored to the apical surface of epithelial cells present in the superficial and intermediate layers[242, 245, 249] and is not expressed on basal cells.[249] MUC1 is monomeric, does not form gels, and has a characteristic membrane peptide domain and properties typical of membrane glycoproteins.[242, 245] The large extra cellular component can be shed via a second cleavage event or physical shear forces.[245] MUC15, another membrane bound mucin, is also expressed in the OMM.[245, 247]

4.1.2. Epithelial Layer

The epithelium of the OMM is a stratified squamous epithelium that provides protection against mechanical, microbial, and chemical damage. It consists of tightly packed epithelial cells with varying degrees of differentiation, and morphology dependent on the region and type of mucosa. [250-252] The epithelial layer contains Merkel cells and two types of dendritic cells, Langerhans cells and melanocytes. [252-254]

4.1.2.1. Squamous Epithelium

Epithelial characteristics at the cellular level include cohesive interaction between cells allowing the formation of continuous cell layers, three types of membrane domains: apical, lateral and basal, the existence of tight junctions between apical and lateral domains, polarized distribution of different organelles and components of the cytoskeleton and the quasi immobility of the group of epithelial cells relative to the local environment.[255]

The epithelium of the OMM is stratified squamous epithelium and can be divided into: masticatory (hard palate and gingival), specialized (dorsal surface of the tongue) and lining (buccal mucosa, ventral surface of the tongue, soft palate, intra-oral surfaces of the lips and alveolar mucosa). The epithelium of the masticatory mucosa of the gingiva and hard palate is keratinized and firmly attached to the underlying bone via a mucoperichondrium.[250, 251, 256, 257] The specialized epithelium on the dorsum of the tongue is firmly fixed to the underlying muscle and can be represented as a mosaic of keratinized and non-keratinized epithelium formed by papillae, termed filiform, fungiform and

circumvallate papillae,[251, 257] the later two associated with taste buds. Circumvallate papillae are the largest papillae and are found in the posterior region of the tongue in a "V" configuration.[251] The remaining lining mucosa, which covers about 60% of the oral cavity, has epithelium that is non-keratinized.[250, 251, 256, 257]

The OMM epithelium relies on epithelial stem cells for tissue renewal. Normal tissue stem cells constitute a life-long reservoir of cells with active mechanisms for self-renewal. Cell division in OMM epithelial cells takes place mainly in the basal layer that contains the adult stem cells.[257]

4.1.2.2. Merkel Cells

Merkel cells are present in the basal layer, usually adjacent to nerve endings.[252, 254, 258] They are commonly seen in masticatory mucosa, but are usually absent in lining mucosa. Merkel cells differ from other nonkeratinocytes in that they are not dendritic. It is believed they play a role in somatosensation, chemosensation and have an endocrine function.[258]

4.1.2.3. Langerhans Cells

Langerhans cells are antigen-presenting cells and part of the immune system.[252-254] Langerhans cells originate from the bone marrow and migrate into the epithelium to perform the function of antigen recognition and presentation. The cell bodies of Langerhans cells sit in the suprabasal region with dendritic processes usually directed towards the surface epithelium and may be found in the stratum corneum.[253]

4.1.2.4. Melanocytes

Melanocytes produce the pigment, melanin.[252, 254, 259] The number of melanocytes in the suprabasal layer of mucosa under normal physiological conditions is the same regardless of ethnicity but the amount of melanin produced is genetically determined. Melanocytes function as stress-sensors having the capacity both to react to and to produce a variety of micro environmental cytokines and growth factors, modulating immune, inflammatory and antibacterial responses. Melanocytes act as neuroendocrine cells producing local neurotransmitters including acetylcholine, catecholamines and opioids, and hormones of the melanocortin system that participate in intracellular and in intercellular signalling pathways,

contributing to tissue homeostasis. Melanin provides protection from environmental stressors such as ultraviolet radiation and reactive oxygen species.[259]

4.1.3. Basement Membrane

The basement membrane (BM) is a nanoscale sheet of extracellular matrices indispensable for tissue architecture, mechanical stability and acts as a protective barrier against cell invasion.[260-263] It is comprised of the plasma membrane of the basal epithelial cell, the lamina lucida with the core structural component laminin 332 (LM332), and the lamina densa formed by collagen IV, nidogens, the heparin sulfate proteoglycans (HSPGs) perlecan and agrin, and the anchoring fibrils. [252, 264] Various growth factors, many belonging to the TGFβ superfamily, are found tethered to the BM and act to provide specific signals to BM-adherent cells. Proteinases and their inhibitors, regulatory macromolecules and serum factors are found associated with the BM. Collectively, these components, provide cell and tissue support and act as complex signalling platforms.[264]

4.1.3.1. Laminin 332

Laminins (LM) are a family of extracellular matrix glycoproteins that constitute a major component of the BM. They are heterotrimeric proteins composed of one heavy chain, α, and two light chains, β and γ. Each laminin chain consists of a globular domain, a rod-shaped, epidermal growth factor (EGF)-like laminin-repeat domain and an α-helical coiled-coil region in the long arm. The α, β and γ chains associate by forming a triple-helical coiled-coil linked by disulphide bonds. Different combinations of the various chains form the laminin isoforms that are cell and tissue specific. At least fifteen distinct laminin isoforms are known.[214, 261, 265]

LM332 (*Figure. 6)* is the major adhesive component of epithelial BMs. It consists of α3, β3 and γ2 chains (*Fig. 6*). The β3 chain and γ2 chain are found only in LM332. All three chains are truncated at the N-terminal preventing self-association.[214, 265-267]

The α3 chain is the largest chain. It contains the long arm on the C-terminal and the short arm on the N-terminal. The C-terminal end is characterized by a large globular domain (G-domain), which is involved in interactions with

cellular receptors such as the integrins and dystroglycans. The N-terminal of the short arm is composed of EGF-like laminin repeats (LE modules) and is truncated compared to the α chain of other laminins.[214, 261, 265]

The β3 chain is characterized by a small interruption of amino acids in the coiled-coil segment termed Lβ (previously called 'α'). It is rich in glycine and cysteine. There is no globular domain at the C-terminal. The short arm N-terminal is similar to the α chain being composed of EGF-like laminin repeats and truncated compared to other isotypes. It contains a single globular domain, LN. [214, 261, 265]

The γ2 chain has a long arm C-terminal without a globular domain and a short arm that contains EGF-like laminin repeats and a globular domain but lacks a LN globular region. [214, 261, 265]

Different genes on different chromosomes encode each chain generating complexity of regulation of genetic expression. The individual laminin subunits are glycosylated with high mannose oligosaccharide side chains at the N-terminus within the rough endoplasmic reticulum, stabilizing the subunits and protecting them from degradation. Laminin trimer assembly begins first by the stable association of the β and γ chains. The α chain then combines with this dimeric complex in order for the trimeric molecule to be secreted. This is the rate-limiting step. The three chains bind together via their long arms forming a triple helical coiled-coil. Toward the extreme N-terminal, the three chains combine through the action of disulphide bridges, thus providing greater stability for the trimer assembly. The laminin trimer undergoes terminal glycosylation within the Golgi organelle and is secreted out of the cell and deposited in its unprocessed form. [214]

LM332 undergoes multiple post-translational modifications that alter its structure and biological activity. A number of different enzymes have been shown to cleave the glycoprotein at various domain sites. Important among these are the serine proteases, matrix metalloproteinases (MMP's), and bone morphogenic protein-1/ mammalian Tolloid metalloproteinase family (BMP).[214, 268-272]

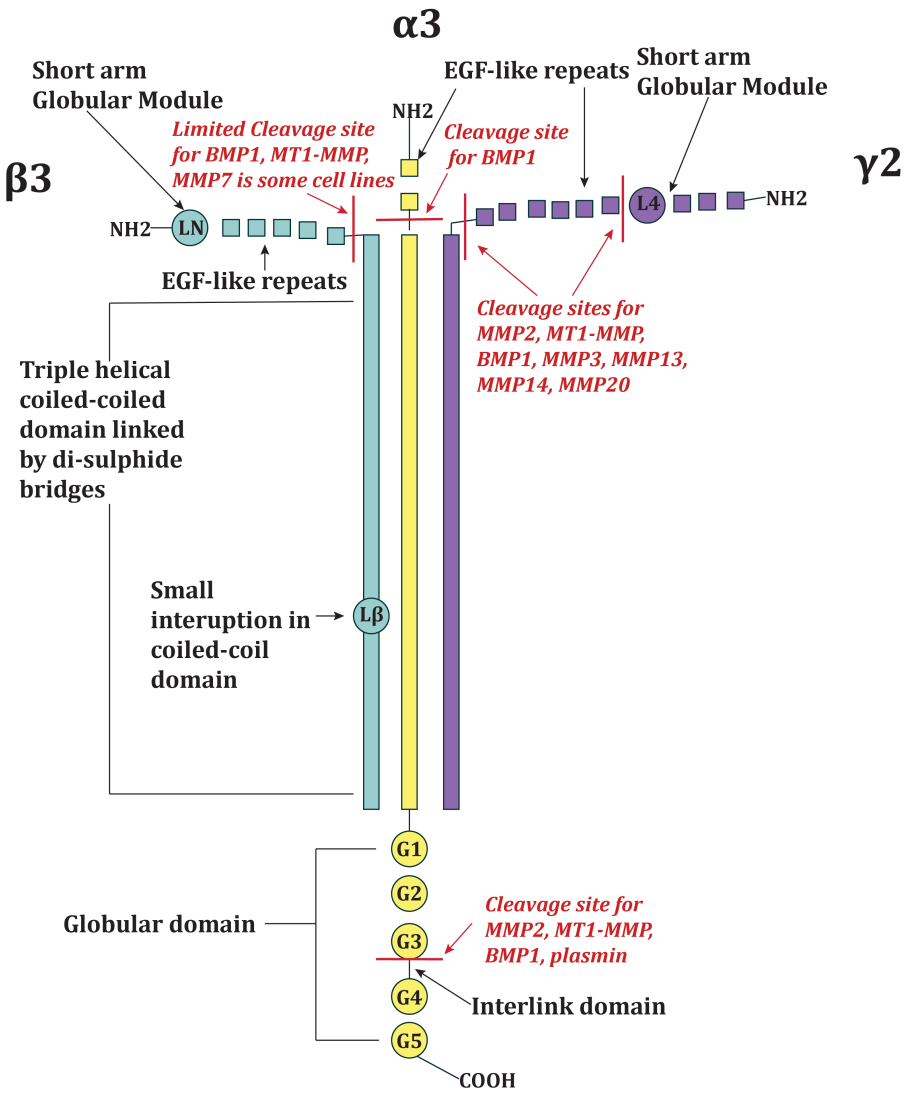


Figure 6 Schematic drawing of the structure of LM332 showing the major sites of cleavage

LM332 is a heterotrimer made up of a heavy α3 chain and β3 and γ2 light chains. Cleavage at different points leads to changes in physiological activity
Original drawing based upon drawings and text description from various references.[214, 230, 261, 263, 265, 268, 272-274]

The majority of α3 and γ2 chains in secreted LM 332 undergo modification. In contrast there appears to be minimal post secretionary processing of the β3 chain.[214, 271, 275]

The α3 chain is cleaved in the globular domain in the hinge region between LG3 and LG4 by plasmin, MMP2, MT1-MMP and BMP1. The LG4-LG5 fragment binds with syndecan and dystroglycan. The exposed LG3 can then interact with the integrin α6β4 on the basal surface of the cells.[214, 268, 272]

The γ2 chain is cleaved in the short arm at the N-terminal end of domain LEb.[214, 268, 270, 271, 276, 277 272] MMP2, MT1-MMP and BMP-1 are able to cleave the chain at this site.[214, 268, 270-272] MMPs 3, 13 and 20 cleave the short arm of the γ2 chain.[12] MMP-14 has been implicated in cleavage of a second site.[271]

LM332 is secreted into the extracellular matrix and undergoes specific proteolysis for different biological activities that are mediated via integrins. Normal biological functions include; mediating ECM-cell and cell-cell interactions, and regulation of cell adhesion, migration, proliferation, survival and differentiation. It thus has a pivotal role in stabilizing the epithelium to the basement membrane and in wound healing.[265, 266, 268, 270, 272]

4.1.3.2. Collagens

Collagens are extracellular matrix molecules used by cells for structural integrity and a variety of other functions. The collagen superfamily comprises 28 members numbered I–XXVIII. The common structural feature of collagens is the presence of a triple helix, consisting of three polypeptide α chains. Further diversity occurs in the collagen family due to several molecular isoforms for some collagen types (e.g. collagens IV and VI). [278, 279]

4.1.3.2.1. Type IV Collagen

Type IV collagen, a non-fibrillar network collagen, is present in all BMs. Non-fibrillar collagens differ from connective tissue fibrillar collagens by the presence of globular or rod like, noncollagenous domains (NC domains). Type IV collagen is made up of 6 different α chains that can assemble into 3 different heterotrimers. Each type IV collagen α chain consists of three domains: an N-terminal 7S domain, a middle triple-helical collagenous domain, and a C-terminal globular noncollagenous (NC1) domain. The collagenous domain provides for the structural integrity of the type IV collagen protomer and suprastructure. Numerous short sequence interruptions in the collagenous domain ensure flexibility. Both the 7S and NC1 domains are critical for type IV collagen network formation. [280-282]

Type IV collagen is secreted from cells in the form of a protomer. Protomers are the building units of type IV collagen network. The NC1 domain limits each a-chain's ability to associate with other α -chains at random.[282]

4.1.3.2.2. Type VII Collagen

Type VII collagen a non-fibrillar collagen that forms a major component of anchoring fibrils involved in attaching the lamina densa to the lamina propia. Type VII collagen is expressed in the BMs of stratified squamous epithelia including the OMM.[278, 279, 283] It is composed of three identical α chains, each consisting of a central collagenous triple-helical portion flanked by non-collagenous domains., at the amino (NC1) and carboxy-terminus (NC2). Two molecules form antiparallel tail-to-tail dimers stabilized by disulfide bonding through an overlap between NC2 domains.[279, 283, 284] Both fibroblasts and squamous epithelial cells have been shown to secrete type VII collagen.[285] It is secreted as a procollagen and matures through cleavage by proteinases, including MMPs.[278]

4.1.3.3. Nidogen

Nidogens are proteoglycans constituting 2-3% of the BM. The nidogen protein core consists of three globular domains, G1 (N-terminal) and G2 and G3 (C-terminal). A long rod-like region separates G1 and G2 and a short link is between G2 and G3. Nidogen's composition is 10% carbohydrates with equal proportions of N-linked and O-linked oligosaccharide chains.[281, 282]

Nidogens are mainly expressed by mesenchymal cells and are deposited into the BM during development. Nidogen has a strong affinity for LM332 and type IV collagen.[281]

4.1.3.4. Perlecan

Perlecan is a heparin sulphate proteoglycan (HSPG) composed of five domains (I-V). Domain I at the N-terminus is covalently linked to three glycosaminoglycan (GAG) chains.[281, 282] Perlecan is one of the few gene products expressed by both vascular and non-vascular tissue. It acts as a strong elastic tether and a modulator of cell adhesion, proliferation and differentiation.[264] These functions are mediated by controlling growth factor signalling and activation of fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs), platelet derived growth factor (PDGF), vascular endothelial growth factors (VEGFs), transforming growth factors (TGFβ), epithelial growth factor (EGF), hepatocyte growth factor (HGF), and insulin-like growth factor

(IGF).[281]

4.1.3.5. TGFβ Family

The transforming growth factor-β (TGFβ) superfamily is a large family of more than 30 structurally related cytokines including three TGFβs, two activins, bone morphogenetic proteins (BMPs), NODAL, growth and differentiation factors (GDF) and anti-Müllerian hormone (AMH). [215, 286-288] Members of this superfamily have an important role in numerous cellular processes, including proliferation, adhesion, motility, extracellular matrix production, angiogenesis, apoptosis, differentiation, and immune regulation.[286-288] Aberrations in these processes have been linked to various diseases, including cancer.[215, 287-294]

All ligands of the family share similar properties regarding their structure, synthesis, signal transduction mechanisms and regulation.[286] Synthesized as precursors, with a large pro-domain and a carboxy-terminal mature domain, the mature ligands are cleaved from the precursor by pro-protein convertases. The ligands then form homomeric or heteromeric dimers, held together by disulphide bonds.[287] All act through binary combinations of transmembrane serine–threonine kinase receptors, grouped into Type I and Type II TGF- β receptors (TβRI and TβRII). The receptors have dual specificity acting as tyrosine kinases in addition to serine–threonine kinases, producing activation of different signalling pathways. [215, 286-288, 295]

4.1.3.5.1. TGFβ

Transforming growth factor-β (TGFβ) exists in three different isoforms, TGFβ1, TGFβ2 and TGFβ3,[291, 296, 297] All are synthesized as homodimeric pro-TGFβ, consisting of TGFβ that is covalently linked to the latency-associated protein (LAP). Pro-TGFβ is then intracellularly cleaved by furin-type enzymes to generate the mature TGFβ which remains non-covalently associated with its LAP, forming a small latent complex (SLC). This association prevents recognition of TGFβ by its high affinity receptors and further downstream signalling. [289, 298, 299]

The majority of cell types secrete TGFβ as part of a large latent complex (LLC), formed within secretory vesicles by establishing a covalent disulfide bond between the SLC and a family member of the latent TGFβ-binding

proteins (LTBP). On secretion the LLC is sequestered in the extracellular matrix (ECM) through the binding of LTBP to other ECM proteins including fibrillin-1 and vitronectin, creating a reservoir of TGFβ in the BM and ECM.[289-291, 298, 300, 301]

4.1.3.5.2. BANGS

Other members of the TGFβ superfamily comprised of bone morphogenetic proteins (BMPs), activins, anti-Müllerian hormone (AMH), NODAL, and growth and differentiation factors (GDF) are collectively known as BANGs.[215, 286-288] These proteins share the same dimeric structure that is classic of the TGFβ superfamily and in a similar fashion to TGFβ subfamily are released as a precursor and become activated on cleavage of the pro-peptide.

BANGs in a similar manner to TGFβs are bound by various binding factors in the ECM.[287, 293] These binding proteins inhibit the signalling of BANGs by preventing binding with TGF-β receptors, however the binding proteins themselves have a complex interplay which can result in agonist activity.[293] The function of extracellular binding proteins in controlling ligand access or acting as ligand reservoirs is an important factor in the establishment of the TGFβ superfamily morphogen gradients that control early embryonic patterning.[287, 293, 302] Regulation of receptor complex formation is a further important mechanism to control BANG mediated signalling.[287, 293]

4.1.4. Lamina Propria

The lamina propria (LP) is an underlying supportive layer of dense connective tissue composed of cells, predominantly fibroblasts, and fibers embedded in ground substance composed of proteoglycans (hyaluronan, heparan sulphate, syndecan, decorin) and glycoproteins (fibronectin, tenascin).[250] Other cells found in varying numbers include leukocytes, macrophages, mast cells, and numerous blood vessels in the form of capillaries that loop into the connective tissue papilla. Lymphatics and neural elements are also present. The latter include intraepithelial nerve fibers, organized nerve endings (lamellar, coiled, or glomerular) and Merkel cell–neurite complexes. [250, 252]

4.1.4.1. Fibroblasts

Fibroblasts are one of the most abundant cell types in connective tissues and

are the major cell of the LP. These spindle shaped cells are mesenchymal in origin and are responsible for tissue homeostasis under normal physiological conditions.[303, 304] Fibroblasts in the LP of the OMM secrete collagens for both the BM and LP, and the proteoglycans and glycoproteins that form the ground substance of the LP. [250] Fibroblasts produce the nidogens found in the basement membrane[305] and are a major producer of MMPs, which are capable of degrading the ECM.[303]

The fibroblast cytoskeleton contains actin, microtubules and the intermediate filament, vimentin. Microtubules are rigid hollow rods composed of the globular protein, tubulin. Tubulin is a dimer consisting of two closely related polypetides, α-tubulin and β-tubulin. Tubulin dimers polymerize to form microtubules around a hollow core. The microtubules are polarized with a fast growing plus end and a slow growing minus end and undergo continual assembly and disassembly within the cell. A third type of tubulin, γ-tubulin is specifically located at the centrosome, located near the nucleus, which anchors the minus end of the microtubules and has a key role in determining the intracellular organization of the microtubules. Microtubules play a central role in fibroblast movement.[306, 307]

4.1.4.1.1. Vimentin

Vimentin is a major constituent of a large family of proteins, intermediate filaments (IF), which are involved in formation of cytoskeletons. It is ubiquitously expressed in normal mesenchymal cells and commonly used to distinguish mesenchymal cells from epithelial cells. Vimentin is sometimes expressed in migratory epithelial cells during wound healing and embryogenesis. It helps to maintain cellular integrity and provide resistance against stress.[127, 308-310]

Vimentin is first expressed during development in the mesoderm between the primitive streak and the proximal endoderm. Initially it is widely expressed in the embryo but is progressively restricted to fewer cell types during terminal differentiation. [311, 312]

Numerous factors that positively regulate vimentin have been identified. These include NF-κB, STAT-1 and STAT-3, c-JUN and TGFβ.[311, 312] The only

direct inhibitors identified are the zinc- finger proteins ZBP 89 and ZBP 99. There is evidence these factors may act via epigenetic modifications.[311] Epigenetic mechanisms are emerging as major pathways for regulation of vimentin expression. Methylation of the gene has been described and histone deacetylase 1 in the promoter region has been demonstrated to repress expression. [308, 311, 313]

Posttranslational modifications play a key role in the regulation of vimentin assembly and disassembly. Known posttranslational modifications include phosphorylation, glycosylation, ubiquitylation, sumoylation, acetylation, farnesylation, transamidation, and ADP-ribosylation. Phosphorylation helps regulate vimentin structural reorganization during cytokinesis and mitosis.[308, 313, 314]

The structure of vimentin confers super-elastic and highly flexible properties to a cell. A single filament can be stretched up to three times its resting length. Under high strain, vimentin tends to harden but shows high flexibility under low strain[313] creating an important mechanical role in the cells in which it is expressed. Vimentin filaments extend from extracellular to the nucleus and interact with other structural materials such as actins and microtubules to provide stiffness to the cytoplasm and transmit mechanical signals to the rest of the cell. This enables change in shape to maintain cellular integrity and maintains position of organelles in the cytoplasm. [308, 313-316]

Fibroblasts display a spectrum of phenotypic entities ranging from the non-contractile fibroblast to the contractile myofibroblast (*Fig. 7)*.[303, 304] Activated fibroblasts show characteristic features that include expression of vimentin in the absence of desmin, α-smooth muscle actin, abundant endoplasmic reticulum and prominent Golgi associated with the synthesis and secretion of ECM molecules as well as proteases capable of degrading the ECM. Cytoskeletal proteins in association with cell surface integrins and the ECM facilitate cell motility and the generation of contractile forces important in tissue homeostasis and wound healing.[303]

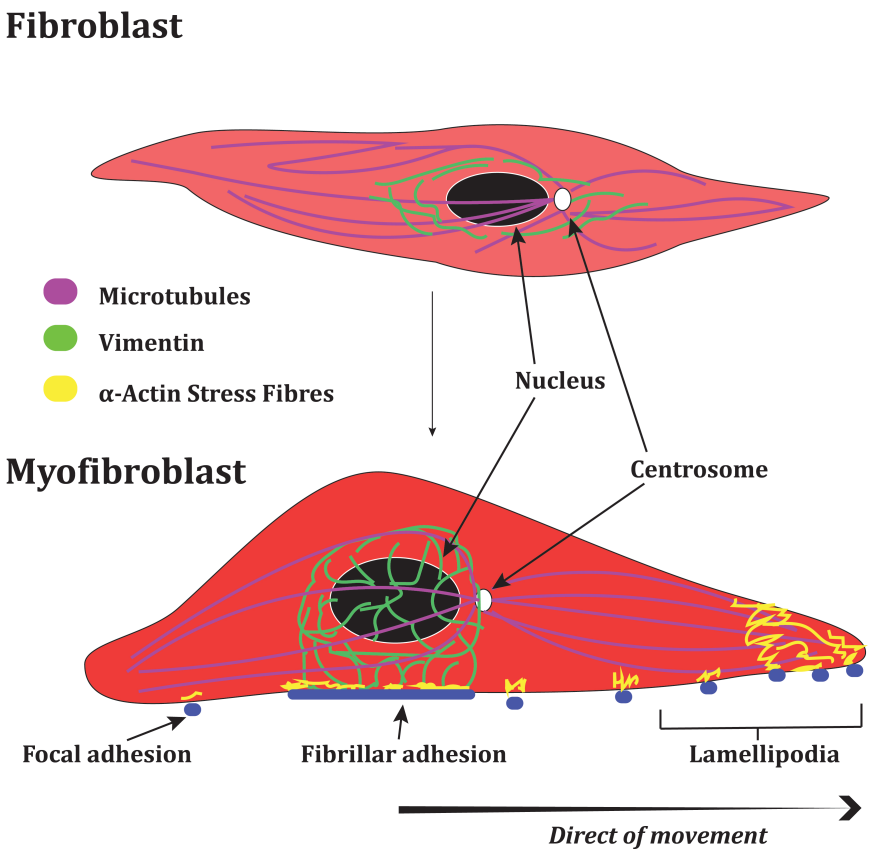


Figure 7 Schematic drawing of fibroblast and activated myofibroblast

Fibroblasts display a range of phenotypes from non-contractile fibroblast to contractile myofibroblast. Compared to non-contractile fibroblasts, myofibroblast express α-actin stress fibres and increased ED-A fibronectin. They have focal adhesions, fibrillar adhesions and lamellipodia. Original drawing based upon drawings and text description from various references.[304, 307, 317-321]

Prototypical myofibroblasts are distinguished by the presence of stress fibres expressing α-smooth muscle actin, bundles of actin fibers cross-linked by α-actinin, linked in a linear fashion through trans-membrane fibronexus junctions to protruding filamentous fibres, increased expression of extra domain A (ED-A) fibronectin and gap junctions. (*Fig.6)* Myofibroblasts are differentiated from smooth muscle cells by their general lack of smooth muscle markers including desmin and smooth muscle myosin.[303, 304] Both fibroblasts and myofibroblasts play a critical role in wound healing by generating traction and contractile forces, respectively, to enhance wound contraction.[303, 304, 322]

Circulating bone marrow-derived fibrocytes and de-differentiation of epithelial cells via the process of epithelial-mesenchymal transition have been shown to contribute to the population of fibroblasts in the LP.[303, 304]

4.1.4.2. Stem Cells

Oral mucosal lamina propria-progenitor cells (OMLP-PCs) are of neural crest origin, are multipotent and identified in the LP. These are phenotypically similar to fibroblasts and are responsible for replacement and/or expansion of fibroblast/myofibroblast populations homeostatically and in wound healing or disease settings.[323-326]

OMLP-PCs have potent immunosuppressive properties, suppressing lymphocyte proliferation through secreted factors. They have been shown to have a direct antibacterial action against both gram-positive and gram-negative bacteria in a bacteriostatic manner through the release of the differentially acting soluble factors osteoprotegerin and haptoglobin.[326]

4.1.4.3. Collagens

The LP of the OMM contains collagen fibres types I, III, V, and VI.[250, 278]

4.1.4.3.1. Fibrillar Collagens

Fibrillar collagens I, III, V contain one major triple-helical domain and have at least one imperfection or interruption in their triple helices.[278, 279] The amino end of a fibrillar collagen, called the N-peptide, contains at least one small minor helix. The collagen molecules are aligned in a quarter stagger arrangement in the growing fibril. Fibrils have an alternating light and dark pattern in electron micrographs, leading to them being called banded fibrils.[279]

4.1.4.3.2. Type VI Collagen

Type VI collagen is a network collagen ubiquitously expressed in ECMs.[278, 279, 327] Five different α chains enables a variety of isoforms. In the type VI α chains, the short collagenous domain is flanked on each side by von Willebrand factor A. To make the type VI collagen supramolecular aggregate, molecules form dimers, the dimers form tetramers, and the tetramers form end-to-end associations that yield the ultrastructural appearance of beads on a string.[278, 279] The major function of type VI collagen is as an anchoring meshwork that connects collagen fibres and other structures such as nerves and blood vessels to the surrounding matrix. Type VI collagen interacts directly with type IV collagen of the BM.[327]

4.1.4.4. Elastic Fibres

Elastic fibers are present in the non-keratinized lining mucosa formed from fibrilin scaffolds and an amorphous substance. [250, 256, 328] In the OMM the elastic fibres, oxytalan and elaunin are more prominent than elastin. Both oxytalan and elaunin consist of fibrillin microfibrils. Elastin is only incorporated in small quantities in elaunin fibrils. Oxytalan fibres are expressed in tissues subjected to mechanical stress, and have a similar role as elastin.[328]

4.1.4.5. Ground Substance

4.1.4.5.1. Proteoglycans

Proteoglycans comprise a core protein to which one or more glycosaminoglycan chains (GAGs) are covalently attached. GAGs are unbranched, long polysaccharides consisting of a repeating disaccharide, generally an acetylated amino sugar alternating with an uronic acid.[329, 330] The main proteoglycans in the LP of the OMM are hyaluronan (hyaluronic acid), heparan sulphate, syndecan, and decorin.[250, 252]

4.1.4.5.1.1. Hyaluronan

Hyaluronan takes part in a number of essential processes in development, repair, and disease. It is synthesized at the cell surface and released to the extracellular environment without passage through the Golgi. Hyaluronan can be modified by covalent linkage to inter-α-inhibitor family members, a group of serine protease inhibitors. Cross linkage is catalyzed by tumour necrosis factor (TNF)–stimulated gene 6 forming a complex that is anti-inflammatory. Hyaluronan interacts non-covalently with a number of ECM components and may be bound at the cell surface by receptors such as CD44. This may be important in enhancing signalling through cell surface growth factor receptors.[329]

4.1.4.5.1.2. Heparan Sulphate

Heparan sulphate is synthesized in the Golgi and under goes many modifications to produce a complex structure. It is important for growth factor sequestration, protection from proteases, and presentation in the correct configuration to cell receptors. A key property of heparan sulfate on the cell surface is the concentration of ligands for signalling through

high-affinity receptors. Heparan sulfate controls morphogen gradients necessary for tissue differentiation via WNT and Hedgehog signalling pathways and is broken down by heparinases.[329]

4.1.4.5.1.3. Syndecans

Syndecans are transmembrane receptors and capable of signalling independently or in combination with other receptors, such as fibroblast growth factor receptors (FGFR) or integrins. Syndecans are highly susceptible to MMPs and other proteinases. [329, 331, 332]

Syndecan-1 expressed in epithelial cells is an important regulator of cell–cell and cell–ECM interactions. Down regulation of its expression in epithelial cells results in loss of cell polarity associated with reduced levels of E-cadherin on the cell surface, suggesting involvement in the epithelial–mesenchymal switch during development and wound healing.[329, 331, 332] Syndecan-2 is expressed by fibroblasts and contains a heparin-sulphate moiety enabling it to bind TGF-β by direct protein-protein interactions.[331, 332] Syndecan-3 is expressed in neural and developing musculoskeletal tissues.[332] Syndecan-4 is widely expressed and has numerous activities including modulation of FGF2 signalling, regulation of cell migration via cross talk with β1 integrin, and control of adhesion through cytoskeletal modifications. Syndecan-4 is involved in regulation of fibronectin signalling and ECM contraction together with tenascin-C. Fibroblasts use integrin receptors and syndecan-4 to induce RHO-dependent spreading in fibronectin.[331]

4.1.4.5.1.4. Decorin

Decorin is a small leucine-rich proteoglycan. It bears a single dermatan or chondroitin sulfate chain and is abundant in dense connective tissues where it associates with, and can regulate the formation of, interstitial collagen fibres. Decorin can bind and inhibit TGF-β.[329]

4.1.4.5.2. Glycoproteins

Glycoproteins, together with the proteoglycans, form the ground substance of the LP of the OMM. The main glycoproteins present are fibronectin and tenascin-c.[127, 250, 333]

4.1.4.5.2.1. Fibronectin

Fibronectin is a ubiquitous ECM glycoprotein that is assembled into a fibrillar matrix in all tissues throughout all stages of life.[127, 334] It is produced by many cell types, such as epithelial cells, fibroblasts, and mononuclear cells.[127] Fibronectin provides not only physical support for surrounding cells, but also critical biochemical and mechanical cues that dictate cell behaviour, mediates cellular interactions with the ECM and is important in migration, differentiation, growth, and adhesion of cells.[333, 335]

A single gene codes fibronectin. Alternative splicing of pre-mRNA results in the inclusion of one or both of two additional domains, extra domain-A and -B (EDA and EDB) to produce multiple forms. Splicing of the EDA and EDB domains is tightly regulated during embryogenesis with a high-degree of tissue-specific expression that decreases with the age. Most healthy adult tissue is devoid of these extra domains until periods of active tissue remodelling, with re-expression following tissue injury and during wound healing.[334, 335] The OMM however, has an increased baseline expression of fibronectin-EDA, similar to that found in foetal skin.[328]

Fibronectin fibrils form linear and branched meshworks around cells and connect neighbouring cells. Thin fibrils predominate early in production and as the matrix matures these fibrils cluster together into thicker fibril bundles.[334] Cells mediate fibronectin matrix assembly via α5β1 integrin binding to the cell-binding site. Fibronectin has binding sites for fibrinogen, heparin, collagen, bacterial proteins, TGFβ LAP thrombospondin, and tenascin-C.[334]

4.1.4.5.2.2. Tenascin-C

Tenascin-C (TNC) is an ECM protein involved in regulation of cellular response with a structural relationship to fibronectin, but an opposing regulatory function. TNC inhibits binding of fibronectin to its co-receptor syndecan-4, preventing cell spreading.[322] TNC is highly expressed during embryonic development in the developing central nervous system, migrating neural crest cells and at epithelial–

mesenchymal interaction sites. In adult tissues, TNC expression is tightly regulated and generally repressed, although certain connective tissues like periosteum, ligaments, tendons and smooth muscles are positive for TNC. Cells within epithelia are essentially negative for TNC. A striking up regulation is observed under conditions of tissue regeneration such as wound healing, inflammation[336] and mechanical stress.[322]

4.2. Environment

The OMM's major function is to protect underlying tissues and the body from environmental elements whilst allowing passage of ingested and inhaled substances required for sustenance of the whole-body organism. The major environmental factors interacting with the μ_{abcd}(omm) are mechanical and abrasive forces, extremes of temperature, microorganisms, and chemical and toxic materials.[242-244, 250, 251]

Mechanical forces that the OMM is exposed to include compressive pressure from biting, shearing from chewing, and abrasion from food particles.[256, 337, 338] Exposure to extremes of temperature occurs with consumption of hot beverages, cooked foods, and sub-zero frozen desserts and ice.[337] Ingested and inhaled substances can trigger chemical reactions, or may contain toxins that are required to be blocked or neutralized.[242-245]

The oral cavity possesses a diverse array of microflora consisting predominantly of streptococcus, prevotella, porphyromonas and fusobacterium species.[242, 248] Pathogenic microorganisms can enter the oral cavity via numerous mechanisms. The OMM needs to prevent both colonization of the surface and penetration into deeper tissues to maintain a healthy state.[242, 245, 248, 254, 326]

The OMM must interact with the internal environment, as it is reliant upon it to provide the substrates required for metabolism and mechanisms for removing by-products or isolating pathogens that breach the barrier. Humoral and cell-mediated immunity are key internal environmental interactions of the OMM. TGFβ1 within the OMM when activated exhibits pleiotropic effects on a variety of immune cells including the inhibition of proliferation and differentiation of effector T cells, inducing B cell apoptosis, inhibiting immunoglobulin secretion and decreasing surface immunoglobulin expression in stimulated human B cells.[288, 289, 291, 301, 339, 340]

The OMM feeds back to the rest of the body through efferent nerve endings. Substances or pathogens that breach the OMM may gain systemic entry through lymphatics, blood vessels and direct spread.[242, 244, 245, 254, 337]

Exposure to external environmental elements influences the development of structural features such as keratinization, increased rete pegs, firm fixation to underlying tissues and formation of a mucous salivary pellicle. Interaction with the internal environment contributes to the structure of the LP. These are discussed in detail in the next section.

4.3. Structure

The structure of the of μ_{abcd} (omm) consists of the endostructure, generated by the relationship and collection of bonds between the different components that enable self-organization of μ_{abcd}(omm), and the exostructure, the collection of bonds between μ_{abcd}(omm) and the environment that modulate the inputs and outputs of μ_{abcd}(omm).

4.3.1. Endostructure

The endostructure of the of μ_{abcd}(omm) is the organization of the different components within the different layers, and the way the layers bond together to form the FTU.

4.3.1.1. Mucous Salivary Pellicle

The MSP formed by secreted gel-forming mucins is structured into a 3-dimensional web-like network with high water holding capacity achieved through arrangement of monomers in homo-oligomeric structures.[242-245, 248] The extended conformation caused by dense glycosylation enables the molecules to occupy large volumes, with secreted oligomeric mucins occupying volumes equivalent to those of small bacteria.[245] Average thickness of the MSP, calculated from the residual volume of saliva after swallowing, varies between 70 and 100µm.[244] It acts as physical barrier and provides lubrication.[248]

4.3.1.2. Epithelium

The epithelial cells are highly polarized along an apical–basal axis, perpendicular to the plane of the epithelium, and bind together through cell-

cell-adhesions enabling them to form a continuous sheet. This ensures mechanical integrity of the tissue and establishes mutually exclusive apical and basal membrane domains.[255, 341]

Epithelial cell-cell adhesion systems include adherens junctions, desmosomes, and tight junctions.[255, 342] Cadherins are transmembrane adhesion receptors whose homophilic binding to each other on the surface of adjacent cells is required to form and maintain adherens junctions.[342] E-cadherin is the founding member of the cadherin superfamily and has pivotal roles in epithelial cell behaviour, tissue formation, and suppression of cancer. [32, 342-348]

4.3.1.2.1. E-Cadherin

E-cadherin is a type 1 cadherin, composed of a cytoplasmic domain, an extracellular domain and a single trans membrane domain (*Fig.8)*.[343, 344, 349, 350] It is coded by the *CDH1* gene located on chromosome 16. [32, 343, 344, 347] Precise transcriptional control of E-cadherin gene expression is essential during developmental reprogramming, cellular differentiation, and cancer progression.[343, 344, 347, 348] The promoter for the *CDH1* gene contains a CCAAT box, a C-G rich sequence, several E-boxes (enhancer box) and a SMAD binding element that allow the direct binding of transcriptional regulators. [32, 344, 347-349, 351, 352] The CCAAT box is dominantly active and promotes transcription of the *CHD1* gene in epithelial cells.[32, 344, 347] The E-boxes are a major site for many transcriptional regulators that reduce expression of the *CHD1* gene. Key transcriptional factors identified to repress E-cadherin expression through its promoter include SNAIL, TWIST1, ZEB1 and 2, and E2A. [347-352]

Epigenetic mechanisms impact expression of E-cadherin. The most common is methylation of a C-G domain of the promoter. [344, 347-349, 351] Histone acetylation maintains chromatin in a state that is accessible for transcription machinery. Several factors have been shown to increase expression of E-cadherin via histone acetylation including E1A (p300), FOXA3 and RUNX1.[347, 349] In addition to direct binding to E-box elements, the transcriptase repressors SNAIL, TWIST and ZEB1 and 2 recruit various DNA/histone modification complexes that produce histone deacetylation resulting in condensation of chromatin, blocking transcription.[347, 349]

Histone methylation via lysine residues has been associated with gene silencing.[349] Histone demethylation has the reverse effect and increases expression of the E-cadherin.[349]

MicroRNAs (miRNAs), highly conserved, small RNA molecules processed from much longer primary non-coding transcripts, control gene expression at the post-transcriptional level by specifically interacting with target mRNAs. Members of the miRNA200 family reduce expression of the inhibitors ZEB1 and ZEB2[347, 349, 351, 353] and inhibit methylation of the C-G sequence of the *CDH1* promoter. [349] miR9 directly represses the expression of E-cadherin, driving the up regulation of mesenchymal and angiogenic genes.[351]

E-cadherin is synthesized as a propeptide in the endoplasmic reticulum. Post-translational processing takes place in the Golgi network before it is transported and integrated into the plasma membrane of the cell.[343, 344]

The cytoplasmic domain forms a complex with β-catenin that produces stabilization and anchors the glycoprotein to the actin cytoskeleton. The juxtamembrane portion binds with p120 catenin. [343, 344, 349] The extracellular domain is structured in tandemly arrayed blocks of repeating extracellular cadherin domains. Each of these domains forms a β-barrel structure. A *cis* dimerization occurs between the sides of adjacent E-cadherin molecules on the same cell, followed by a *trans* interaction with E-cadherin molecules on a neighbouring cell to form an adhesive bond (*Fig. 8)*. These interactions are major contributors to adherence junctions in epithelial tissues.[108, 343, 344, 349, 350] E-cadherin contributes to the apical basal polarity of epithelial structures.

E-cadherin plays an indirect role in intracellular signalling by sequestering β-catenin, p120 and plakoglobin at the cell surface and facilitating formation of a complex between β-catenin and caveolin-1. Both these actions prevent β-catenin translocation to the nucleus where it acts as an intracellular transducer of the canonical WNT signalling pathway and as a transcriptional co-activator enabling transcription of genes that inhibit apoptosis.[343, 344]

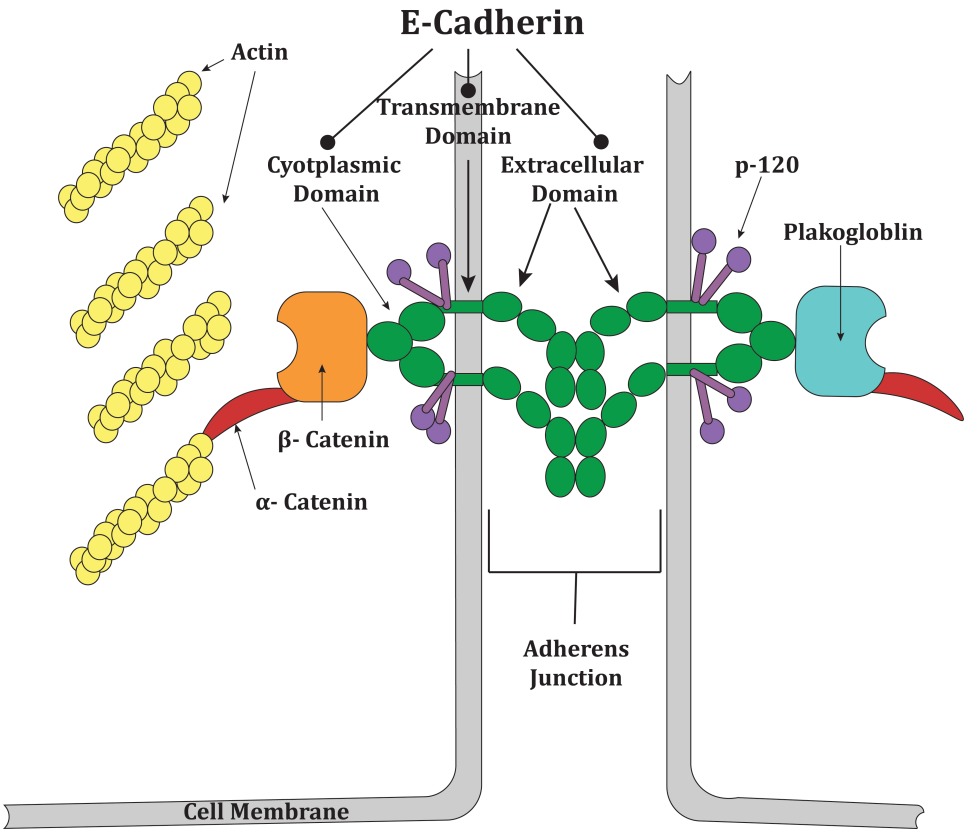


Figure 8 E-Cadherin and adherens junction

E-Cadherin is a key component of adherens junctions that forms cell-cell adhesions in epithelial cells. The cytoplasmic domain forms a complex with β-catenin / plakoglobulin and p120 which anchors to the actin cytoskeleton via α-catenin. Extracelluar domains of adjacent molecules form a dimer, which then interacts with E-cadherin dimers on neighbouring cells.
Original drawing based upon drawings and text description from various references. [108, 343, 344, 349, 350]

p120 acts as a scaffold protein to regulate cadherin adhesive strength by clustering cadherins to specific sites on the cell surface. It regulates cadherin turn-over by adapting the amount of cadherin available for adhesion on the surface.[344] Over expression of p120 in the cytoplasm modulates small GTPases and produces a branching phenotype.[343, 344]

E-cadherin has been shown to inhibit ligand activation of epithelial growth factor receptor (EGFR), ERBB2 receptor, insulin-like growth factor receptor (IGF-1R) and the hepatocyte growth factor (HGF) receptor, c-MET.[343] The same interactions can conversely lead to endocytosis of E-cadherin and

degradation in the presence of calcium deprivation and phosphorylation of E-cadherin via activation of c-SRC.[343]

Various layers are identified in the oral epithelium with cells increasing in size and becoming flatter as they progress from the basal layers to the superficial layers.[244] Keratinized epithelium displays a basal layer, a spinous layer, a granular and a keratinized layer. Non-keratinized areas are composed of a basal layer, an intermediate layer and a superficial layer. Non-keratinized epithelium shows tolerance to compression and distension to accommodate chewing, swallowing, and speech.[244, 251, 252]

Differentiating keratinocytes in the regions of the gingiva and hard palate accumulate proteins in the form of keratohyalin granules and lipids in the form of lamellar granules.[244, 254] In the uppermost granular layer, the bounding membrane of the lamellar granule fuses into the cell plasma membrane and the contents are extruded into the intercellular space. The extruded lipid is largely a mixture of phospholipids, glucosylceramides and cholesterol. Hydrolytic enzymes delivered by the lamellar granules act on the extruded phospholipids and glucosylceramides to produce ceramides and fatty acids generating a permeability barrier that limits the penetration of non-polar groups.[244, 254]

Keratohyalin granules contain profilaggrin, a high molecular weight, histidine-rich highly phosphorylated protein that is proteolytically cleaved to produce filaggrin. Filaggrin causes aggregation of the keratin filaments into dense bundles of keratin that lie parallel to the plane of the epithelium. This collapse of the cytoskeletal network is associated with extreme flattening of cells in the stratum corneum. After the aggregation of the keratin, filaggrin is broken down into its component amino acids which together with lactate produced by anaerobic glycolysis generates an extremely high osmolarity within the cornified cells facilitating the removal of all free water from the intercellular spaces of the stratum corneum.[254]

Epithelial cells in non-keratizing regions do not produce keratohylin or lamellar granules[254] but produce two secretory organelles, a cored granule and a lamellate granule. Contents of these granules are secreted into the intercellular

spaces about 2/3 of the way from the basal layer to the surface. The barrier function is less effective than that of the keratinized regions. [244, 254]

4.3.1.3. Bonding between Mucous Salivary Pellicle and Epithelium

Bonding between the MSP and epithelium produces the wet mucosal surface and initial barrier function of μ_{abcd}(omm).[243, 244, 248, 249] The apical surfaces of the epithelial cells is covered by cell membrane ridges, microplicae.[243, 249] Membrane anchored mucins, notably MUC1, is expressed on the microplicae of the superficial epithelial cells and on the plasma membrane of the cells in the upper and intermediate layers of healthy oral mucosa.[243, 249] Both the microplicae and expressed MUC1 contribute to the bonding between the epithelial and MSP layers through crosslinking.[243] Other membrane-associated mucins are secreted from granules into the intercellular spaces in upper parts of the epithelium, increasing impermeability to non-polar substances.[242, 244, 245]

4.3.1.4. Basement Membrane

The components of the BM are organized into the lamina lucida, just below the basal surface of the basal epithelial cells, and the lamina densa, an electron dense zone that is between the lamina lucida and the LP.[264]

Laminins are central organizers in the BM and the main structural component of the lamina lucida.[214, 264] LM332 is unique in that it anchors cells to the BM via hemidesmosomes.[214, 261, 264] LM332 is orientated so that the LG domain of the α3 chain can interact with the hemidesmosome transmembrane integrin α6β4 of the basal epithelial cells. The LG domain of the α3 chain is in the lamina lucida whilst the cruciate shaped short arms and coiled-coil domain are within the lamina densa.[262, 263, 354] The long axis of the molecule lies at a 27° angle to the lamina densa border with the γ2 chain being furthest away from the epithelial cells.[354]

The β3 chain binds to LM311 and 321 and type VII collagen via its LE domain, helping in stabilizing the BM.[275] The β3 chain is relatively resistant to proteolytic cleavage. [214, 261-263, 271]

The γ2 chain can associate with collagen VII, nidogen and fibulin. Through the association with nidogen it is connected to the extensive collagen IV network. Association with other laminins allows it to form heteropolymers within the ECM.[214, 261-263, 283]

Type IV collagen forms the lamina densa. It self assembles to form predominantly three sets of triple-helical molecules that self associate via their NC1 domains and their middle triple-helical regions via covalent and non-covalent bonds with adjacent monomers, to form three dimensional spider web-like scaffolds. This network interacts with the laminin network via nidogen and probably perlecan to form a basic BM scaffold.[278-280]

Nidogens bind to type IV collagen and LM332, connecting the two zones of the BM. It binds to perlecan, fibrinogen, and fibronectin.[282] Perlecan has various binding sites for nidogen/entactin, type IV collagen, integrins, and heparin in its protein core.[282]

Type VII collagen forms part of an attachment complex comprised of hemidesmosomes in the basal epithelial cells, anchoring filaments in the BM, and anchoring fibrils that reach from the BM down into the LP. Type VII collagen is the major component of anchoring fibrils, which merge into the banded collagen fibrils of the LP.[278, 279, 281]

4.3.1.5. Lamina Propria

The superficial or papillary region of the LP consists of connective tissue papillae that interdigitate with the rete ridges of the overlying epithelium. Deep to papillary region lies the reticular layer with its fibre network. [250, 252, 338] The rete ridges have two main functions: enlarging the contact area between epithelium and LP to enhance adhesion between the two and help scatter mechanical masticatory stresses; and provide physical protective niches where keratinocyte stem cells can reside at the bottom of rete ridges.[338] The relative height of the connective tissue papillae to the thickness of the epithelium is generally similar for the different regions of OMM however the number of connective tissue papillae varies with resultant differences in the surface area of this junction. In masticatory mucosa, which is subjected to compressive and frictional forces, approximately 1.5 to 2.5 times more connective tissue

papillae/ mm2 of mucosal surface can be found compared to lining mucosa and up to seven times more connective tissue papillae/ mm2 than the floor of the mouth. [250, 256] The rete ridges of masticatory mucosa are longer than in the lining mucosa.[338]

The reticular layer has a small variation between lining and masticatory mucosal areas. Lining mucosa is more flexible and has the highest level of elastic tissue. Collagen fibres are thin and loosely arranged compared to the masticatory mucosa where they are arranged in bundles.[250, 256]

Numerous blood vessels in the form of capillaries loop into the connective tissue papilla along with lymphatics and neural elements.[250, 252] The latter include intraepithelial nerve fibres, organized nerve endings and Merkel cell–neurite complexes that are present at the tips of the papillations just below the BM.[250, 252, 258]

4.3.1.6. Anchoring Complex between Epithelium, Basement Membrane and Lamina Propria

The anchoring complex (*Fig. 9*) comprised of hemidesomsomes at the basal surface of the basal epitheilial cells, the anchoring filaments linking the hemidesmosomes to the BM, and the anchoring fibrils connecting the BM with the underlying LP, stabilizes the epithelium to the BM and LP. [355, 356]

4.3.1.6.1. Hemidesmosomes

Hemidesmosomes are multiprotein complexes that enable bonding between the epithelium and BM. These highly specialized integrin mediated epithelial attachment structures help epithelial cells to firmly adhere to the extracellular matrix by establishing a link between the underlying BM and the internal mechanical stress-resilient keratin intermediate filament network.[264, 355] The hemidesmosomes found in the OMM are made up of five elements: integrin α6β4, plectin isoform 1a (P1a), tetraspanin CD151, bullous pemphigoid antigen 1 isoform e (BPAG1e) and BPAG2.[355, 357]

Hemidesmosomes along the BM display a characteristic tripartite structure with inner and outer electron dense plaques and a less dense intervening zone (*Fig. 9*) The outer plaque is slightly larger and lies on the plasma

membrane. P1a and BPAG1e bind with the basal cell keratins and form the inner plaque. The outer plaque is formed by integrin α6β4, CD151 and BPAG2. Critical links are created between the components of the inner plaque and outer plaque.[355]

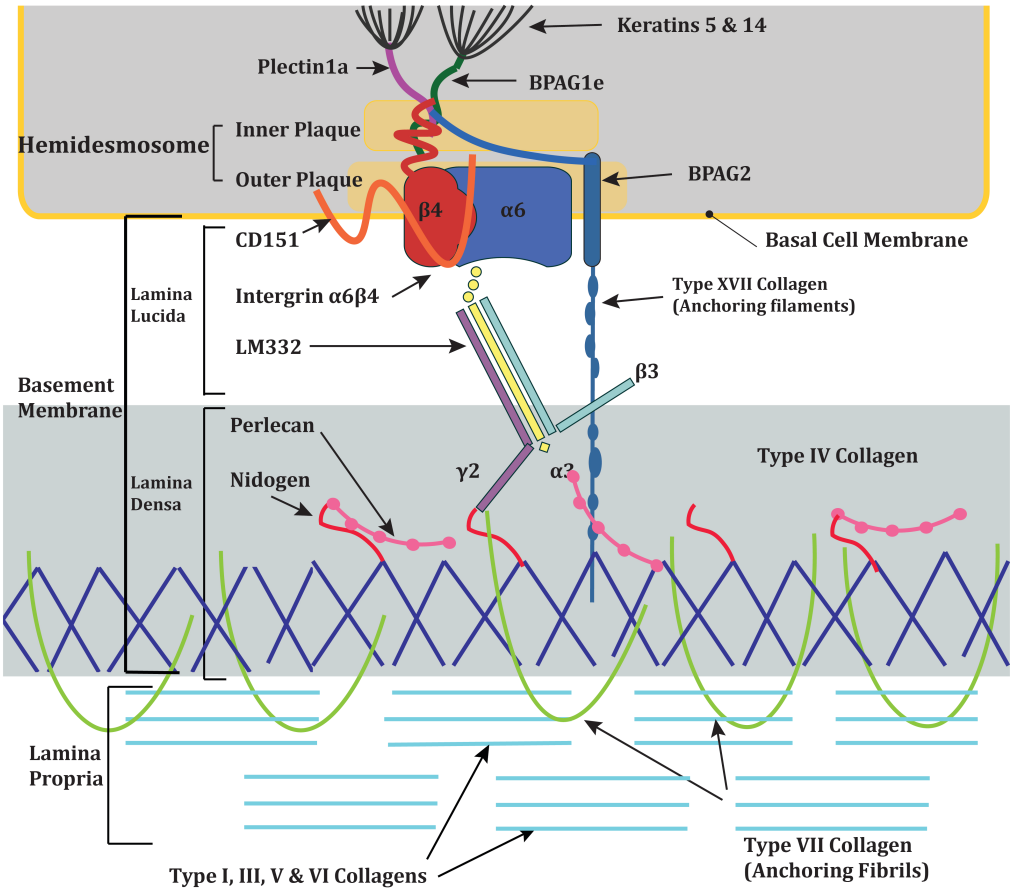


Figure 9 Schematic Drawing of Anchoring Complex

The anchoring complex is composed of hemidesmosomes, anchoring filaments and anchoring fibrils. Hemisdesmosome in the oral mucous membrane is comprised of integrin α6β4 (red and bright blue), CD151 (orange), BPAG2 (dark blue), Plectin1a (dark pink) and BPAG1 (dark green). Plectin1a and BPAG1 connect the hemidesmosome to the keratin of the cytoskeleton, BPAG2 has 3 components, intracellular, trans membrane and anchoring filaments composed of collagen XVII. The β4 subunit of the integrin has an intracellular tail that binds to Plectin1a and BAPG2. Laminin 332 is a central structure in the basement membrane. The globular domain of the α3 chain (bright yellow) binds to the α6 subunit of the integrin after cleavage in the hinge region. The unprocessed γ2 chain of LM332 interacts with nidogen (orange-red) which connects the laminin to the cross hashed Type IV collagen fibres (purple) in the lamina densa. It interacts with the Type VII collagen anchoring fibrils (light green) that sweep up from the laminin propria. The role of the β3 chain is less defined. Perlecan (hot pink) associates with nidogen, Type IV collagen and the Type XVII collagen anchoring filaments of BAPG2. The Type VII collagen anchoring fibrils help to fix the collagens in the laminia propria to the other structures. Through these multiple interactions the oral mucosal epithelium is firmly adhered to the underlying lamina propria
Original drawing based upon drawings and text description from various references. [214, 250, 261-263, 266, 268, 271, 272, 355-357]

Integrin α6β4 is widely expressed in epithelial tissues in association with hemidesmosomes (*Fig. 9)*.[355] The binding site on the integrin receptor is formed by a combination of its α and β chains.[214, 268] Integrin α6 is a conventional integrin α subunit, whereas integrin β4 has a unique cytoplasmic domain, much larger than that of other integrin β subunits, that mediates most of the intracellular interactions of the receptor, including those with P1a and BPAG1e, and BPAG2.[262, 355, 357] The extracellular domain of the integrin α6β4 acts as a receptor for LM332, binding to the cleaved LG domain of the α3 chain.[214, 263, 266, 268, 355, 357] Integrin α6 has binding sites for BP180 and CD151.[355]

BPAG2 is the second transmembrane component of the hemisdesmosome and is a homotrimeric transmembrane protein with an intracellular N terminus (*Fig. 9)*. Each molecule consists of three type XVII collagen α1 chains and an extracellular C-terminal domain. BPAG2's intracellular domain lies within the outer plaque of the hemidesmosome and the extracellular domain spans the lamina lucida with the tail residing in the lamina densa.[355, 357] This tail of collagen contributes to the formation of the anchoring filaments.[262, 355] The extracellular domain of BPAG2 is constitutively shed from the cell surface by the action of ADAM proteases and becomes incorporated into the BM.[355]

CD151 is a cell surface protein that belongs to the tetraspan superfamily of transmembrane proteins. These proteins are involved in cell adhesion, migration and signalling.[355] All tetraspanin proteins share a similar structure characterized by four transmembrane domains forming a small and a large extracellular loop, with short intracellular N- and C- terminal tails. CD151 interacts with integrin α6 via its large extracellular loop (*Fig. 9)*.[355]

P1a and BPAG1e are both members of the plakin family of cytoskeletal linker proteins. P1a binds directly to the cytoplasmic tail domain of integrin β4. BPAG1e has been shown to bind to integrin α6β4, BPAG2 and keratins K5 and K14.[355]

4.3.1.6.2. Laminin 332

Laminin332 interacts with integrin α6β4 of the hemisdesmosome via the globular region of its α3 chain. The α3 chain is cleaved in the hinge region of the globular domain between LG3 and LG4 allowing the exposed LG3 to interact with the α6 component of integrin α6β4.[214, 268, 272] The β3 chain binds to LM311 and 321, and type VII collagen via its LE domain.[214, 261-263, 271] The L4 domain of the short arm of the unprocessed γ2 chain is important for deposition and incorporation of the LM332 molecule into the ECM.[271, 358] The γ2 chain associates with the type VII collagen of anchoring fibrils, connecting LM332 to the LP. Association with nidogen connects LM332 to the collagen IV network (*Fig. 9).* [214, 263, 266, 268, 355, 357]

4.3.1.6.2.1. Anchoring Fibrils

Beneath the lamina densa, most of the type VII collagen molecules form semi-circular loop structures, known as anchoring fibrils that originate and terminate in the lamina densa.[250, 262, 356] The amino-terminal, non-collagen-like interacts with collagen IV and LM332 of the BM, and with the anchoring filaments, The fibrils link or encircle collagen fibers or other components of the LP, providing tight anchorage of the basal lamina to the underlying structures (*Fig. 9)*.[355-357]

4.3.2. Exostructure

The exostructure is the relationship of the OMM with its environment. The exostructure determines how the environment acts upon the system (input) and how the system acts upon the environment (output). The system boundary is formed by the components that have a direct relationship with the environment. In the μ_{abcd}(omm) these are the MSP, which interacts with the external environment, and the LP, which interacts with the internal environment.

4.3.2.1. The Salivary Mucous Pellicle and External Environment

The MSP in healthy oral mucosa forms the μ_{abcd}(omm), which interacts with saliva in the external environment. The pellicle, which is tightly bound to the epithelium, acts as a template for further protein/mucin assembly via this interaction with saliva.[242, 337] Saliva mainly consists of water (95–99% per

weight), enzymes, inorganic salts, lipids, and is a source of non-membrane bound mucins which form the mucous salivary pellice,[244] primarily MUC5B and MUC7.[242, 248, 337]

MUC5B and MUC7 are secreted with an intrinsically high osmotic pressure created by their glycosylated regions and counterion concentrations. Adsorption of water from saliva leads to rapid expansion of the mucins.[337] MUC5B's complex structure allows it to cross-link with the membrane bound mucin, MUC1,[249] and interact with an array of different salivary proteins and microbes to maintain a healthy oral cavity.[242, 248]

Constant interaction between saliva and the mucous salivary pellicle enables the washing away of components that have been sheared off by mechanical forces, prevents the formation of bacterial biofilms, and facilitates turnover and renewal of the pellicle.[242, 245, 248, 337] Saliva therefore plays an important role in the barrier function of the oral mucous membrane.[242, 244, 245, 248]

4.3.2.2. The Lamina Propria and Internal Environment

The structural relationship of the junction of the reticular layer of the LP with the underlying tissues varies depending on the type of mucosa. In the masticatory mucosa, the gingiva and anterior aspects of the hard palate, the LP is bound to periosteum and tooth and there is no submucosa. [250, 252] These areas are exposed to the significant physical forces associated with mastication and movement of food. This structural arrangement provides a greater level of rigidity, reducing movement of the mucosa and providing protection against tensile stresses.[256] In the posterolateral aspects of the hard palate the LP is bound to a fibrous submucosa containing salivary glands and fat.[250, 252]

The LP of lining mucosa is attached to a submucosa of connective tissue associated with muscles (lips, cheeks, tongue, and soft palate), fat (soft palate, cheek, and labial mucosa) and salivary glands (lips, cheeks, palate, and tongue).[250, 252] The submucosa is composed of a mixture of connective and adipose tissue and contains the larger blood vessels, lymphatics, and nerves contributing to the neurovascular network of the LP. This enables a broader systemic relationship. Within the submucosa there are minor salivary glands in the areas of the lips, tongue, and cheek[250, 252]

4.3.2.3. Gingiva and Teeth

There is a specialized attachment of LP to the teeth. Teeth are the only structures that perforate epithelium in the body.[359] Junctional epithelium is located at the junction of the periodontal soft tissue and hard tissue and is composed of cells with low differentiation and higher regeneration ability that attach directly to cementum surface junction via a basement membrane.[360]

4.4. Mechanisms

Mechanism(s) refers to the collection of processes that enable the μ_{abcd}(omm) FTU to form, function and maintain function in response to alterations in environment. The principle mechanisms are morphogenesis, growth, homeostasis, and repair. These are orchestrated and sustained by a number of common processes in response to biochemical and biophysical cues from the microenvironment of the FTU.

Morphogenesis is the process that generates spatial distribution of cells to produce tissue organization and shape in response to timing and patterning cues during development[255, 361] It is a major mechanism in the emergence of any FTU through determination of the components of the FTU, self-organization of those components to produce the endostructure and exostructure, and the response to environmental morphogens, morphostats and physical stressors during formation both in any given individual and on a larger evolutionary basis.[361] Synergy between epithelial and mesenchymal cells is important and necessary for morphogenesis, in particular for formation of BMs. This is mediated by diffusible factors such as cytokines and growth factors secreted by epithelial cells or fibroblasts, which reciprocally modulate the programme of each other cell type. [305]

Growth refers to an increase in tissue mass or size and is achieved by an increase in cell size and or cell number through cell division. Energy input required for growth is obtained from the whole organism level whilst tissue level mechanisms help to control growth. [362] The extracellular matrix forms the three-dimensional tissue scaffold that defines tissue boundaries, and provides an adhesive substrate for cell migration and, by binding morphogens and growth factors creates concentration gradients.[363] When released these factors form ligands with cell receptors and trigger cell division.[215, 362]

Cell competition whereby fast growing cells out compete slow growing cells and results in apoptotic elimination of the slow cells and their engulfment by the fast-growing cells has a key role in buffering tissue growth.[362] Cell crowding limits growth in epithelia by triggering delamination; a process by which cell-cell adhesions are lost and cells are squeezed out.[364] Cell crowding can inhibit growth through cell-cell signalling pathways by increasing localization of various transcriptases in the cytoplasm rather than the nucleus.[362, 365] Tissue growth occurring during morphogenesis and after birth is controlled at the tissue level through a balance of cell division, cellular competition and apoptosis.[362]

Tissue homeostasis is the functional and structural maintenance of the tissues and organs of the body. It is dependent upon genetic, molecular and cellular processes involved in the repair of structures and restoration of functions that have been damaged by intrinsic or environmental agents. Tissues are maintained in steady state equilibrium where cell loss is balanced by cell production.[366] The OMM is a dynamic structure with high turnover of epithelia and mucous. The normal turnover of the barrier is essential if an ongoing and viable defense is to be maintained.[242]

Repair of a FTU in response to injury from either the internal or external environment is critical to maintaining function. The OMM in performing a barrier function is particularly prone to injury.

A number of common processes varied by contextual, spatial and temporal differences underlie the mechanisms of morphogenesis, growth, homeostasis, and repair utilize relevant to the FTU level under consideration. These are discussed in the following sections.

4.4.1. Cell Division

Cell division is a key process required for morphogenesis, growth, homeostasis and repair. Timing and frequency of cell division is directly regulated internally by the cell cycle, and indirectly by paracrine growth factor signals from other cells and surrounding extracellular matrix, typically acting on cyclin expression.[367-372]

4.4.1.1. Cell Cycle

There are four phases of the cell cycle. The first phase of the cell cycle, gap phase 1(G1 Phase), occurs just after cell division. During this phase the

daughter cells undergo growth and detect environmental cues, which determine if the cell cycle is continued, paused or exited.[369, 372, 373] This serves as a checkpoint for DNA damage.[372, 374] The next phase is the synthesis phase (S phase) with DNA replication and repair, followed by a second gap phase (G2 phase), serving as another DNA damage checkpoint before the cell enters into the fourth and final phase of mitosis (M phase).[373, 374] Numerous cell cycle regulators control these phases.[373, 374]

4.4.1.1.1. Cyclins and Cyclin Dependent Kinases

Cyclin dependent kinases (CDKs) are serine/threonine protein kinases that require a regulatory subunit, a cyclin, for their enzymatic activity. Binding of cyclins to the relevant CDK generates transformation in shape, exposing the enzymatic site and stabilizing the structure.[375] Cyclin-CDK pairs drive the transitions between the different stages of the cell cycle. CDK1-cyclinB transitions the cell into mitosis, CDK1-cyclinA progresses the cell cycle through G2 phase, CDK2-cyclinA transitions the cell into S-phase, CDK2-cyclinE promotes DNA replication and S-phase, and CDK4-cyclinD and CDK6-cyclinD transition the cell through early G1 phase[376, 377] Other Cyclin-CDK pairs are involved in regulating transcription.[375, 377]

Cell-cycle-related CDKs are negatively regulated by binding to small proteins of the INK4 or Cip/Kip families of inhibitors. INK4 proteins ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$) are specific for the CDK4 subfamily and interact with the monomeric CDKs. They function by distorting the cyclin interface and the ATP-binding pocket, thus preventing activation of CDK4 and CDK6 by D-type cyclins or by CD activating kinase (CAK). Members of the Cip/Kip family of inhibitors ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$) contact both the CDK and cyclin subunits and are able to inhibit CDK-cyclin heterodimers, giving additional levels of regulation once these complexes have already formed.[375]

4.4.1.1.1.1. Cyclin D

CyclinD is of particular importance in the OMM due to its induction by mitogens and association with tumorogenesis.[378-380] Binding with CDK4 and CDK6, cyclinD acts primarily to phosphorylate protein from retinoblastoma gene (pRB). pRB binds with $E2F_{1-3}$ transcription factors

to repress transition of the cell cycle. On phosphorylation by CKD4/6-cyclinD complex, pRB release the $E2F_{1-3}$ transcription factor enabling the activation of genes for G1-S-phase transition and S-phase.[375, 377, 379, 380]

Expression of cyclinD is promoted by mitogenic signalling by growth factors and integrins via MAPK and PI13/AKT pathways.[369, 379, 381] Inflammatory signalling via tumour necrosis factor α (TNFα) increases transcription via nuclear factor κB (NF-κB).[379] STAT 3 and 5 increase transcription in response to interleukins.[381] NOTCH signalling, Hedgehog signalling and c-MYC can all increase transcription of cyclinD.[379, 381] Glycogen synthase kinase 3β (GSK3β) down regulates transcription[381] but facilitates phosphorylation of cyclinD, allowing its nuclear export followed by polyubiquitination and proteasomal degradation.[379]

Phosphatase and tensin homolog (PTEN) plays an important role in maintaining DNA integrity and regulates cell cycle through decreasing expression of cyclinD and increasing p53 in the nucleus whilst increasing p27Kip2 in the cytoplasm, inhibiting CDK2.[373] PTEN controls the G2–M transition to prevent bypass of the G2 checkpoints and premature entry into mitosis and in response to genotoxic stress, PTEN functions to activate the DNA damage checkpoint by suppressing PI3K/AKT-dependent CHK1 phosphorylation and maintaining its nuclear localization and stability.[373]

4.4.1.1.2. Retinoblastoma Protein and E2F Transcription Family

Retinoblastoma protein (pRB) is a member of the pocket protein family, containing a 'pocket' for functional binding of other proteins.[382, 383] pRb is a stable protein and instead of being degraded it undergoes cyclic phosphorylation and dephosphorylation, causing pRb to oscillate between active and inactive states. Activity of pRB is controlled by CDKs.[374, 382, 383] In the cell cycle, pRB modulates the activity of the E2F family of transcriptases.[372, 374, 384]

The E2F transcription family is comprised of six polypeptides, E2F1-6. E2F1-3 are activators of cell cycle and interact only with pRB.[372, 374, 384] They positively target the gene for cyclinE, which activates CDK2 and further

phosphorylates pRB to create a positive feedback loop. E2F1 transcription is self-stimulated, creating a feed forward regulation loop, and by c-MYC.[372]

E2F4-5 are mainly nuclear during G0 and G1phases and act as repressors of cell cycle. They interact not only with pRB but the other pocket proteins as well. It is proposed they play a role in holding the cell at various checkpoints in the cell cycle allowing for DNA repair, and forcing cell cycle exit if required.[372, 374, 384]

4.4.1.1.3. WNT Pathway

The WNT signalling pathway is central to organ morphogenesis and growth in multicellular organisms.[221, 385, 386] WNTs are intercellular growth stimulatory factors not found in free form but in secretory vesicles or exosomes, limiting the range of action.[385] WNTs bind to the WNT membrane receptor, Frizzled (FZD) to activate the intracellular pathway[385] leading to cell proliferation and impacting the cell cycle at various points.[221, 385, 387]

WNT signalling has the distinct property of giving shape to growing tissues while inducing cells to proliferate, acting in the process as directional growth factors.[221, 385, 387, 388] WNT signalling instructs new cells to become allocated in a way such that organized body plans are generated not only by changes in gene expression but in effects on the cytoskeleton and the mitotic spindle, contributing to polarity, motility and differentiation.[385, 388] WNT signalling is important in maintaining tissue architecture and epithelial stem cells in adult tissues and has a role in myofibroblast differentiation.[221, 385]

WNT initiates activation of several major signalling pathways, the canonical WNT/β-catenin pathway, and the non-canonical WNT pathways.[215, 221, 385, 387] β-catenin is phosphorylated by GSK3β in the cytoplasm preventing translocation into the nucleus and enabling its destruction. Activation of WNT signalling leads to the inhibition of GSK3β, stabilization of β-catenin and translocation into the nucleus. In the nucleus β-catenin acts as a transcriptional co-activator binding to T-Cell Factor (TCF) and lymphoid enhancing factor (LEF) transcription factors and dissociates repressors such as Groucho. This enables transcription of genes that inhibit apoptosis.[215, 343,

[344, 385, 388-391] WNT signalling pathways independent of β-catenin include the Planar Cell Polarity (PCP), c-Jun amino-terminal kinase (JNK), RHO, and calcium signalling pathways.[221, 386, 387]

4.4.1.1.4. Growth Factors, Receptor Tyrosine Kinases and Their Intracellular Pathways

Growth factors, or mitogens, are substances that stimulate cell growth, division and differentiation, critical processes in morphogenesis, growth, homeostasis and repair. The major factors in the OMM are epidermal growth factor (EGF), transforming growth factor α (TGFα) and fibroblast growth factor (FGF). They act via receptor tyrosine kinases (RTK), transmembrane protein receptors that trigger intracellular cascades to promote cell cycle and cell division.[13, 95, 350, 392-395]

4.4.1.1.4.1. Epidermal Growth Factor

EGF is found in saliva, platelets and macrophages. In the OMM it is integral in maintaining completeness of the mucosal barrier with roles in healing of ulcers, stimulation of DNA synthesis and protection of mucosa from damage caused by physical, chemical and bacterial agents. [396, 397]

The release of EGF into saliva involves exocytosis by fusion of the secretory granule membrane with the apical cellular membrane (exocrine). A small amount of the EGF accumulated in submandibular glands ends up in the blood (endocrine). The production and secretion of EGF in the submandibular gland is dependent on androgen levels and sympathetic system status.[396]

EGF has a high affinity for the RTK epidermal growth factor receptor (EGFR). Ligand binding activates a number of intracellular pathways, including MAPK, PI3k/AKT and JAK/STAT.[387, 396-398] EGF activation of EGFR increases receptor expression.[399]

EGF plays a role in maintaining OMM stem cells by supressing epithelial differentiation and enabling turnover related to homeostasis and proliferation in response to injury.[396, 398] EGF stimulated OMM stem cells promote fibroblast migration and proliferation which assist in wound

healing.[398] Increased EGF increases LM332 expression, particularly an over expression of LMγ2 which contributes to increased cell motility in wound healing and pathological states.[95]

4.4.1.1.4.2. Transforming Growth Factor

TGFα is a member of the EGF family of ligands. Like EGF it is transcribed in a pre-pro form and undergoes posttranslational modification to its active form but has a slightly lower affinity for EGFR than EGF. A common action of TGFα is proliferation. In the OMM it may have a function in mediating mucous production.[399]

TGFα is secreted in an autocrine fashion in both a membrane tethered form and a soluble form. It may function in a juxtacrine fashion. TGFα activation of EGFR increases its own translation and mRNA cleavage. The pro-form localizes to the basolateral aspects of polarized cells where it is further cleaved by metalloproteinase ADAM17 to the active form.[399]

4.4.1.1.4.3. Fibroblast Growth Factor

The FGF family is comprised of eighteen secreted proteins, grouped into seven subfamilies based on biochemical function, sequence similarities, and evolutionary relationships. Five of the subgroups, FGF1, FGF4, FGF7, FGF8 and FGF9, function as secreted signalling proteins acting as ligands for RTKs.[400]

FGFs are involved in regulating cellular processes that include positive and negative regulation of proliferation, survival, migration, differentiation, and metabolism. In development FGFs are important in regulating the of formation of primitive endoderm and subsequently organogenesis.[400] FGF4 may induce differentiation through autocrine signalling.[401] In adult tissues, FGFs have an essential role in injury response and repair.[400]

Heparin and heparin sulfate proteoglycans (HSPG) in the ECM bind FGFs, controlling their function. Heparin sulphate (HS) also serves as a co-factor in receptor activation. Specialized adaptor proteins such as fibroblast growth factor receptor substrate 2α (FRS2α) and regulators

of the RAS-MAPK and PI3K-AKT pathways are involved in regulating the intracellular cascades triggered by RTK activation.[400]

4.4.1.1.4.4. Receptor Tyrosine Kinases

RTKs are a family of trans membrane receptors that respond to growth factor ligands and trigger intracellular signalling cascades. RTKs share a common structure and all have protein tyrosine kinase activity. They are composed of an extracellular ligand binding region, a single trans membrane helix and a cytoplasmic region.[395, 397, 402]

Activation is by ligand dimerization[395, 397, 402], with the exception of the insulin receptor in which ligand binding produces an allosteric transition.[402] Dimerization produces auto phosphorylation in the intracellular region.[395, 397, 402] Auto-phosphorylation is followed by recruitment of cytoplasmic signalling molecules containing SRC homology-2 (SH2) and phosphotyrosine-binding (PTB) domains. [395, 397, 402]

4.4.1.1.4.4.1. Epidermal Growth Factor Receptor

EGFR (ERB-B1) belonging to the family of ERB-B receptor,[395, 403] is of particular importance in epithelial tissues.[13, 350, 387, 403, 404] EGFR has multiple auto phosphorylation sites enabling the activation of multiple signalling molecules.[395] This is regulated by protein tyrosine phosphatases (PTP), inhibitors of auto- phosphorylation.[395]

Trafficking of EGFR is complex and plays a major part in signal variation and regulation.[13, 397, 405-408] Receptors are removed from the surface primarily by clathrin-coated pits via endocytosis. Clathrin independent mechanisms of endocytosis also occur. [397, 407, 408] Both result in formation of an early endosome in which activated signalling continues. The receptor can then be trafficked to a late endosome and degraded by lysosomes or ubiquitination, recycled back to the cell surface or trafficked into the nucleus or mitochondria.[397, 403, 405-408] EGFR translocated to the nucleus can directly influence cell proliferation, DNA replication, repair and transcription.[13, 395, 397] EGFR

induces transcription of CCND1 gene and acts as a co-activator of other transcription factors such as STAT proteins.[13]

Cellular stresses alter trafficking. Hypoxia-inducible factor 2α (HIF-2α) increases transcription of EGFR. UV irradiation, hypoxia, oxidative stress, TNFα and cisplatin, all which activate the p38 MAPK pathway, promote non-degradation and translocation into the nucleus and mitochondria.[407]

Inactive EGFR is subject to endocytosis but is rapidly returned to the plasma membrane.[397] Inactive EGFR in endosomes promotes autophagy whereas activated EGFR inhibits autophagy and increase cell survival.[407]

4.4.1.1.4.4.2. Fibroblast Growth Factor Receptor

The fibroblast growth factor receptors (FGFRs) regulate important biological processes including cell proliferation, differentiation, survival and tissue repair.[400, 409, 410] There are four receptors in the family, FGFR-1, FGFR-2, FGFR-3, and FGFR-4.[400, 409] Alternative splicing enables different isoforms, which is an important mechanism in determining ligand-binding specificity. [351, 400, 409, 411] FGFR2 is the most common receptor in the OMM epithelium.[400, 409, 412]

Sprouty (SPRY) and similar expression to FGF (SEF) participate in FGFR signal regulation. Following activation, FGFRs are often ubiquitinated by E3 ubiquitin protein ligase (CBL) to facilitate clathrin-mediated endocytosis and receptor degradation.[400, 409]

4.4.1.1.4.5. Receptor Tyrosine Kinase Intracellular Pathways

The primary intracellular pathways initiated by RTK activation in the OMM include extracellular signal regulated kinase mitogen-activated protein kinase (ERK MAPK), the Pi3K/AKT and the Janus kinase (JAK) / signal transducer and activator of transcription (STAT) pathways.

4.4.1.1.4.5.1. ERK MAPK Pathway

A major signalling pathway of the RTKs is extracellular signal

regulated kinase mitogen-activated protein kinase (ERK MAPK) pathway that activates the small GTP-ase protein RAS.[395, 398] Activation of RAS initiates a multistep phosphorylation cascade that leads to the activation ERK1 and ERK2 by dual phosphorylation. [215, 393, 398, 409, 413] ERK1 and 2 subsequently translocate to the nucleus and regulate transcription and cell proliferation through phosphorylation of nuclear transcription factors that increase transcription of cyclins.[215, 393, 398, 409, 413, 414]

Inhibitors of the pathway are induced by RTK signalling but in a delayed fashion.[395, 409] RAS and RAF are able to increase the expression of SNAIL and RHO GTPases, creating an important cross-talk pathway for epithelial mesenchymal transition.[215]

4.4.1.1.4.5.2. PI3-Kinase AKT Pathway

The PI3Kinase (PI3K)/AKT pathway is another major intracellular pathway activated by RTKs. AKT, also called protein-kinase B, is involved in down-stream pathway of PI3-kinase and supervises the balance of apoptosis and cell survival by inhibition of pro-apoptotic proteins. [13, 215, 393, 398, 400, 409, 413]

PiIK catalyzes the conversion of PIP2 to PIP3 allowing phosphorylation of AKT. Activated AKT phosphorylation of a multitude of effectors mediate its anti-apoptotic/pro-survival function and induce cell growth and protein translation. AKT inhibits GSK3β, diminishing its ability to phosphorylate SNAIL and other transcriptases, reducing its degradation of cyclinD and freeing β-catenin to enter into the nucleus. It inhibits the pro-apoptotic factor BAD, and TSC1/2, which allows increased activity of mammalian target of rapamycin (mTOR) kinase to block apoptosis and autophagy.[215, 387, 398, 400, 413, 415-417]

mTOR integrates signals from nutrients, growth factors, energy and stress to regulate growth during early development, and ageing during adulthood.[387, 418-420] mTOR forms the catalytic unit of two distinct complexes, mTORC1 and mTORC2. mTORC1 is essential for

cell growth and proliferation playing a key role in protein, nucleotide and lipid production, and glucose metabolism.[215, 418, 419] mTORC1 suppresses catabolic pathways such as autophagy.[418-420] mTORC2 controls proliferation and survival. Its primary function is phosphorylation of AKT in response to IGFR signalling. It functions in regulating ion transport, cytoskeleton changes and cell motility and cell survival, being implicated in suppressing apoptosis.[419-421]

AKT increases the expression of NF-κB, increasing transcription of cyclinD, c-MYC and MUC1.[215, 379, 413, 415, 422] It promotes the expression of the anti apoptotic protein BCL-2 and MDM2 which block the tumour suppressor p53. [423, 424]

PTEN is an inhibitor of the PI3K/ AKT pathway. It prevents PI3K from activating AKT by blocking PIP2 to PIP3.[373, 415-417]

4.4.1.1.4.5.3. Stress Activated Protein Kinase JNK and p38MAPK Pathways

The stress activated protein kinases c-Jun amino N-terminal protein kinases (JNKs) and p38MAPK belong to the superfamily MAP-kinases and become activated by environmental stress, growth factors and pro inflammatory cytokines.[158, 215, 393, 425, 426] JNK and p38 MAPK family members function in a cell context-specific and cell type-specific manner to integrate signals that affect proliferation, differentiation, survival and migration.[427]

Stress signals are delivered by small GTPases of the RHO family, RHO, RAC and CDC42.[425-430] RHO proteins belong to the RAS superfamily and only become activated when bound to GTP. RHO GTPases are involved in cytoskeleton rearrangements and cell motility, and in cell proliferation, transformation and differentiation through their activation of JNK and p38MAPK pathways.[430]

EGFR and FGFR both activate JNK and p38MAPK pathways.[215, 413] TGFβR complexes also can activate this pathway. [215, 413, 425, 426]

Transient JNK signalling promotes cell survival however prolonged signalling has a direct pro-apoptotic effect.[427] The JNKs regulate activity of multiple other transcription factors through phosphorylation. These include ELK, p53, c-MYC and HSF-1. Phosphorylation of p53 inhibits ubiquitin-mediated degradation stabilizing the levels of p53. JNKs interact with the BCL-2 family to promote apoptosis.[158, 425-427]

p38MAPK can exert apoptotic or mitogenic effects that are dependent on cell type and context. [393] p38MAPK negatively regulates cell cycle progression both at the G1/S and the G2/M transitions by several mechanisms, including the down regulation of cyclins, up regulation of cyclin-dependent kinase (CDK) inhibitors and modulation of the tumour suppressor p53. Inducing cell arrest may enable DNA repair and lead to cell survival. Interaction with BCL-2 family members occurs and can promote apoptosis.[427, 431]

p38MAPK may have an important role in differentiation programs in embryogenesis and morphogenesis with a key role in proliferation arrest that occurs at the start of differentiation. It can directly phosphorylate and modulate the activity of several transcription factors involved in tissue-specific differentiation.[427]

There is significant crosstalk between the JNK, p38MAPK and ERK MAPK pathways. This cross talk contributes to the contextual impact of the various signalling pathways determining the ultimate fate of the cell.[158, 393, 427, 431]

4.4.1.1.4.5.4. JAK STAT Pathways

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway represents a major mechanism used to transmit signals from extracellular receptors to the nucleus. The canonical pathway consists of JAK and STAT proteins activated by receptor ligation and inactivated by negative regulators, including SH2-containing protein tyrosine phosphatase (SHP) and suppressor of cytokine signalling (SOCS) proteins. Cytokines and growth promoter

factors stimulate STAT proteins by acting on their specific receptors leading to the recruitment and phosphorylation of Janus kinase 1 and 2 (JAK-1 and JAK-2). These in turn phosphorylate STAT proteins at specific tyrosine residues promoting their homo- and hetero-dimerization. [387, 432]

JAKs contain SH2 domains that enable them to interact with RTKs. They have a N-terminal FERM domain, which allows interactions with other cytokine receptors such as interleukin 6 (IL-6) in inflammation. [215, 432] STATS consist of a unique N-terminus, with roles in nuclear translocation and protein interactions, followed by coiled-coil, DNA-binding, SH2 and variable TAD domains.[432]

Among STAT family members, STAT1 and STAT3 are often phosphorylated in serine residues, which further activate STATs. STAT dimers translocate to the nucleus where they bind to consensus DNA sequences and activate the expression of growth promoting genes, such as c-MYC and cyclinD.[387, 432]

4.4.1.2. Cell Senescence

Cell senescence is a hypo-replicative state characterized by irreversible cell cycle arrest, expression of anti-proliferative molecules (e.g., $p16^{INK4a}$), activation of damage sensing signalling pathways (e.g., p38MAPK) and nuclear factor κB (NF- κB).[366, 433] Differentiated cells, unlike stem cells, undergo a finite number of replications, likely controlled by an internal molecular clock reliant upon telomeres, repetitive DNA sequences at the end of chromosomes. Telomeres prevent DNA degradation and recombination, shortening with each cell division.[219, 351, 366] Telomerase is active, synthesizing and lengthening telomeres in stem cells, but is lost with differentiation. Shortening of telomeres triggers senescence to prevent the development of genetic instability and abnormalities in differentiated cells.[366]

Stresses including oxidants, gamma radiation, UVB light, genotoxic drugs, and oncogenic stimuli can trigger persistent DNA damage response (DDR) or stress signalling through p53 or $p16^{INK4a}$ –RB pathways.[366, 433] Chronic stress favors

senescence over apoptosis as part of tissue remodeling to prevent tissue loss and loss of function.[366]

Senescent cells increase in number with age[433] accompanied by increased lysosomal β-galactosidase activity responsible for the characteristic senescence-associated β -gal (SA- β -gal) staining. Senescent cells secrete pro-inflammatory cytokines and can modify their tissue microenvironment by adoption of both autocrine and paracrine features.[219, 366, 433]

4.4.2. Elective Cell Death

Development and homeostasis depend on the balance between cell survival and cell death. Cell death is a fundamental process that is regulated by multiple interconnected signalling pathways.[434-436]

4.4.2.1. Apoptosis

Apoptosis is a process of programmed cell death regulated by complex molecular signalling circuits that results in an orderly, energy-dependent enzymatic breakdown into characteristic molecular fragments, DNA, lipids and other macro-molecules which are easily consumed and eliminated by phagocytes without disturbing the normal function of surrounding tissues. This process is mediated by caspases, cysteine aspartate proteases. Apoptosis has a stereotypical morphological appearance including nuclear condensation and cell shrinkage, and generally does not elicit an immune response.[366, 436, 437]

Apoptosis can be initiated through two separate pathways, the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway. Both pathways converge and utilize caspases as their downstream effectors of death, as these proteases have thousands of targets and orchestrate the final stages of apoptosis.[366, 436, 437]

4.4.2.1.1. Caspases

Caspases are proteases that contain cysteine in the active proteolytic centre and are specific for aspartic acid.[437] Two subsets, known as effector and initiator caspases, are key regulators of apoptosis. The initiator caspases (caspases-2, -8, -9, and-10) are activated through a conformational change

produced by an upstream multiprotein complex. The effector caspases (caspases-3, -6, and -7) are the mediators of cell destruction through the proteolysis of thousands of cellular substrates. The initiator caspases cleave the inactive monomer forms of the effector caspases causing them to form active dimers and initiate cell death cascades.[436, 437] Caspase-9 is essential for the intrinsic or mitochondrial mediated pathway. Caspase-8 is involved in the death receptor pathway. Both act upon caspase-3.[437]

4.4.2.1.2. BCL2 Family

B-cell lymphoma 2 (BCL2) proteins are important regulators of cell death consisting of pro-apoptotic and anti-apoptotic proteins with three subgroups based on function and structure. Pro-apoptotic members, including BAX, BAD, BID, and BCLX$_S$, promote apoptosis in response to cellular insults. [436-438] BAD and BAX form pores in the outer mitochondrial membrane, releasing cytochrome c. [436, 437] Anti-apoptotic members, such as BCL2, BCLX$_L$, and BCLW, inhibit initiation of apoptotic machinery.[436-438] They limit permeability of the mitochondrial outer membrane, maintaining integrity of mitochondria and block release of different apoptosis-activating molecules such as cytochrome c, AIF and Endo G.[437]

The third group includes BAD, BID, BMF, PUMA and NOXA, which contain only the BH3 domain. The anti-apoptotic members, BCL2, bind directly to the BH3 domain. This inactivates BAD and BID. [436, 437, 439] The relative ratios of pro-apoptotic and anti-apoptotic BCL2-family proteins determine the sensitivity or resistance of cells to apoptotic stimuli.[438] PUMA and NOXA account for the pro-apoptotic action of p53 by binding to BCL2 and allowing the release of BAD and BID.[439]

4.4.2.1.3. P53

The p53 protein plays a critical role in the cell cycle via its action as a transcriptional regulator promoting cell cycle arrest and apoptosis. [440-443] It has since emerged as an important tumour suppressor. [18, 101, 113, 116, 417, 424, 437, 440, 444-456]

p53 is only activated when cells are stressed or damaged. The p53 protein shuts down the multiplication of stressed cells, inhibiting progress through

the cell cycle and will trigger apoptosis if cellular stress or damage persists.[417, 424, 436] This is achieved by functioning as a transcriptase, binding to particular DNA sequences and activating the expression of adjacent genes, which, directly or indirectly, lead ultimately to cell death or inhibition of cell division.[424]

The amount of p53 protein in cells is determined by the rate at which it is degraded rather than made. Degradation is achieved by MDM2 and MDMX. MDM2 ubiquitinates p53 and leads to proteasomal degradation, keeping p53 levels and activity low in unstressed cells.[423, 424] Hetero-oligomerization of MDM2 and MDMX via their RING domains is crucial for the suppression of p53 activity during embryonic development.[423]

The MDM2 gene is a target of p53, which increases its transcription, creating a negative feedback loop. As the levels of p53 increase, the levels of MDM2 increase lowering the levels of p53 and reducing transcription on MDM2, which then allows the levels of p53 to rise again. MDMX is induced by p53 only under certain conditions.[423, 424]

The DNA damage response pathway enables cells to sense and signal problems in DNA, arrest cell cycle at the cell-cycle checkpoints, activate appropriate DNA repair mechanism or eliminate cells with genomes beyond repair.[424, 436, 457] Sensors of DNA damage activate members of the phosphatidylinositol-3-kinase-like kinase family (PIKKs). These kinases phosphorylate p53 close to the MDM2-binding region of the protein, which do not affect its DNA- binding abilities, but influences its affinity for MDM2 and subsequent degradation.[423, 424, 457]

Aberrant growth signals activate p53. This occurs through stimulating the transcription or stabilization of $p14^{ARF}$, which then binds to MDM2 and inhibits activity. $p14^{ARF}$ is located within the nucleolus and appears to sequester MDM2 into this sub compartment, keeping MDM2 away from p53 within the nucleus where it can activate the transcription of its target genes.[424]

p53 targets $p21^{Cip1}$ which as a CDK inhibitor blocks G1-S phase and G2-M

phase.[375, 424] REPRIMO, another downstream effector gene of p53, induces cell cycle arrest at G2-M phase.[424, 458] Protein 14-3-3σ is expressed primarily in epithelial cells and is a target of p53. It has a positive role in regulating p53 and mediates a G2/M checkpoint following DNA damage.[424, 459]

p53 can trigger apoptosis through a number of mechanisms. The major mechanism is through targeting transcription of PUMA, which competes for binding with BCL2 leading to the release of BAD and BID. Transcription of NOXA has a similar effect.[424, 439] p53 targets p53-induced death domain (PIDD) in response to extrinsic death receptor signalling.[424, 436] Finally, p53 may cause death by directly stimulating mitochondria to produce an excess of highly toxic reactive oxygen species.[424]

4.4.2.1.4. Death Receptors

The tumour necrosis factor receptor (TNFR) superfamily is a family of cytokine receptors characterized by the ability to bind tumour necrosis factors (TNFs) via an extracellular cysteine-rich domain.[436, 437] Extracellular cues working through a subset of the TNFR family, the death receptors, activate the extrinsic apoptotic pathway. Ligand binding causes death receptors to recruit the adaptor molecule Fas-Associated protein with Death Domain (FADD). Inactive monomers of caspase-8 are then recruited and dimerized into active caspase-8 homodimers [436] to form the death-inducing signaling complex (DISC). Activation releases the caspase-8 homodimer from DISC, allowing it to activate caspase-3 and -7 to promote apoptosis. Forming a heterodimer with its catalytically inactive homologue c-FLIP competitively blocks formation of the caspase-8 homodimer and inhibits its action.[436, 437]

4.4.2.2. Necroptosis

Necroptosis is regulated necrosis that is dependent on receptor interacting protein kinase1 or 3(RIPK1, RIPK3) activity and occurs when a cell is unable to die via apoptosis, such as when ATP levels are low. It is characterized by cell swelling, swelling and rupture of mitochondria, rupture of cellular membranes, and spilling of the cytoplasmic contents to the extracellular space and cell lysis. Unlike apoptosis, necroptosis usually elicits an inflammatory recaction.[434-437]

The extrinsic apoptotic pathway and necroptopic pathways are intrinsically linked. Both are triggered by death receptors and mediated by caspase-8, which is pro-apoptotic and anti-necroptotic.[436, 437]

4.4.2.3. Autophagy

Autophagy is an evolutionarily conserved catabolic process that enables self-digestion of non-essential and/or unwanted cellular components to provide essential nutrients during periods of stress and starvation. It removes damaged and dysfunctional organelles, mis-folded proteins and foreign particles, including microorganisms, thereby protecting cells against infections. Autophagy modulates health and longevity through quality control functions affecting the regulation of innate and adaptive immunity, neurodegeneration, ageing and cell death.[158, 419, 435, 437].

Autophagy is triggered in response to a variety of internal and external stress stimuli like nutrient deficiency, hypoxia, endoplasmic reticulum stress, and oxidative stress.[435, 437]Regulation is strongly connected to signalling pathways that promote both cell proliferation (i.e. RAS, PI3-K/AKT) and cellular metabolism.[419, 437] Hyperactivation produces cell death.[435, 437]

4.4.3. Epithelial Mesenchymal Transition

Epithelial mesenchymal transition (EMT) is the process by which epithelial cells can trans differentiate, partially or fully, into mesenchymal cells. [215, 413, 460, 461] EMT is essential for development and maintenance of complexity in multicellular organisms.[255, 341, 413, 460-463] Mesenchymal-epithelial transition (MET) is the reverse process.[215, 460, 461] Both processes are transitional with cells not necessarily oscillating between full epithelial and mesenchymal states but moving through a spectrum of intermediary phases.[464] Surprisal analysis, an information-theoretical analysis technique that integrates and applies principles of thermodynamics and maximal entropy to relate microscopic to macroscopic properties, has demonstrated altering thermodynamic behaviours that are phenotype specific as a cell passes through different phases in the EMT process.[465]

EMT has been sub classified into three types by The EMT International Association (established 2003). Type 1 occurs during implantation, embryogenesis and organ development. Type 2 is associated with tissue

regeneration, wound healing and organ fibrosis and type 3 is associated with cancer progression and metastasis.[350, 460, 462, 466]

Type 1 EMT that occurs during development enables morphological plasticity and increased complexity of organisms.[341] The process is reversible and several rounds of EMT and MET are necessary to achieve the final differentiation of specialized cell types and complex three-dimensional structures. It drives the processes of implantation, gastrulation and neural crest migration. [341, 467] Type 1 EMT occurs without inflammation with WNT and NOTCH signalling being important pathways.[221, 341, 413, 466]

Type 2 EMT is a normal process for homeostasis and tissue maintenance in adult tissue. It occurs in wound healing, tissue regeneration and organ fibrosis in the setting of inflammation, the first response of mature epithelium to injury.[215, 221, 460, 466] Inflammatory cells and fibroblasts play a key role in mediating the process along with TGFβ1, an essential factor in both healing and fibrosis. [207, 350, 460, 462, 466]

Type 3 EMT is a biologic process that allows a polarized epithelial cell, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype. This includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of extra cellular matrix (ECM) components. The completion of EMT is signalled by the degradation of underlying basement membrane and the formation of a mesenchymal cell that can migrate away from the epithelial layer in which it originated. [350, 351, 460, 462, 466]

The three different types of EMT share a common set of genetic and biochemical events. Variability is determined by cell type and context.[460, 466] The first steps of EMT are disassembly of epithelial cell-cell junctions. There is loss of apical–basal cell polarity, replaced by front-rear polarity. Secondly there is re-organization of the cytoskeleton architecture with changes in cell shape and acquisition of motility through the formation of lamellipodia, filopodia and invadopodia. Expression of MMPs enables breakdown of the BM and ECM. Finally there is repression of epithelial genes and an activation of expression of mesenchymal genes, resulting in a mesenchymal phenotype.[215, 413, 460]

Whilst epithelial cells are dependent on their environmental context and cell-cell junctions for survival, growth and division, the change to a mesenchymal phenotype protects the cell from apoptosis after delamination.[341] The characteristic hallmarks of EMT are loss of E-cadherin, reduced tight junction proteins, such as occludin, and cytokeratins associated with an increase in mesenchymal markers such as vimentin, fibronectin, fibroblast specific protein 1 (FSP-1), α-smooth muscle actin (α-SMA), and N-cadherin.[127, 215, 315, 341, 413, 460-462, 466]

EMT is driven by a number of transcription factors that repress epithelial gene and promote mesenchymal gene expression. The major factors are the SNAIL Transcriptase family, the basic helix-loop-helix (bHLH) factors TWIST1 and TWIST2, and the zinc-finger-E-box-binding transcriptase (ZEB).[215, 341, 413, 460, 462, 463, 467-470]

4.4.3.1. SNAIL Transcriptase Family

The SNAIL family of transcriptional regulators has an essential role in cell differentiation and cell survival.[471] They are important in development of mesoderm and neural crest during embryogenesis.[349, 471, 472] The SNAIL superfamily of transcription factors is subdivided into SNAIL and Scratch families.[349, 471, 473, 474] SNAIL plays an important role in EMT during morphogenesis, wound healing and carcinogenesis.[127, 215, 350, 352, 469, 471, 473-475]

Transcriptional and translational regulation of SNAIL expression occurs through multiple signalling pathways including TGFβ SMAD-dependent and SMAD-independent pathways, WNT signalling, NOTCH signalling and growth factors acting through RTKs.[215, 471] SNAIL represses epithelial genes by binding to E-boxes through their carboxy-terminal zinc-finger domains, specifically to the CAGGTG sequence.[352, 416, 473] E-boxes form part of the promoter regions for genes coding for E-cadherin[127, 215, 347, 348, 352, 469, 471, 473, 476, 477], RAF kinase inhibitor protein (RKIP), a suppressor of metastasis [471], PTEN which acts as a tumour suppressor [471], occludin[215, 471], claudin-1, -3, -4, and -7[215, 471] which bind with occludin to form tight junctions, and MUC1.[471, 473] MUC1 through its association with β-catenin plays a role in cell signalling. Desmoplakin and cytokeratins 8 and 18 are repressed.[215, 471, 473]

SNAIL binds the E-box in the promoter region for the α subunit, α3, of LM332 in epithelial cells.[469] β and γ subunits are not associated with E-box sequences in promoter regions and are not impacted by SNAIL. SNAIL has been associated with reduced expression of α5 and increased expression of α4 laminin subunits in stromal cells.[478]

Expression of mesenchymal markers vimentin, fibronectin and N-cadherin is upregulated by SNAIL.[215, 471, 473] Increased expression is associated with an increase in the mRNA levels of MMP-2 and -9 and RHO- GTPases.

E-box binding with repression of epithelial genes, primarily E-cadherin, and increased transcription of mesenchymal genes induces EMT. [127, 350, 352, 469, 471, 472, 479] SNAIL increases the expression of TWIST 1, and ZEB1 and 2 further driving the EMT process.

GSK3β-mediated phosphorylation inactivates SNAIL transcriptional activity and leads to its ubiquitination and degradation. Restriction of GSK-3β to the cytoplasm after WNT–β-catenin activation prevents the destabilizing phosphorylation of SNAIL1 [215, 413, 460]

4.4.3.2. Basic Helix-Loop-Helix Transcriptase Family

The basic loop-helix-loop (bHLH) transcription factor super family of transcriptional regulators is essential in cellular differentiation, growth and pathogenic processes.[215, 480, 481] They contain a basic domain for DNA binding and the HLH domain to interact with another bHLH factor to form homodimeric or heterodimeric complexes.[215, 480] Class I bHLHs, or E proteins, and Class II bHLHs including TWIST recognize promoter E-boxes. Class V, or inhibitor of differentiation proteins (ID), lack a DNA binding domain.[215, 482, 483]

The E proteins are expressed in most tissues and can form homodimers or heterodimers.[480, 482] The E2A bind specifically to the E-box site on DNA, including the E-box of the promoter for E-cadherin. [482, 483] ID proteins bind preferentially with E2A proteins leading to down regulation of their activity.[481]

TWIST and other Class II bHLHs show tissue-specific expression.[480, 484] They preferentially form heterodimers with the E2A proteins. TWIST proteins

behave as transcriptional repressors through recruiting histone deacetylases or inhibiting acetyl-transferases, or as transcriptional activators.[480, 484] TWIST proteins directly interact with several other transcription factors, including p53 and NF-k B, and inhibit their activities.[480, 484]

TWIST is generally expressed in mesoderm-derived tissues including placenta, heart and skeletal muscles. Expression in brown fat has been found to be essential in adaptive thermogenesis, a process that consists of metabolizing fat as heat.[480, 484] Renewed expression of TWIST in other tissues is promoted by a number of mechanisms. Hypoxia-inducible factor 1α (HIF1α) induces TWIST expression under hypoxic conditions, promoting EMT. Mechanical stress induces TWIST expression via integrin-mediated activation of β-catenin. [215, 484] Increased TGFβ decreases expression of ID proteins that in turn increases the availability of E2A proteins to form heterodimers with TWIST.[215, 481, 484] SNAIL transcriptase increases TWIST expression.

4.4.3.3. Zinc- Finger E-Box Binding Transcriptase Family

Zinc-finger-E-Box-binding transcriptase 1 and 2 (ZEB1 and ZEB2) belong to a family of transcription factors characterized by the presence of 2 clusters of C2H2 type zinc fingers that mediate binding to the specific DNA sequences in the E-box promoter of numerous genes.[215, 463, 485] By recruiting co-suppressors or co-activators through other protein binding domains they can either down regulate or up regulate the expression of target genes.[347, 463]

ZEB1 and ZEB2 are crucial in T cell differentiation in the thymus and skeletal differentiation of limbs, sternum, ribs and craniofacial region during embryogenesis.[347, 463] In healthy adult OMM the expression of ZEB1 and ZEB2 is minimal.[463]

ZEB1 can bind directly to E-box of the CDH1 gene encoding E-cadherin, producing suppression of transcription. In conjunction with co-repressors, ZEB transcriptase factors contribute to remodelling of chromatin, such as histone demethylation and interacting with histone deacetylase to promote EMT.[215, 347, 463]

The miRNA200 family induces terminal epithelial differentiation, suppressing

EMT and reducing stem cell phenotypes through inhibiting translation of mRNA for ZEB1 and ZEB2. ZEB1 and ZEB2 down regulate the expression of miRNA200 creating a double- negative feedback loop between ZEB and miR-200 expression.[215, 470, 485, 486]

Expression of ZEB transcriptase factors is modulated by a number of pathways. TGFβ signalling increases expression through SMAD signalling and increased expression of SNAIL1 and via RAS-MAPK pathway and activation of ETS1. NOTCH and WNT, pathways can increase ZEB expression.[215, 347, 463, 469, 470, 485, 486] ZEB1 and the Hippo pathway effector YAP interact directly, shifting ZEB1 from a repressor to a transcriptional activator of mesenchymal genes.[487]

Epithelial–mesenchymal transition (EMT) progression is regulated by a number of signalling pathways that can cooperate to induce full EMT responses by increasing the expression of EMT promoting transcriptase factors.[215]

4.4.3.4. WNT Pathway

Activation of WNT signalling pathway promotes EMT. Due to the dual role of β-catenin in cellular adhesion and acting as an intracellular transducer of the canonical WNT signalling pathway, activation of WNT favours the promotion of mesenchymal phenotype. [343, 344] Additionally inhibition of GSK3β by WNT signalling prevents phosphorylation of SNAIL transcriptase. This allows it to accumulate and induce EMT.[388] Reduced degradation of cyclinD promotes cellular proliferation. [215, 387, 398, 400, 413, 415-417,215, 413, 460]

4.4.3.5. TGFβ Pathways

TGFβ signalling regulates a variety of key events in normal development and physiology is the most well characterized pathway that is known to induce EMT.[95, 215, 350, 413, 460, 486] Signalling is typically activated by the TGFβ superfamily of ligands, discussed in Section 4.1.3.2.

In development, TGFβ ligands play essential roles in organogenesis by determining the direction and extent of mesenchymal differentiation in part through modulating type 1 EMT. TGFβ1 and TGFβ2 expression is associated with EMT-like events in the formation of endocardial cushions, whereas TGFβ3 drives EMT that mediates palate fusion.[215, 340] NODAL helps maintain

pluripotency, specify cell fates, govern cell-sorting behaviour, and induces EMT.[287, 293, 302, 488] BMP participates in EMT that is required in heart cushion formation.[215] AMH has been demonstrated to mediate EMT in coelomic epithelium during Müllerian duct regression.[215]

In postnatal tissues TGFβ1 helps to maintain mesenchymal and epithelial stem cells in an undifferentiated state in preparation for tissue repair.[290] Its expression and activation are rapidly induced in response to injury.[290, 298] At the site of injury, epithelial cells undergo partial or complete EMT associated with an increase in motility as a result of increased TGFβ1 signalling, and then subsequently reacquire their epithelial phenotype.[215, 289, 290, 293, 349] TGFβ1 increases synthesis of tissue inhibitor of metalloproteinase (TIMP), which inhibits MMP expression, creating a negative feedback loop on MMP activation of latent TGFβ1 enabling the wound healing process to be self-limiting. [290, 298, 489]

Liberation of biologically active TGFβ requires its dissociation from LLC.[289, 298, 299, 301] This process of latent TGFβ activation is mediated by proteolytic cleavage of LLC by numerous factors including BMP-1, a variety of matrix metalloproteinases (MMPs), including membrane-type- 1 (MT-1) MMP, MMP-2, MMP-3, MMP-9, and MMP- 13, plasmin, urokinase-type and tissue-type plasminogen activators, thrombin, elastase, and by cathepsin and integrins on epithelial cells and fibroblasts.[291, 298, 301] Mechanical stress activates latent TGFβ independent of any proteolytic activity.[289, 298, 299, 301]

4.4.3.5.1. TGFβ Receptors

The TGFβ ligands exert their influence on cells, including the induction of EMT, via acting on TGF-β receptors. TGF-β receptors are grouped into Type I and Type II TGF-β receptors (TβRI and TβRII).[287, 295, 296] There are seven TβRIs called activin receptor-like kinases (ALK)[287, 295, 296], and five TβRIIs: TGF-βRII, two activin receptors (ACTR and ACTRB), anti-Müllerian hormone receptor (AMHR), and bone morphogenetic protein receptor (BMPR).[287, 295]

The receptors work in tandem with ligands binding two TβRI and two TβRII receptors to form heterotetrameric complexes. This ligand-bound receptor complex allows TβRII to cross- phosphorylate TβRI. Phosphorylation

activates TβRI and provides a binding site for the downstream substrates. Some ligands require additional co-receptors for optimal binding to the TβRI/ TβRII complex.[287, 288, 295, 296]

TGF-β receptors primarily activate SMAD signalling but also activate RTK intracellular pathways, including the mitogen-activated protein kinase (MAPK), phosphatidy-linositol-3-kinase (PI3K)/AKT, and RHO-like GTPase signalling pathways to produce EMT.[215, 288, 295, 296]

TGF-β receptors can function independently of downstream signalling molecules. TβRII interacting with phosphorylate PAR6 recruits the ubiquitin E3 ligase SMURF1 to degrade RHO-A, leading to loss of tight junctions and epithelial-mesenchymal transition.[293, 413, 460, 490] Polyubiquitination of TβRI by ubiquitin ligase tumour necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) promotes cleavage that releases a intracellular domain (TβRI-ICD). TβRI-ICD enters the nucleus and associates with p300 to regulate the expression of target genes such as SNAIL and MMP2.[491]

Regulation of receptor complex formation is an important mechanism to control TGF-β mediated signalling. TGF-β receptors exist on the membranes as monomers in resting cells and undergo dimerization on TGFβ stimulation.[295] TGF-β co-receptors facilitate TGFβ signalling by helping presentation of the TGFβ ligands to TβRII.[287, 295]

Extracellular BMP and activin membrane bound inhibitor (BAMBI) and the ETV6-NTRK3 chimeric tyrosine kinase attenuate TGFβ signalling by interfering with the heterocomplex formation of TGFβ receptors.[287, 295] Numerous extracellular soluble antagonists impact on TGFβ receptor signalling and a number of the TGFβ superfamily are antagonists themselves. GDF3 inhibits BMPs and BMP3 is a soluble antagonist for both other BMPs and GDFs.[287]

Phosphorylation is required for activation. TβRIIs are constitutively active and can undergo auto-phosphorylation producing activation or an inhibitory effect depending on which serine residue is phosphorylated.[295] TβRII activates TβRI through the phosphorylation of its GS domain.[287, 288, 295,

[296] The immunophilin FKBP12 binds to this GS domain preventing phosphorylation and activation from occurring.[295] Protein phosphatases play a role in regulation of signalling and generate negative feedback loops.[295]

The inhibitory SMAD, SMAD7 mediates ubiquitination of TGF-β receptors through the recruitment of E3 ligases SMURF1, SMURF2, NEDD4-2, or WWP1 to the receptor. Multiple proteins and mechanisms control the process.[295] Small ubiquitin-like modifier (SUMO) can modify TβRI by sumoylation in ligand-dependent manner that enhances the recruitment and activation of SMAD3, and consequently regulates TGF-β responses.[492]

TGF-β receptors are partitioned between the lipid raft micro-domains and non-raft parts on the plasma membrane, regulated by the extracellular domain of TβRII.[493] TGF-β receptors located in the non-raft part of the plasma membrane are internalized via clathrin-dependent endocytosis of the receptors to early endosomes which promote r-SMAD activation and SMAD complex formation.[295, 494] Trafficking of internalized receptor complexes includes recycling of receptors back to the plasma membrane or directing them to lysosomes for degradation.[295, 494] Insulin promotes increased translocation of TGF-β receptors from intracellular stores to the cell surface.[495]

Distribution of TGF-β receptors in lipid-raft microdomains is necessary for activation of the MAPK pathways, ERK and p38.[496] SMAD signalling is not activated. Endocytosis of TGF-β receptors in lipid-raft microdomains is associated with Caveolin-1, which inhibits TGF-β signalling by interacting with TβRI and promotes TβRI degradation in a SMAD7/SMURF2- dependent manner.[295, 494]

4.4.3.5.2. SMAD Signalling

The SMADs are a group of intracellular signalling molecules activated in response to TGF-β receptor complex activity. They are classified into three groups: receptor-regulated SMADs (R-SMADs) SMAD1, 2, 3, 5, and 8, the common mediator SMAD (Co-SMAD), SMAD4, and the inhibitory SMADs (I-SMADs), SMAD6 and 7.[293, 340, 411, 497, 498]

R-SMADs and SMAD4 share a similar structure consisting of a N-terminal Mad Homology 1 (MH1) domain that mediates DNA binding, a C-terminal MH2 domain that mediates oligomerization, and a linker domain.[293, 411, 497] SMAD2 contains an extra insert in the MH1 domain and does not bind to DNA.[293, 497]

The I-SMADs lack a phosphorylation motif in the MH2 domain. This makes them unable to bind with SMAD4 and subsequently act as inhibitors of the pathway.[293]

The specific combination of TβRI and TβRII determines which R-SMAD and which intracellular SMAD signalling pathway is activated. In general TGFβs, activins and NODAL activate SMAD2/3 whereas BMPs and GDF's activate SMAD1/5/8.[287, 293, 295, 296, 340] Recent research has shown that TGFβ does also induce phosphorylation of SMAD1 and SMAD5 in many cell types in addition to SMAD2 and SMAD3.[287, 340]

Phosphorylation plays a central role in controlling the duration and magnitude of SMAD signalling.[293, 497] This can occur at the in MH2 COOH tail or in the linker region. The site of phosphorylation helps to direct physiological activity.[497] Phosphorylation in the linker regions serves an important function in regulating stability, activity and transport of R-SMADs.[497]

Phosphorylation of TβRI enables receptor-mediated phosphorylation of the R-SMADs MH2 tail allowing formation of heteromeric complexes with SMAD4.[287, 291, 293, 446, 497] The I-SMADs compete with the R-SMADs for binding to TβRI, inhibiting signalling and leading to receptor degradation. [215, 293, 415, 497]

Activated SMAD2/SMAD3 or SMAD1/5 complexes bind with SMAD4 for transport into the nucleus. Phosphorylation of the COOH-tail of SMAD3 via TβRI in normal epithelial cells suppresses proliferation by reducing expression of c-MYC and inducing expression of the CDK inhibitors $p15^{INK4b}$ and p27Kip1 preventing cell cycle progression.[411, 415, 497] In contrast,

phosphorylation of SMAD3 via the RAS/MAPK pathways occurs at the linker region. This results in an increase in c-MYC expression that blocks the CDK inhibitors, advancing cell cycle and promoting cell survival. Phosphorylation of the linker region also prevents phosphorylation of the COOH-tail by TβRI.[411, 497] Persistent TGFβ signalling can enable additional phosphorylation of nuclear located phosphorylated SMAD2 and SMAD3 at the linker region by cyclin-CDK complexes, increasing cell growth and increasing expression of MMPs.[497]

The R-SMADs and SMAD4 constantly shuttle between the cytoplasm and nucleus. Nuclear accumulation of active SMAD complexes is achieved by a decrease in SMAD nuclear export rate and an increase in import rate.[340, 497] SMAD protein complexes require active transport into and out of the cell nucleus. This is facilitated by karopherins (importins and exportins) and nucleoporins.[293, 340]

Within the nucleus SMAD complexes target specific promoters to regulate gene expression. SMAD4 and all the R-SMADs except for SMAD2, bind to DNA via SMAD binding elements (SBE) found in the E-Box of various gene promoters. SMADS have low affinity for the SBE sites and rely on concentration or direct interaction with high affinity DNA binding proteins, such as FOXH1, for binding.[293, 460, 497] Co-regulators that promote DNA transcription include basic chromatin remodelling complexes and histone modifying acetlytransferase, whilst histone deacetylases, SKI and SKIL, reduce transcription.[293]

SMAD2/SMAD3/SMAD4 complex increases the expression of the EMT transcriptases SNAIL, TWIST and ZEB1 and ZEB2. In addition, the activated complex binds directly to the E-box of E-cadherin to reduce expression.[215, 288, 293, 413, 497]

In combination with ATF-3 transcriptional repressor, SMAD signalling blocks expression of inhibitor helix-loop-helix 1 (ID1) protein. The ID family are inhibitors of differentiation and form dimers with bHLHs to inhibit their activity. Blocking ID1 enables E2A dependent repression of E-cadherin.[215, 483]

SMAD2/SMAD3/SMAD4 positively regulates the expression of all three genes for LM322. The LAMα3 gene has a promoter that contains 2 E-boxes with SBEs. LAMβ3 and LAMγ2 do not have promoter associated E-boxes. The mechanism of transcriptional regulation is via SMAD4 binding to API sites with API proteins as co-regulators, creating a divergent mechanism of regulation of expression.[469, 499]

SMAD1/SMAD5/SMAD8 promotes expression of ΔNp63 in epithelial cells.[447] In addition, ΔNp63 acts a co-regulator with SMAD1/SMAD5/SMAD8 complex and synergistically enhances the effects of BMP signalling including binding to the promoter of inhibitor of differentiation (ID2), a pro-survival BMP target gene, and other genes that promote EMT.[500]

4.4.3.5.3. Non-SMAD Signalling

TGFβ ligands complement their SMAD signalling through inducing non-SMAD signalling pathways via TGF-β receptors. Activation of these pathways produces non-transcription changes and cooperation with SMAD-mediated gene expression during EMT and directly regulates the stabilities and activities of SMADs.[215, 288, 290, 293, 350, 413, 460]

TβRII phosphorylates PAR6, a cell polarity protein, with subsequent recruitment of SMURF1 that marks RHO-A for degradation by RHO-A GTPase. Loss of RHO-A promotes EMT through changes in organization of actin filaments to favour formation of lamellopodia, loss of adherens junctions, decreased cell adhesions and increased motility. Loss of RHO-A may increase SNAIL expression.[293, 413, 460, 490]

TGFβ induces ERK MAPK signalling through the adaptor protein SRC homology 2 domain-containing-transforming A (SHCA), which associates with the TβRI receptor.[215, 413] TβRI can activate the p38MAPK and JNK cascades via the E3 ligase member TRAF6. Association of TRAF6 with TGF-βRI causes activation of TGF-β–activated kinase 1 (TAK1) postulated to promote NF-κB signalling and EMT. TRAF6 is also involved in stimulation of EMT by disrupting junction assembly at the plasma membrane.[293, 413, 415, 460]

TGFβ family proteins activate the PI3K-Akt pathway, leading to activation of TOR complex 1 (mTORC1) and mTORC2 and enhanced protein synthesis. In TGFβ-induced EMT, mTORC1 contributes to the increased cell size, protein synthesis, motility and invasion that take place. mTORC2 is required for transition from an epithelial to a mesenchymal phenotype. [215, 413, 460]

TGFβ stimulates reactive oxygen species (ROS) production by various mechanisms including increasing mitochondria and activation of NADPH oxidases (NOX) family members.[292, 297] These in turn, engage a number of downstream signalling pathways including SMAD and MAPK signalling. ROS increases expression and activation of TGFβ1, and modulates TGFβ1 signalling to favour MAPK over SMAD signalling. [292, 297]

4.4.3.6. Receptor Tyrosine Kinase Pathways

Receptor tyrosine kinase receptor (RTK) signalling has a major role in EMT both independently and synergistically with TGFβ signalling.[215, 350, 351, 411, 460] RTK signalling increases TGFβ expression producing increased autocrine TGFβ signalling.[215, 411] TGFβ in turn increases expression of betacellulin that is a signalling ligand for EGFR[351] and promotes FGFR isoform switching. [215, 411]

Activation of the PI3K-AKT signalling by EGFR or IGFR increases expression of mTOR, promoting cell growth, replication and survival through its two complexes mTORC1 and mTORC2.[215, 387, 418, 419, 421, 501] AKT inhibits GSK3β resulting in stabilization of SNAIL transcriptase, reduction of E-Cadherin expression and reduction in SMAD ubiquitylation.[215, 351, 460] AKT increases expression of NF-κB, which increases the transcription of cyclinD, c-MYC and MUC1.[215, 379, 413, 415, 422]

EGFR interacts directly with β-catenin in hemidesmosomes producing endocytosis of E-cadherin. This releases β-catenin enabling its relocation to the nucleus where it acts to increase transcription of SNAIL and TWIST via the WNT signalling pathway.[215, 351] EGFR increases expression of MMP-9 and MMP-2 via the ERK MAPK pathway that degrades E-Cadherin, breaking down hemidesmosomes with further release of β-catenin and increased cell motility.[215, 350]

FGFs are EMT inducers in development enabling generation of mesoderm and neural crest migration. In adult epithelial tissue, FGF stimulation of MAPK in the presence of TGFβ signalling during EMT produces mesenchymal features.[215, 351] TGFβ signalling down regulates epithelial splicing regulatory proteins 1 (ESRP1) and ESRP2, switching IIIb isoforms of FGFRs to IIIc isoforms. IIIc isoforms, associated with mesenchymal tissue, are more sensitive to FGF2 and produce autocrine signalling.[215, 411]

Insulin like growth factor (ILGF) acting via IGFR increases expression of ZEB1 and 2 via the ERK MAPK pathway.[215, 460] IGFR can form complexes with E-cadherin and av-integrins promoting cell migration.[215, 351]

RAS and RAF, activators of the ERK-MAPK pathway, are able to increase the expression of SNAIL and RHO GTPases, creating an important cross-talk pathway for EMT.[215, 411, 460]

4.4.3.7. NOTCH Signalling

NOTCH signalling is an evolutionarily conserved pathway essential in many processes during development and in adult tissues. It is involved in cell fate control during development, stem cell self-renewal and postnatal tissue differentiation, proliferation, apoptosis and has a role in EMT.[205, 486, 502]

NOTCH signalling enables communication between contiguous cells via trans membrane ligands and receptors. There are four NOTCH receptors, NOTCH1, 2, 3 and 4, which are single-pass type I trans membrane receptors.[205, 486, 503] The receptors exist as a non-covalently linked heterodimer at the cell surface.[205] Trans membrane ligands, Delta-like 1, 3 and 4, and Jagged1 and 2, located on adjacent cells activate the NOTCH receptors.[205, 486, 502]

On activation, the NOTCH receptor is cleaved at the plasma membrane by a disintegrin and metalloprotease (ADAM) 10 or 17[205, 486, 502] to produce an active NOTCH intracellular fragment (NIC). NIC is translocated to the nucleus where binds to DNA transcriptional repressors, displacing co-repressors and recruiting co-activators This enables expression of genes such as those coding for NF-κB, AKT, p21 and c-MYC.[205, 413, 486]

EMT is promoted by NOTCH signalling through direct increased expression of SNAIL. It also induces expression of hypoxia-inducible factor 1α (HIF-1α), which binds to the promoter of LOX (encoding lysyl oxidase) increasing its transcription. LOX stabilizes SNAIL.[215, 413, 486] NOTCH indirectly mediates E-cadherin suppression in a SNAIL dependent manner and increases β-catenin activation.[413, 486]

NOTCH is inhibited by ΔNp63, which maintains epithelial expression.[504] miRNA200 targets JAG1 to down regulate NOTCH signalling in a feedback loop. miR200 itself is inhibited by the intracellular cascade produced by binding of JAG2 to NOTCH receptors, which favours EMT.[413, 486]

NOTCH signalling is regulated by ubiquitination. E3 ligases are responsible for ubiquitination of NOTCH ligands, which is required for proper trafficking and presentation of active ligands on the cell membrane. NOTCH receptors are also ubiquitinated.[205]

4.4.3.8. Hedgehog Signalling

Hedgehog signalling (HH) is another highly evolutionary conserved pathway important in EMT.[215, 413, 486, 502, 505] It is important in tissue patterning during development[215, 502, 506, 507] whilst in post-natal tissues it has a role in regulating cell fate specification, differentiation and tissue homeostasis.[504, 506, 507]

HH signalling is transmitted via trans membrane receptors patched homolog 1 (PTCH1) and PTCH2. Unlike other pathways, unbound receptors actively inhibit activity of Smoothened (SMO), a member of the G-protein coupled receptor (GPCR) superfamily. SMO is an obligate transducer of the HH signal and its inactivation causes the complete loss of HH target gene expression.[413, 502, 504, 506, 507]

There are three ligands: Sonic Hedgehog (SHH), Desert Hedgehog (DHH), and Indian Hedgehog (IHH). Ligand binding with PTCH1 and PTCH2 causes release of SMO and activation of the HH pathway. SMO initiates an intracellular signalling cascade that leads to activation and nuclear localization of GLI transcription factors, driving expression of HH target genes.[413, 502, 504, 506, 507] Cytoplasmic suppressor of fused homolog (SUFU) modulates the HH pathway

by inhibiting nuclear translocation of GLI proteins and facilitating processing into repressor forms.[502, 504, 506, 507]

Target genes are involved in proliferation, survival, angiogenesis and stem cell maintenance.[224, 502, 507] HH signalling promotes EMT in epithelial tissues by increasing the expression of SNAIL, induces increased secretion of TGFβ1 and increases cell survival by increasing expression of the anti-apoptotic BCL2.[413, 506, 507]

4.4.4. Epithelial Stratification, Turnover and Healing

Forming a vital and dynamic protective barrier is an emergent property of μ_{abcd}(omm). Stratification of the OMM epithelium, formation of tight junctions, constant turnover over of cells through terminal differentiation and replacement, and rapid healing are key mechanisms used for maintenance of on-going and viable barrier function. [242-244, 257, 324, 328]

Despite sharing common morphological features when compared to skin, wound healing in the OMM is associated with less inflammation, more rapid re-epithelialization and less formation of scar tissue.[324, 328] Turnover is also more rapid. Average turnover time of OMM epithelium is about 14 days, with variation depending on site. The turnover of the OMM is faster in the lining than in the masticatory regions, and can be as quick as 6 days.[244, 257]

Neural crest cells contribute to formation of structures in the craniofacial region during development, including the OMM. Epithelial stem cells in the basal layer of epithelium and OMLP-PCs are integral to the mechanisms of rapid healing and turnover that contribute to the barrier property of the OMM These adult stem cells originate from neural crest.[244, 257, 323, 325, 326, 328, 508]

4.4.4.1. Neural Crest Cells

Neural crest cells (NCCs), which emerge from the dorsal aspect of the neural tube during early development, contribute to formation of structures in the craniofacial region including bones, teeth, oral muscles and muscles of facial expression, tongue, and craniofacial nerves.[325, 509] In the OMM NCCs contribute to specialized cells such as melanocytes, Merkel cells and adult stem cells in the basal epithelial layer, the LP and in the junctional epithelium around dentition.[258, 323-325, 360, 508-510] These adult stem cells are integral to the

mechanisms of rapid healing and turnover that contribute to the barrier property of the OMM.[244, 257, 323, 325, 326, 328, 508]

Stem cells provide a life-long reservoir of cells for tissue renewal.[257] They make up a very small fraction of the total number of epithelial cells in the OMM and have a low proliferation rate with specific spatial ordering.[511] Asymmetric division of stem cells results in the persistence of the normal stem cell and generation of one amplifying transitory cell (ATC). ATC cells are slightly more differentiated than stem cells yet highly proliferative. They continue to split between three and five times before undergoing terminal differentiation/maturation into the functional cells of the tissue. The number of ATCs in the epithelium at any one time can vary dramatically.[257, 511] ATCs expand laterally to occupy the parabasal layer and do not, under normal circumstances, move into the more superficial layers. It is proposed that stem cells form proliferative clusters with the coordinated and alternating activity of the stem cells within the group with ATCs continually proliferating in the parabasal layer to provide the cells that renew the epithelium, thus conferring an antitumor mechanism by keeping stem cell proliferation rate low whilst maintaining homeostasis.[511]

Stratification of the epithelium, as described previously, enables the OMM to utilize terminal differentiation with shedding and on-going renewal from the epithelial adult stem cells in the basal layer as a barrier mechanism.[257, 324, 511] Stratification and maintenance of the stem cell population in epithelium has been shown to depend on the expression of p63.[503]

4.4.4.2. P63

p63 and p73, identified in the late 1990's, are homologues of p53. [445, 512] Both p63 and p73 are essential for development of normal tissues. p73 has a role in the development of neuronal and pheromonal pathways[440, 442, 512, 513] [514] whilst p63 is crucial for epithelial tissues that show stratification. [4, 27, 30, 32-37] Unlike p53, neither p63 nor p73 function primarily as tumour suppressors and are rarely mutated in cancers.[442, 449-451, 515, 516]

p63 controls the expression of specific genes involved in epithelial tissue

integrity and homeostasis, cell survival, as well as migration and EMT related features in wound healing and in cancer cells. It has distinct roles in development of stratified epithelial structures and ectodermal appendages, such as teeth and salivary gland. p63 is essential for craniofacial and limb development and maintenance of the regenerative capacity of stratified epithelial structures including oral and upper aerodigestive tract mucosa in adult tissue. [440, 444, 445, 449, 450, 455, 503, 515-518]

p63 shares the hallmarks of p53: an acidic, amino-terminal transactivation (TA) domain, a core domain for DNA- binding (DBD) and a carboxy-terminal oligomerization domain (ISO). The homology in the DBD region of the three genes is over 60% including preservation of all the essential DNA contact residues.[442, 444, 445, 453, 512, 519] The ISO domain is highly preserved between p53, p63 and p73. This area is important in allowing the proteins to oligomerize to generate the correct tetramer conformation to activate the DBD. [442, 447]In addition p63 isoforms may have a protein-protein interaction domain, sterile α motif (SAM) and a transactivation inhibitory domain (TID) (*Fig. 10)*.[440, 442, 444, 445, 447, 449, 450, 512, 519]

Different isoforms of p63 are produced by alternative promoter usage and alternative splicing of the mRNA. Activation of P1 promoter leads to transcription of the TA domain at the N-terminal. Transcription through the second promoter, P2, results in mRNA that lacks the TA region. This isoform is denoted by the ΔN (ΔNp63). The lack of the TA domain leads to different biological behaviour. [442, 443, 520] Alternative splicing occurring at the 3′ end of the TP63 RNA generates at least three C-terminal variants (α, β, and γ) for both TAp63 and ΔNp63. β and γ isoforms are truncated at the C-terminal and lack the SAM and TID domains. ΔNp63α, which will be denoted by ΔNp63, is the predominant isoform and in normal mature OMM the only isoform detected.[442, 447, 449, 450, 503, 521-524]

The TA domain of TAp63 isoform leads to transactivation of target genes that arrest cell cycle and induce apoptosis, very similar to that of p53.[442-445, 447, 449, 450, 503, 515, 525] Under normal physiological conditions TAp63 expression is restricted to the oocytes of the ovaries. It is not readily detected in stratified epithelial tissues, including the OMM.[449, 504, 525, 526] TAp63 is activated in adult tissue in response to UV damage, with a corresponding reduction in ΔNp63,

and coordinates with p53 to drive apoptosis.[447, 525, 527]

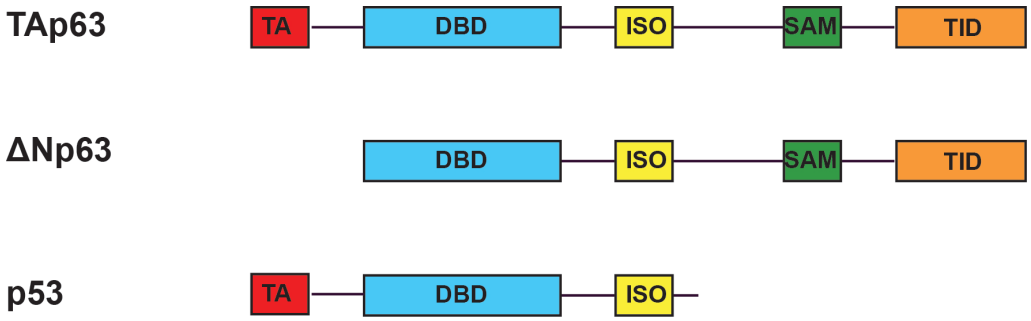


Figure 10 Schematic diagrams of p53 and p63 isoforms

p53, p63 and p73 (not shown) are homologues of the same family. Even though p53 was discovered first it has most likely evolved from the p63/p73 gene sequences. The highest homology is in the DNA binding domain (DBD, light blue), followed by the carboxy-terminal oligomerization domain (ISO, yellow). p63 has a protein-protein interaction domain, a sterile α motif (SAM, green) and a transactivation inhibitory domain (TID, orange). The TAp63 isoform contains, as does p53 an acidic, amino-terminal transactivation domain (TA, red) that is involved in transactivation of target genes that arrest cell cycle and induce apoptosis. TAp63 in expressed in oocytes and is not usually detectable in stratified epithelium under normal physiological conditions. The p63 gene has a number of promoters to produce the different isoforms. Transcription commenced at P2 results in RNA lacking in the TA domain to produce ΔNp63, the most common isoform in stratified epithelium, being found in the basal layers. Lack of the TA domain enables the SAM and TID domains to compete with p53 and TAp73 producing an opposing (anti-apoptotic) biology effect. ΔNp63 has its own set of target genes.
Original drawing based upon drawings and text description from various references. [440, 442, 444, 447, 449, 450, 503, 519, 521]

ΔNp63 is a major influencer of a number of key pathways employed by the OMM during development and in maintaining its function (*Fig. 11*).[503, 523] In contrast to TAp63, lack of the TA domain and presence of the SAM and TID domains in ΔNp63 leads to an opposing action on p53-targeted genes. ΔNp63 competes with p53 and binds to the promoters of the same target genes. It promotes progression through the cell cycle and proliferation by binding to the promoter of the CDK inhibitor $p21^{Cip1}$, a target of p53, and inhibiting expression. It directly inhibits p53 and TAp73, blocking their pro-apoptic actions as the dominant negative regulator (*Fig. 11*).[442-445, 447, 449, 450, 503, 515, 525] ΔNp63 also inhibits apoptosis in a p53 independent manner and has it's own set of target genes.[442, 503, 525]

During morphogenesis, ΔNp63 induces p53 effector related to PMP-22 (PERP), a desmosomal component that is critical for cell adhesion in the epidermis,

soon after cells have adopted an epidermal fate. [522-524] Subsequently, ΔNp63 leads to the expression of the embryonic basement membrane component, Fraser syndrome 1 (FRAS1) [522] and then of Ik B kinase- α (IKKα), which drives differentiation [522, 523, 526] and is essential for cell cycle withdrawal during spinous layer development in stratified epithelium.[522]

ΔNp63 and NOTCH signalling interact to maintain a balance between stemness/ proliferation and differentiation. Opposing gradients of ΔNp63 and NOTCH activation govern the balance between self-renewing and transit-amplifying cells. In development, during early differentiation they synergize to produce the terminal differentiation marker keratin 1.[503, 522, 523, 525] NOTCH signalling increases expression of interferon regulatory factor 6 (IRF6), required for proper palate closure during embryonic development and a key determinant of the keratinocyte proliferation-differentiation switch.[449] IRF6 is a direct transcriptional target of ΔNp63.[504, 523] When up regulated by ΔNp63, IRF6 promotes proteasome-mediated degradation of ΔNp63, thereby creating a negative feedback loop to control the expression of both ΔNp63 and itself.[449, 523] In mature tissue NOTCH is down regulated by ΔNp63 in cells that have high renewal potential and NOTCH in turn down regulates ΔNp63 to permit differentiation (*Fig. 11*).[503, 523, 525]

HH signaling is another key pathway regulating cell fate, differentiation and tissue homeostasis and is also involved in regulating p63 expression. [504, 506, 507] HH signaling has been shown to increase TAp63 and decrease ΔNp63 during development.[504] TAp63 increases expression of IHH, promoting proliferation whilst ΔNp63 inhibits its expression, keeping cells in a quiescent state.[503] In mature OMM HH signaling increases the expression of ΔNp63, which in turn increases the expression of SUFU.[506] SUFU inhibits the effector GLI proteins by inhibiting nuclear translocation and facilitating processing into their repressor forms creating a negative feedback loop.[502, 504, 506, 507] Loss of SUFU in mature tissues suppresses differentiation (*Fig. 11*).[506]

Interactions between ΔNp63 and the WNT pathway help to maintain epithelial stem cells. ΔNp63 maintains stemness through the direct transcriptional control of the FZD7 receptor [503, 504] and, to a lesser extent, of the WNT5B ligand (*Fig. 11*).[503] Activation of the WNT pathway allows translocation of β-catenin

into the nucleus where it acts as a transcriptional co-activator binding to T-Cell Factor (TCF) and lymphoid enhancing factor (LEF) transcription factors and dissociates repressors such as Groucho. This enables transcription of genes that inhibit apoptosis.[215, 343, 344, 385, 388-391] The promoter for ΔNp63 has also been identified as a target of LEF/TCF with β-catenin co-activation.[504]

ΔNp63 has a key role in maintaining epithelial cell adhesion. ΔNp63 induces expression of PERP and leads to increased expression of K14, envoplakin and BPAG1, an important component of hemidesmosomes.[503, 526] Integrins β1 and β4 are also enhanced by ΔNp63.[503, 523] Integrin β4, alongside BPAG1, combines with integrin α6 to form part of hemidesmosomes. [262, 355, 357] Integrin β1 preserves the stem cell population by balancing symmetric and asymmetric stem cell divisions through the control of spindle orientation.[503] This process is also important for the development of stratification.[528, 529]

Epithelial-mesenchymal interaction in the OMM is important in maintaining the structural and functional integrity of its barrier function and responding to environmental factors. ΔNp63 located in the basal epithelial cells on the basement membrane enables mesenchymal fibroblasts to provide instruction through various growth factors and vice versa. Interleukin1α (Il1α), a ΔNp63 target gene, induces growth factors including fibroblast growth factor 7 (FGF7) that are important in epithelial development and homeostasis in fibroblasts. [412, 504, 523] These bind to receptors on the basal epithelial cells to promote proliferation.[504, 523] In addition to increasing expression of FGFR ligands from the ECM, ΔNp63 can induce the expression of FGFR2.[412, 504] FGF7 binding to FGFR2 also increases expression of ΔNp63 completing the positive feedback loop (*Fig. 11*). [412, 504]

ΔNp63 expression is promoted by EGFR signaling through activation of the PI3K pathway in both normal keratinocytes and SCC cells.[442, 504] The STAT3 growth-stimulatory pathway, triggered by EGFR, may also increase expression of ΔNp63.[504] In turn, EGFR itself is a transcriptional target of ΔNp63, implying positive feedback regulation through ΔNp63 (*Fig. 11*).[442] PTEN, which inhibits the PI3K/AKT pathway, is down regulated by ΔNp63, allowing increase in anti-apoptosis activity.

BMP signaling through the SMAD1/5/8 complex is a negative regulator of stem cell proliferation. It increases expression of ΔNp63α that in turn leads to transcription of BMP7. In normal stratified epithelium ΔNp63-mediated activation of BMP7 signaling governs stem cell activity and plasticity.[447, 500, 503, 518] Adult stem cells do not have the capacity for unlimited divisions. This pathway favors epithelial stem cell quiescence and prevents premature stem cell exhaustion (*Fig. 11*). [503, 518] [500]

ΔNp63 interacts with SMAD1/5/8 complex to activate the promoter of inhibitor of differentiation 2 (ID2). The ID family, are helix-loop-helix proteins that lack a DNA-binding domain and function as a dominant-negative regulator of bHLH through the formation of inactive heterodimers with intact bHLH. Important differences in their expression pattern generate binding specificity for bHLH. ID1 blocks E2A, a repressor of E-Cadherin expression. ID2, considered a prosurvival BMP target gene, helps to maintain stemness.[500, 518] ΔNp63 induced expression of ID3 has been demonstrated to inhibit ETS-1 induced matrix-metalloproteinase-2 (MMP-2) and reduce motility (*Fig. 11*).[483]

Though the TGFβ pathway primarily involves activation of SMAD2/3/4 complex, it has also been shown to activate the SMAD1/5/8 complex through the TβRI, ALK5. This has the ability to activate the intracellular BMP pathway in a ΔNp63 dependent manner during EMT.[500, 518] ΔNp63 acts as a co-regulator for SMAD2/3/4 in promoting transcription of various factors required for EMT. The presence of ΔNp63 has been shown to be required for full activation of SNAIL (*Fig. 11*).[500]

SNAIL inhibits the expression of ΔNp63 [472, 481, 523] indirectly by interacting with CAAT/enhancer binding protein (C/EBP) that is required for ΔNp63 promoter activity.[481] Down regulation of ΔNp63 during EMT has been associated with increased motility and invasion of cells (*Fig. 11*).[345, 481, 483, 523, 530]

ΔNp63a directly interacts with the promoter region of ALOX12, a lipoxygenase involved in barrier formation. The lipid-peroxidizing enzyme stereospecifically inserts molecular oxygen into polyunsaturated fatty acids, contributing to the lipids extruded into the intercellular space during both epidermal development and epidermal terminal differentiation. Positive expression of ALOX12 is

dependent on extracellular Ca2+, whose concentration is the highest in the granular layer, where components of the epidermal barrier are produced.[531]

Micro RNAs (miRNA), short RNA molecules that act as posttranscriptional regulators, are also involved in regulation of ΔNp63.[504, 523, 525] miRNA203 inhibits the expression of ΔNp63 in suprabasal cells through direct targeting of its 3'UTR for degradation and promotes differentiation by restricting proliferative potential and promoting cell cycle exit to create a demarcation between progenitor cells and differentiated epithelial cells.[504, 523] A feedback

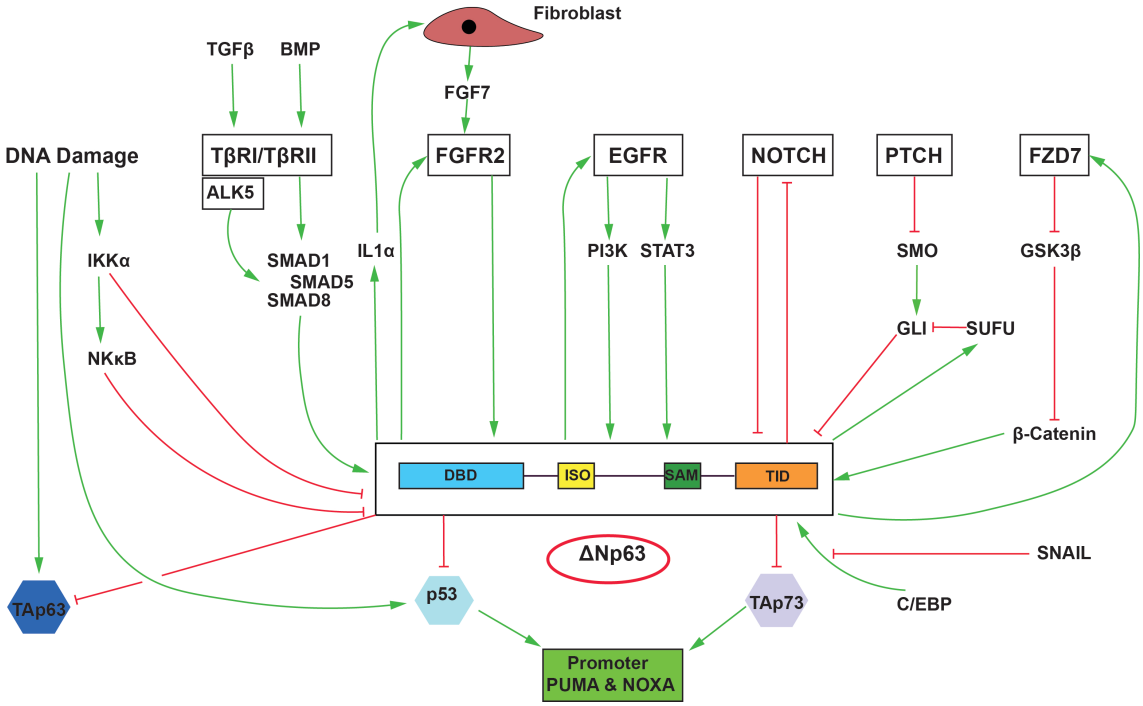


Figure 11 Schematic diagram of ΔNp63 interactions with major signalling pathways

ΔNp63 is involved in a large number of intracellular signalling pathways during morphogenesis and in adult tissue. ΔNp63 competitively inhibits p53 and TAp73, subsequently reducing pro-apoptotic gene transcription. Green arrows represent up regulation and red represent down regulation. TGFβRI/ TGFβRII is transforming growth factor β receptor and SMAD signalling pathway, Receptor tyrosine kinases (RTKs), EGFR, epidermal growth factor receptor and FGFR, fibroblast growth factor receptor positively influence ΔNp63 transcription in positive feedback loops, NOTCH signalling inhibits ΔNp63 in a negative feedback loop, WNT signalling via FZD7, frizzled 7 receptor, inhibits GSK3β allowing translocation of β-Catenin to the nucleus where the promoter for ΔNp63 is a target, Hedgehog signalling via PTCH, patch receptor, in mature oral mucous membrane tissue increases expression of ΔNp63. DNA damage via various sources inhibits ΔNp63 and promotes apoptosis.
Original drawing based upon drawings and text description from various references. [345, 412, 442-445, 447, 449, 450, 481, 483, 500, 502-504, 506, 507, 515, 518, 523, 525, 526, 528-531]

loop between p63 and an inhibitory member of the apoptosis stimulating protein of p53 family, iASPP, critical for epidermal homeostasis, is modulated

by miRNAs. In this loop, iASPP is a direct transcriptional target of ΔNp63 that positively regulates ΔNp63 via the repression of miRNAs 754-3p and 720 to allow for proliferation. Blocking iASPP expression allows for differentiation via up regulation of miRNAs 754-3p and 720, which down regulate ΔNp63α.[504, 523] miRNAs are also regulated by ΔNp63α. miRNA-34a and miRNA-34c, associated with cell cycle withdrawal, are negatively regulated by ΔNp63α.[523] In contrast, miRNA17, miRNA20b, miRNA30a, miRNA106a, miRNA143, and miRNA455-3p are positively regulated by ΔNp63 and are critical for the onset of keratinocyte differentiation via modulation of the MAPKs.[523, 525] Other miRNAs are involved in ΔNp63 expression and their roles are still being explored.[504]

Regulation of ΔNp63 is contributed to by ubiquination by E3 ligases. ITCH places a major role in maintaining a steady state.[504, 523, 525, 526] The scaffold proteins STXBP4 and RACK1 interact to contribute to regulation. A decrease in STXBP4 allows RACK1 to interact with ΔNp63 and facilitate the binding of E3 ligases.[525]

4.4.5. Extracellular Matrix Assembly, Dynamic Reciprocity and Remodelling

The extracellular matrix (ECM) is an important part of the μ_{abcd}(omm). A complex three-dimensional meshwork of proteins, it provides support, tensile strength, scaffolding that guides morphogenesis, and biochemical and biomechanical signals. The mechanisms that underlie ECM assembly, remodelling and interaction with the other components of the μ_{abcd}(omm) are critical for morphogenesis, growth, homeostasis and repair.[239, 281, 532, 533]

The ECM is synthesized and secreted by embryonic cells from the earliest stages of development defining tissue boundaries, providing tissue integrity and elasticity, and functioning as an adhesive substrate to direct migrating cells through haptotactic gradients. [264, 355, 534-536] It comprises two distinct biochemically and structurally different forms, the BM and the interstitial, or stromal, ECM.[334, 534, 537] The BM is a highly structured[214, 264, 282, 354, 534, 535] and serves as a boundary between tissue compartments, whereas the components of the interstitial ECM are normally randomly arranged in threadlike fibrils that form a fibrous and porous network surrounding cells. [334, 534]

4.4.5.1. Basement Membrane Assembly

Assembly of a BM is one of the most conserved attributes of multicellular organisms.[214, 264, 282, 535, 538, 539] BMs influence morphogenesis and cell fate, enforcing apico-basal polarity of the epithelium and its quiescent phenotype.[264, 355, 534, 535]

Laminins are the critical element in assembly of BM. In early embryogenesis, LM111 is expressed and secreted by ectodermal and endodermal cells.[214, 264, 535, 539, 540] Long arm tethering to laminin binding cell surface receptors, which include integrins, α-dystroglycan, heparan sulfates and sulfated glycolipids, initiates spontaneous self-assembly through calcium dependent interactions of the three short arms to form a polygonal lattice network. Subsequently collagen IV is recruited, which self polymerizes to form a network. Nidogen and percelan stabilize the BM.[282, 535, 538, 539]

Different laminin subunits are expressed as development proceeds, contributing to tissue differentiation and morphogenesis.[281, 282, 538, 540] LM332 is the primary laminin expressed in the OMM and is secreted from epithelial cells as a precursor molecule. [214, 271, 275, 305] LM332 has all three short arms are truncated and cannot undergo self-polymerization. [214, 261-263] Instead, epithelial cells and fibroblasts actively synergize to facilitate assembly of BM through processing of the α3 and γ2 chains, enabling association with basally located integrins on epithelial cells and with other components of the ECM. [214, 261-263, 305]

The α3 chain is central to triggering assembly of the BM.[281] The α3β1 integrin mediates the initial attachment of LM332 to the epithelial cells via the globular domain, LG, of the unprocessed α3 chain. The α3β1 integrin connects to components of the actin cytoskeleton and this interaction produces cell migration. [260, 262, 263, 265, 268, 276] Cleavage of the α3 chain exposes the LG3 domain, allowing the ligation to be transferred to the α6β4 integrin.[262, 263] Activating the intracellular portion of this integrin is crucial in the formation of hemidesmosomes.[214, 266, 268, 272, 541] This binding also promotes the cadherin dependent cell-cell adhesions,[268] inhibits the effects of α3β1 integrin binding through ERB-B2 phosphorylation and initiation of the PI-3K pathway,[214, 268] and is important in establishing and/or maintaining integrity of the BM. [262, 263] The

LG domain of the α3 chain can interact with non-integrin cellular receptors, including dystroglycan, heparin sulphate proteoglycans, syndecan 3 and 4.[214, 265]

The L4 domain of the short arm of the unprocessed γ2 chain is important for deposition and incorporation of the LM332 into the ECM.[271, 358] The γ2 chain associates with nidogen, fibulin, collagen VII, and is a key component of the anchoring fibrils.[355-357] Post secretory processing of the γ2 chain depends on the presence of fibroblasts.[305] Processing is associated with increased cellular motility. Cleavage at the N-terminal end exposes EGF-like repeats, which can bind with the EGF receptor, ERB-B1, suppressing tyrosine phosphorylation of integrin β4 and causing disruption of hemidesmosomes. It also activates MMP2 expression, which further increases γ2 chain processing.[214, 270]

There is minimal post secretion processing of the β3 chain.[214, 271, 275]The β3 chain can polymerize with LM311, LM321 and collagen VII [214, 261-263, 542]

The laminin scaffold enables recruitment and assembly of the remaining components of the basement membrane. Nidogen strongly associates with the γ2 chain and forms a bridge connected to the extensive collagen IV network.[214, 261-263] Perlecan binds nidogen, αDG and collagen IV[264, 281] and acts as a strong elastic tether and a modulator of cell adhesion, proliferation and differentiation.[264] Collagen IV self-assembles into a network-like polymer[280-282] providing mechanical stability.[264, 281] Integrin-nidogen-LM332 complexes may have a role in the regulation of the stem cell niche and epidermal maintenance.[281]

In quiescent tissues the unprocessed form of LM332 γ2 chain dominates. Local inflammatory mediators activate quiescent cells so in inflammatory conditions and wound healing there is increased γ2 chain processing. Neutrophil elastase cleaves all 3 chains of LM 332 and in particular cleaves the γ2 chain similar to MMP2, producing migration. The released fragments are chemotactic for neutrophils. [214, 271, 281]

Deposition of LM332 during wound healing regulates the polarization of the cells. The leading cells form a migratory population characterized by

deposition of LM332, failure to communicate via gap junctions, and expression of p16. These cells advance the epithelial edge and provide LM332 for stable adhesion of following cells. Polarization is achieved through binding with the α3β1 integrin on the apical surface of the cell. Deposition of LM332 is restricted to the rear of the leading cells. Hemidesmosomes are then formed with the following cells as the α3 chain is processed.[276]

TGFβ is sequestered and found tethered to the BM. It acts to provide specific signals to BM-adherent cells when released in response to injury, inflammation or mechanical stress. [264, 289, 298, 299, 301]

4.4.5.2. Interstitial Extracellular Matrix Assembly

The LP, composed primarily of fibronectin and fibrillar collagens, represents the interstitial ECM of the OMM.[537] Its role is to impart a tightly controlled environment, which maintains a homeostatic equilibrium regulated by fibroblastic cells responding to growth or temporary environmental insults.[543]

The process of interstitial ECM matrix assembly is similar to assembly of the BM. Multivalent ECM proteins are induced to self-associate and to interact with other ECM proteins to form fibrillar networks. Matrix assembly is initiated by ECM glycoproteins binding to cell surface receptors. In the OMM, fibronectin (FN), similar to LM332 in BM, assembly, has a key role.[328, 334, 536, 537]

FN enters the ECM in a dimer form.[333, 334] Cells mediate FN matrix assembly through α5β1 integrin. Receptor binding stimulates FN self-association and organizes the actin cytoskeleton to promote cell contractility. FN conformational changes expose additional binding sites that participate in fibril formation and in conversion of fibrils into a stabilized, insoluble form. Once assembled, the FN matrix impacts tissue organization by contributing to the assembly of other ECM proteins.[333, 334]

TGFβ1 plays a role in maintenance of the extracellular matrix. [288, 289, 291, 301, 339, 340] and is considered the most important mediator in stimulating synthesis of ECM proteins.[261] Myofibroblasts, key players in tissue repair, respond to cues from TGFβ1 for this function. Disturbances in TGFβ signalling can lead to

fibrotic diseases, which are a result of excessive scarring due to increased ECM deposition by overactive myofibroblasts.[49, 215, 289-291, 301, 340, 346]

The interstitial ECM of the OMM has much higher levels of fibronectin, fibronectin ED-A and chondroitin sulphate when compared to skin. This may contribute to faster wound closure and less scarring.[328]

4.4.5.3. Dynamic Reciprocity

Dynamic reciprocity is the persistent biophysical and biochemical interplay between cells and the ECM that enables morphogenesis, healing in response to environmental insult, and regulates and maintains tissue homeostasis.[239, 533, 534, 543, 544] The ECM provides biochemical signals to cells through growth factors or cytokines that acts as ligands on cell receptors triggering intracellular signalling pathways, many of which have already been discussed in previous sections. The ECM can affect cellular activity via changes to the cytoskeleton of cells and subsequently drive the expression and secretion of matrix remodelling molecules, such as collagen cross-linkers and MMPs. These in turn assist to degrade and remodel the ECM. It is currently understood that this 'mechano-transduction' is modulated through trans membrane cell surface receptors such as integrins, which interact with both the ECM and the cytoskeleton. The engagement of these bi-directional ECM-cell receptors serves as a link that enables the transmission of physical signals, not solely chemical cues, from the extracellular environment to the nucleus. [239, 533, 534, 540, 543, 544]

4.4.5.3.1. Integrins

Integrins are a large family of heterodimeric transmembrane glycoprotein receptors. Eighteen α and eight β subunits combine into around 24 different integrins, each of which has an extracellular portion capable of binding to a specific type of cell surface and ECM protein ligand.[97, 298, 545-549] The intracellular portion interacts with signalling and adaptor proteins that link to the cytoskeleton enabling bi-directional transmission of mechanical forces and regulation of numerous intracellular signaling pathways. Integrins mediate ECM-cell and cell-cell interactions in multicellular organisms, contributing to dynamic reciprocity through gene expression, protein synthesis, actin organization, cell polarity, differentiation, proliferation, migration, and survival. [214, 264, 268, 298, 532] Integrin binding

modulates the signalling of syndecans, growth factors, and other cytokines.[298, 322, 544] Their role in dynamic reciprocity makes integrins key components in development, morphogenesis, homeostasis and repair.[532]

Integrins are categorized into four broad groups based on which cell surface, ECM ligand or inflammatory ligand they bind: laminin binding integrins; leukocyte specific integrins; collagen binding integrins; and integrins that recognise the triplet sequence arginine-glycine-aspartate (RGD) motif found in many ECM proteins including fibronectin and collagen.[548, 550] The recognition/-binding site on the integrin receptor is formed by a combination of its α and β subunits The α subunit is thought to contribute more to ligand specificity. [214, 322, 551]

Expression of integrins is limited to the basal layer in the OMM. The laminin-binding integrins, α6β4 and α3β1, have the highest level of expression.[214, 264, 547, 550, 552] α3β1 forms part of focal adhesions, binding to LM332 and collagen. α6β4 forms part of the hemidesmosomes, anchoring the cell cytoskeleton to the ECM through binding with LM332. [214, 355, 357, 550, 552]

Fibroblasts in the LP express primarily RGD-binding integrins. The main integrins are α5β1 that binds to fibronectin, and αvβ3, which binds to vitronectin and is a major component of focal adhesions.[322] In the OMM, fibronectin-EDA is expressed.[328] Integrins, α4β1, α4β7 and α9β1 have been identified as receptors for the EDA domain and play a role in mediating the fibronectin-EDA stimulated myofibroblast differentiation.[322, 335, 553]

Integrins rely on recruitment of multi-protein integrin adhesion complexes to their cytoplasmic domains to mediate functions and transduce bidirectional signals. Talin is a major intracelluluar activator of integrins. Binding to the cytoplasmic tails near the plasma membrane generates a change in shape of the integrin complex, increasing the affinity for ligand binding. Kindlin, paxillin, α-actin and vinculum also mediate connection to the actin stress fibers and intracellular pathway activation.[298, 322, 547, 549, 551, 554-557] Ligand binding produces a clustering of the intracellular portion of integrins, adaptor proteins and actin stress fibres to form focal adhesions.

These complexes activate signaling proteins including focal adhesion kinases (FAK), RHO GTPases and MAPK pathways to effect cellular activities.[214, 298, 322, 557]

The cytoplasmic tail of the β unit binds to the cytoskeleton creating a bridge between intracellular cytoskeleton and ECM, and forming a connection from the plasma membrane to the nuclear membrane. [214, 322, 355, 551, 555, 556, 558] Binding to the cytoskeleton is facilitated by integrin binding kinase (ILK).[547, 554, 557, 559] The β4 subunit has a unique cytoplasmic domain that is much larger than that of other β subunits. It mediates most of the intracellular interactions of the α6β4 receptor.[262, 355, 357] The β4 subunit binds to keratin filaments of the cytoskeleton rather than actin, inhibiting migration.[355, 551]

Activation of α6β4 integrin complex by the α chain of LM332 results in ERB-B2 phosphorylation, initiation of the PI- 3K pathway and inhibition of α3β1 integrin.[214, 268, 550] α6β4 signaling increases cadherin cell–cell adhesion, inhibiting cellular movement. [214, 268] Processing of LMγ2 alters α6β4 signaling and is associated with increased cellular motility.[214, 270]

RGD-binding integrins, αvβ6 on epithelial cells and $\alpha_v\beta3$ and $\alpha_v\beta5$ in fibroblasts, mediate mechanical stress activation of latent TGFβ, BMP, and other growth factors.[298, 322, 547, 560, 561] Activation is dependent on the β subunit being anchored to the actin cytoskeleton. Contraction of the actin fibres against the stiffness of the ECM produces a physical transformation in shape, exposing TGFβ and/ or other bound growth factors to their cell membrane receptors, activating growth factor pathways. [289, 298, 299, 301, 322, 547, 561] RGD-binding integrins can serve as a common docking point for TGFβ LLC and MMPs, enabling MMP cleavage activation of LLC and release of latent TGFβ.[298, 561]

4.4.5.3.2. Cytoskeleton Networks

Actomyosin stress fibres, microtubules and intermediate filaments have distinct and complementary roles in integrating the nucleus of a cell into its environment and influencing its mechanical state.[562] Control of actin cytoskeleton contractility is a fundamental mechanism for moulding shape changes in development [563, 564], wound healing [544], response of cells to their

environment and cells influencing their surrounding ECM.[239, 322, 543]

The cytoskeleton is anchored to the cell membrane, which enables force transmission and force integration between cells and tissues.[322, 555, 565] Adhesion molecules can be grouped into two main classes. The first group is the integrins, discussed above, which connect cells to the ECM.[214, 544, 555, 565, 566] The second is the cadherins that mediate intercellular adhesions.[565]

Cadherins comprise a large family of trans-membrane associated glycoproteins that mediate specific cell-cell adhesions and influence morphogenesis of a variety of organs. E-cadherin has pivotal roles in epithelial cell behaviour, tissue formation, and suppression of cancer. [32, 343-348] Other cadherins associated with the actin cytoskeleton include type I cadherins, N-Cadherin and DE-Cadherin and type II cadherins VE-Cadherin, Cadherin-7 and Cadherin-8. Desmonal cadherins are associated with desmosomes and are connected to intermediate filaments.[344]

The linker of nucleoskeleton and cytoskeleton (LINC) complex mechanically links the cytoskeleton of a cell to its nucleus allowing forces at the cell membrane to be transmitted to the nucleus and intranuclear structures.[562, 567] LINC is a two-membrane adhesive assembly capable of transmitting mechanical force across the nuclear envelope. It is composed of KLARSICHT, ANC-1, and SYNE homology (KASH) domain in the outer nuclear membrane, which binds to different cytoskeletal elements including actin, microtubules, and intermediate filaments, and SAD1 and UNC-84 (SUN) domain in the inner nuclear membrane. The KASH proteins project into the perinuclear space between the inner and outer nuclear membranes, interacting with the SUN proteins to prevent diffusion out of the outer nuclear membrane.[567] SUN proteins anchor the LINC complex in the nucleus by interactions with A-type lamins, chromatin-binding proteins, and other proteins.[567]

Actin is the most abundant protein in human cells. Participation in various protein-protein interactions contribute to actin switching between a monomeric (G-actin) and filamentous (F-actin) state to influence many cellular functions including, cell motility, cell shape and polarity and regulation of transcription.[568] Actin, together with myosin and intermediate

filaments, is a crucial building block for the cytoskeleton.[562, 563, 565, 568]

Non-muscle myosins are a large family of motor proteins expressed in every cell.[563, 565] Whilst most myosins are monomeric, members of the myosin II sub-class form hexamers consisting of two heavy chains, two essential light chains and two regulatory light chains.[565] Type II non-muscle myosins bind to actin and control contraction of the cytoskeleton by hydrolyzing ATP to generate mechanical forces.[563, 565] Phosphorylation of myosin light chains, which increases myosin ATPase activity, regulates the contractile force generated.[322, 335, 555, 563, 565] This enables type II non-muscle myosin to perform a number of functions, such as generating cortical tension, mediating cytokinesis and mediating cell shape changes during development.[563]

Intermediate filaments are composed of one or more members of a large family of proteins involved in formation of the cytoskeleton. Six types are recognized. Types I and II are acidic and basic located in the cytoplasm. Type III are cytoplasmic intermediate filaments and include vimentin, expressed in mesenchymal cells, desmin, noted in skeletal and cardiac muscle, glial fibrillary acidic protein (GFAP) expressed in astrocytes and other glial cells and peripherin seen in peripheral neurons and cranial nerves. Type IV are neurofilaments, located in the cytoplasm and expressed in mature neurons. Type V, lamins, are located in the nucleus. Type VI filaments are associated with embryogenesis and stem cells. Nestin is found in stem cells of the CNS and developing muscle. Filensin is a protein expressed during development of the epithelium of the lens of the eye.[308, 313, 314, 569]

RHO-GTPases are involved in cell cytoskeleton organization, migration, transcription, and proliferation. [322, 430, 563, 565] RHO proteins belong to the RAS superfamily and are small molecules that become activated when bound to GTP. RHO-GTPases work as sensitive molecular switches existing either in an inactive, GDP-bound form or an active GTP-bound form.[430] RHO-GTPase signalling plays a key role in controlling actino-myosin contractility.[322, 430, 563, 565]

RHO-GTPases are activated by guanine nucleotide exchange factors (GEFs).

GEFs catalyze the conversion of GDP to GTP in response to activity of integrins, growth receptors and cytokine receptors.[430, 563] RHO-GTPases are inactivated by intrinsic GTPase activity that hydrolyzes GTP to GDP, or by RHO guanine nucleotide dissociation inhibitors (GDIs), which bind to RHO GTPases and block binding to effectors. [430]

RHO-A is involved in regulation of actinomyosin contractility. Activation of RHO-A leads to actin polymerization and formation of stress fibres[322, 430] and to activation of RHO-associated serine/threonine kinase (ROCK). ROCK increases myosin II activity by phosphorylation of the myosin light chain and inhibiting myosin light chain phosphatase.[322, 430, 563, 565] Integrin dependent RHO-A/ROCK mediated contractility enables cells to sense the stiffness of the ECM and rearrange their cytoskeleton accordingly.[322, 430, 534, 563]

RHO-GTPase dependent changes in cytoskeleton during morphogenesis contribute to cell shape, orientation, directional migration and differentiation.[534, 563] In mature tissues, the RHO-A/ROCK pathway is important in homeostasis and wound healing. It enables fibroblasts to form mature focal adhesions and stress fibers in response to changes in the ECM and in conjunction with growth factors, activates fibroblasts to myofibroblasts.[322, 534, 543, 555]

Cells can internally activate the pathway to send stress signals to the ECM [425-430], contributing to remodelling of the ECM with release of growth factors and bioactive peptides.[532] Chemical activation of the RHO-A/ROCK pathway by inflammatory mediators can produce a cytoskeletal pre-stress and increase the sensitivity of cells to mechanical changes.[322] Loss of RHO-A promotes EMT through changes in the organization of actin filaments with dissolution of tight junctions and adherens junctions, and loss of cell adhesions.[293, 413, 460, 490]

4.4.5.4. Extracellular Matrix Remodelling

Cells constantly turnover and remodel the ECM through synthesis, degradation, reassembly and chemical modification to enable development, growth, and

maintenance of homeostasis in response to internal and environmental stresses.[537, 570] Turnover is the normal physiological breakdown and replacement of ECM proteins whereas remodelling involves the breakdown and synthesis of proteins leading to a change in architecture.[571] Assembly and dynamic reciprocity have been discussed in the preceding sections. Breakdown and modulation of organization of the ECM mediated by proteases and accompanied by the release of growth factors, cytokines and chemokines is the final major mechanism involved in ECM turnover and remodeling.[532, 534, 537, 543, 570]

4.4.5.4.1. Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) belong to the metzincin superfamily of metalloproteinases and are the main enzymes involved in irreversible ECM degredation.[483, 489, 534, 537, 572] MMPs activate and inactivate of variety of cytokines, chemokines, growth factors, and other proteinases at the cell surface and within the ECM.[537, 570, 573] They facilitate organogenesis and branching morphogenesis.[537]

Activities of MMPs are tightly regulated. [572, 573] Expression is controlled at the transcription level and is increased in response to inflammatory cytokines, hormones and various growth factors including TGFβ, EGF and TNF-α.[573] Hypoxia, hyperglycaemia, ROS and pH influence transcription in a cell and state specific manner. [573] Increasing ECM stiffness can increase MMP transcription.[532]

MMPs are produced and secreted into the extracellular space as inactive zymogens.[489, 573] A subclass of membrane bound MMPs (MT-MMPs) are expressed on the cellular membrane and activated intracellularly by pro-protein convertases such as furin.[489] MMP-2 can be activated intracellularly by phosphorylation.[573] The small GTPase RAC regulates secretion and/or activation of MMPs in response to shear stress.[430]

Secreted MMPs are activated at the cell surface or in the ECM.[489, 573] MMP-2 is activated by MT-MMP whilst other MMPs are activated by CD151.[489] In the ECM serine proteases and other MMPs activate zymogens through proleolytic cleavage.[489, 532, 537, 573] α2-macroglobulin from plasma and tissue

inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of MMPs. [489, 573]

Activities of MMPs are tissue and state specific.[573] MT-MMPs act on collagen, fibronectin, laminin, tenascin C, vitronectin and growth factor receptors on the cell surface. Cleavage of collagen fibres allows reorganization into parallel bundles forming microtracts that enable cell migration.[534] MMP-1 expressed by fibroblasts degrades fibrillar collagen.[534, 570] MMP–9, also called gelatinase B, and MMP-2 are involved in the digestion of type IV collagen in the BM.[534, 572]

MMP-2 and MT1-MMP are involved in processing the α3 chain and short arm of the γ2 chain of LM332, influencing motility of epithelial cells. [214, 268, 270-272] MMPs assist in cleaving pro-collagen to form mature collagen and converting structural proteins of the ECM into signalling molecules, including latent TGFβ activation. [291, 298, 301, 532] Active bio-products of ECM proteins released by MMP activity further regulate MMP activity with both positive and negative feedback, supporting the tight control of MMPs in complex multicellular systems.[532, 573]

4.4.5.4.2. Adamlysins

Disintegrin and metalloproteinases (ADAMs) and ADAMS with thrombospondin motif (ADAMTs) are secreted metalloproteinases of the metzincin superfamily.[489, 491, 571, 574, 575] ADAMs are involved in homeostatic turnover of the ECM, producing protein ectodomain shedding from the cell surface.[537, 571, 575] ADAMs adhere to integrins through a binding sequence that mostly contains an aspartic acid-containing sequence.[575] Various ADAMs bind to cytokine and growth factor receptors.[537, 575] They then cleave the transmembrane protein ectodomains adjacent to the cell membrane releasing the complete ectodomain of cytokines, growth factors, receptors and adhesion molecules. [537, 571, 575] Shedding of ectodomains influences cell-cell and cell-ECM contacts, produceing autocrine and paracrine effects.[575] Some ADAMs are proteolytic. ADAM-17, also known as TNF alpha converting enzyme (TACE), releases membrane bound tumor necrosis factor (TNF)-precursor to a soluble form. ADAM-10 functions to produce ECM degradation, as well as localized shedding of various cell surface

proteins, and influences cell signaling patterns. [575] Similar to MMPs, ADAMs activity is inhibited by TIMPs.[575]

ADAMTs are secreted proteinases that possess thrombospondin type I-like repeats in their carboxy-terminal sequences and act on primarily on substrates in the ECM.[537, 576] They process procollagen enabling the deposition of normal collagen fibrils into the ECM. ADAMT-13 cleaves the large multimers of von Willebrand factor, contributing to normal coagulation. [537, 576] ADAMTs are inhibited by TIMPs, binding to or lack of substrate in the ECM and by endocytosis by the cell that produced them.[489]

4.4.5.4.3. Meprins

Meprin-α and meprin-β are multidomain metalloproteases belonging to the astacin subfamily of the metzincin superfamily.[537, 571, 577-579] Meprin-α is secreted as a soluble protease. Meprin-β is membrane bound.[537, 577-579]

Regulation of meprins occurs at the transcriptional level by specific factors. Meprin-α is activated by furin. [537, 577-579] Meprin-β expression is increased by activator protein 1 (AP-1) in response to cytokines and growth factors activating MAPK pathways.[577, 578] Various other metalloproteinase and serine proteases are involved in meprin activation.[537] Fetuin-A and cystatin-C, members of the cystatin superfamily of protease inhibitors, are the only demonstrated endogenous inhibitors of meprins.[579]

Meprins cleave procollagens III and I enabling formation of mature collagen fibrils in the ECM. Collagen IV, nidogen, and tenascin_C are cleaved by meprins [537, 577, 578] which can cause disruption of the BM and interstitial ECM, enabling infiltration by immune cells.[577]

Meprins are further involved in inflammation through release and maturation of cytokines and proteoglycans. [577, 578] Meprin-β induces production of pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-18 (IL-18) and IL-6 in macrophages, and leukocyte migration by processing of the adhesion molecule CD99.[577] Conversely, IL-6, a key regulator in inflammation, is cleaved by both meprin-α and meprin-β leading to reduction in activity.[577]

4.4.5.4.4. Lysyl Oxidase

Lysyl oxidase (LOX) is a copper-dependent oxidative enzyme that modifies lysyl residues through oxidative deamination, to produce cross-linking of the ECM proteins.[534, 570, 580-582] It is expressed in various cell types such as basal and suprabasal keratinocytes, fibroblasts, adipocytes, osteoblasts, smooth muscle cells, and endothelial cells.[580-582]

LOX expression is regulated at many levels.[582] TGFβ promotes LOX mRNA expression through SMAD and MAPK pathways. FGF down regulates transcription.[582] Various cytokines, such as TNFα, chronic inflammation, hypoxia and ROS induce increased LOX expression.[582] Numerous cell and tissue specific micro-RNAs are involved in decreasing transcription.[582] LOX is secreted as an inactive pro-enzyme. Cleavage by extracellular metalloproteases, such as BMP-1, produces a mature active form and a propeptide (LOX-PP).[581-583]

LOX initiates cross-linking of ECM collagen and elastin fibres, vital for connective tissue strength and integrity, and important in development, growth and wound healing.[534, 580-582] Cross-linking of the ECM modulates its stiffness, feeding into ECM-cell mechanotransduction and dynamic reciprocity via regulating integrin, and consequently SRC, signalling.[581, 582]

LOX and LOX-PP have autocrine and paracrine functions. High LOX expression in wound healing is associated with decreased E-cadherin facilitating Type 2 EMT. LOX acts a positive transcriptional regulator on the promoters of collagen, elastin and cyclinD.[582] The LOX-PP is biologically active and inhibits RAS signaling, MAPK/ERK signaling and EMT and negatively regulates the cyclinD promoter. [581-583]

The 'Oral Mucous Membrane Functional Tissue Unit' has been qualitatively defined using the CESM equation from **Principle 1**:

$$\mu_{abcd}(omm) = \{C_a(omm), E_b(omm), S_c(omm), M_d(omm\}$$

5. Application of the Framework for Assessment of Margins to Predict Recurrence in Oral Squamous Cell Carcinoma

The opening sections highlighted the importance of adequate margins in preventing tumour recurrence in HNSCC.[57, 73, 74] Determining what is an adequate margin through morphological assessment with standard H&E, whilst useful, has reached its limits. The functional consequence of wider surgical margins in HNSCC means simply taking more is not an answer. Predictive value of histological features such as invasive front, inflammatory infiltrate and perineural invasion support the importance of the underlying biological state.[88] This has led to an increased recognition and interest in the role of biological and molecular markers in assessment of surgical margins.[57, 73, 107, 109-114]

5.1. Defining the OSCC System

The OSCC system is defined by employing **Principle 2** and **Principle 9**. 'Oral Squamous Cell Carcinoma System', denoted as μ_{abcd}(oscc), arises from the 'Oral Mucosal Membrane Functional Tissue Unit', μ_{abcd}(omm) which was defined in the previous Chapter.

$$\mu_{abcd}(omm) = \{C_a(omm), E_b(omm), S_c(omm), M_d(omm)\}$$

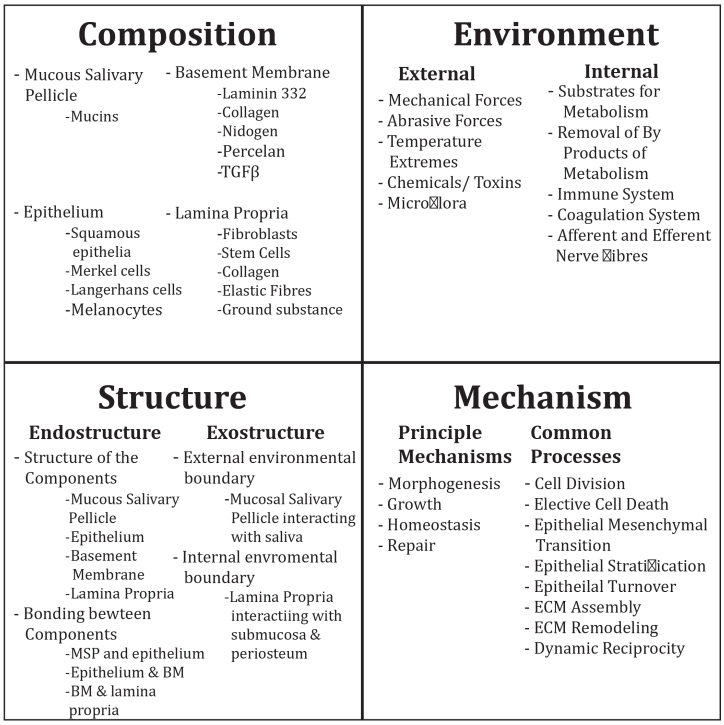
is replaced by

$$\mu_{abcd}(oscc) = \{C_a(oscc), E_b(oscc), S_c(oscc), M_d(oscc)\}$$

The properties, or functions diminished or lost are the barrier function and lubricated passage of ingested or inhaled substances. The initial new emergent property that defines the presence of OSCC is 'invasion' with loss of the BM. A comparative four-square CESM is used identify the changes that have led to the emergence of the cancer system (*Fig. 12*).

In accordance with **Principle 9** all components of μ_{abcd}(omm) are accounted for in μ_{abcd}(oscc). The external environment is altered as change of system properties and interaction with saliva leads to loss of normal MSP formation allowing microflora colonization. Loss of barrier function and lubrication triggers a greater immune response to protect deeper tissues from environmental insults and increases sensitivity of afferent neural structures. Increased metabolic demand of the new system and persistent inflammation increases demand on the internal environment

Oral Mucous Membrane FTU



Oral Squamous Cell Carcinoma System

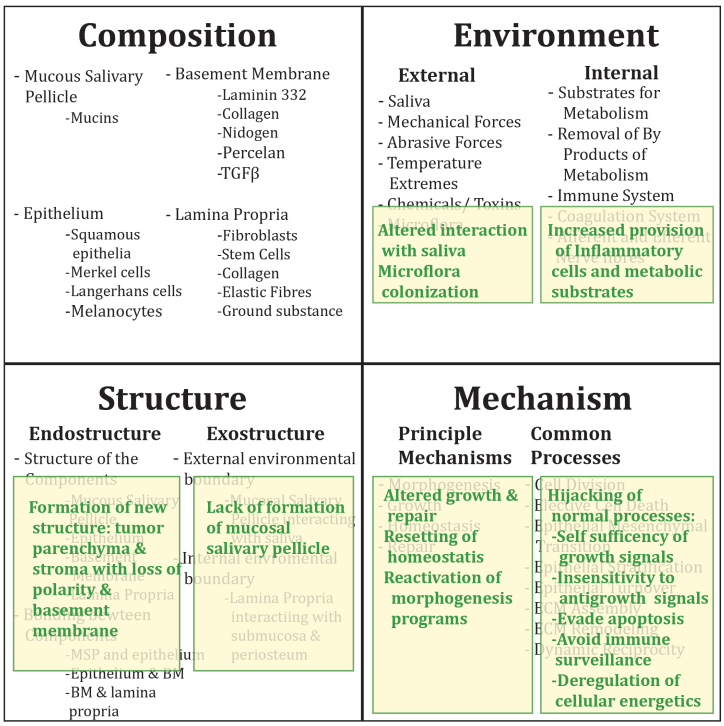


Figure 12 Four-square CESM Oral Mucous Membrane compared to Oral Squamous Cell Carcinoma

The normal FTU is compared to the cancer system and differences identified. Original diagram.

promoting angiogenesis. The components are rearranged with loss of endostructure and exostructure, to produce a new structure of tumour and stroma lacking a BM and polarity of epithelial cells. Many mechanisms are similar but have been hijacked to produce cancer hallmarks of self-sufficiency of growth signals, insensitivity to anti-growth signals, ability to avoid apoptosis and immune surveillance and deregulation of cellular energetics.[142, 146]

The clinical question to be addressed is once an OSCC has emerged, what are the on going changes in morphology and physiology over time that alter its behaviour and increase the risk of recurrence? In an emergence framework this question relates to cancer progression as per **Principle 11**, which states "Cancer progression is the deterministic development of a sequence of rapidly adapting emergent systems, each with identifiable patterns of morphology, physiology and behaviour, the dynamics of which can be studied via a state-space approach." (*Fig. 13*)

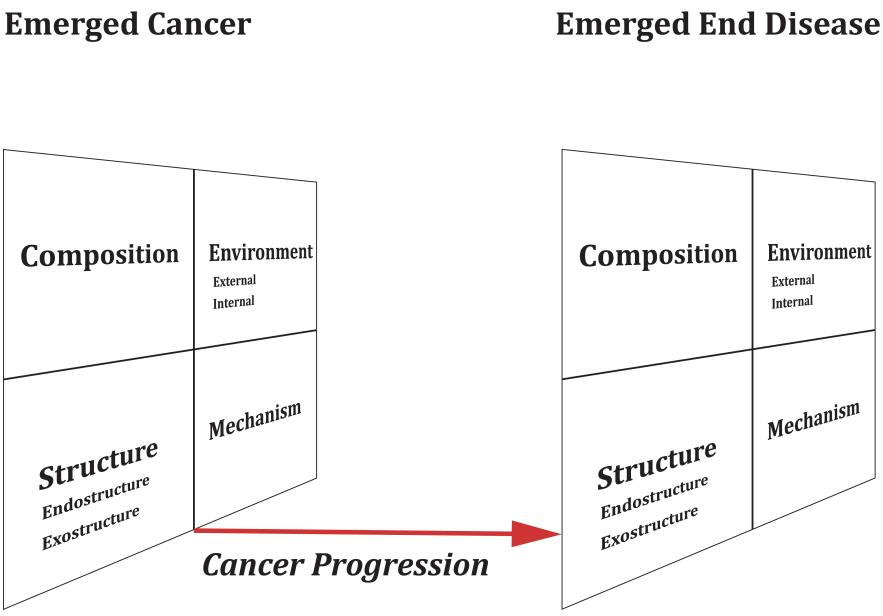


Figure 13 Applying Principle 11 to cancer progression

To assess for risk of increased recurrence, Principle 11 is applied to create a state space equation of how a cancer progresses over time from the initial diagnosis of cancer to the end disease state of metastatic disease.
Original diagram.

Morphological assessment, as discussed earlier, is currently the standard method for assessment of resection margins, primarily as a physical measurement. Histological features, such as pattern of invasion, perineural invasion and lack of lymphocytic response, are now being taken into account in some instituions.[87, 88] The aim of this

study is to apply "An Emergence Framework of Carcinogenesis" to identify and subsequently test 'biological' markers that are reflective of changes in physiology indicating progression to more aggressive disease. Comparing the CESM for μ_{abcd}(omm) and μ_{abcd}(oscc), changes in C, E and S, are most likely to be reflected in morphological changes currently assessed. This directs focus to changes between M_d(omm) and M_d(oscc) to identify tissue level biological markers reflecting changes in physiology.

5.2. Identification of Biological Markers

A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention.[584] Cancer progression, as per **Principle 11**, can be studied using a state-space approach. Using this approach for the purpose of predicting recurrence from surgical margins, the 'function' is the risk of recurrence and the quantitative properties are the biomarker(s). The complex and heterogeneous nature of OSCC biology makes it unlikely that analysing a single biomarker will be adequate to consistently enable comment on biological margins and prognosis, therefore multiple biomarkers may need to be considered.[62, 93, 103, 121, 123-131]

Using the equation:

$$F = \{A, B, C \ldots\ldots Z\}$$

Where:

F= '% risk of recurrence over 5 years'

A, B, C … Z = potential biomarkers

The next step is to identify which specific biomarker(s) are potentially relevant. Challenges of biomarkers in cancer research are numerous and include tumour heterogeneity, sub-clonal variation, sample handling and processing, assay validity, biomarker validation, bioinformatics, and appropriate trial design.[585] Discovery and application of biomarkers are typically modelled as a feature selection problem. The aim is to identify the most discriminating features for a given classification task, for example, distinguishing between different tumour stages.[584] Many feature selection techniques have been proposed but often do not necessarily identify the same feature subsets in the biomarker discovery process. This issue is amplified when the search is targeted at the genomic level. The same data, with different techniques can result in different groups of genes, raising questions about the true biological

significance.[584, 585]

In HNSCC, HPV p16+ in oropharyngeal SCC as of 2018 has been incorporated in to the HNSCC staging system and is the only recognized biomarker accepted as a factor in treatment decision-making in HNSCC.[26] EBV, present in a large number of nasopharyngeal carcinomas, is recognised as a prognostic marker but as yet is not used to determine specific treatment.[129, 586, 587] Other biomarkers have been proposed including EGFR, cyclinD1 (CCND1 gene), BCL-2, p27 (KIP1), VEGF, PIK3CA, LM γ2, HIF-1, immune checkpoints, p53, IL-8 and IL-10, cytokeratins I and II, miRNAs and various gene arrays.[93, 99, 103, 118, 123, 126-129, 131, 404, 588-601] Reviews have found that reported conclusions about biomarker prognostic or predictive value by the different research groups is not always consistent. Even newer work from the same group may be contradictory.[126, 128, 129, 404, 588, 592]

Numerous factors contribute to different results being produced by different laboratories, including true clinical variability in patient cohorts, the level of detection of a marker such as gene, mRNA or protein, and variations in the assay including technological platforms for detection and measurement, sources of reagents, use of fresh tissue or fixed, scoring procedures, and cut points.[128, 588] All these variations are identified as risks in progressing biomarkers into clinical trials.[585]

The current trend for focusing on genomics and molecular profiling has produced huge volumes of data. Data-driven analysis without context can produce results that do not translate into the clinical setting.[167, 590] Cetuximab is a classic example. EGFR is overexpressed in 80% of HNSCC yet response rate to treatment is 10%.[129]

Genomics, and some molecular profiling, is based on the hypothesis that cancer is a 'thing' and there is a causal relationship where the lower level components drive development of cancer.[240, 590] In an emergence framework of carcinogenesis, cancer is an emergent complex system manifesting at tissue level. It is a process. Unless a gene or molecule influences the FTU or the cancer arising from the FTU, changes at the genetic or molecular level are outside the logical intersect and do not form part of clinical translation. For example, Tsuchiya et al, showed focusing on genes or on their expression was not useful in accounting for the destiny of blood cells towards specific functions, but patterns identifiable at the level of m-RNA was.[602] In this example, the

coding genes fall outside the logical intersect. An example in a cancer system is the epigenetic silencing of *MGMT* (O^6-methylguanine–DNA methyltransferase), a DNA-repair gene, by promoter methylation which compromises DNA repair, in glioblastoma. Its presence has been associated with longer survival in patients with glioblastoma who receive alkylating agents.[603] This epigenetic mechanism would fall within the logical intersect in a CESM analysis.

The parameters required of the biomarker(s) in this framework to address this specific clinical question are:

- to be used to predict risk of early recurrence for OSCC with the aim of identifying the cohort most at risk and streamlining preventive management early, which may include new therapies
- be assessable at the tissue level
- relate to progression of oral squamous cell carcinoma
- have a validated antibody for detection
- have a method that is repeatable for assessment
- must add additional information to current morphological assessment of margins
- be cost effective

Determining which biomarkers fit these parameters using a systems approach requires analysing how hijacked mechanisms, M_d(oscc), produce or express a product(s) that changes in the state-space system as the tumour progresses through to end-stage disease. Using M_d(omm) as the starting point an extensive literature search was undertaken to identify and map how these mechanisms change in M_d(oscc) and interact to drive OSCC progression (*Fig. 14*). Four potential biomarkers that meet the required criteria were identified.

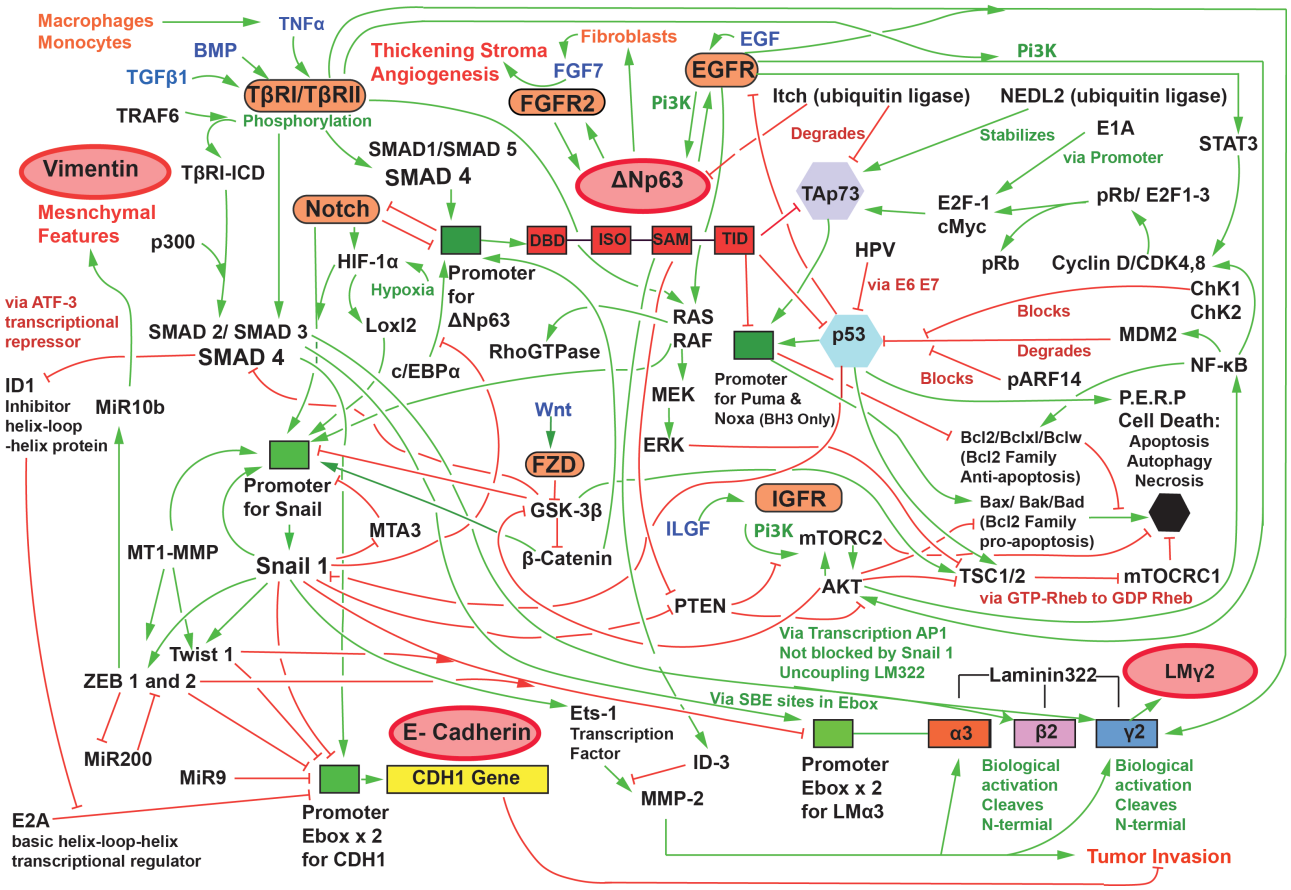


Figure 14 Schematic drawing of interacting mechanisms in OSCC system and influence on components and structure to identify potential biomarkers identifiable at tissue level

Key mechanisms and pathways are mapped showing the complexity of interactions, for which tissue level biomarkers appear to be end points and how the interacting mechanisms and pathways can influence the level of expression of potential biomarkers. This is purposefully not presented in a linear fashion to highlight that complexity is not linear. Not all pathways and all points are included.
Orange text: interacting inflammatory cells and fibroblasts
Dark Blue text: Growth factors and cytokines. TGFβ1, transforming growth factor beta 1; BMP, bone morphogenic protein; TNFα, tumour necrosis factor alpha; EGF, epidermal growth factor; FGF7, fibroblast growth factor 7; Wnt, wnt cytokines; ILGF, insulin like growth factor
Orange bullets: Epithelial cell membrane receptors. TβRI/TβRII, transforming growth factor beta dimeric receptor signal to SMAD and non- SMAD intracellular pathways; FGFR2, fibroblast growth factor receptor 2, EGFR, epidermal growth factor receptor and IGFG, insulin growth factor receptor trigger various receptor tyrosine kinase pathways; Notch, notch receptor triggers NOTCH pathway; FZD, frizzled receptor triggers WNT pathway
Green box: Promoters for CDH1 gene (E-Cadherin), LMα3, SNAIL transcriptase, ΔNp63 and Puma and Noxa
Hexagons: represent keys in apoptosis: light blue, tumour suppressor p53; purple, ΔTAp73 in its tumour role; black, the end point, apoptosis/ cell death
Black text: Intracellular transcriptases, proteins, mRNAs, and enzymes
Green arrows and text: represent positive influence
Red arrows and text: represent negative influence
Bright Red text: the progression histology seen of invasion, thickening of stroma and angiogenesis and loss of epithelial features and acquisition of mesenchymal features
Red circles: identify potential end-point biomarkers visible at tissue level
Original drawing based upon drawings and text description from various references. [13, 49, 95, 127, 158, 215, 219, 221, 255, 258, 287-291, 293, 295, 296, 298, 299, 301, 315, 323-325, 340, 341, 343-347, 350, 352, 360, 366, 373-375, 377-383, 385-409, 411-417, 419-421, 423-433, 436, 437, 442-445, 447, 449, 450, 457, 460-466, 469, 471, 473-475, 480, 481, 483, 484, 486, 489, 497, 498, 500, 502-504, 506-510, 515, 518, 523, 525, 526, 528-531, 581-583, 6046, 12-16, 23, 31, 32, 35, 49, 59, 68, 73, 89, 90, 92, 93, 95, 97, 99-101, 103, 107, 108, 112-115, 118, 123, 124, 126, 127, 129, 130, 231, 238, 257, 310, 350, 352, 358, 387, 393, 394, 404, 451-453, 479, 505, 511, 515, 548, 589, 591, 592, 594, 595, 599, 600, 605-643]

5.2.1. P63

ΔNp63a, which is important in stratified epithelial structures for maintenance of its regenerative capacity and control of expression of genes involved in epithelial tissue integrity, homeostasis and cell survival,[440, 444, 445, 449, 450, 455, 503, 515-518] has been shown to be overexpressed in 80% of HNSCC.[412, 442, 447, 449, 450, 515, 644]

The mechanism mapping (*Fig. 14*) and *Fig 11* in section 4.4.4.2 show how ΔNp63 expression is a convergence point of many key pathways that underlie the mechanisms of μ_{abcd}(omm) and μ_{abcd}(oscc). In μ_{abcd}(oscc), increased ΔNp63 is associated with suppression of apoptosis and cell death via competitively inhibiting p53 and TA-p73, and blocking the promoters for transcription of PUMA and NOXA.[442, 447, 450, 453] Expression is increased in response to numerous growth factors including TGFβ, BMPs, and EGFR.[442, 447, 449, 453, 500, 645]

Increase in expression of ΔNp63 is an early event in pathogenesis of OSCC seen in premalignant conditions and before morphological changes.[442, 449, 450, 644, 646] Increasing expression is correlated with tumour progression and worsening prognosis.[453, 515, 644] Increased ΔNp63 has been associated with increasing loss of E-caherin expression.[617] Loss of ΔNp63 expression is seen in late EMT and correlates with even worse prognosis [453, 472, 481, 608, 644], and to chemoresistance.[450, 451, 454, 481, 608, 647, 648]

ΔNp63 is easily seen with standardized immunohistochemistry with light microscopy and is located in the nucleus of the cell.[116, 442, 448, 450, 451, 453, 516, 608, 644] In normal tissue about 10% of cells in the basal layer stain positive for ΔNp63[453] though this baseline has been shown to vary between different subsites in the oral cavity by as much as 2.5 fold.[516]

5.2.2. LMγ2

Breach of the BM is the histological feature that defines and differentiates cancer from dysplasia.[69, 98, 649] In the mid 80's Lance Liotta and his group proposed a three-step hypothesis of tumour cell invasion, where the tumour cells first attach to laminin on a BM, locally degrade BM type IV collagen with tumour-associated proteinase, and then finally migrate into the interstitial stroma.[650] Recent studies

have supported this theory and have shed light on the dynamic, complex role of the BM and the ECM in cancer invasion and tumour development. [98, 230, 266]

In OSCC interaction with LM332 is a critical step in carcinogenesis. LM332, particularly the γ2 chain, has been found to be over expressed in OSCC and associated with enhanced tumorogenesis.[230, 266, 651] It accumulates at the interface of tumour and the surrounding stroma. Notably, over expression of the γ2 chain mRNA and protein has found to be invariably associated with budding cancer located at the invasive front of tumours whilst cancer cells deeper in the tumours are often negative. The γ2 chain monomer accumulates intracellulary in the cytoplasm.[230, 265, 266, 652, 653] Some cancers secrete the monomeric form and circulating levels of the γ2 chain have been correlated with stage of disease both *in vitro* and in patients with pancreatic cancer and oral cancer.[654-656]

Different genes located on different chromosomes encode each chain of LM332, producing a divergence in regulation of the genetic expression.[214, 499] SMAD4 has a positive regulation effect via TGF-β family on all 3 chains of LM332 and promotes a morphological change of mesenchymal-like tumors cell back to their epithelial form. The gene for the α3 chain has a promoter which contains 2 E-box motifs that respond positively to SMAD2, 3,4 complex and is inhibited by SNAIL, TWIST and ZEB1 and 2 in a manner that is similar to E-cadherin. Promoters for the β3 and γ2 chains do not contain E-box motifs. The mechanism by which SMAD4 promotes transcription of these chains is different. Increasing SNAIL expression blocks SMAD modulated transcription of the α3 chain through the E-box motifs, but not the β3 or γ2 chains leading to an uncoupling of transcription and increasing expression of γ2 chain, which accumulates in the cytoplasm.[469, 478, 499] (*Fig 14*) TGFβ1 and TNFα secreted from inflammatory cells seem to inhibit formation of α3 chain, but not the β3 or γ2 chains. TGFβ1 in cancers has been observed to activate the γ2 mRNA subunit promoter with over-expression of γ2. These may be due to activation of non-SMAD pathways.[653] Loss of E-cadherin in EMT results in disassociation of hemidesmosomes enabling the passage of β-catenin into the nucleus where is has been associated with up regulation of LMγ2 and increased expression of MT1-MMP. [73, 134]

In well differentiated cancers LM332 as a heterotrimer is over expressed whereas as in invading tumours that have changed their morphology from epithelial to

mesenchymal like it is the γ2 chain which is over expressed and accumulated intracellularly, making it a target as a tumour marker.[266] Increasing LMγ2 chain immunoreactivity at the invasive front has been correlated with increasingly poor prognosis in HNSCC and OSCC.[5, 117, 118, 120, 121, 223, 230, 265, 266, 592, 653, 657-660]

5.2.3. E-Cadherin

E-cadherin is considered to be tumour suppressive due to its key role in forming epithelial adherens junctions with β-catenin. Reduction and loss of expression of E-cadherin, associated with translocation of β-catenin to the nucleus, is a hallmark feature of EMT in epithelial cancers. This is associated with loss of cell-cell interactions and loss of apical-basal cell polarity enabling the acquisition of motile and invasive cell behaviour. [32, 103, 121, 127, 237, 343, 344, 347-352, 469, 580, 608, 612, 617, 661]

A number of different mechanisms for E-cadherin inactivation in malignancy have been elucidated. Mutations in the CDH1 gene,[343, 349] and activation via various pathways of transcriptase repressors SNAIL, TWIST and ZEB1 and 2 that bind specifically to the E-box in the CDH1 (*Fig.14)*, have been associated with numerous epithelial cancers.[127, 215, 347, 352, 390, 416, 463, 469, 471, 473, 474] Epigenetic silencing via the recruitment of various DNA/histone modification complexes that produce histone deacetylation and condensation of chromatin, blocking transcription and via promoter hypermethylation has been observed. [347, 349]

Increased endocytosis and degradation of E-cadherin can occur by activation of proto-oncogenes, such as c-MET, cSRC and EGFR.[343] Degradation of E-cadherin is associated with dislocation of p120 to the cytoplasm. This up-regulates expression of MT1-MMP at the mRNA and protein levels, and increases the migratory and invasive abilities of the cell.[343]

Cleavage of the E-cadherin ectodomain by MMPs, including MMP3, MMP7, MMP9, and MT1-MMP, plasmin, ADAM10 and kalikriens affect normal cell-cell adhesion and cell migration. Soluble E-cadherin ectodomain fragments may block normal E-cadherin function by forming pseudoligands.[343] Cleavage of the cytoplasmic domain by capsases blocks formation of stable adherence junctions. The intracellular fragments disturb β-catenin subcellular localization, enabling translocation into the nucleus and stimulation of cyclinD1 expression.[343]

In HNSCC, the expression pattern of E-cadherin is variable not only among different HNSCC tumours but within the same tumour. The most aggressive cells in HNSCC appear at the invasive front of HNSCC, which has been shown to exhibit decreased, or loss of expression of E-cadherin with increased expression of vimentin, the hallmark features of EMT. [32, 103, 127, 309, 629]

Reduced E-cadherin expression in OSCC has been associated with a more invasive tumour pattern,[310, 593] nodal metastasis,[121, 593, 617, 662, 663] distant metastases,[629] radioresistance[664] and poor prognosis in terms of both disease free interval and overall survival.[32, 103, 309, 593, 599, 661, 662, 665, 666] Whilst there is an abundance of evidence that supports the use of E-cadherin as a prognostic biomarker in OSCC there is still no uniform system in place for its measurement to enable standardized use. [599] In addition, a negative marker requires a consistent baseline. If this is fluctuant or variable between individuals or different tumours, assessment becomes more complicated. [599]

5.2.4. Vimentin

The expression of vimentin in cancer cells of epithelial origin, in conjunction with the loss of expression of E-cadherin, is considered a hallmark of EMT.[127, 215, 308, 313, 315, 351, 461] Its expression in cancer cells is associated with a change in morphology involving loss of hemidesmosomes, elongation of cells and appearance of invadopodia, membranous protrusions rich in MMPs that degrade BMs and enable migration, invasion and metastasis.[313, 315]

The EMT process in cancer cells is progressive and complex with many cellular pathways and epigenetic mechanisms driving the increase expression of vimentin, as seen in *Fig. 14.* Vimentin expression has been shown to be transactivated by β-catenin/ TCF, an endpoint of the WNT/ β-catenin signalling pathway, binding upstream of the transcription initiation site of vimentin promoter.[309, 664] MT1-MMP has been demonstrated to induce progressive increase in expression of vimentin inducing cells that exhibit cancer-stem-cell characteristics of low proliferation, self-renewal and resistance to apoptosis and chemotherapy.[612] TGF-β, EGFR signalling, NOTCH signalling and hypoxia have all been demonstrated to increase vimentin expression in various cancers.[127, 308, 313, 349, 664]

The recognition of EMT at the invasive front in HNSCC has lead to specific assessment of vimentin expression and its correlation with metastatic potential and prognosis. [49, 89, 127, 310, 350, 469, 479, 632] An increase in vimentin expression has been observed as OSCC progresses from dysplasia to invasive carcinoma.[310] The level of expression of vimentin at the invasive front has been identified as a prognostic predictor of recurrence and survival [309] An association between vimentin expression and distant metastatic risk has been observed. [629] Higher vimentin expression in regional nodal disease has been demonstrated to be associated with poorer prognosis in HNSCC. [663, 667]

Vimentin expression is emerging as one of the key markers for identifying tumour cell phenotype that is representative of malignant potential. Chen et al[479] demonstrated vimentin was expressed in 90% of cancer stem cells derived from HNSCC cell lines and subsequently highlighted the importance of mesenchymal markers in detecting circulating tumour cells.[632]

A major limitation of vimentin as a biomarker is that stromal cells derived from connective tissue express vimentin creating a challenge in discerning between stromal cells and cancer cells expressing vimentin at the invasive front.[103, 127, 308, 350]

It is now widely accepted that in any specific OSCC tumour the invasive front is the most clinically relevant area to consider with regards to biological activity and prognosis.[5, 31, 32, 89-106]

From the literature, the change in expression of the selected biomarkers can be estimated and graphed relative to the increasing aggressiveness of the tumour (*Fig. 15*).

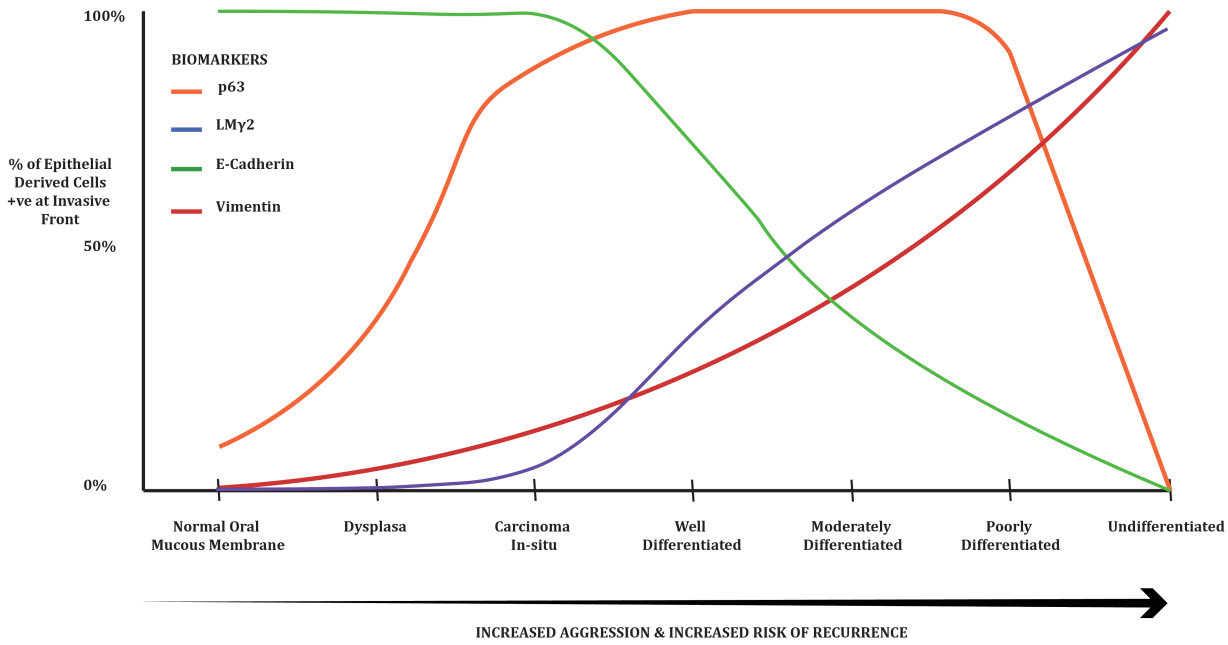


Figure 15 Schematic two-dimensional graphs of predicted changes of expression of potential biomarkers at the invasive front in OSCC with increasing aggressiveness

The expression of p63, LMγ2, E-Cadherin and vimentin change as OSCC emerges from the oral mucous membrane functional tissue unit and as OSCC evolves towards end stage disease. Assessment of the biomarker expression at any point in time has the potential to identify how progressed an individual OSCC is towards end-stage and help predict recurrence risk

Original diagram based upon various references. 5, 31, 32, 49, 89-106, 116-118, 120, 121, 127, 214, 215, 223, 230, 237, 265, 266, 308-310, 313-315, 343, 344, 347-352, 390, 412, 416, 442, 447-451, 453, 454, 461, 463, 469, 471-474, 478, 479, 481, 499, 501, 515, 516, 580, 592, 593, 599, 608, 612, 617, 629, 632, 644, 646-648, 652-670

E-Cadherin as a negative marker requires a consistent baseline. If this is fluctuant or variable between individuals or different tumours, assessment becomes more complicated. [599] In addition, the mechanisms that produce inhibition of E-box promoters and suppression of E-cadherin expression, primary by SNAIL, also produce the uncoupling of LM332 chain production through inhibition of the E-box promoter of LMα3.[499] From this, it can be extrapolated that decrease in E-cadherin expression is inversely proportional to the increase in accumulation of cytoplasmic LMγ2. For these reasons E-cadherin was removed as a potential biomarker. This enables the creation of a state-space equation and the hypothesis that:

'% risk of recurrence over 5 years' = {p63, LM γ2, vimentin}

All three biomarkers can be detected by simple validated immunohistochemistry.

5.3. Immunohistochemistry as a Tool for Assessing Margins

Haematoxylin and eosin (H&E) staining of tissue, a technique that has been in use for at least a century, remains the primary tool for recognizing various tissue types and the morphological changes that form the basis of contemporary cancer diagnosis.[77-79] Physiological and functional changes may occur in cancer cells, resulting in altered biological behaviour that is not reflected in morphological changes detected by H&E staining, enabling these cells to appear as unaffected or disguise their aggressiveness.[77, 107, 108] Consequently, there is a growing interest in the use of biological and molecular markers in the assessment of surgical margins.[57, 73, 107, 109-114]

Immunohistochemistry and different DNA techniques have been shown to have potential. Protein biomarkers can be detected via immunohistochemistry, a technique that is relatively cheap, easy to perform and widely available. DNA techniques may be more sensitive, but are more laborious, require a relatively high level of expertise and have a higher cost.[109, 110, 116] These factors make immunohistochemistry staining for protein biomarkers presently a more attractive option for wider use.

Immunohistochemistry analysis in conjunction with traditional pathological assessment is accepted as a feasible technique. Immunohistochemistry is widely available and can be performed using either manual or automated techniques. These techniques use equipment that is available in pathology labs worldwide.[73, 107, 109, 111, 113, 115-122]

5.3.1. Impact of Heat on Immunohistochemistry

The biomarkers identified can all be detected through immunohistochemistry. This is attractive as the technique is relatively cheap, easy to perform and widely available. [109, 110, 116] On the other hand, a study by Folz et al [671] in 1999 suggested that these proteins may be denatured by heat generated through the resection method (laser, diathermy,or coblation). To assess whether this was a barrier or not, an assessment of glottis tumors resected via laser was performed.

This is presented in the following article.[4, 5, 49, 57, 62, 72-74, 77, 82, 84-89, 98, 107-109, 111-119, 121, 122, 127, 214, 223, 230, 257, 261, 263, 265, 266, 268, 270, 272, 276, 305, 350, 354, 469, 478, 479, 499, 541, 542, 592, 606, 613, 620, 625, 649, 651, 653, 654, 657-660, 671-692]

ORIGINAL ARTICLE

Surgical margins in head and neck squamous cell carcinoma: Effect of heat artifact on immunohistochemistry as a future tool for assessment

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ABSTRACT: *Background.* Margins in head and neck squamous cell carcinoma (HNSCC) are determined by morphological changes assessed via hematoxylin-eosin staining. Physiological changes may not be detected by this technique. The purpose of this study was to determine if a protein biomarker, laminin-332γ2, overexpressed in cancer cells at the invasive front in HNSCC, remains unaffected by heat produced during resection, supporting a role for immunohistochemistry assessment of margins.

Methods. Archived tissue blocks from glottic squamous cell carcinomas (SCCs) resected by CO2 laser likely to contain both cancer cells and artifact were identified; 129-paired slides were obtained. One slide of each pair was stained with hematoxylin-eosin; the second stained for laminin-332γ2. The presence of cancer cells, artifact, and positive laminin-332γ2 staining was recorded. Twenty-seven pairs met the inclusion criteria.

Results. Immunohistochemistry staining of laminin-332γ is preserved in presence of heat artifact.

Conclusion. This study supports use of immunohistochemistry to assess margins. ©2016 Wiley Periodicals, Inc. *Head Neck* **38:** 1401–1406, 2016

KEY WORDS: CO2 laser, laminin-332γ2, tissue artifact, laser artifact, head and neck squamous cell carcinoma, glottis squamous cell carcinoma, biomarkers

INTRODUCTION

Adequate resection margins in head and neck squamous cell carcinoma (HNSCC) are considered to be an essential element in achieving the best possible outcomes for patients.[1–3] Current accepted assessment of margins in HNSCC is with traditional hematoxylin-eosin staining and light microscopy. This method looks at the structure and morphology of the cells. Increasingly, evidence in the literature demonstrates that this method of assessment does not reveal the full picture.[2,4–7] Physiological and functional changes may occur in cancer cells resulting in altered biological behavior, particularly in early phases of epithelial mesenchymal transition, which are not reflected in morphological changes detected by hematoxylin-eosin staining, enabling these cells to appear as unaffected or disguise their aggressiveness.[8–10] This undetected change in biological nature may explain why positive margins are not always associated with local recurrence and a clear margin does not guarantee cure.[2–5,7] This has led to an increasing interest in the use of biological and molecular markers in the assessment of surgical margins.[3,4,6,9,11–15]

Immunohistochemistry and various DNA techniques have both been shown by multiple authors to have potential. Protein biomarkers can be detected via immunohistochemistry, a technique that is relatively cheap, easy to perform, and widely available. DNA techniques may possibly be more sensitive, but are more laborious, require a relatively high level of expertise, and have a higher cost.[4,6,16] These factors make immunohistochemistry staining for protein biomarkers a more attractive option for wider use. However, a study by Folz et al[17] in 1999 suggested that these proteins may be denatured by heat generated through the resection method (laser, diathermy, or coblation). To the authors' knowledge, no other study has investigated whether heat generated during resection of a tumor may cause coagulation and denaturing of protein biomarkers resulting in potential limitation of their use in the assessment of surgical margins.

The tissue effects from the use of lasers have been well-described.[18–20] These include a very fine carbonization zone, a necrotic zone where cells become protein conglomerates and cell debris, and an edematous zone. Denaturing of collagen and coagulation artifact is also seen on hematoxylin-eosin staining.

The primary purpose of this study was to determine if immunohistochemistry staining of intracellular protein

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biomarkers associated with carcinogenesis in HNSCC is affected by heat that is generated during resection of the primary tumor.

Artifact can be produced during the tissue handling, preparation, and processing. These factors may also interfere with a pathologist's ability to accurately assess the tumor margin in such a specimen.[19,21] There is, to the authors' knowledge, no study to assess whether the staining of intracellular protein biomarkers is adversely affected by other tissue and processing artifacts. The secondary purpose of this study was to assess the impact, if any, of tissue and processing artifacts on the staining of protein biomarkers.

Numerous biomarkers have been proposed in HNSCC and, in all likelihood, a combination, rather than a single biomarker, may be required for future clinical application. The purpose of this study was not to investigate or promote any particular biomarker as a tool for margin assessment or prognostic predictive value but to simply identify a representative biomarker to assess the impact of heat from resection on detectability. Criteria applied to determine suitability were that the biomarker needed to be detectable via immunohistochemistry, was cytoplasmic in location, had been demonstrated to be overexpressed at the invasive front of HNSCC, and, to be associated with carcinogenesis, is not overexpressed in normal tissue or normal background stroma. Based on these criteria and after review of the literature, laminin γ2 (LMγ2), sometimes referred to as LAMC2, was selected as the biomarker for this study.

Laminins are a family of extracellular matrix (ECM) glycoproteins that constitute a major component of the basement membrane. The laminin family are heterotrimeric proteins composed of 1 heavy chain, α, and 2 light chains, β and γ, linked by disulfide bonds. Laminin 332 (LM332), previously termed laminin 5, kalinin, nicein, epiligrin or ladsin, consists of α3, β3, and γ2 chains, and is the major adhesive component of epithelial basement membranes, being therefore distributed in skin and epithelial tissues.[22–25]

LM332 is secreted into the ECM and undergoes specific proteolysis for different biological activities that are mediated via integrins. These normal biological functions include mediating ECM–cell and cell–cell interactions, and regulation of cell adhesion, migration, proliferation, survival, and differentiation. Postsecretory cleavage of the α3 chain promotes formation of hemidesmosome and cadherin-dependent cell–cell adhesions. In contrast, postsecretory cleavage of the γ2 chain increases cell motility. Thus, LM332 has a pivotal role in stabilizing the epithelium to the basement membrane and in wound healing.[24–29]

The γ2 chain (LMγ2) is unique to LM332.[22,23,27,30–40] In noncancerous tissue, LMγ2 can sometimes be detected in an extracellular regular linear pattern corresponding to the basement membrane of normal squamous cell epithelium.[41] LMγ2 becomes overexpressed in HNSCC and many other epithelial tumors.[33,42–46] Significantly, overexpression of the γ2 chain mRNA and protein with intracellular accumulation has been found to be invariably associated with budding cancer located at the invasive front of tumors, generally in cells that have changed their morphology from epithelial to mesenchymal-like, whereas cancer cells deeper in the tumor are often negative.[22,27,32,34,41,42,45,47–50] Ono et al[44] described both quantitative and qualitative methods for assessing the level of expression. Many authors have found correlation between the level of overexpression of LMγ2 and aggressiveness or prognosis of the tumor.[27,33,43,48,51–57]

Glottic squamous cell carcinomas (SCCs) resected by CO2 laser were the specimens to be used in this study. The small size of the resections enabled the invasive front to be easily identified. Smaller margins increased the likelihood of the resection margin being close enough to the tumor for heat artifact to occur and enable adequate samples with heat, coagulation, processing, and tissue handling artifacts to be identified.

MATERIALS AND METHODS

Approval was obtained from Monash Health (formerly Southern Health) Human Ethics Committee, Monash University Ethics Committee, and the Victorian Cancer Research Biobank. All glottic cancers removed by laser resection by the Otorhinolaryngology, Head and Neck Unit, at Monash Health, from January 2007 through January 2011 were identified and a cross-check of patient consent by Victorian Cancer Research Biobank was conducted. In the pathology reports, 143 blocks from 63 resections were recorded to contain artifact. Pathology review excluded 14 samples because of insufficient tissue in the remaining block to produce the slides required. Paired slides were then cut from the remaining 129 blocks; 1 slide of each pair was stained with hematoxylin-eosin using standard techniques and the remaining slide(s) were stained for LMγ2, based on the method described by Ono et al[44] and Pyke et al,[45] using Dako mouse monoclonal LMγ2 antibody (code NR. M 7262, monoclonal mouse anti-human laminin 5, gamma 2 chain, clone 4G1). Sections of paraffin-embedded tissue were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval was performed by microwaving the slides in citrate buffer (pH 6.0) for 10 minutes. This was followed by blocking endogenous peroxidase activity by a 15-minute incubation in 1% hydrogen peroxide.

A protein block with normal serum was performed for 30 minutes, followed by incubation with LMγ2, the primary antibody, at a concentration of 1:25 at 4°C overnight. Staining was detected using the mouse Vectastain Elite ABC mouse kit (cat #PK6101) from Vector Laboratories (Burlingame, CA), in accord with the manufacturer's instructions. Diaminobenzidine solution (Fast DAB tablets cat #D4293-50SET; Sigma–Aldrich) was used for color detection and then the sections were counterstained with hematoxylin, dehydrated, and mounted.

Slides derived from an HNSCC previously proven to stain positively with LMγ2 antibodies were used as the positive control and slides derived from resected, noncancerous tonsil tissue, previously demonstrated to not stain, were used as the negative control for the immunohistochemistry staining.

A second pathologist, who had not previously seen any of the specimen blocks, reviewed the paired slides. The hematoxylin-eosin stained slides were reviewed first; the presence of laser, processing, tissue handling, or crush

TABLE 1. Summary of pathology assessment.

Hematoxylin-eosin slide			LMγ2 antibody				
Cancer cells present	No.	Artifact present	Cancer cells present	No.	Artifact present	LMγ2 antibody positive	Included/ excluded
Yes	17	Laser	Yes	17	Laser	17	Included
Yes	1	Processing	Yes	1	Processing	1	Included
Yes	1	Tissue	Yes	1	Tissue	1	Included
Yes	10	Laser and tissue	Yes	10	Laser and Tissue	10	Included
Yes	11	None	Yes	11	None	11	Excluded
Yes	6	Laser	No	6	None	0	Excluded
Yes	2	Tissue	No	2	None	0	Excluded
Yes	2	Laser and tissue	No	2	None	0	Excluded
Yes	7	None	No	7	None	0	Excluded
No	41	None	No	41	None	0	Excluded
No	4	Tissue	No	4	None	0	Excluded
No	17	Laser	No	17	None	0	Excluded
No	2	Laser	No	2	Laser	0	Excluded
No	1	Laser and processing	No	1	Laser	0	Excluded
No	1	Laser and tissue	No	1	Laser and Tissue	0	Excluded
No	7	Laser and tissue	No	7	None	0	Excluded
Total pairs	**129**					**Total pairs included**	**29**

Abbreviation: LMγ2, laminin γ2.

artifact was noted and the presence of cancer cells was recorded as a "yes," "no," or "unsure." The matching LMγ2 antibody stained slide(s) were subsequently reviewed, and again the presence of artifact and the presence of cancer cells were recorded as for the hematoxylin-eosin stained slides. The type of artifact was classified based on well-recognized histopathological patterns.[58,59]

Paired slides were included when both the hematoxylin-eosin and LMγ2 antibody stained slide in a pair contained identifiable cancer cells and identifiable artifact. Paired slides were excluded when cancer cells were not positively identified in either the hematoxylin-eosin slide or the LMγ2 antibody stained slide, or where one or both of the paired slides contained no identifiable artifact.

RESULTS

The results of the pathology assessment are shown in Table 1. Of the 129 paired slides, 29 paired slides met the inclusion criteria. Eleven of the paired slides contained identifiable cancer cells in both slides but no artifact was present. LMγ2 staining was present in all 11 of the LMγ2 stained slides. In 10-paired slides, the hematoxylin-eosin contained artifact and cancer cells, but neither artifact nor cancer cells were present in the LMγ2-stained slides with no positive staining, leading them to be excluded. A further 7 pairs were seen to have cancer cells on the hematoxylin-eosin slide without any artifact with no cancer cells or artifact being seen on the corresponding LMγ2 stained slides. In all of the 29 paired slides meeting the inclusion criteria, LMγ2 antibody staining was positive in the presence of artifact: 17 had laser artifact only; 10 had laser and tissue handling artifact; 1 contained processing artifact only; and 1 contained tissue artifact only.

The 27 paired slides that contained laser artifact and cancer cells in both slides of the pair demonstrated positive immunoassay staining with LMγ2 of the cancer cells in the area of the artifact. Figure 1 is a sample of a set of paired slides in which tissue and laser artifact are present. The predominant artifact created by laser is coagulation. Figure 1A is stained with traditional hematoxylin-eosin staining, and Figure 1B is stained with LMγ2 monoclonal antibody immunohistochemistry. The results show that positive LMγ2 staining is seen in areas of identified artifact and does not seem to be affected by heat or by tissue/processing handling artifacts.

DISCUSSION

Adequate removal is essential for therapeutic treatment of the cancer when surgery is the treatment of choice. In HNSCC, assessing a surgically resected specimen with hematoxylin-eosin staining and determining whether an "adequate" margin is achieved can be challenging. Variation in specimen size from millimeters to many centimeters, the 3D anatomy of a resected specimen from the head and neck region, which can include multiple air-tissue interfaces, and/or inclusion of bone or cartilage in a resection, all add various levels of complexity. Artifact can be produced by the method used for resection, such as laser, diathermy, or coblation. Determining where the cancer ends and the surrounding stroma starts can be very difficult in HNSCC when an infiltrative pattern is present. Finally, as summarized by Hinni et al,[3] there is lack of clear consensus on what is determined to be an "adequate" margin.

Correlation between the status of resection margins determined by hematoxylin-eosin and recurrence varies.

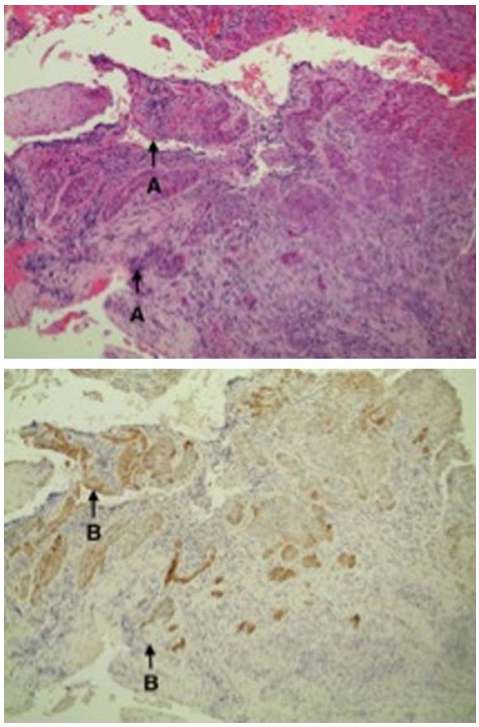

FIGURE 1. Paired slides stained with hematoxylin-eosin (A) or laminin γ2 (LMγ2) (B). Positive staining for LMγ2 (arrow B) is seen in the areas containing laser artifact (arrow A). (A) Hematoxylin-eosin original magnification ×20. (B) Laminin γ2 monoclonal antibody immunohistochemistry original magnification ×20. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Iseli et al[2] found that although positive margins in oral tongue SCC increased the likelihood of recurrence, 33% of tumors that had been deemed to be clear of margins recurred, prompting the question whether larger resections are required. At the other end of the spectrum, Sigston et al[7] found that the recurrence rate in 18 patients from a series of 52 early glottic SCCs who had close, suspicious, or positive margins, as determined by hematoxylin-eosin assessment, was only 16% (3 patients). Peretti et al[60] also found that reported positive margins with hematoxylin-eosin staining for early glottis cancers had no statistical impact on 5-year survival: 23 of 45 patients with positive margins had further treatment in the form of re-resection or radiotherapy, with no difference in survival between those who received additional treatment and those who did not.

Increasing evidence points to the histological features, such as perineural invasion, lymphatic invasion, vascular invasion, poorly differentiated infiltrative front, presence of satellite lesions, and tumor thickness being more important in determining prognosis than physically measured margins.[61–66] Moving beyond this, our expanding understanding of the heterogeneity of an individual tumor, the importance of the invasive front/stromal interface, epithelial-mesenchymal transition, and cancer stem cells point to the need to be able to assess biological function, not just morphology, in assessment of HNSCC margins.[4,6,8,9,16,53,67–78]

Two major types of techniques have been proposed to assess biological margins in HNSCC by various authors: immunohistochemistry[4,14,16,48,51,54,55,79,80] and DNA extraction techniques.[4,9,11,13,16,78,79] Each technique has its advantages and disadvantages. DNA techniques have a higher sensitivity, but are also associated with a high false-positive rate.[4,16,78] Concern has also been raised that genetic changes do not necessarily relate to expression or biological activity. The techniques used are laborious, require a high level of expertise, and are more costly than immunohistochemistry. Immunohistochemistry is more rapid, more widely available, and less expensive, can be performed manually, and is potentially applicable to frozen-section specimens. The limitations are the need to identify the appropriate biological markers and for an antibody to the selected marker to be available or developed.

A further consideration for immunohistochemistry is the potential for the selected biological marker to be altered or denatured by the resection method; technological advances have seen the wide use of various instruments to replace the scalpel during resection. These include monopolar and bipolar electrosurgery (diathermy, Coblation, LigaSure, Harmonic Scalpel™ and other devices), and various lasers, which all create varying degrees of thermal damage. The heat produced can result in precipitation of protein causing coagulation and torn appearance, separation of epithelium from basement membrane, or connective tissue with nuclei assuming a spindled palisading configuration.[18–20,58,59,81,82] Lippert et al[19] suggested that a CO2 laser did cause denaturing of proteins within a small radius of the excision.

Tissue preparation and processing may also produce artifact that can interfere with the assessment of histological specimens. There is shrinkage of tissue with fixation, but some tissue, such as collagen, can expand. Tissue handling during time of resection, such as injection or crushing, can cause disrupt cells, cause loss of cytological detail, or generate misplacement of tissue. Folding, curling, and diffusion of unfixed material can all impede assessment.[58,59]

To our knowledge, there are no other studies looking specifically at the impact of thermal artifact on the immunohistochemistry staining of biomarkers. Impact of tissue handling and processing also does not seem to have been considered. Both of these are important factors to consider if immunohistochemistry is to play an important role in the assessment of margins in HNSCC.

In our study, we elected to use glottic SCC tissue samples removed by CO2 laser to maximize the likelihood of the presence of heat and/or tissue handling or processing artifact in the presences of cancer cells and LMγ2 as the biomarker. LMγ2 overexpression has been demonstrated to be consistently present at various levels at the invasive front of HNSCCs, including glottis tumors, by numerous authors. There is also evidence suggesting that the pattern of staining may be correlated with invasiveness and migratory properties of a tumor. Even more recently,

there is emerging evidence that LMγ2 overexpression may be a marker of early epithelial mesenchymal transition.[27,34–36,42,44,47,51,54,73,79,80,83–88] A commercially available and validated monoclonal antibody is available through Dako (code NR. M 7262, monoclonal mouse anti-human laminin 5, gamma 2 chain, clone 4G1) and the technique for staining can be performed manually, as in this study, but also has the potential for automation. The staining is easily visible with light microscopy. Therefore, LMγ2 was deemed to be a highly suitable biological marker for the purpose of this study.

In this study, by identifying both cancer cells and artifact together over a reasonable number of samples, the preservation of LMγ2 staining with monoclonal antibody in areas of heat and tissue artifact seems to be confirmed. This supports the validity of considering immunohistochemistry staining of biological markers as a technique to improve margin assessment in HNSCC. The heterogeneity and complex biological functioning of HNSCC at the invasive front would suggest that a combination of a number of biological markers together is more likely to be able to provide information about adequate biological resection and possibly prognosis, rather than any single biological marker on its own. Further research is being undertaken in this area.

CONCLUSION

Immunohistochemistry staining of LMγ2 does not seem to be affected by tissue changes produced by heat from a CO2 laser and we have been able to show that the staining persists in the presence of laser, tissue, or processing artifact. LMγ2 is present at the invasive front of SCCs and is easily seen with light microscopy. This technique may have a role in assessing the adequacy of biological margins of HNSCC, either on its own or as a part of a suite of biological markers. The next step is to establish whether the use of biological markers would improve concordance in assessment of biological margins in HNSCC. The technique has the potential to be applied to any resected head and neck cancer, and may, in fact, offer the advantage of being able to identify cancer cells that are not phenotypically evident on traditional hematoxylin-eosin staining, thus not only improving assessment of margins in complex specimens, but also determining the true biological margin of head and neck cancers. Further research is being undertaken to explore this notion.

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5.3.2. Dual Immunohistochemistry

A number of potential issues exist with using multiple markers. One is matching cells on sequential slides stained with the different markers to determine which markers each cell is expressing. Another is that each sequential slide moves away from the initial slide and that some cells present in the first slide may not be present in the final slide of a series. An additional challenge with choosing vimentin as a marker is determining if there is staining within a cancer cell or the stroma.

To minimize these issues a dual immunohistochemistry staining technique amenable to either automated or manual staining was developed. p63 is expressed in the nucleus and LMγ2 and vimentin are both expressed in the cytoplasm. This enables p63 to be co-stained with either of the other two markers simultaneously. The advantages are that staining can be done over 2 slides instead of 3, the p63 can be used as the matching identifier and p63 will distinguish, in most cases, tumour cells from stroma assisting with identifying vimentin staining in the tumour cells.

p63 and vimentin have been used widely in pathology laboratories primarily to establish the epithelial or mesenchymal origin of tumours for many years. Techniques for these stains are highly standardized and well validated. LMγ2 immunohistochemistry staining is not routine. Monoclonal antibody with high specificity, optimized for immunohistochemistry with validated protocols was sourced from Dako (Clone 4G1; Dako Corporation, Cat. No: M7262).

The protocol for LMγ2 staining was manually confirmed on slides obtained from a hypopharyngeal SCC. Additional slides from this tissue block were used as the positive control. Tonsil tissue was used as a negative control for LMγ2.

5.3.2.1. Dual Immunohistochemistry Protocol

Three sequential slides in total sliced radially, perpendicular to the border of the specimen are taken from the invasive front of the tumour. The first slide is stained with standard H&E (*Fig. 15 A*). The second slide is for dual staining of p63/LMγ2 (*Fig. 54 B*) and the third for dual staining of p63/vimentin (*Fig. 15 C and D*).

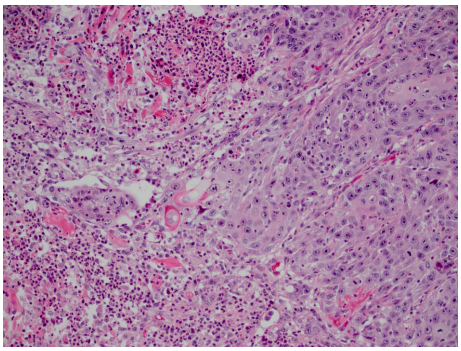
5.3.2.1.1. P63/LMγ2 Dual Immunohistochemistry Staining

The dual-immunohistochemistry stain is performed on a Ventana Bench-Mark Ultra automated stainer. Tonsil tissue is used as the negative control for LMγ2 and a pre-tested upper aerodigestive tract SCC (in this case a hypopharyngeal tumour) confirmed to stain positively for LMγ2 is used as the positive control with each run. Sections of formalin-fixed, paraffin-embedded tissue cut at 4 microns, are floated onto a 45°C water-bath and picked up on 3-aminopropyltriethoxysilane AAS-coated slides. The slides are then melted at 60°C and allowed to cool. After on-board dewaxing, Heat Induced Epitope Retrieval for 52 minutes at 95°C is applied to the sections before application of the first antibody. The first primary antibody, p63 (Clone 4A4; Ventana Medical Systems, Cat No: 05867061001), is applied for 28 minutes and visualized using the Ventana Ultra View DAB detection system (Ventana Medical Systems, Cat No: 05269806001).

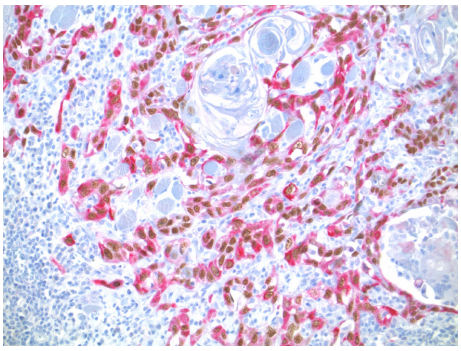
After the first antibody, a denaturation step removed and neutralized any unreacted and unbound antibody in preparation for the second antibody. The second primary antibody, Laminin-5, Gamma-2 Chain (Clone 4G1; Dako Corporation, Cat. No: M7262) is applied at a dilution of 1:100 for 60 minutes. It is visualized using the UltraView Universal Alkaline Phosphatase Red (Ventana Medical Systems, Cat. No: 05269814001). A signal amplification step is performed to enhance the Laminin antibody (Ventana Medical Systems, Cat. No: 05266114001). A final Hematoxylin II counterstain (Ventana Medical Systems, Cat. No: 05277965001) and Bluing reagent (Ventana Medical Systems, Cat No: 05266769001) are then applied. The slides are then dehydrated, cleared and mounted, ready for microscopic evaluation (*Fig. 15 B*).

5.3.2.1.2. P63/Vimentin Dual Immunohistochemistry Staining

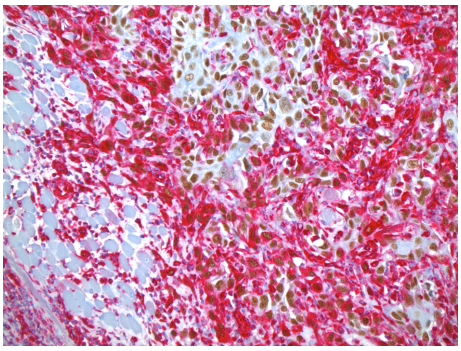
The dual-immunohistochemistry stain is performed on a Ventana Bench-Mark Ultra automated stainer. Sections of formalin-fixed, paraffin-embedded tissue cut at 4 microns, are floated onto a 45°C



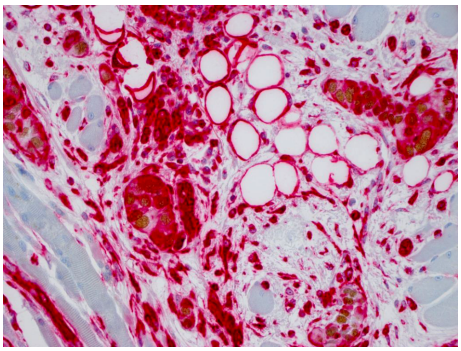
A.



B.



C.



D.

Figure 16 Dual immunohistochemistry staining

H&E staining (A) and dual-immunohistochemistry staining for p63 and LMγ2 (B) or p63 and vimentin (C and D). A, Invasive tumour front stained with traditional H&E at 200x magnification. B, Co-expression of p63 in the nucleus staining brown and LMγ2 in the cytoplasm staining red at 200x magnification. C, Co-expression of vimentin in the cytoplasm of cancer cells, identified by the p63 staining in the nucleus in brown, is seen staining red at 200x magnification. The stromal cells stain for vimentin. D Co-expression of vimentin, red, in the cytoplasm with p63, brown, in the nucleus of cancer cells at the invasive front at higher magnification 400x

water-bath and picked up on 3-aminopropyltriethoxysilane AAS-coated slides. The slides are then melted at 60°C and allowed to cool. After on-board dewaxing, sections Heat Induced Epitope Retrieval for 52 minutes at 95°C is applied before application of the first antibody. The first primary antibody, p63 (Clone 4A4; Ventana Medical Systems, Cat No: 05867061001), is applied for 28 minutes and visualized using the Ventana Ultra View DAB detection system (Ventana Medical Systems, Cat No: 05269806001).

The second primary antibody, vimentin (Clone V9; Roche, Cat. No: 05278139001), was applied for 60 minutes. It is visualized using the

UltraView Universal Alkaline Phosphatase Red (Ventana Medical Systems, Cat. No: 05269814001). A final Hematoxylin II counterstain (Ventana Medical Systems, Cat. No: 05277965001) and Bluing reagent (Ventana Medical Systems, Cat No: 05266769001) are then applied. The slides are then dehydrated, cleared and mounted, ready for microscopic evaluation (*Fig. 15 C and D*).

5.4. Assessment of Margins by Dual Immunohistochemistry to Predict Recurrence

The emergence framework of carcinogenesis and the state-space equation has been applied to form the hypothesis. A novel dual immunohistochemistry technique for assessing the hypothesis has been developed so the state-space equation is modified and working hypothesis becomes:

'The risk of recurrence over 5 years in oral squamous cell carcinoma can be predicted as a function of the % of p63/LM γ2 positive staining cells and/or p63/vimentin positive staining cells at the invasive front, seen in the field by light microscopy at a magnification of 40x represented by the equation:

'% risk of recurrence over 5 years' = {p63/LM γ2, p63/vimentin}'

5.4.1. Aim

The aim is to assess the application of dual immunohistochemistry staining with p63/ LMγ2 and p63/vimentin at the invasive front of OSCC to predict the risk of recurrence and to compare this to the predictive power of resection margins and other morphological features including differentiation, pattern of invasion, lymphocytic infiltrate, perineural invasion and lymphovascular invasion.

5.4.2. Method

Approval was obtained from Monash Health (formerly Southern Health) Human Research Ethics Committee (HREC), Monash University Ethics Committee (Reference 07057A) and the Victorian Cancer Research Biobank (VCRB) (Reference 7057A 27/6/2007).

Oral cavity cancers, with and without bony invasion, surgically resected by the Otorhinolaryngology, Head & Neck Unit at Monash Health, from January 2007

through to January 2011, were captured prospectively and a cross check of patient consent by VCRB was conducted. Recurrent tumors were excluded. 106 cases were identified and followed up for minimum of 5 years or until death. 4 patients were lost to follow up. Age, gender, site, side and stage of tumour, date of initial surgery and whether or not neck dissection was performed, date of any re-resection, adjunct therapy (radiotherapy, chemoradiotherapy or chemotherapy alone), date of any recurrence, date of death or date of last follow up were recorded.

5.4.2.1. Histological Assessment

A review of original pathology reports was used to select the appropriate tissue block for each resected tumour. In instances were the invasive front was not easily determined from reports, several tissue blocks were selected. The original histology reports were used to record the closest histological margin as positive, close (.1mm to 1mm), 1.1 to 4.9mm, 5mm or more. Three sequential slides were taken from the invasive front of identified tissue blocks. In 16 cases the required slides could not be obtained for various reasons including inadequate remaining tissue or poor quality of remaining tissue block.

The first slide from each block was stained with standard H&E and reviewed by a single pathologist. The histological grade (well differentiated, moderately differentiated, poorly differentiated), lymphocytic infiltrate (none, light, moderate, heavy), presence or absence of perineural invasion or lymphovascular invasion, and the invasive front pattern (pushing or infiltrative) were recorded. 14 cases were excluded due to no cancer cells being present.

The second slide from each block was submitted to dual staining of p63/LMγ2 using the protocol described in 5.3.2.1.1. The slides were then assessed for the percentage of p63/ LMγ2 co-staining, and the presence of LMγ2 staining without p63 in advance of the co-staining. The third slide from each block was submitted to dual staining of p63/vimentin using the protocol described in 5.3.2.1.2. These slides were assessed for the percentage of p63/vimentin co-staining. Comparing slides 2 and 3 for each tumour, co-staining of vimentin and LM γ2 was inferred. 14 slides failed the dual staining process and were

excluded. In 3 cases one of the paired slides were missing and these cases were excluded.

5.4.2.2. Statistical Analysis

All analyses were performed using Stata software version 14 (StataCorp, Texas, USA). Time to disease recurrence was the primary end point. Univariate and multivariate analyses were performed using Cox proportional hazards regression to determine the association between demographic and surgical factors with disease recurrence. Results from the regression analyses were reported as hazard ratios and 95% confidence intervals (95% CI). All calculated P values were two-tailed. $P<0.05$ indicated statistical significance.

5.4.3. Results

Results are presented in *Table 11*. There were 55 cases that met the inclusion criteria, had the full set of slides and required data set. The age range was from 30 to 93 years with a median age of 65years. The ratio of male to female was about 2:1 (36:19), consistent with cohorts published in the literature. The most common site was oral tongue (29/55, 53%). 25 (45%) of cases were T4 and involved the mandible. Out of the 55 cases, 17 (31%) cases were early stage (I and II) with the remaining 38 (69%) presenting with late stage disease (III).

45 of the 55 (84%) cases included a neck dissection with the primary resection and 69% had postoperative adjunct treatment with 16 receiving radiotherapy and 21 had chemo-radiotherapy. None received postoperative chemotherapy alone

Surgical margins were 5mm or greater in 17 of the 55 cases (31%), 14 had margins greater than 1mm but less than 5mm (25%), 11 had close margins (20%) of which 6 (55%) were re-excised and 11 (20%) had a positive margin, with re-excision being performed in 10 (91%). One patient refused further surgery. In 2 cases, the margins were not reported in millimetres, only as clear and could not be stratified.

23 (46%) cases had a recurrence. 12 of the recurrences were local, 10 regional and 1 distant to the lungs. The time of recurrence ranged from 5 months to 37 months. There were no recurrences after 37 months. The median time of

recurrence was 10 months. The median time of recurrence for regional disease was 9.8 months whilst recurrence of local disease was later at 22.5 months. The sole distal recurrence occurred at 12 months. 16 of the 23 (70%) had received adjunct post operative, 6 radiotherapy and 10 chemo-radiation, comparable to the group with no recurrence in which 21 (66%) received adjunct therapy and 11 did not. Of the 10 regional recurrences only 3 did not have treatment to the neck, being neck dissection and/or adjunct therapy, at the time of treatment of the primary tumour.

Analysis demonstrated that p63/vimentin was significantly associated with disease recurrence (P=0.003) (*Table 12*). For a 1% increase in p63/vimentin co-staining, the risk of disease recurrence increases by 2%. The only other relevant factor to reach significance was inferred LMγ/vimentin co-expression (P=0.05). None of the other potential morphological or biological features, including resection margins, p63/ LMγ co-staining, LMγ in advance of p63, infiltrative invasive front, lymphocytic infiltrate, perineural invasion or lymphovascular invasion reached statistical significance. Of note, the P value for margins was bordering on significance (P=0.088) suggesting a trend toward increased risk of disease recurrence. Multivariate analysis showed that p63/vimentin co-staining was an independent predictor of disease recurrence (HR: 1.02, 95% CI:1.00-1.04; P=0.01) after adjusting for margins and re-resection (*Table 13*). In addition, p63/vimentin co-staining showed an independent association with disease recurrence (HR: 1.02, 95% CI: 1.01-1.04; P=0.004) after adjusting for p63/LMγ2 expression and LMγ in advance of p63 (*Table 14*).

Gender	Age	Neck	Adjunct Treatment	Re-Excision	Recurrence	Time to Recur	Site	Side	T	N	M	Histo	Margin	Invasive Front	LI	PI	LR	P63/ LMγ2	P63/ VIM.	LMγ2 VIM. CO-EXPRESSION	LMγ2 ADVANCE OF P63
M	30.5	Y	CHEMO-XRT	N	N		TONGUE	R	2	1	0	MOD	5+	INFILTRATIVE	N	N	HEAVY	75	75	Y	N
M	35.9	Y	CHEMO-XRT	Y	N		TONGUE	R	3	0	0	MOD	CLOSE	INFILTRATIVE	N	N	NONE	90	70	Y	N
F	36.8	Y	NIL	Y	N		TONGUE	R	1	0	0	WELL	.1-4.9	INFILTRATIVE	N	N	MOD	50	50	Y	Y
M	41.0	Y	CHEMO-XRT	N	N		TONGUE	L	2	3	0	WELL	5+	INFILTRATIVE	N	N	HEAVY	60	20	Y	N
M	44.0	Y	CHEMO-XRT	N	METASTATIC	12	TONGUE	L	0	2	0	WELL	5+	INFILTRATIVE	N	N	NONE	25	5	N	N
M	45.1	Y	CHEMO-XRT	Y	N		TONGUE	R	1	1	0	POOR	CLOSE	INFILTRATIVE	N	Y	LIGHT	80	65	Y	N
M	45.1	N	CHEMO-XRT	N	N		TONGUE	R	4	1	0	MOD	POS	INFILTRATIVE	N	N	NONE	75	33	Y	N
M	47.3	Y	XRT	N	N		TONGUE	R	1	0	0	WELL	5+	PUSHING	N	N	MOD	75	25	Y	N
F	50.5	Y	CHEMO-XRT	N	N		TONGUE	R	2	0	0	WELL	5+	INFILTRATIVE	N	N	LIGHT	50	30	Y	N
M	51.1	Y	XRT	Y	N		TONGUE/ FOM	R	1	0	0	MOD	5+	INFILTRATIVE	N	N	HEAVY	90	3	N	Y
F	51.3	N	CHEMO-XRT	Y	REGIONAL	15	TONGUE	L	1	0	0	WELL	CLOSE	INFILTRATIVE	N	Y	MOD	80	80	Y	N
M	51.3	Y	NIL	Y	N		FOM	L	1	0	0	POOR	.1-4.9	INFILTRATIVE	N	N	LIGHT	80	3	N	N
F	52.0	Y	NIL	N	N		FOM	L	2	0	0	MOD	.1-4.9	PUSHING	N	N	MOD	3	0	N	N
M	54.0	Y	CHEMO-XRT	Y	REGIONAL	6	TONGUE	R	3	3	0	MOD	CLOSE	INFILTRATIVE	N	N	NONE	92	75	Y	N
F	54.4	Y	XRT	Y	REGIONAL	9	RMT	L	4	3	0	MOD	5+	INFILTRATIVE	N	N	MOD	75	40	Y	N
M	55.4	Y	CHEMO-XRT	N	N		FOM	L	1	4	0	WELL	POS	PUSHING	N	Y	NONE	20	3	N	N
M	56.6	Y	CHEMO-XRT	N	LOCAL	8	FOM	L	4	0	0	MOD	CLOSE	INFILTRATIVE	N	N	NONE	80	10	Y	Y
F	57.8	Y	NIL	Y	N		FOM	L	2	0	0	WELL	CLOSE	INFILTRATIVE	N	N	NONE	50	5	N	N
F	58.7	Y	CHEMO-XRT	N	LOCAL	10	ALVEOLAR	L	4	3	0	MOD	POS	INFILTRATIVE	N	N	LIGHT	75	75	Y	N
M	60.9	Y	CHEMO-XRT	N	N		RMT	L	4	0	0	MOD	CLOSE	PUSHING	N	N	MOD	3	0	N	N
F	62.0	Y	XRT	Y	N		TONGUE	R	1	0	0	MOD	.1-4.9	PUSHING	Y	N	MOD	0	0	N	N
M	62.7	Y	CHEMO-XRT	N	N		RMT/ FOM	R	4	1	0	POOR	POS	PUSHING	Y	N	LIGHT	7	0	N	N
M	64.0	Y	CHEMO-XRT	N	LOCAL	15	TONGUE/ FOM	L	4	0	0	WELL	5+	INFILTRATIVE	N	N	NONE	80	0	N	Y
M	64.7	Y	NIL	N	N		TONGUE	L	1	1	0	WELL	5+	INFILTRATIVE	N	N	HEAVY	20	20	Y	N
M	64.7	Y	CHEMO-XRT	N	REGIONAL	10	TONGUE	L	2	1	0	WELL	.1-4.9	PUSHING	N	N	HEAVY	60	80	Y	N
M	64.8	Y	CHEMO-XRT	N	LOCAL	31	ALVEOLAR	L	4	0	0	WELL	POS	PUSHING	N	N	NONE	50	0	N	N
M	66.5	Y	CHEMO-XRT	N	LOCAL	35	TONGUE	L	2	0	0	MOD	POS	INFILTRATIVE	N	N	LIGHT	90	10	Y	Y
M	67.0	N	NIL	Y	REGIONAL	5	TONGUE	L	1	0	0	POOR	.1-4.9	INFILTRATIVE	N	N	NONE	50	80	Y	Y
M	67.0	Y	XRT	N	N		ALVEOLAR	R	4	0	0	MOD	5+	INFILTRATIVE	N	N	NONE	50	0	N	N
F	67.7	Y	XRT	N	LOCAL	37	BUCCAL	L	2	0	0	POOR	.1-4.9	INFILTRATIVE	N	N	LIGHT	10	20	Y	N
M	68.1	Y	NIL	N	N		TONGUE	L	2	3	0	MOD	5+	INFILTRATIVE	Y	N	MOD	80	10	Y	Y
F	68.5	Y	NIL	N	N		TONGUE	L	1	3	0	WELL	5+	PUSHING	N	N	MOD	10	0	N	N
M	68.5	N	CHEMO-XRT	N	N		TONGUE	R	4	1	0	WELL	.1-4.9	INFILTRATIVE	N	N	NONE	75	10	Y	Y
M	70.7	Y	CHEMO-XRT	N	REGIONAL	6	BUCCAL	L	4	3	0	WELL	5+	PUSHING	Y	N	NONE	75	40	Y	N
M	71.2	Y	XRT	N	N		RMT	R	4	1	0	POOR	.1-4.9	INFILTRATIVE	N	N	LIGHT	75	10	Y	N
M	73.2	Y	XRT	Y	REGIONAL	25	FOM	R	4	1	0	MOD	POS	INFILTRATIVE	N	N	NONE	60	60	Y	N
M	73.5	Y	XRT	N	N		FOM	R	4	0	0	MOD		INFILTRATIVE	N	N	LIGHT	90	0	N	N
F	73.6	Y	XRT	N	N		TONGUE/ FOM	M	4	3	0	MOD	POS	INFILTRATIVE	N	N	LIGHT	90	50	Y	Y
M	73.7	N	NIL	Y	LOCAL	33	TONGUE/ FOM	R	2	0	0	WELL	POS	PUSHING	N	N	MOD	3	0	N	N
M	74.2	Y	CHEMO-XRT	N	N		TONGUE	L	3	0	0	WELL	CLOSE	INFILTRATIVE	N	N	LIGHT	66	5	Y	N
F	74.9	N	NIL	N	LOCAL	7	TONGUE	L	1	0	0	MOD	.1-4.9	PUSHING	N	N	HEAVY	0	40	N	N
M	76.5	Y	NIL	N	N		FOM	R	4	0	0	MOD	5+	PUSHING	N	N	LIGHT	75	0	N	N
F	77.9	Y	NIL	N	N		FOM	R	4	2	0	MOD	5+	PUSHING	N	N	NONE	3	0	N	N
F	78.4	N	NIL	N	REGIONAL	5	TONGUE	L	1	0	0	MOD	5+	INFILTRATIVE	N	N	NONE	10	10	Y	N
F	79.8	Y	XRT	N	N		RMT	R	3	3	0	POOR	.1-4.9	INFILTRATIVE	N	N	HEAVY	80	25	Y	N
M	80.4	N	NIL	Y	REGIONAL	10	TONGUE	L	1	0	0	WELL	POS	INFILTRATIVE	N	N	MOD	92	92	Y	Y
M	80.5	N	NIL	Y	LOCAL	36	ALVEOLAR	L	4	0	0	WELL		INFILTRATIVE	N	N	NONE	60	33	Y	Y
F	82.1	Y	XRT	N	REGIONAL	7	TONGUE	R	3	3	0	MOD	CLOSE	INFILTRATIVE	N	N	HEAVY	80	92	Y	Y
M	83.3	Y	NIL	N	LOCAL	9	RMT	L	4	0	0	MOD	POS	INFILTRATIVE	N	N	LIGHT	90	5	Y	N
F	83.4	Y	XRT	N	N		FOM	M	4	0	0	MOD	5+	INFILTRATIVE	N	N	LIGHT	50	33	Y	N
F	84.0	Y	XRT	N	LOCAL	34	BUCCAL	L	4	3	0	POOR	.1-4.9	INFILTRATIVE	N	N	LIGHT	80	40	Y	Y
M	84.7	Y	XRT	N	LOCAL	15	FOM	R	4	3	0	POOR	.1-4.9	INFILTRATIVE	N	N	NONE	50	0	N	N
M	85.8	Y	NIL	N	N		ALVEOLAR/ FOM	L	4	1	0	WELL	CLOSE	PUSHING	N	Y	LIGHT	0	0	N	N
M	86.1	Y	XRT	N	N		TONGUE	R	1	0	0	WELL	CLOSE	PUSHING	N	Y	LIGHT	10	10	Y	N
F	93.6	Y	NIL	N	N		BUCCAL	R	4	0	0	MOD	.1-4.9	INFILTRATIVE	N	N	NONE	92	3	N	Y

Table 11 Dataset of cohort

Gender M=male, F=female. Age is in years Neck refers to neck dissection at time of primary operation Adjunct treatment is post-operative Recurrence is recorded as yes or no Type of recurrence is local, regional or metastatic disease. Time to recurrence is in months from time of original operation. Site is the primary site of origin Side L= left and R=right M=midline TNM is staging at time of initial operation. Histo is the histological grading of the tumour as well, moderately or poorly differentiated Margins refer to closest margin in resection and recorded as positive, close (.01-1mm), 1.1-4.9mm and 5mm or more. Invasive front is assessed as broad and pushing, pushing, or infiltrative. LI is lymphovascular invasion, PI is perineural invasion. LR is lymphocytic response. P63/LMγ2 and p63/vimentin are the % of cells in the field at 40x that co-stain the biomarkers. LMγ2 vimentin co-expression is inferred staining for both markers in cells at invasive front. LMγ2 in advance of p63 is the presence of cells staining positively for LMγ2 but not p63

Variable	n	mean	std	max	q3	median	q1	min	HazardRatio	Low95	High95	P value
p63/vimentin	55	26.27	29.38	92	40	10	0	0	1.02	1.01	1.03	0.003
LMγ2/vimentin coexpression	55	61.8%	0.49	1	1	1	0	0	2.72	0.98	7.56	0.050
Margins	53	1.30	1.14	3	2	1	0	0	1.39	0.95	2.03	0.088
LMγ2 inadvance of p63	55	25.5%	0.44	1	1	0	0	0	1.82	0.73	4.57	0.191
Lymphoctyic_Response	55	1.15	1.06	3	2	1	0	0	0.83	0.55	1.27	0.388
p63/LMγ2	55	55.29	31.84	92	80	66	20	0	1.01	0.99	1.02	0.395
Infiltrative Invasive Front	55	70.9%	0.46	1	1	1	0	0	1.53	0.55	4.24	0.403
Perineural Invasion	55	9.1%	0.29	1	0	0	0	0	0.51	0.07	3.97	0.513
N Stage	55	0.98	1.28	4	2	0	0	0	1.04	0.74	1.48	0.804
Lymphovascular Invasion	55	7.3%	0.26	1	0	0	0	0	0.85	0.11	6.58	0.871
T Stage	55	2.65	1.34	4	4	3	1	0	1.02	0.73	1.42	0.910

Table 12 Univariant Analyses

P63/vimentin co-expression was highly significant in predicting recurrence P= 0.003 and 95% CI. LMγ2/vimentin co-expression was just significant but most likely related to the power of prediction of vimentin. Of note, margins were not found to reach significance as a predictor of recurrence

Variable	HazardRatio	Low95	High95	P value	Outcome
p63/vimentin	1.02	1.00	1.04	0.010	Disease Recurrence
Margins	1.37	0.90	2.07	0.136	Disease Recurrence
Re-excision	0.77	0.27	2.17	0.614	Disease Recurrence

Table 13 Multivariate analyses with margins and re-excision

p63/vimentin is shown to be a predictor of recurrence independent of margins and re-excision

Variable	HazardRatio	Low95	High95	P value	Outcome
p63/vimentin	1.02	1.01	1.04	0.004	Disease Recurrence
p63/LMγ2	0.99	0.97	1.01	0.409	Disease Recurrence
LMγ2 in advance of p63	1.97	0.73	5.30	0.172	Disease Recurrence

Table 14 Multivariate analyses of biomarkers

p63/vimentin is shown to be a predictor of recurrence independent of p63/LMγ2 and LMγ2 staining in front of p63

5.4.4. Discussion

The risk of recurrence in oral squamous cell carcinoma (OSCC) has been reported to vary from 9% up to 75%.[24, 59, 61, 72, 75, 84, 85, 87, 88, 682, 693-696] Factors that have been related to the risk of recurrence include tumour stage at presentation[24, 59, 693-696], perineural invasion[87, 88, 697, 698], invasive front[31, 88, 94, 102, 104, 105] and surgical resection margins.[57, 59, 61, 72, 74, 75, 84, 87, 683] Various biomarkers detected via a number of different methods have been proposed and investigated with the aim of assessing prospectively the risk of recurrence. [57, 73, 107, 109-114] In contrast to p16, used as a marker for HPV in oropharyngeal SCC for prognosis and therapeutic streaming, no biomarker for OSCC has been adopted into everyday practice. [57, 73, 107, 109-114]

In line with our increased appreciation of the heterogeneity of tumours and an increasing interest in personalized medicine, there has been an increased investment into molecular and genetic profiling in an attempt to provide tools that may aid clinicians in identifying which patients with OSCC are at risk of recurrence.[73, 92, 93, 106-112, 116, 122, 123, 125, 126, 168, 404, 588, 590, 594, 597, 598, 626, 699-701] Whilst techniques of detection vary what most have in common is the notion that cancer is a genetic disease.[125, 126, 404, 694] To date, this has not translated to have any meaningful impact in the clinical setting.

Applying an emergence framework of carcinogenesis to understand OSCC as a complex emergent system shifts the focus from genes to the level of the functional tissue unit. This has enabled a large body of research available in the literature to be analysed via a systems approach by first defining the normal functional tissue unit in terms of components, environmental interactions, structure and mechanism, then comparing this to the OSCC system and finally evaluating how the OSCC system continues to evolve. Systems mapping of the key mechanisms enabled the identification of biomarkers visible at the functional tissue unit level and, using evidence from the literature, to predict how their expression changes during tumour progression to create a state-space formula that quantitatively relates these changes to risk of recurrence.

Once the biomarkers were identified, the limitations of each marker were examined which led to the development of dual immunohistochemistry staining techniques to negate these limitations. Co-staining removes the need for a known quantitative baseline expression of the individual markers. Co-staining of p63/vimentin enabled the identification of vimentin staining in tumour cells. Co-staining of p63/LMγ2 identified in some specimens LMγ2 staining in front of the p63, which may represent cancer cells that have detached from the main body of the tumour and have lost p63 expression.

The limitation in this study was the reduction of the cohort for various reasons to 55. Despite this, p63/vimentin co-staining was highly significant in predicting recurrence (P=0.003) with a very high confidence levels. This was more significant than any single morphological feature, including excision margins and remained significant in multivariate analyses. A larger cohort may have demonstrated added significance of LMγ2 staining in front of p63 but as all the cases in this cohort had positive p63/vimentin the additive prognostic information may be limited.

p63 and vimentin have been used in immunohistochemistry for many years, have standardized protocols, are cheap and the staining can be performed with standard automated strainers or by-hand. This makes this technique inexpensive and able to be employed in any pathology laboratory worldwide.

5.4.5. Conclusion

Application of an emergence framework of carcinogenesis led to the design of a study, which has identified a simple, standardized, inexpensive dual immunohistochemistry technique that does not require any additional skills or expensive equipment that is highly predictive of recurrence in OSCC. For every 1% of p63/vimentin co-staining at the invasive front of an OSCC, there is a 2% increase in risk of recurrent disease.

Expansion of the retrospective cohort to re affirm these findings is the next step before employing in a prospective setting. This simple dual immunohistochemistry technique may help to identify early patients at high risk of recurrence and direct management toward newer therapies earlier.

6. Summary, Conclusion and Future Directions

6.1. Summary

The aim of the research embodied in this thesis was to identify a simple, cost effective method to assess biological margins in OSCC that could improve ability over standard H&E staining in predicting recurrence from tumour margins. This has been achieved. Whilst this has significant potential to influence future management of OSCC, and HNSCC in general, the bigger future impact of this work is on the way cancer and cancer research is approached, specifically relating to translation research, with the development of a new framework for understanding carcinogenesis.

The field of genomics has exploded since 1988 with the Human Genome Project. $3.8 billion US was invested into the project by the American government between 1988 and 2003 and has enabled the full sequencing of the human genome.[702, 703] Since then many more billions have been poured into this area from both public and private funding.[702] In 2017 $1.9 billion had been invested in genomic start up companies within the first 6 months.[704]

In cancer research, genomics has been driving predictive models and the concept of precision medicine.[106, 129, 168, 694, 705-707] Despite the billions of dollars invested into this research significant progress that is meaningful in the clinical setting is limited.[708] This should serve as a timely reminder that complexity is not explained by simply looking at the parts.

Many highly regarded scientists, researchers, and philosophers including Denis Noble, Mario Bunge, Carlos Sonnenschein, Anna Soto, Björn Brucher, Sydney Brenner and Simon Baker have been advocating for a move away from a gene-centric model of cancer for many years.[3, 132, 136, 137, 141, 150-152, 155, 159, 178, 181-183, 709] Systems biology refers to quantitative analysis of the dynamic interactions among several components of a biological system with the aim to understand the behaviour of the system as a whole.[710] Systems biology has become one of the most rapidly growing fields in the last decade.[106, 143, 155, 157, 160, 161, 165, 168, 170, 225, 708, 710-716]

Carl Sagan, an American astronomer, cosmologist, astrophysicist, astrobiologist, author, science popularizer, and science communicator in astronomy and other natural sciences, in one of his last interviews commented, "Science is more than a body of knowledge. It is a way of thinking."[708] The work in this thesis adds to our knowledge and more importantly, to our way of thinking. "An Emergence Framework of Carcinogenesis" provides a new way to think about cancer, as an emergent complex system. The framework through '12 Principles' provides a unified, practical approach for bi-directional translational research.

In the second part of this thesis "An Emergence Framework of Carcinogenesis" has been applied to address the clinical question of how resection margins in oral cavity squamous cell carcinomas can be better assessed to predict the risk of recurrence. This provides a strong example of the benefit of using this framework as opposed to following a more traditional research method in which it is common for researchers to focus on a particular 'thing', and become the expert on that 'thing'. An example is Joe McCord, who, with Irwin Fridovich, discovered super oxide dismutases (SODs), enzymes that convert superoxide radicals to molecular oxygen and hydrogen peroxide..[717] Dr McCord is recognized as an expert in SODs and whilst a huge contribution has been made to understanding redox biology and in particular understanding of reperfusion injury, huge amounts of time and money invested into trying to develop SOD into a clinically useful therapeutic agent to protect against inflammation, reperfusion and oxidative damage, has failed to achieve clinical success.[718] As alluded to in the introduction, my 'thing' was LMγ2. I believed it would provide the answer to better assessing margins in OSCC. Every paper I read about it supported this hypothesis. This was my intent when I started my PhD; to prove that LMγ2 was the answer. Even when doing the CESM mapping, a small part of me was excited to see LMγ2 have a place.

The process of CESM mapping helped to take the blinkers off, reduce bias and look at the broader picture. Developing the detail was extremely challenging. The majority of literature is not written from a systems view, which makes it difficult to stay in the systems frame of thinking. I experienced a distinct discomfort in constantly correcting the way I was thinking and the process was mentally exhausting. However without going through this process I would never have recognized the importance of p63 and vimentin as potential markers, nor understood the interplay between E-cadherin, LMγ2, p63 and vimentin. I would have likely ended up with a

qualitative process for margin assessment that may have added only marginal additional benefit.

Instead, application of "An Emergence Framework of Carcinogenesis" has resulted in the development of a simple dual immunohistochemistry-staining test that provides a quantitative measure of risk of recurrence in OSCC using biomarkers that are already widely used, easily available and relatively inexpensive. Despite a smaller than desired cohort, the P value on both univariant and multivariant analysis was highly significant, P=0.003 and P=0.01, with a 95% confidence interval. Of particular note, this was independent of the adequacy of surgical resection margins which have to date been considered the primary determining factor in recurrence. This was not the anticipated outcome and without following the thought process provided through the framework and trusting in the process this significant finding could have been missed.

6.2. Conclusion

Taking Max Planck's advice and changing the way we look at cancer has led to the development of a systems orientated framework of carcinogenesis, "An Emergence Framework of Carcinogenesis" [719], in which cancer is looked at as an emergent complex system. This framework can be utilized in any area of cancer research with its major benefit in translational research, providing clear guidance on linking basic research with clinical research through the identification of 'functional tissue units', that are defined in a consistent manner.

The application of this framework has been demonstrated through the biological assessment of margins in OSCC with the successful development of a simple, cost effective dual immunohistochemistry technique. Using this technique it has been demonstrated that for every 1% of co-staining in OSCC of p63 (marker for epithelial cells) and vimentin (marker for mesenchymal cells) there is a 2% increase risk of recurrent disease within 3 years.

6.3. Future Directions

Future directions are two-fold: those that relate to "An Emergence Framework of Carcinogenesis", and those that relate to the use of p63/vimentin dual immunohistochemistry-staining in HNSCC.

The first is to actively promote "An Emergence Framework of Carcinogenesis" through various forums, seeking wider feedback and encouraging others to employ this model in their work. To assist, a short handbook, "An Emergence Framework of Carcinogenesis: a guide to practical implementation" will be written. I foresee an online "Functional Tissue Unit" library that serves as a resource for researchers, which can be added to and modified as new discoveries come to light. This avoids the need for individual researchers or research groups to constantly define the relevant functional tissue unit from scratch and provide a common point of basic and clinical research.

The second is to expand the cohort of OSCC's reviewed and to implement a prospective study. There is the capacity for this to be a multicentre trial. I envisage that early identification of those with a high risk of recurrence to enable a redirection in management early to trial therapies, such as immunotherapies, rather than waiting for the recurrence. It is likely that a similar pattern may be seen in other HNSCCs and could be expanded to those tumours as well.

There may very well be many other future directions that become apparent with input from others. As we change the way we look at cancer and cancer research, who can say what possibilities may be seen. They may well be limitless.

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