DOCTOR OF PHILOSOPHY

THE ROLE OF TGFB1 IN ORAL CANCER CELL MIGRATION VIA ACTIVATING SMAD AND NON-SMAD SIGNALLING PATHWAYS

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BDS, MSc

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Thesis submitted for the degree of Doctor of Philosophy in Dentistry / Oral Surgery

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DEDICATION

This thesis is dedicated to my devoted parents, Edress M. Shalgm and Fatima Basher, whose love, support, and prayers have enabled me to attain such accomplishment and honour.

To my wife Muneera Ebrahem, who has shown me unending love, patience, and kindness.

To my daughters Fatima Shalgm and Khadija Shalgm, who make my life joyful and happy.

To my siblings, who have been a tremendous source of inspiration and support.

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My parents, siblings, wife, and all of my other family members have encouraged and supported me in growing amounts, and I am incredibly appreciative of them.

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Finally, I would also like to acknowledge Sebha University, Libyan Ministry of education and Libyan Cultural affairs in London for providing the financial support for this project.

DECLARATION

I declare that I am the author of this thesis and that I have consulted all the references cited. The work of which this thesis is a record has been accepted for a higher degree. This work has been carried out in the Cell and Molecular Biology laboratories of Dundee Dental School, under the supervision of Dr Sarah Jones, Dr Ian Ellis and Prof. Michaelina Macluskey.

Signature: …….

Date: 22/05/2023

Basher Shalgm.

CERTIFICATE

I hereby certify that Basher Edress M. Shalgm has fulfilled the condition of Ordinance 39 of the University of Dundee and is qualified to submit this thesis for the degree of Doctor of Philosophy.

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ABBREVIATIONS

SCC: Squamous Cell Carcinoma.

HPV: Human Papilloma Virus.

IHANCE: International Head and Neck Cancer Epidemiology.

ECM: Extracellular matrix.

CAF: Cancer associated fibroblasts.

EMT: Epithelial to mesenchymal transition.

MET: Mesenchymal to epithelial transition.

S/TKRs: Serine/threonine kinase-linked receptors.

PTKRs: Protein tyrosine kinase-linked receptors.

TGFβ: Transforming growth factor Beta.

EGFR: Epidermal growth factor receptor.

EGF: Epidermal growth factor

FGFR: Fibroblast growth factor receptor.

FGF: Fibroblast growth factor.

AMH or MIS: Anti-muellerian hormone.

BMPs: Bone morphogenic proteins.

GDFs: Growth differentiation factors.

mTOR: Mammalian target of rapamycin.

RTK: Receptor tyrosine kinase.

IL-6: Interleukin-6 signalling.

GRHL2: Grainyhead-like 2.

MMP-10: matrix metalloprotase-10.

PTHrP: Parathyroid Hormone-related Proteins.

FCS: Foetal Calf Serum.

SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis.

HRP: Horse radish peroxide.

DMSO: Dimethyl sulfoxide solvent.

ALK: activin receptor-like kinase receptors.

PKBα: protein kinase B.

MAPK: Mitogen-activated protein kinases.

RIPA: Radioimmunoprecipitation assay buffer.

EDTA: Ethylenediaminetetraacetic acid.

PBS: Phosphate buffered saline.

PBS-T: Phosphate buffered saline with Tween.

TBS: Tris buffered saline.

TBS-T: Tris buffered saline with Tween.

PVDF: Polyvinylidene fluoride

MeOH: Methanol

ABSTRACT

Introduction: In general, a malignant tumour formed from the oral mucosal epithelium is referred to as oral cancer. Squamous cell carcinoma has been identified in more than 90% of cases of oral cancer. Transforming growth factor beta (TGFβ) participates in a variety of biological processes, including cellular differentiation, organogenesis and halting the growth of tumours. However, tumour cells use TGFβ signalling systems to their advantage to develop new features, such as the capacity for metastasis and invasion. The term "epithelial to mesenchymal transition" (EMT) describes a biological process in which an epithelial cell undergoes modifications that cause it to have a mesenchymal cell phenotype, such as the capacity to migrate and invade, resist apoptosis, and produce extracellular matrix components.

Aims: This study sought to determine whether TGFβ-1 contributed to the ability of oral cancer cell lines to migrate or not by triggering Smad or non-Smad signalling pathways. Additionally, the investigation included the mechanism of how TGFβ1 controls the migration of oral cancer cell lines via EMT.

Materials: In this project, three cell lines, HaCaT developed from adult human skin keratinocytes, TYS developed from oral adeno-squamous cell carcinoma of the minor salivary gland and SAS-H1 obtained from a poorly differentiated squamous cell carcinoma from human tongue, were utilised. TGFβ-1, epidermal growth factor (EGF), and several signalling pathway inhibitors, including TGFβ-RI Kinase Inhibitor VII, MK-2206, PD98059, and SB431542, were used as test conditions.

Methods: The behaviour and features of the cell lines under test conditions were evaluated using three types of migration assay (the Scatter assay, Gap closure assay and Scratch assay). Western blotting was used to assess the expression of various cell signalling molecules such as Smad, Akt, Erk/MAPK and Rac1/Cdc42. Additionally, to evaluate the expression and localisation of migratory markers including E-cadherin and N-cadherin, immunocytochemistry and western blotting were used.

Results: Both normal and oral cancer cell lines exhibited both individual and collective cell migration triggered by TGFβ-1. Cell adhesion was broken down when TGFβ-1 caused the re-localisation of E-cadherin and β-catenin molecules

from the cell membrane to the cytoplasm. N-cadherin, a mesenchymal marker, was increased by TGFβ-1 in both normal and oral cancer cell lines.

Conclusion: TGFβ1 induces migration of oral cancer cell lines via EMT. TGFβ1 regulates cell migration by Smad, PI3K/Akt and Erk/MAPK signalling pathways in normal and oral cancer cell lines.

Chapter 1: Introduction and Literature Review

1.1 Introduction:

In general, the term oral cancer refers to a malignant tumour developed from the tissues of the oral cavity (Speight and Farthing, 2018). Oral cancer worldwide is among the ten most common cancers (Rivera, 2015). It has been classified as the sixth most common cancer in the world (Kumar *et al.*, 2016). More than 90% of oral cancers are diagnosed as squamous cell carcinoma (SCC), however, a distinction needs to be made between different tumours of the oral cavity based on site, aetiology and prognosis (Speight and Farthing, 2018).

Multiple terms are used to describe oral cancer due to the lack of consensus on the definition of oral cancer in the literature. This is linked to the different cancer subsites including the oral cavity. One example is the difference between attributing the anatomical site to the oral cavity or to oropharynx (Conway, Purkayastha and Chestnutt, 2018; Bugshan and Farooq, 2020). The impact of determining the distinction between the two the tumours is related to the aetiology. For example, human papillomavirus (HPV) is a risk factor for oropharynx carcinoma, but not for oral cancer. According to the Scottish Cancer Registry, oral cavity cancer is a subgroup of head and neck cancer, which includes lip, tongue (excluding lingual tonsil), gingiva, floor of the mouth, hard palate, uvula, cheek mucosa and the lateral walls of oropharynx (Conway, Purkayastha and Chestnutt, 2018).

1.2 Epidemiology of Oral Cancer:

In 2012, the global estimation analysis carried out by World Health Organisation (WHO) showed 202,000 newly diagnosed cases of oral cavity cancer per year. In the same year, the global estimated age-standardised rate for oral cancer was 2.7 per 100,000 with a higher incidence rate in men compared to women (Salehiniya and Raei, 2020). The recent global estimation by WHO in 2020 showed the yearly report of new cases of oral cancer reached 377,713 and 177,757 new deaths in 185 countries. The incidence and mortality rate of oral cancer are higher in men than women. Moreover, in countries with a lower human development index, the age-standardised rate of oral cancer was 10.2 per 100,000 due to the high burden of disease in India (Sung *et al.*, 2021). The records of epidemiological trend of oral cancer has changed over this period of time (Sarode *et al.*, 2020).

In the United Kingdom, the incidence rate of oral malignancy has risen over the past 20 years by 68% (Chaturvedi *et al.*, 2019). In Scotland, the analysis carried out by the cancer registry agency showed a 10% increase in oral cavity cancer between 2001 and 2012. In England, the increase in the incidence rate of oral cavity cancer per annum, was by 3% in females and 2.8% in males. Data from 2016 showed the number of cases diagnosed with oral cancer as follows England (2016) 3,088 cases, Scotland (2016) 400 cases, N. Ireland (2016) 80 cases and Wales (2015) 174 cases. According to the UK cancer registries, 52,829 cases of oral cancer were recorded in the period between 2006 to 2016. The incidence rates of oral cancer in Scotland have been stable at high levels, while for England and Wales, the rates have steadily risen (**Table 1**) (Conway, Purkayastha and Chestnutt, 2018).

Table 1: Age-standardised incidence rates of oral cancer from cancer registries of UK countries.

Country	Gender	Oral cavity cancer incidence rate	Year
Scotland	Male	10 per 100,000	2016
	Female	5.6 per 100,000	
England	Male	7.3 per 100,000	2016
	Female	4.8 per 100.000	
Wales	Male	7.4 per 100,000	2016
	Female	3.7 per 100,000	
N. Ireland	Male	5.9 per 100,000	2015
	Female	3.9 per 100,000	

1.3 Risk Factors for Oral Cancer:

1.3.1 Tobacco Smoking and Alcohol Consumption:

Both smoking of tobacco and consumption of alcohol are heavily associated with oral cancers (Chaturvedi *et al.*, 2019). On the other hand, collection of datasets from the international head and neck cancer epidemiology (IHANCE) showed a significant number of cases of oral cavity cancer in patients with no previous history of smoking and/or alcohol consumption. To solve this confusion, a few points were taken into consideration including the establishment of an accurate risks estimation, analysis of tobacco-alcohol interaction, assessment of

smokeless tobacco and investigating the impact of stopping smoking and alcohol consumption (Gupta *et al.*, 2017).

The estimated risk for oral cancer was two-fold high among smokers with no history of alcohol consumption compared to non-smokers. This estimation of risk was positively correlated with the frequency and duration of tobacco smoking. Similarly, the estimated risk for oral cavity cancer was two-fold among alcohol drinkers, who consume more than three drinks a day, with no smoking history. The highest risk was recorded among those who were smokers and heavy alcohol consumers, which showed an estimated increase in the risk of the disease by five-fold (Conway, Purkayastha and Chestnutt, 2018).

Data from INHANCE shows a strong association of a dose-response relationship between duration and frequency of smoking tobacco and alcohol consumption, and the risk for oral cancer. For oral cavity cancer, the duration of smoking is far more crucial than the frequency, which is similar to the risk for lung cancer. This correlation indicates that smoking less cigarettes per day over a long period of time has a higher risk than smoking more cigarettes over a short period of time. On the other hand, the frequency of alcohol intake is far more important than the duration of consumption, which implies consuming more than three drinks a day over a short period of time has a higher risk for oral cancer than low consumption over a long period of time. The link of risk factors for oral cancer and specific age group is based on the observation of the consumption of alcohol and smoking tobacco of young adults. Both of those risk factors have no low safety limit for the risk of oral cancers (**Figure 1**) (Conway, Purkayastha and Chestnutt, 2018).

1.3.2 Betel Quid:

Betel quid or paan comprises of areca nut plus or minus tobacco wrapped up in betel leaf (**Figure 1**). Betel quid is used by being placed in the mouth, which is common in the South and East-South of Asia. Data showed that betel quid without tobacco increases the risk for oral cancer by five-fold (Ernani and Saba, 2015).

1.3.3 Smokeless Tobacco:

The INHANCE dataset showed a positive correlation between the increase of the oral cancer risk and smokeless tobacco consumption including powered snuff or chewing (Niaz *et al.*, 2017). Smokeless tobacco increases the risk for oral

tumours by two-fold even among people with no history of smoking cigarettes (**Figure 1**) (Conway, Purkayastha and Chestnutt, 2018).

1.3.4 Socioeconomic Status:

Low socioeconomic status, low education levels and low income have been associated with increasing the risk for oral cancer at similar levels to tobacco smoking and alcohol consumption, which is two-fold (**Figure 1**) (Conway, Purkayastha and Chestnutt, 2018).

1.3.5 Diet:

Reports of high consumption of fresh fruits and vegetables has been associated strongly in reducing the risk of oral cancer by half (Conway, Purkayastha and Chestnutt, 2018; Rodríguez-Molinero *et al.*, 2021). In addition, the data suggested that people with low body mass index have a higher risk for oral cancer, especially those less than 30 years old (**Figure 1**) (Conway, Purkayastha and Chestnutt, 2018).

1.3.6 Genetics:

A Few genetic variations showed an association with an increased risk for oral cancer (Ali *et al.*, 2017; Sreekumar, 2019), which might include alcohol metabolism, DNA repair and metabolism of nicotine. In addition, family history of oral cancer showed moderate association as a risk factor for the disease (**Figure 1**) (Conway, Purkayastha and Chestnutt, 2018).

1.3.7 Oral Health:

The INHANCE dataset showed that regular dental attendance and routine daily brushing of the teeth may decrease the risk for oral cancer. On the other hand, a slight increase in the risk for oral cancer has been linked to prolonged use of mouthwash (Gupta *et al.*, 2017).

1.3.8 Obesity:

Height in present and previous smokers, waist circumference or waist-to-hip ratio regardless of smoking history, and BMI in non - smokers were all strongly correlated with risk of Head and neck cancer. These data give additional evidence of the impact of excessive adiposity in the development of cancer (Gaudet *et al.*, 2015).

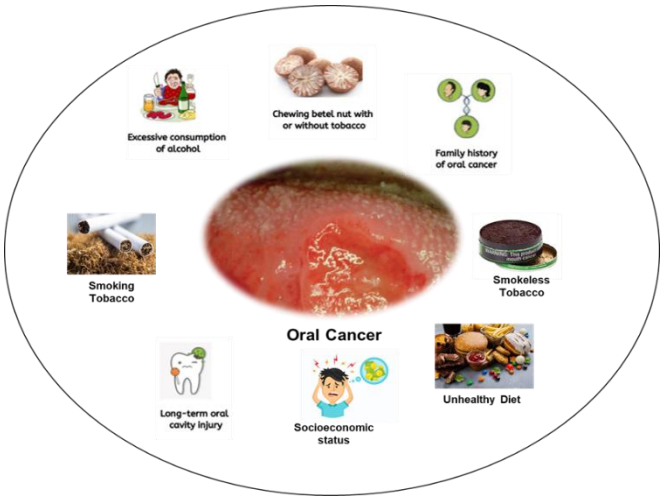

Figure 1: Risk factors for oral cancer

This figure shows the different risk factors for oral cancer (Lewis, 2018). Permission has been granted to use the figure by Courtesy of Professor M. A. O. Lewis.

1.4 Oral Cavity:

1.4.1 Anatomy of the Oral Cavity:

Oral cavity and oropharynx compose the upper part of the aerodigestive tract. These two components are separated by four structures which are pterygomandibular raphe, tonsillar pillars, soft palate and circumvallate papillae of the tongue (Laine and Smoker, 1995). The normal anatomy of the oral cavity is comprised of lips, vestibule, gingivobuccal mucosa, alveolar ridges, teeth, tongue (only anterior two thirds), floor of the mouth and hard palate (Laine and Smoker, 1995; Yates and Phillips, 2001). Anatomically, the oral cavity can be divided into two main components the vestibule and oral cavity proper (Laine and Smoker, 1995). Vestibule refers to the area between the dental arches and the lips/cheeks anteriorly and laterally, while the oral cavity proper refers to the lining of the alveolar ridges of both the mandible and the maxilla(Laine and Smoker, 1995; Yates and Phillips, 2001). Both the vestibule and oral cavity proper connect via interdental spaces and the area posterior to the last molars of both upper and lower alveolar arches. The buccal mucosa is a description of the mucosal lining of both lips and cheeks, while gingiva refers to the mucosal surface of the alveolar ridges. Both mucosae meet at the gingivobuccal sulcus (**Figure 2**) (Laine and Smoker, 1995).

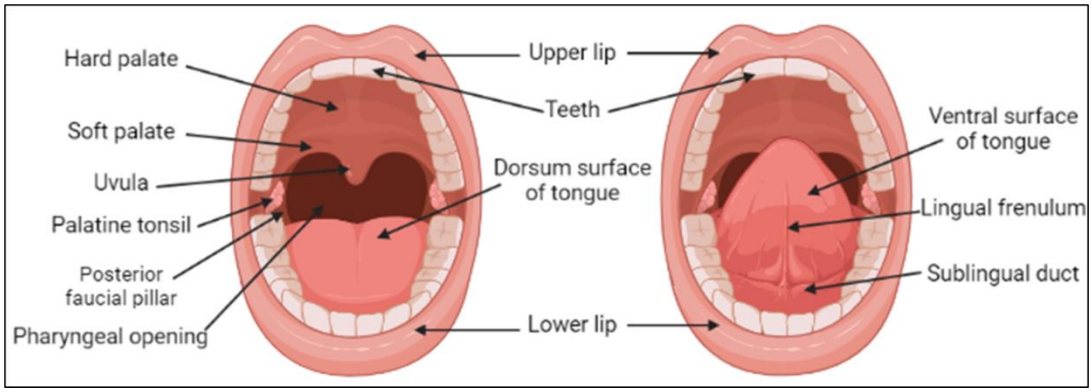

Figure 2: Anatomy of the oral cavity.

This figure (Created in BioRender.com) shows the anatomical structures and landmarks in oral cavity (Nanci, 2017).

1.4.2 Histology of the Oral Cavity:

Oral mucosa or oral mucous membrane refers to the lining of the oral cavity, which shows some properties of both skin and intestinal lining. The most common feature in all of them is that they comprise an epithelial layer supported by underlying connective tissue. The epithelial layer or oral epithelium is a stratified squamous epithelium supported by the lamina propria, which is a connective tissue layer. These two layers interface, which results in irregular projections of lamina propria called connective tissue papillae toward the epithelial layer. The interdigitation of the oral epithelium are called epithelial pegs. Submucosa refers to the layer under the lamina propria, however, the distinction between them is difficult in the oral cavity. In the different regions of the oral cavity such as lip, cheeks and specific areas of the hard palate, there is a layer of glandular connective tissue which contains blood vessels and nerves or loose fatty tissue. This layer represents the submucosa which separates between the oral mucosa and underlying structures such as bone and muscles. In the case of the attached gingiva and parts of the hard palate, the oral mucosa is connected directly to the periosteum of the underlying bony structures (**Figure 3**). Minor salivary glands and ectopic sebaceous glands can be found in submucosa of the oral mucosa. Different areas of the oral cavity contain nodules of lymphoid tissues and the largest examples can be found in the posterior part of the oral cavity. Waldeyer's ring refers to three large nodules of lymphoid tissues which are lingual, palatine and pharyngeal tonsils (Nanci, 2017).

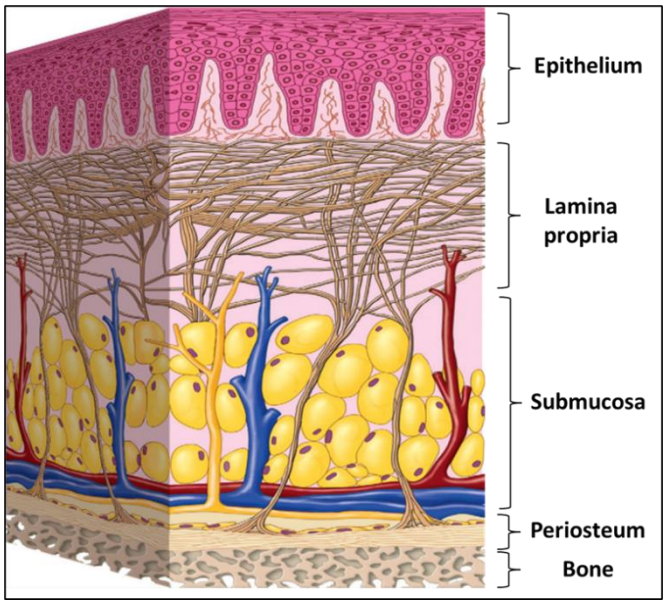

Figure 3: Illustration of histological structure of the oral mucosa.

The diagram illustrates the different layers of the oral mucosa, which includes oral epithelium, lamina propria, submucosa, periosteum and bone (Nanci, 2017). Permission has been granted to use this figure by the author.

1.4.2.a Oral Epithelium:

The oral epithelium provides primary protection for the underlying structures that form the oral environment (Wang *et al.*, 2019). Histologically, the oral epithelium is described as a stratified squamous epithelium (Groeger and Meyle, 2015). The epithelial cells in this tissue are closely attached to each other and organised in specific layers called strata or epithelial layers. This arrangement helps the structural integrity of the oral epithelium by continuous renewal, differentiation/maturation and programmed cell death (apoptosis). The superficial layer of the oral epithelium is surrounded by a protective layer called the cornified cell envelope. This layer contains keratins within an insoluble mixture of proteins wrapped up by lipids (Nanci, 2017).

Progenitor cells in the basal layer of the oral epithelial are responsible for continuous renewal. The number of these layers differs and is dependent on the region in the oral cavity. For example, the number of basal layers in the floor of the mouth is less than the number of basal layers in areas such as the cheek and palate. Two types of progenitor cells are found in the basal layer of the oral epithelial. Based on their functions, they can be categorised as stem cells and amplifying cells. Stem cells have slow cell cycles to maintain tissue proliferative potential, while the amplifying cells increase the number of the cells ready for the

maturation phase. The estimations of the turnover times for epithelial cells in the gingiva and cheeks are 57 days and 25 days respectively. Generally, the turnover rates are faster in non-keratinised buccal epithelium than keratinised gingival epithelium. Different growth factors are linked to proliferation and differentiation phases of the oral epithelium, including epidermal growth factor (EGF) and transforming growth factor beta (TGFβ) (Nanci, 2017).

The differentiation or maturation of the oral epithelium falls under two categories including keratinisation (Keratin formation) or non-keratinisation. The keratinised layer of the oral mucosa can be found in the masticatory surfaces in the oral cavity such as hard palate, gingiva and dorsal surface of the tongue. The keratinised layer gives the masticatory mucosa a few features including inflexibility, toughness, resistance to abrasion and is firmly bound to the underlying connective tissue. The keratinised mucosa contains several distinct strata. The deeper layer of the epithelium next to the connective tissue is called the basal layer or stratum basale. This layer is formed with columnar or cuboidal cells above the lamina propria. The layer just above the basal layer is called stratum spinosum or prickle cell layer, which is formed of several layers of epithelial cells which have a spherical shape. The stratum basale and stratum spinosum represent a third of the thickness of the oral epithelium. The stratum granulosum or the granular layer sits above the prickle cell layers and it is formed from large flattened cells containing keratohyalin granules. The most superficial layer of the oral epithelial layers is the stratum corneum or keratinised layer, which constituted of flat cells referred to as squamous cells, which have no nuclei and is also called ortho-keratinised layer. Other variations of keratinisation of the oral epithelium can be seen in the oral cavity, which is referred to as para-keratinised epithelium. The keratinised layer here, contains keratin and cells that retain their nuclei (Nanci, 2017; Chen *et al.*, 2021).

The non-keratinised layer of the oral cavity can be found as the lining mucosa for the soft palate, buccal mucosa, floor of the mouth, ventral surface of the tongue, lips and alveolar mucosa. Some areas of the oral cavity have a thicker non-keratinised oral epithelium such as the buccal mucosa and lips compared to other non-keratinised lining mucosa. In general, the histological representation of both basal and prickle cell layers resembles those in the keratinised oral epithelium. There are no major differences between the prickle cell layer and the layer above

it. The last two outer layers of non-keratinised mucosa are referred to as the intermediate layer or stratum intermedium and superficial layer or stratum superficiale. The superficial layer of non-keratinised contains cells with nuclei, while the granular layer is not present. In addition to epithelial cells, different types of cells can be found in the oral epithelium such as melanocytes, Langerhans cells, Merkel cells and different types of inflammatory cells (**Figure 4**) (Nanci, 2017; Chen *et al.*, 2021).

Lamina propria refers to the underlying connective tissue that supports the oral epithelial layer. It contains different types of cells such as fibroblasts, macrophages, mast cells and lymphocytes. Also, the lamina propria is composed of blood vessels, nerves, fibres and components of the extracellular matrix (Nanci, 2017).

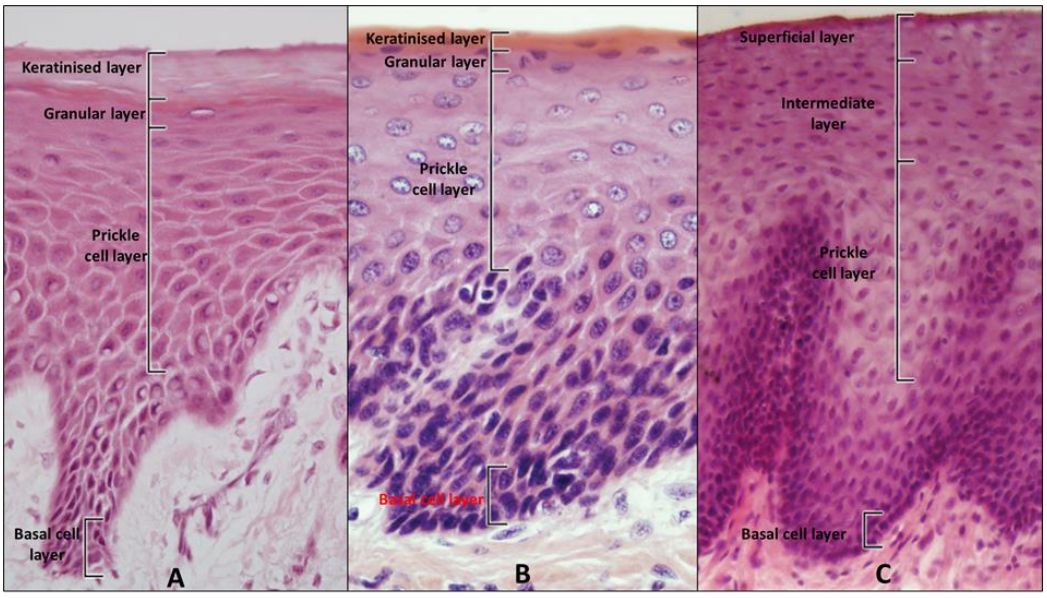

Figure 4: Histological sections of three different variations of oral epithelium.

The figure shows three different types of oral epithelial. Picture (**A**) shows ortho-keratinised oral epithelium of the gingiva. Picture (**B**) shows para-keratinised type of oral epithelium of gingiva. Picture (**C**) shows non-keratinised oral epithelium of buccal mucosa (Nanci, 2017). Permission has been granted to use this figure by the author.

1.4.2.b Intercellular Junctions in Oral Mucosa:

The junctions between cells or between cells and the extracellular matrix (ECM) are referred to as specialised junctions, which can be categorised into three groups: tight junctions (zonula occludens), adhesive junctions and communicating junctions (gap junctions). There are different types of adhesive junctions including cell to cell, which has subtypes zonula adherens and macula

adherens (desmosome), and cell to matrix, which includes focal adhesion and hemidesmosome (**Figure 5**) (Kingsley and Kourtidis, 2023).

Tight junctions are formed by complexed structures of different transmembrane adhesive proteins including the occludin and claudin family. The main feature of the tight junction is to encircle the cells at the apical part of the lateral surface, in a belt like pattern, where the intracellular space is completely closed. The pattern of tight junctions helps to establish a barrier to control the transportation of water, ions and other small molecules. The structure of claudin establishes and controls the transport function as para-cellular channels (Groeger and Meyle, 2019). Also, other cytoplasmic proteins at the cell end of the transmembrane proteins are linked to other cellular functions such as cell polarity, vesicular transport, transcription factors and tumour suppressor proteins. A few of the tight junction proteins are attached to actin filaments, one of the cell's cytoskeletal proteins. Another important function of the tight junctions is to maintain the apical and basolateral surfaces of the cell membrane (Nanci, 2017; Kingsley and Kourtidis, 2023).

The presence of adhesive junctions is a distinctive feature of epithelial tissues (Groeger and Meyle, 2019). The main function of the adhesive junctions is to hold the epithelial cells together or to the extracellular matrix. With cell-to-cell junctions, the intercellular space is retained and this type of adhesive junction plays a crucial role in cell signalling. The cytoplasmic components of the adhesive junctions interact with the cell cytoskeleton, which might promote changes in cell morphology or acquisition of motility. Also, those molecules have the ability to be interact with tumour suppressor molecules or act as transcription factors. The loss of any type of adhesive junction might lead to different cellular responses including apoptosis, loss of cell polarity, cell differentiation or uncontrolled cell proliferation (Nanci, 2017). Cell-to-cell adhesive junctions or desmosomes form the apical adhesive structures and are aligned to the cell membranes of the neighbouring cells and their locations are basal to the tight junctions. One important component of cell-to-cell adhesive junctions includes transmembrane proteins, which belong to the cadherin family. Cadherins are linked to the actin cytoskeleton by the catenin family of proteins and other molecules such as vinculin and actinin. Therefore, the homophilic link between the extracellular domains of E-cadherin of the neighbouring cells establishes the cell-to-cell

junction. Moreover, the cytoplasmic domain of E-cadherin is linked to β-catenin and interacts with p120-catenin. The cadherins in the desmosomes are desmoglein and desmocollin, while desmoplakin, plakoglobin and plakophilin represent the catenins (Groeger and Meyle, 2019).

In the case of cell-to-matrix junctions, there are two types of junctions, focal adhesion and hemidesmosomes. Both types of cell-to-matrix junctions show resemblance in their structural organisation to cell-to-cell adhesive junctions, however, different molecules establish the junction between cells and the extracellular matrix (Hanein and Horwitz, 2012). A molecule of the integrin family is the transmembrane protein in the focal adhesion, while the cytoplasmic adapter proteins are actin-binding proteins such as α-actinin and vinculin. These proteins link the transmembrane integrins to the cytoskeleton (Mishra and Manavathi, 2021). The interaction between integrin and extracellular matrix proteins such as collagen, laminin and fibronectin, leads to remodelling of the actin cytoskeleton. The second type of cell to extracellular matrix adhesive junction are hemidesmosomes, which attach the cell to the basal lamina and some extracellular molecules to the extracellular matrix. Integrin $\alpha_6\beta_4$ represents the transmembrane adhesive protein in hemidesmosomes, which specifically attaches to the glycoprotein laminin and collage XVII in the basal lamina (Nanci, 2017).

The third type of cell junction is the gap junction, where the intercellular space between two adjacent cells becomes around 2 to 3 nm. The transmembrane protein for these type of cell junctions is connexin family, which forms a channel between the cytoplasm of two adjacent cells (**Figure 5**) (Nielsen *et al.*, 2012; Nanci, 2017).

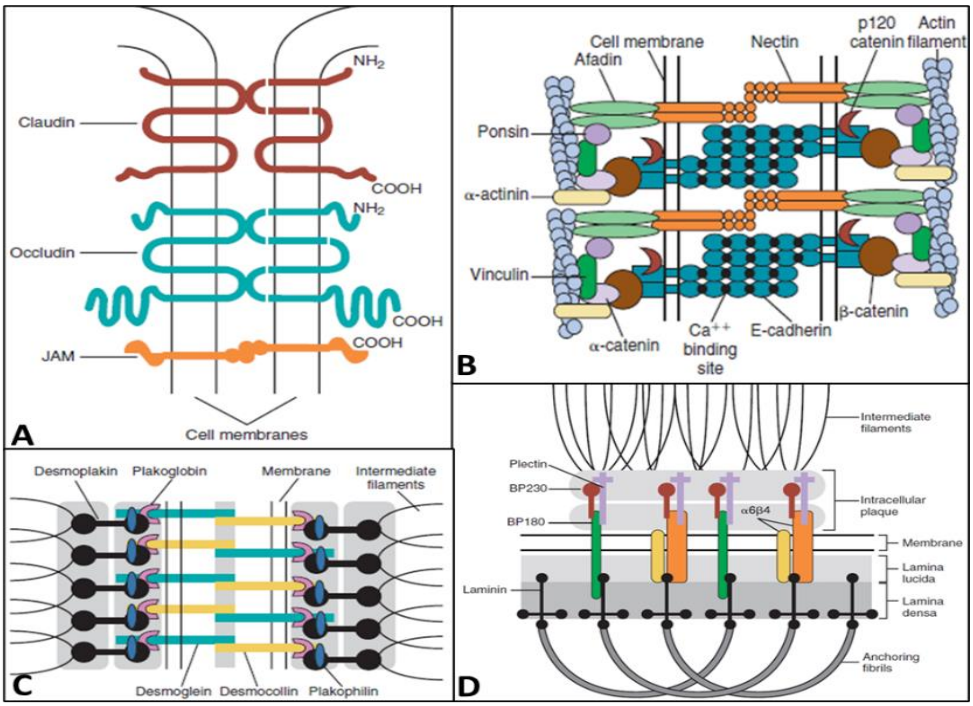

Figure 5: Illustration of the molecular structure of different types of intercellular junction.

This figure shows the different types of intercellular junctions (**A**) Tight junction, (**B**) Adhering junction, (**C**) Desmosome and (**D**) Hemidesmosome (Nanci, 2017). Permission has been granted to use this figure by the author.

1.5 Oral Squamous Cell Carcinoma:

Over 90% of the malignant tumours that arise from the oral cavity are squamous cell carcinomas (SCC), which originate from the mucosal epithelium (Lo Muzio *et al.*, 2007; Speight and Farthing, 2018). Oral squamous cell carcinoma (OSCC) has specific histopathological characteristics, which include the invasion of epithelial tumour cells to the underlying tissues, after breaching the basement membrane. The tumour invasion may begin with a few cells or small group of epithelial cells breaking through the basement membrane. The progression of the disease might reach the stage where tumour cells migrate as sheets or islands to reach the submucosa or bony structures (Speight and Farthing, 2018). As a result, clinically, three major signs of OSCC appear, first induration (the lesion gets harder), second fixation and finally ulceration (Markopoulos, 2012; Speight and Farthing, 2018). The ulceration of the lesion happens because of the ability of the OSCC to become large, develop its own blood supply or being traumatised (**Figure 6**) (Lewis, 2018).

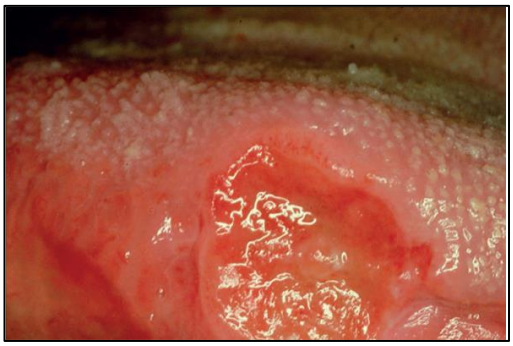

Figure 6: Squamous cell carcinoma of the tongue.

The diagram shows the clinical presentation of squamous cell carcinoma of the tongue (Lewis, 2018). Permission has been granted to use this figure by Courtesy of Professor M. A. O. Lewis.

Under the microscope, SCC shows the invasive component of tumour either as sheet or island of cells in the submucosal layers (**Figure 7**). In addition, some types of oral tumour have the ability to grow above the level of the surface mucosa, which results in exophytic component of the lesion. This phenomenon may give rise to the clinical presentation of rolled margins of OSCC as one of the characteristics of the disease (Bagan, Sarrion and Jimenez, 2010; Speight and Farthing, 2018).

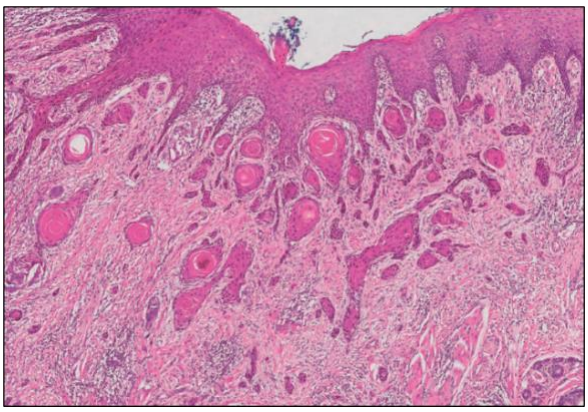

Figure 7: Histological presentation of squamous cell carcinoma of the oral cavity.

This figure shows a histological section of oral squamous cell carcinoma, which includes tumour islands in the connective tissue (Speight and Farthing, 2018). Permission has been granted to use this figure by Prof. Paul M Speight.

There are a few variants of OSCC including verrucous carcinoma, basaloid squamous cell carcinoma, spindle cell carcinoma, adeno-squamous carcinoma and papillary squamous cell carcinoma (Pereira *et al.*, 2007). Verrucous carcinoma occurs most commonly in the buccal mucosa and appears clinically as an exophytic verruciform surface (Liu *et al.*, 2012). The histopathological examination of verrucous carcinoma normally shows well differentiated cells and a cohesive invasive front with wide rete pegs. The superficial layer of the lesion

appears heavily keratinised. This variant of OSCC rarely metastasises and has a good prognosis (Speight and Farthing, 2018). For basaloid squamous cell carcinoma, the tumour is commonly located on the tongue with poor prognosis. Under microscopic examination, the lesion appears as dark basaloid cells in the shape of islands with peripheral palisading (Cho *et al.*, 2017).

Also, a few histopathological features are observed in this variant of OSCC including area of conventional SCC, prominent pleomorphism, areas of necrosis. Spindle cell carcinoma is commonly located on the tongue with pedunculated or polyploid appearance. Moreover, spindle cell carcinoma has poor prognosis. Examination under the microscope shows sheets of malignant spindle cells including areas with conventional carcinoma and overlying dysplasia. Adeno-squamous carcinoma shows features of both SCC and adenocarcinoma and is most common on the tongue and the floor of the mouth. Similar to spindle cell carcinoma, adeno-squamous has a poor prognosis. The most common sites of papillary squamous cell carcinoma in the oral cavity are gingivae and tongue. Under the microscope, papillary squamous cell carcinoma shows exophytic lesions similar to benign squamous papilloma with stromal invasion (Speight and Farthing, 2018).

1.5.1 Histological Grading of OSCC:

The histological grading system helps to determine tumour prognosis, setting up appropriate management plan by the clinician and correlation to survival (Speight and Farthing, 2018; Cariati *et al.*, 2022). The classification of tumour differentiation histologically is based on their resemblance to the normal epithelial tissue, which can be categorised into three groups: well-differentiated, moderately differentiated and poorly differentiated (Lindenblatt *et al.*, 2012; Dik *et al.*, 2018). Generally, 60% of oral cancers are moderately differentiated, 30% well-differentiated and 10% poorly differentiated. According to a study carried out in the UK, the five-year disease specific survival for patient with well-differentiated OSCC was 89%, for moderately differentiated was 68% and for poorly differentiated was 45% (Speight and Farthing, 2018).

Well-differentiated carcinomas show few features of squamous epithelium, which makes them easily identified as epithelial tumours. These features include formation of keratin pearls and tumour islands, which are formed by stratified layers of basal cells with central eddies of keratin. The tumour islands of well-

differentiated OSCC are well defined and usually connected to the superficial epithelial layer. A cohesive invasive pattern is observed, where large intact rete pegs invade the submucosal layers. Cell atypia or dysplasia are not prominent in well-differentiated OSCC (Speight and Farthing, 2018).

Moderately differentiated OSCC shows less similarities to the normal squamous epithelium such as keratin formation in the lower degree and more prominent cellular atypia. Compared to well-differentiated carcinomas, moderately differentiated has less cohesive pattern of invasion, which can be observed as small islands, sheet or cords of cells penetrating the underlying connective tissues. The characteristics of cellular atypia are nuclear pleomorphism, cellular pleomorphism and hyperchromatic nuclei (Speight and Farthing, 2018).

Despite the lack of resemblance to squamous epithelium, poorly differentiated carcinomas can still be identified as tumours with an epithelial origin. They show significant number of cellular atypia and pleomorphic cells. Also, they show a non-cohesive invasive pattern, which can be seen as individual cells or small islands into the underlying connective tissues. The extreme version of poorly differentiated carcinoma is difficult to identify as a tumour with epithelial origin, which can be referred to as anaplastic or undifferentiated (Speight and Farthing, 2018).

1.5.2 Invasion of Oral Squamous Cell Carcinoma:

Four different patterns of tumour invasion are found with oral squamous cell carcinoma. Type I pattern is referred to as a cohesive invasive pattern, that is characterised by pushing bulbous rete pegs into the underlying connective tissues. Those rete pegs appear histological to be at the same level depth wise. The type II invasive pattern features a cohesive invasion in the form of cords, strands or large islands, which are a continuation of the main mass of the tumour. The type III invasive pattern is similar to type II with the main difference being those islands and cords are separated from the main tumour, which results in a non-cohesive mode of invasion. The type IV pattern of invasion is identified as groups of individual cells or small islands infiltrating the underlying connective tissues, which leads to a non-cohesive invasive pattern (Speight and Farthing, 2018; Mishra *et al.*, 2022).

The recommendation from the UK Royal Collage of Pathologists is to grade the invasion of oral squamous cell carcinoma into two patterns either cohesive pattern or non-cohesive pattern (Odell *et al.*, 1994; Woolgar, 2006). A study of OSCC of the anterior tongue included 47 samples showed 74% of carcinomas had a non-cohesive invasive pattern of metastasis, while only 16% of had a cohesive pattern metastasis (Speight and Farthing, 2018).

The invasion and metastasis of OSCC along nerves has been shown to influence carcinoma recurrence and survival of patients. Correlation between the disease and perineurial invasion is observed in around 60% of OSCC, especially with the involvement of large nerves. In addition, lymph node metastasis and OSCC poor prognosis are associated with the invasion of lymphatic and blood vessels. Perineurial, lymphatic and blood vessels invasions are linked heavily to poorly differentiated OSCC, with a non-cohesive invasive pattern and large tumours (Jardim *et al.*, 2015; Speight and Farthing, 2018).

Around 50% of oral cancer cases are presented with tumours in the cervical lymph nodes. The lymph nodes in the cervical region can be divided into five different levels, where tumour cells metastasise. In 90% of oral cancers, positive lymph nodes are found in level I, just below the mandible in the submandibular area, and level II, the upper aspect of the sternomastoid muscle. The location of the tumour also has an influence on the distribution of nodal metastases e.g. tumours in the cheek or floor of the mouth are linked to level I, while tumours in the tongue are linked to level II. The assessment of lymph nodes is essential for the TNM staging scheme (Jones *et al.*, 2009; Speight and Farthing, 2018).

1.5.3 Tumour Stroma:

The interactions and the complex dynamic between the host cells and the tumour cells is referred to as tumour microenvironment. This microenvironment includes cancerous cells in addition to cells of connective tissues and the host immune system. It has been known for some time, that strong responses from the immune system of the host towards a tumour, suggests a good prognosis. On the other hand, some evidence of inflammation indicates poor prognosis, especially when it includes tumour associated macrophages. An example of the complex dynamic between the epithelial and mesenchymal environment is the creation of cancer associated fibroblasts (CAF). CAFs are a collection of populations including resident fibroblasts and other cells such as tumour cells having undergone

epithelial to mesenchymal transition (EMT). CAF has role in inducing cancer cell proliferation, migration and invasion (Hale, Hayden and Grabsch, 2013; Speight and Farthing, 2018).

1.6 Tumour Staging System (TNM):

To identify the stage of malignant tumours, TNM staging is used to assess the management and prognosis of the disease (Speight and Farthing, 2018; Almangush *et al.*, 2020). According to the latest report of UICC 8th edition of staging manual, oral cancer and oropharyngeal cancer were given different criteria for both tumour size (T) and lymph node(s) involvement values (Huang and O'Sullivan, 2017; Mattavelli *et al.*, 2020). For oral cancer, T refers to the tumour size in terms of diameter and invasion depth (Caldeira *et al.*, 2020), while N refers to positive or negative lymph nodes and M stands for distant metastasis (Amin *et al.*, 2017; Speight and Farthing, 2018; Tirelli *et al.*, 2018).

Table 2: TNM staging system for oral cancers.

T= Assessment of the size of the primary tumour			
T category	**Diameter**	**Depth of invasion**	
T1	≤ 2 cm	≤ 5 mm	
T2	≤ 2 cm	5-10 mm	
or	2-4 cm	≤10 mm	
T3	2-4 cm	>10 mm	
or	>4 cm	<10 mm	
T4a	>4 cm	>10 mm	
or	Tumour in cortical bone of mandible or maxilla		
or	Tumour invades the skin of the face.		
or	Tumour invades inferior alveolar nerve from the lip		
or	Tumour invades floor of the mouth from the lip		
or	Tumour invades the skin of the chin and nose from the lip		
T4b	Tumour invades masticator space		
or	Tumour invades pterygoid plate		
or	Tumour invades skull base		
or	Tumour encases internal carotid artery		
N= Involvement of the locoregional lymph node(s)			
N category	**Description**	**Size of metastasis**	**Extra nodal extension (ENE)**
N0	No nodal metastasis		No
N1	Metastasis in a single ipsilateral lymph node	≤ 3cm	No

N2a	Metastasis in a single ipsilateral lymph node	3-6 cm	No
N2b	Metastasis in multiple ipsilateral lymph nodes	≤ 6 cm	No
N2c	Metastasis in multiple bilateral or contralateral lymph nodes	≤ 6 cm	No
N3a	Metastasis in a single lymph node	>6 cm	No
N3b	Metastasis in a single or multiple lymph nodes	Any	Yes

M= Assessment for distant metastasis	
M category	**Description**
M0	Cancer has not spread to other parts of the body
M1	Cancer has spread to other parts of the body

Tumour staging	
Stage category	**Description**
Stage 0	Carcinoma in situ
Stage 1	T1, N0 and M0
Stage 2	T2, N0 and M0
Stage 3	T3, N0 and M0
or	T1, 2 or 3, N1 and M0
Stage 4a	Any T, N1 and M0
or	Any T, N2a and M0
Stage 4b	Any T, N3 and M0
or	T4b, any N and M0
Stage 4b	Any T, any N and M1

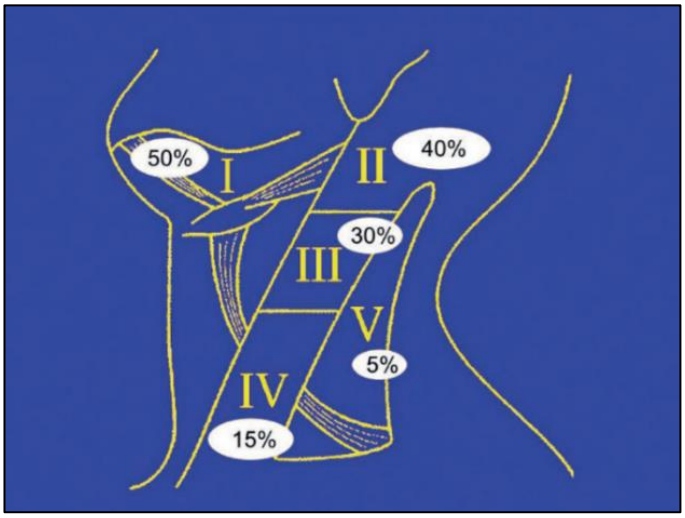

Figure 8: Five anatomical levels of the neck from a lateral view.

The figure shows the different anatomical levels of the neck and where oral cancer metastasis occurs (Speight and Farthing, 2018). Permission has been granted to use this figure by Prof. Paul M Speight.

An oral cancer study illustrated that the five-year disease survival for T1 carcinomas was 95.7%, T2 carcinomas was 80.5%, T3 carcinomas was 69.1% and T4 carcinomas was 60%. The same study showed the five-year disease survival for N0 carcinomas was 89.6%, N1 carcinomas was 58.8%, N2 carcinomas was 57.1% and N3 carcinomas was 56.5%. Based on those findings, it was estimated that the survival for oral carcinoma patients was over 80% for Stage I and II, while it was less than 60% for Stage III and IV. This percentage of five-year survival was worse in cases with distant metastasis, at less than 20% (Speight and Farthing, 2018).

1.7 Cell Migration:

Cell migration is essential for a wide variety of biological processes (Lauffenburger and Horwitz, 1996; Webb, Zhang and Horwitz, 2005). In embryogenesis, cell migration occurs regularly starting from gastrulation to the development of the neural tissues. During human adulthood, cell migration is involved primarily in different processes including normal physiology and pathology, such as inflammatory responses and tumour metastasis. Cell migration is regulated by different molecules and coordinated spatially and temporally. The process of cell migration is a combination of different coordinated steps which range from morphological polarisation, formation of membrane extensions, the formation and stabilisation of attachment to the cell-substratum, contractile force and traction and rear release of attachment (Lauffenburger and Horwitz, 1996).

Morphological polarisation refers to cells acquiring a spatial asymmetry, which allows them to generate intercellular forces and promotes cell body translocation. Morphological polarisation is presented with a distinct cell morphology at front compared to the cell rear. Two types of membrane extension are observed at the front end of migratory cells during cell migration, lamellipodia and filopodia, which are associated with actin polymerisation. The formation of a membrane extension is followed by the establishment of new adhesion points at the front end of the cell and continue to the rear end. Beside the protrusive force for creating the membrane extensions, a contractile force is generated also by a locomoting cell, which is required for moving the cell body forward. The contractile forces can be controlled by different independent mechanisms within the front and the rear ends

of the cells. The process of cell migration is concluded by the rear release of the focal adhesion of the migratory cell (Lauffenburger and Horwitz, 1996).

There are two modes of cell migration that have been reported, individual or single cell migration and collective cell migration. The main distinction between the two modes of cell migration is during collective cell migration, cells maintain their cell-cell contact and move together. The phenomena of cells migrating collectively may result in a few features for example, carrying other immobile cells. Also, collective cell migration has been associated with incomplete epithelial to mesenchymal transition, which is linked strongly with single cell migration after complete transition (Rørth, 2009).

1.8 Cell Migration during Cancer Metastasis:

Cancer metastasis describes the process of dissemination and migration of cancerous cells away from their primary site to a distant secondary site. Metastasis of tumour cells is a major factor in causing death among patients with cancer. This process includes two main features i.e., cell migration and invasion, which allows tumour cells to invade to neighbouring tissues and intravasation into the blood and/or lymphatic system. The ability of cancerous cells to migrate is linked with the upregulation in expression of genes that induce cell motility (Yamaguchi, Wyckoff and Condeelis, 2005).

The classic view on how cancer cells migrate, is based upon single cell migration, where cells separate from the primary tumour, invade vessels and colonise at a secondary site. However, other reports have suggested that single cell migration is not the only way for cells to migrate, they may also migrate by collective cell migration (Rørth, 2009). This claim was supported by the presence of clusters of cancerous cells in the blood and lymphatic vessels. Also, histopathological samples of different carcinomas showed evidence of collective cell migration (Friedl and Gilmour, 2009). Studies also reported different features between single and collective cell migration. In breast cancer, tumour cells were able to disseminate relatively quickly from the primary tumour, which is linked to the single cell mode of migration, to create lung metastases via blood circulation. However, collective cell migration via the lymph vessels was slower to cause metastases. The change in the mode of migration between single cell to collective might require the influence of Transforming Growth Factor beta (TGFβ) and Smad4 (Trepat, Chen and Jacobson, 2012).

1.9 Epithelial to Mesenchymal Transition:

Epithelial to mesenchymal transition (EMT) refers to the biological process that encompasses several biological and chemical changes that may happen to an epithelial cell. Normally, epithelial cells adhere to other epithelial cells or to the basement membrane. However, during EMT those cells acquire mesenchymal cell phenotype, that may include the ability to migrate, invade, resist apoptosis and produce extracellular matrix components (Kalluri and Weinberg, 2009).

The outcomes of complete EMT are breakdown of the basement membrane and the migration of the mesenchymal-like cells to the underlying connective tissues. The regulation of EMT requires a set of specific molecular processes from the initiation to the final phase. These molecular processes include expression of cytoskeletal proteins, change in expression of specific cell-surface proteins and production of enzyme that degrade extracellular matrix components. A reverse process to EMT, has also been observed and is known as mesenchymal to epithelial transition (MET). This process describes the conversion of mesenchymal cells to epithelial cells (Kalluri and Weinberg, 2009).

A classification of the EMT process was proposed based on its biological role in physiological and pathological conditions. Type I EMT refers to the EMT process that is associated with implantation of the embryo and organ development during embryogenesis. In this subtype of EMT, neither fibrosis nor cell invasion will occur as result, which might lead to systemic spread of cells because of their ability to undergo MET (Kalluri and Weinberg, 2009).

The main characteristic of mesenchymal cells generated by type I EMT is having the ability to undergo MET to produce secondary epithelial cells. These cells are referred to as secondary epithelial cells because they have undergone EMT to become mesenchymal cells followed by MET to become epithelial cells. Type II EMT describes the process that occurs during wound healing, tissue generation and fibrosis. Type II EMT is mainly involved in repair programs, which results in generating fibroblasts and other cells that help in tissue reconstruction, especially following inflammation. However, this subtype of EMT can exist with prolonged or chronic inflammation, which might eventually lead to tissue destruction. Type III EMT is associated with tumour cells that have gone through genetic and epigenetic changes especially those related to growth and development of localised tumours. This phenomenon has been observed mainly in carcinomas,

where cells that undergo EMT, gain the ability to metastasise and invade, eventually causing cancer progression. The most significant aspect of this subtype of EMT is that cancer cells undergo EMT to different extents, which range from maintaining epithelial traits to acquiring mesenchymal traits. Although, the clear mechanism of EMT during cancer progression is not fully understood, the signals from the tumour stroma associated with the primary carcinoma have a significant role in type III EMT (Kalluri and Weinberg, 2009; Debnath *et al.*, 2022).

1.10 EMT and Cancer Metastasis:

The initial phase of carcinoma development is characterised by overgrowth and proliferation of epithelial cells accompany by angiogenesis (Kalluri and Weinberg, 2009). The later phase of carcinoma progression features cells acquiring migration ability and breakdown of the basement membrane, which leads to tumour metastasis and life-threatening conditions, (Kalluri and Weinberg, 2009) including oral squamous cell carcinoma (Vallina *et al.*, 2021). Different studies suggested that it is crucial for carcinoma cells to acquire mesenchymal characteristics via EMT for cancer to progress. *In vivo* studies showed that carcinoma cells acquired a mesenchymal phenotype especially at the invasive front of the primary tumour. However, the full understanding of the molecular and signalling programs that control EMT is not yet clear (Kalluri and Weinberg, 2009).

Certain growth factors, which are produced by the tumour stroma of the primary tumour, induce signalling pathways that have been linked EMT (Kalluri and Weinberg, 2009). The most notable growth factors, which are generated by the tumour stroma and that are associated with EMT, are TGFβ (Zavadil and Böttinger, 2005; Suzuki *et al.*, 2019), EGF(Choi and Myers, 2008), Nerve growth factor (Alkhadar *et al.*, 2020), VEGF (Islam *et al.*, 2014) and HGF (Kalluri and Weinberg, 2009). These growth factors activate a series of intracellular signalling pathways and signal-transducing proteins to implement EMT. Those signalling molecules include Smads, PI3K-Akt and MAPK/Erk (Kalluri and Weinberg, 2009). Eventually, signalling pathways induce EMT-inducing transcription factor activation such as Snail (Batlle *et al.*, 2000), Slug and Twist (Kalluri and Weinberg, 2009).

Another characteristic of the EMT program is that cells undergo reorganisation of their cytoskeletal structures in a way that enables them to be motile. The main

feature of migratory cells, which undergo cytoskeletal rearrangement, is creating cell membrane extensions called lamellipodia and filopodia. Also, cells that undergo EMT shows an increase in cell contractility and the formation of actin stress fibres. The dynamics of actin and actin rearrangement during EMT is regulated mainly by Rho GTPase singlling molecules. The formation of actin stress fibres is induced by RhoA, while the formation of lamellipodia and filopodia is promoted by Rac1 and Cdc42 (Lamouille, Xu and Derynck, 2014).

In addition, the disruption of cell-to-cell adhesion and cell to basement membrane are crucial elements in activation of the EMT program (Kalluri and Weinberg, 2009). The integrity of the epithelial tissues is maintained through specialised cell surface protein complexes via different types of cell junction (Lamouille, Xu and Derynck, 2014). At the initiation of EMT, tight junctions, adherens junctions and desmosomes undergo re-localisation or degradation, which results in deconstruction of those junctions too (Kudo *et al.*, 2004; Lamouille, Xu and Derynck, 2014). During EMT, the dissolution of the tight junctions is manifested, by the decrease of expression for certain proteins such as claudin and occludin (Lamouille, Xu and Derynck, 2014). In the case of adherens junctions, E-cadherin is cleaved at the cell membrane and subsequently degraded (Ulrich and Heisenberg, 2009), which leads to the destabilisation of the junction (Birchmeier, Birchmeier and Brand-Saberi, 1996; Lamouille, Xu and Derynck, 2014). Therefore, E-cadherin ceases to interact with β-Catenin, which results in β-Catenin been degraded or nuclear translocation. For the EMT program to progress, transcriptional repression of the cell junction proteins is constantly maintained, which prevent cells from generating epithelial junctions (Lamouille, Xu and Derynck, 2014).

Changes in the expression of certain proteins occurs during EMT including the proteins related to cell junction complexes and the cell cytoskeleton. Cells that undergo EMT show downregulation of epithelial proteins and upregulation of mesenchymal proteins, which induces cytoskeletal rearrangement and cell migration (Lamouille, Xu and Derynck, 2014). One of the hallmarks of EMT is the downregulation of the major cell junction proteins, E-cadherin (Imamichi and Menke, 2007), claudins, occludin, desmoplakin and plakophilin and the upregulation of induce mesenchymal proteins (Lamouille, Xu and Derynck, 2014). The most prominent change in expression of epithelial and mesenchymal

proteins is the downregulation of E-cadherin and upregulation of N-cadherin, which is referred to as the cadherin switch (Lamouille, Xu and Derynck, 2014). This switch of cadherins has been observed in OSCC samples and is considered a hallmark for EMT (Angadi *et al.*, 2016). N-cadherin is a mesenchymal neural cadherin. This switch of cadherins leads to the loss of adhesion or an alteration in cell adhesion (Lamouille, Xu and Derynck, 2014). The cell adherent junction through homotypic N-cadherin interactions is much weaker than E-cadherin homotypic interactions, which allows for cell migration and invasion (Chang *et al.*, 2002; Lamouille, Xu and Derynck, 2014). Another example of the switch between epithelial and mesenchymal proteins, is the repression of cytokeratin and expression of vimentin, a major constituent of the intermediate filament family. Also, the interactions between cells and the extracellular matrix play a role in EMT program initiation or progression (**Figure 9**) (Lamouille, Xu and Derynck, 2014).

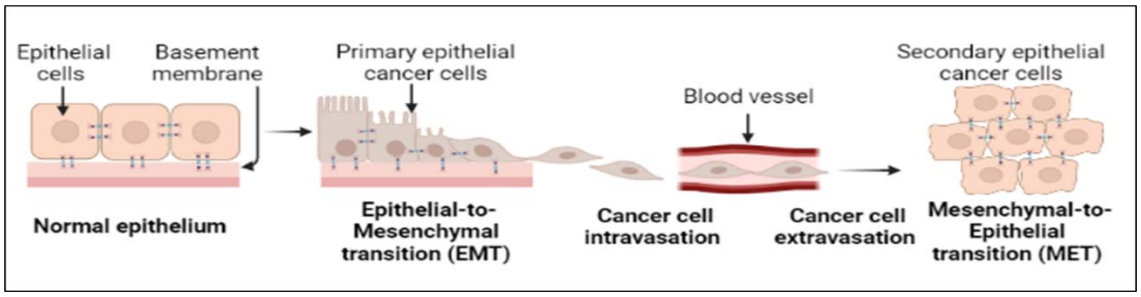

Figure 9: Illustration of epithelial to mesenchymal transition during tumour metastasis.

This figure (Created in BioRender.com) shows the transition of epithelial cells to mesenchymal cells to enabling them to migrate away from the primary tumour (Kalluri and Weinberg, 2009).

1.11 Overview of Cell Signalling Transduction:

Cells communicate with each other on a regular basis via two main modes electrical signals or chemical signals. The electrical signal mode of communication depends upon the presence of gap junctions, which allows the signals to pass directly to the neighbouring cells. Cell communication via chemical signalling represents the major form of communication between cells. This type of communication is generated by the cells releasing a chemical stimulus such as growth factors or hormone, which regulate or alter the cellular activities of the targeted cells. The chemical stimulus is received by receptors at the cell membrane, which transfers the signal to intracellular signalling pathways to induce cellular activity. Receptors help water-soluble stimuli such as growth factors and hormones, which cannot cross the cell membrane, to regulate cellular activities (Berridge, 2014).

The concept of cell signalling mechanisms is about receiving an external stimulus and transferring it to internal signalling pathways to activate both the sensor and the effector, which eventually leads to changes in cell response. There are two mechanisms of cell signalling ON mechanisms, where the flow of the signalling starts at the receptor in response to external stimuli, and OFF mechanisms that regulate the “switching off” of the signalling pathways as soon as the external stimuli cease to active the receptor. Once the external stimuli interact with the receptor, the information is transferred to different transducers and amplifiers to generate intracellular messengers or second messengers. The role of these messengers is to induce sensors and effectors, which stimulate varieties of cellular responses (**Figure 10**) (Berridge, 2014).

Among the different types of receptors, there are single membrane-spanning receptors, which include Serine/threonine kinase-linked receptors (S/TKRs) and Protein tyrosine kinase-linked receptors (PTKRs). S/TKRs have an extracellular domain that binds to members of transforming growth factor (TGF) superfamily. The cytoplasmic domain of the receptor acts as transducer and amplifier, which upon activation phosphorylates Smad proteins. PTKRs also have an extracellular domain which binds to growth factors and a cytoplasmic domain, which contains tyrosine kinase. Tyrosine kinase is the transducer, which is responsible for initiation of signal transduction. The main feature of PTKRs is the capability of transferring the signal to a different number of transducers and amplifiers. There are different examples of PTKRs such as Epidermal growth factor receptor (EGFR) and Fibroblast growth factor receptor (FGFR) (Berridge, 2014).

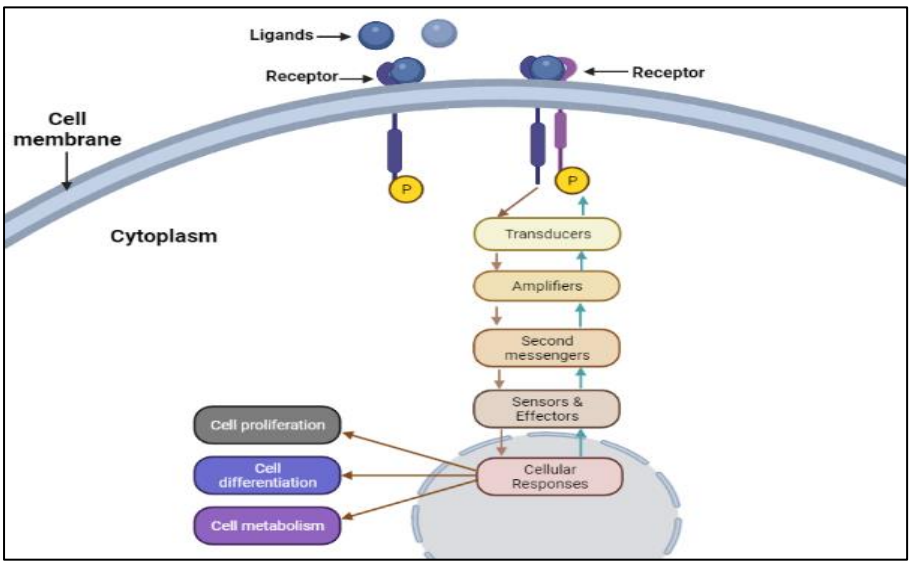

Figure 10: General description of the cell signalling pathway sequence.

This figure (Created in BioRender.com) shows the interaction between the stimuli with the cell receptor, which leads to trigger the activation of the signalling pathway (Berridge, 2014).

1.12 Transforming Growth Factor Beta:

In the human body, over 30 growth factors are considered members of the TGFβ superfamily. These family members can be categorised into two subgroups. Activin, lefty, myostatin, nodal and TGFβ fall under one group, while growth factors such as anti-mullerian hormone (AMH or MIS), Bone morphogenic proteins (BMPs), and various growth differentiation factors (GDFs) represent the other branch of the family (Massagué, 2008). The sequence similarity and activation of certain signalling pathways are common characteristics of TGFβ family (Shi and Massagué, 2003). All those factors are involved in different cellular processes as key regulators. For example, in organogenesis and cellular differentiation of stem cells in an embryo, growth factors such as BMPs, AMH, and GDFs are the key regulators (Massagué, 2008).

However, in the adult the role of these growth factors is different than in the embryo. For instance, GDF9 regulates gonadal functions, myostatin inhibits muscles development and BMPs are responsible for both growth and repair of bone. Moreover, different members of the TGFβ family show different spatial and temporal expression patterns. The best example of a difference in spatial expression is the variation in expression between TGFβ and myostatin, the former shows expression in many cell types, while the latter expresses in only a few types. The difference in temporal expression between TGFβ family members,

is clear as AMH is expressed briefly in development while BMPs exhibit sustained expression throughout life (Massagué, 2008).

TGFβ has a significant role in maintaining homeostasis by being produced at low levels. This level of production might change depending on environmental conditions. In the case of both injured tissues and tumours, the production of the growth factor, TGFβ, suddenly increases as an urgent measure to prevent escape of regenerative cells. This outcome may justify the early presence of the growth factor in the tumour microenvironment as an important factor preventing tumour progression. The expression of this cytokine in different tumours has been reported in many studies. Various sources of TGFβ have been suggested during tumour growth and progression such as leukocytes and macrophages infiltrate the tumour at the same time when levels of TGFβ are high. Also, during bone invasion by cancer cells, TGFβ stores in the bone matrix are displaced from their sites as osteolytic effects of the invasion takes place (Massagué, 2008).

Cancer cells have the ability to alter the suppressive effect of TGFβ by two mechanisms. First, the alterations in one of the key components of the signalling pathway of the cytokine as TGFβ receptors. The second method is by modifying the pathway downstream, which leads to knocking out the suppressive arm. This method allows tumour cells to take advantage of the growth factor (TGFβ) to acquire different properties such as invasive ability, synthesis of autocrine mitogens, or producing growth factors related to tumour metastasis. Therefore, disabling of the growth inhibitory arm of the TGFβ pathway results in tumour growth and progression. In addition, the effect of the cytokine on the tumour stroma, which is produced by the cancer cells, helps the tumour evade immune surveillance and produce chronic inflammation, leading to create a pro-tumorigenic environment (Massagué, 2008).

TGFβ1, TGFβ2, and TGFβ3 represent the variant forms of TGFβ (Massagué, 2008). The bioactive form of TGFβ is a dimer, which is stabilised primarily by hydrophobic interactions and additionally by disulfide bridges between the subunits (Shi and Massagué, 2003). The chain is separated from a precursor by certain enzymes such as furins or other convertases (Massagué, 2008). Once the active dimer is cleaved from the precursor, the TGFβ response is initiated by bringing together two receptors, which are two pairs of serine/threonine kinases TGFβRl, also known as activin receptor-like kinase, and TGFβRll (Massagué,

2008; White, Malkoski and Wang, 2010). Reports have described that the human genome encodes twelve types of receptor serine/threonine kinases (Shi and Massagué, 2003; Massagué, 2008). Seven members are TGFβRI, while the remaining five members are TGFβRII. These twelve respond only to the TGFβ family (Massagué, 2008).

The activation of the TGFβ pathway is triggered when the cytokine binds to the receptors (Zhu and Burgess, 2001; Shi and Massagué, 2003). This interaction promotes the phosphorylation of TGFβRI by the TGFβRII receptor. However, not all members of the TGFβ family shows the same mode of activating the TGFβ singling pathway. For example, both BMP2 and BMP4 have a high affinity for TGFβRI, whereas TGFβ and Activin show high affinity for TGFβRII and at the same time do not interact with TGFβRI. The mechanism of receptor activation starts when the dimeric ligand binds to the extracellular domain of TGFβRII, which induces conformational change and close proximity of the intracellular kinase domains of the receptors eventually causes activation of TGFβRI. TGFβRI contains a certain sequence known as the GS domain, SGSGSG sequence. During TGFβRI activation, the GS domain is phosphorylated by TGFβRII. The GS region acts as a regulatory domain for the TGFβ signalling pathway, which is targeted by immunophilin FKBP12 inhibiting the TGFβ pathway. Two types of extracellular molecules control access of the TGFβ ligands to their receptors, by different modes of action. The first group contains soluble proteins and the small proteoglycan decorin and protein α2-macroglobulin. These molecules act as ligands traps, which isolates the growth factor from the receptors. The second group of proteins work as accessory receptors or co-receptors, promoting ligands to bind to the receptors. This class contains the membrane anchored proteoglycan betaglycan, which is known as TGFβRIII or TGFβ3. TGFβ3 has been identified as a regulator of the TGFβ signalling pathway, it is strongly associated with TGFβ2, and does not interact with either BMPs or Activins. In addition, intracellular regulators are associated with the TGFβ ligands, including FKBP12 and inhibitory Smads e.g. Smad7 (Shi and Massagué, 2003).

1.13 Smad-Dependent Pathway:

The Smad family of transcription factors regulate the canonical TGFβ signalling pathway (Massagué, 2008). The Smad pathway includes eight types of Smad molecules such as Smad1, Smad2, Smad3 and others. These eight Smads are

categorised into three classes. The first group is composed of receptor-activated Smads (R-Smads) such as Smad1, Smad2, Smad3, Smad5, and Smad8. The second group includes inhibitory Smads e.g. Smad6 and Smad7. The last group contains the common Smad or co-mediator Smad (Smad4) (Shi and Massagué, 2003; White, Malkoski and Wang, 2010). However, because there are two groups of TGFβ molecules, each group phosphorylates certain set of Smads. The branch of the TGFβ family, which includes TGFβ and myostatin, phosphorylates Smad2 and Smad3. The other group, which includes BMPs and AMH, phosphorylates Smad1, 5, and 8. Once a group of Smads are activated, the receptor substrate Smads (RSmads) forms a complex with Smad4, which is a common partner to all RSmads, for translocation to the nucleus (Massagué, 2008). Also, Smad7 known as the inhibitory Smad, has two methods of interception TGFβ signalling, first by competing with R-Smad for binding sites on TGFβRl. The second method utilises ubiquitin ligase (E3 polyubiquitin) to break down both R-Smad and TGFβRl (White, Malkoski and Wang, 2010).

Although Smad proteins obtain DNA binding ability, targeting specific genes with high selectivity requires cofactors to increase DNA binding capability. Different transcription factors facilitate the binding between Smad proteins and the targeted genes (White, Malkoski and Wang, 2010). In the regulatory regions, the targeted gene presents a unique binding sequence, this sequence helps Smad4-RSmad- cofactor to identify the target. The complex not only induces the expression of the targeted genes, it also upregulates the activity of the transcription factors that promote EMT (Massagué, 2008). By Smad3-dependent transcription, TGFβ activates Snail1 protein expression, while the myocardin-related transcription factor induces Snail2 expression via Smad3. Repression of genes that encode E-cadherin and occludin, which are the hallmarks for EMT, is accomplished by cooperation between the Smad3-Smad4 complex and Snail2 in response to TGFβ. In addition, TGFβ activates the MAPK signalling pathway, which upregulates ZEB1 expression. Also, the Smad3-Smad4 complex cooperates with ZEB1 and ZEB2 to control TGFβ-regulated gene expression. Additionally, the Smad3-Smad4 complex and activating transcription factor3 interaction leads to repression of inhibition of differentiation1 (ID1) and then increases the expression and the activity of Twist. Both Smad3 and Smad4 in TGFβ-activated EMT induce the expression of high mobility group A2 (HMGA2), which activates both transcription factors Twist and Snail. On the other hand,

some variations of gene expression during EMT occur without involvement of either EMT transcription factors or TGFβ-activated Smads. Smad proteins can induce the expression of a few mesenchymal genes such as vimentin, fibronectin, and collagen αI (**Figure 11**) (Lamouille, Xu and Derynck, 2014).

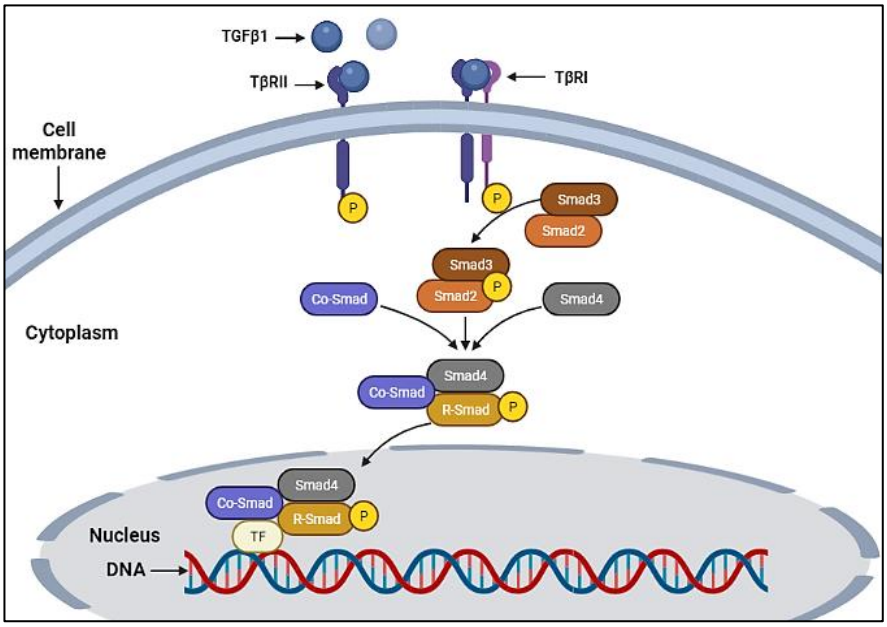

Figure 11: Illustration of the Smad-dependent pathway.

The figure (Created in BioRender.com) shows an illustration of the Smad-dependent pathway (Shi and Massagué, 2003).

1.14 Smad-Independent Pathways:

Recent evidence suggested that the various effects of TGFβ signalling are regulated by the Smad-dependent pathway or by cross talk with other signalling pathways activated by TGFβ receptors (**Figure 12**) (Zhang, 2009). Smad-independent pathways, which are activated by direct interaction or phosphorylation in response to TGFβ via TGFβR activation, are Phosphatidylinositol/Akt, MAP kinases, and Rho GTPase. All of these pathways may be involved in tumour cell progression and growth after dysregulation of Smad dependent pathway (Massagué, 2008).

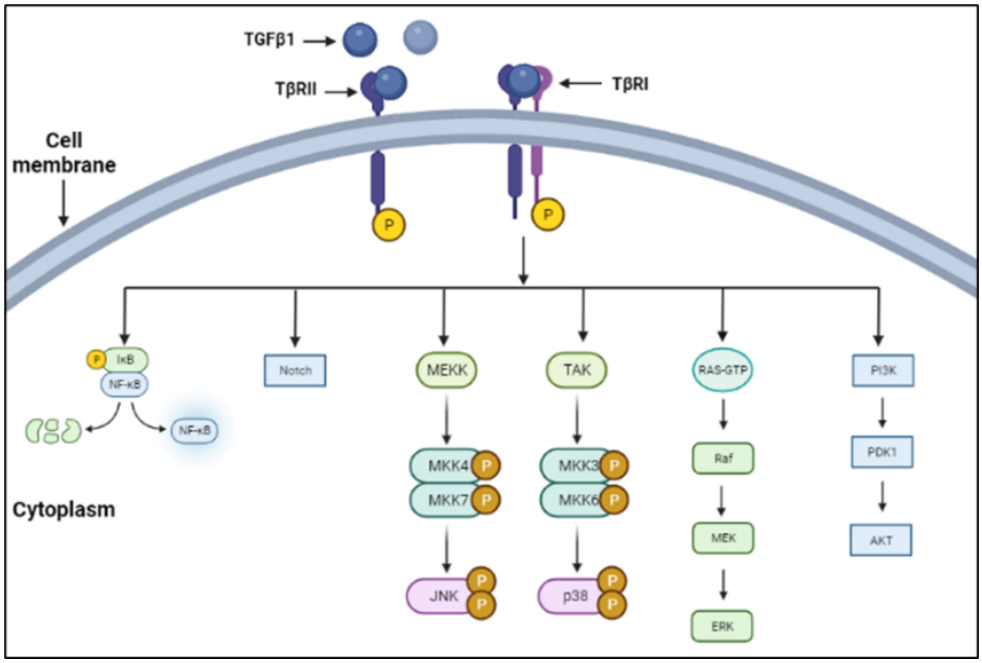

Figure 12: Illustration of the Smad-independent pathways.

The figure (Created in BioRender.com) shows an illustration of the Smad-independent pathways (Shi and Massagué, 2003).

1.14.1 PI3K/Akt Pathway:

The cytokine TGFβ rapidly actives PI3K by phosphorylating its effector Akt, which is independent of the main pathway Smad2/3. P85, which regulates the subunit of PI3K, is strongly associated with TβRII. On the other hand, the association between p85 and TβRI requires the stimulation of TGFβ. Therefore, in the presence of a chemical inhibitor of TβRI, the activation of the PI3K/Akt signalling pathway will be prevented. Also, TGFβ might activate the PI3K/Akt signalling pathway by an indirect stimulus. The stimulus starts by TGFβ promoting TGF-α expression, which leads to the activation of EGF receptor signalling. However, this indirect stimulus can also down-regulate PI3K/Akt signalling, when TGFβ activates Smad-dependent expression of the lipid phosphatase SHIP. SHIP might be responsible for the transient nature of TGFβ induced phosphorylation of Akt (Zhang, 2009).

As a Smad-independent pathway, the PI3K/Akt pathway participates in EMT as a result of TGFβ stimulation. Studies suggested that PI3K plays a role in cell migration and reorganisation of actin filaments in response to TGFβ by use of chemical inhibitors. However, other studies presented that with certain concentrations of the chemical inhibitors, a decrease in phosphorylation of Smad2 by TβRI and TβRII occurs (Zhang, 2009). A few regulatory factors are

involved in TGFβ-induced EMT via the PI3K/Akt pathway (Zhang, 2009; Hsu *et al.*, 2020). These factors at least mediate part or most of the pathway which includes downstream Akt and the mammalian target of rapamycin (mTOR). mTOR is an important factor in the regulation of protein synthesis by phosphorylating S6 kinase(S6K) and eukaryotic initiation factor 4E-binding protein1 (4E-BP1). Apart from EMT, PI3K is also involved in both morphological transformation and proliferation of fibroblasts by downstream tyrosine kinase c-Ab1 (Zhang, 2009).

The effects of the Smad pathway, which is activated by TGFβ, can be inhibited through the PI3K/Akt pathway. For instance, both apoptosis and growth inhibition of cells can be prevented through activation of PI3K or Akt. These outcomes have been suggested as result of interaction between Smad3 and Akt. This interaction stops the phosphorylation and nuclear localisation of Smad3, which is mediated by TβRI. Also, other factors play a role in this interaction such as forkhead transcription factor and FoxO (Zhang, 2009).

1.14.2 Erk Activation and Tyrosine Phosphorylation:

In response to the growth factor TGFβ, both rapid and delayed activation of Erk were reported in different types of cells and conditions (Zhang, 2009). The activation of the receptor tyrosine kinase (RTK)/Ras/Erk pathway starts when the cytokines interact with their RTKs (Zhang, 2009; Sun *et al.*, 2015). This interaction results in dimerisation and activation of RTK, which leads to phosphorylation of tyrosine residues in RTK at the cytoplasmic domain. These residues can act as a docking site for many signalling molecules in association with either Src homology (SH2) or PTB (phospho – tyrosine binding). One example of PTB is Grb2 (growth factor binding protein 2), which is an adaptor protein. In this case there is no stimulus, Grb2 binds to Sos in cytoplasm. Once RTK becomes phosphorylated, the complex Grb2/Sos is utilised by the RTK. RTK brings the complex to the membrane, which results in activation of Ras by replacing GDP with GTP. When Ras is in the GTP-Bound state, it is able to bind to Raf of both MAPK cascades MEK and Erk (Zhang, 2009).

During epithelial mesenchymal transition (EMT) the Erk cascade is activated. It was reported that in the late stage of cancer, the growth factor TGFβ induces tumour growth by facilitating tumour cells to undergo EMT. In these tumour cells, combining the effects of both Smad-dependent and Smad-independent pathways

might result in EMT. In the presence of TGFβ, Erk is an important element in both breaking down cell junctions and promoting cell motility. By using genetic screening programs, it was reported that Erk mediates a set of genes that have been well-known to influence cell motility and cell–matrix interactions. During the pro-oncogenic activity of TGFβ, Erk is a crucial component in invasiveness and metastasis of tumours (Zhang, 2009). Also, Erk regulates the activity of Smads by phosphorylating their receptors (Javelaud and Mauviel, 2005; Zhang, 2009). It was observed that in cell culture, Erk can phosphorylate and inhibit Smad activity. AP-1 family members and Erk substrates mediate gene expression by interacting with Smads (Zhang, 2009).

1.14.3 Rho-GTPases:

The Rho-GTPases includes different factors such as RhoA, Rac, and Cdc42 (Zhang, 2009). These factors are involved in many cellular responses such as gene expression, cell motility and regulating cytoskeletal arrangement (Schmitz *et al.*, 2000; Zhang, 2009; Duff and Long, 2017). RhoA has a significant role in TGFβ induced EMT. This role is observed when the growth factor rapidly activates the RhoA-dependent pathway, which induces stress fibre formation in the epithelial cells with other mesenchymal characteristics (Zhang, 2009). TGFβ activates Cdc42 GTPase then p21-activated kinase (PAK) 2, which appears to occur independently of the Smad pathway, in order to form lamellipodia and filopodia (Zhang, 2009; Lamouille, Xu and Derynck, 2014). Guanine nucleotide exchange factors, guanine nucleotide dissociation inhibitors and GTPase activating proteins regulate the activation of Rho GTPase. The activation of Rho at the early stage of EMT produces a series of actions that effect the organisation of the cell cytoskeleton. ROCK (Rho associated kinase) participates in promoting actin polymerisation with formin diaphanous 1 (DIA1). ROCK also has a role in enhancing acto-myosin contractility. Additionally, after being activated by Rac1 or Cdc42, PAK1 (kinase p21 activated kinase 1) induces the targets that are responsible for cell motility and spreading (Lamouille, Xu and Derynck, 2014).

1.14.4 JNK/P38 Activation:

JNK/P38 signalling cascades are classified also as Smad-independent pathways. Similar to the Erk pathway, Both JNK and p38 represent the tertiary layer of the MAPK signalling pathway. These signalling cascades are activated by MAP kinase kinases (MKKs). TGFβ immediately activates JNK by MKK4, while it

activates p38 by MKK3/6 in different cell types. This activation occurs independently of Smad proteins. Upstream this starts with TGFβ activated kinase 1 (TAK1) inducing MAP3Ks, which leads to the upregulation of MKKs. Therefore, it was reported that TAK1 is important for TGFβ signalling to induce the JNK pathway. Other elements are found to be crucial in the JNK/p38 pathway, including TRAF6, which has a primary role in activation of TAK1 via two types of receptors: interleukin-1 receptors and Toll-like receptors (Zhang, 2009).

Even though it was reported that the activation of JNK/p38 occurs independently from Smad, both signalling pathways associate together to regulate the downstream responses of the growth factor (Zhang, 2009). For example, when TGFβ induces apoptosis in tumour cells as a tumour suppression activity, both Smad-dependent and JNK/p38 pathways are involved (Zavadil *et al.*, 2004; Zhang, 2009). In addition, the JNK/p38 pathway is crucial in EMT, which was observed by inhibiting p38. The inhibition of p38 leads to preventing TGFβ inducing cellular changes in shape or cytoskeletal rearrangement of targeted cells. Also, TGFβ inducing EMT was blocked by knocking-down TRAF6 expression (Zhang, 2009).

1.15 Transforming Growth Factor Beta and Oral Squamous Cell Carcinoma:

TGFβ1 induces oral tumour cell metastasis and invasion (Jin *et al.*, 2001; Sun *et al.*, 2008; Pang, Tang and Liang, 2018), while it inhibits cell proliferation (Hasina *et al.*, 1999; Mincione *et al.*, 2008; Ellis, 2021). TGFβ1 was found to be overexpressed in head and neck cancers (Logullo *et al.*, 2003; Rosenthal *et al.*, 2004; Tang *et al.*, 2004). TGFβ1 phosphorylates the Smad pathway, which induces N-cadherin expression in both oral normal epithelial and oral cancer cells during *in vitro* experiments (Diamond *et al.*, 2008). The increase in TGFβ1 expression by betel leaf extract leads to activation of both Smad-dependent pathway and Smad-independent pathways, which induces the expression of matrix metalloproteinase-9 (MMP-9) (Chang *et al.*, 2019). The elevation of MMP-9 levels leads to OSCC metastasis and invasion *in vitro* (Dang *et al.*, 2004; Chang *et al.*, 2019). TGFβ1 induces tumour metastasis via EMT in OSCC cell lines by downregulating epithelial markers and upregulation of mesenchymal markers. On the contrary, it has been found that bone morphogenic protein-2 (BMP-2) induces the expression of Smad1/5/9, which leads to upregulation of cytokeratin 9 (an

epithelial marker) and downregulation of N-cadherin (a mesenchymal marker). TGFβ1 reduces BMP-2-induced epithelial gene expression by downregulating Smad1/5/9. These findings suggest that TGFβ1 regulates oral cancer cell metastasis and BMP-2 induces their colonisation via mesenchymal to epithelial transition (MET) (Chiba *et al.*, 2017).

The activation of the TGFβ signalling pathway in OSCC results in an aggressive tumour, where cancer cells have undergone EMT and resistance to therapeutic drugs (Chen *et al.*, 2012; Xuan, Jin and Yang, 2020). One example of TGFβ signalling pathways is Interleukin-6 signalling (IL-6), which regulates the role of TGFβ in oral cancer by activating a major downstream signalling molecule such as Akt (Chen *et al.*, 2012). Carcinogenesis and epithelial plasticity, induced by TGFβ signalling, are inversely correlated with the expression of Grainyhead-like 2 (GRHL2). GRHL2 is an epithelial- specific transcription factor that is involved in epithelial cells differentiation. In an *in vitro* experiment, the overexpression of GRHL2 reduced TGFβ signalling in OSCC cell lines, however, it increased the activity of Erk and JNK MAPK (Chen *et al.*, 2018). TGFβ1 upregulates the invasive ability of OSCC cell lines by increasing the expression of matrix metalloprotase-10 (MMP-10) via a Slug-dependent manner, which is mediated by non-canonical Wnt signalling pathways (Joseph *et al.*, 2009; Hino *et al.*, 2016).

TGFβ induces bone invasion of OSCC (Park *et al.*, 2017) via activation of a Smad-dependent pathway, which regulates transcription factor Gli2. Gli2 has a crucial role in OSCC bone invasion in the mandibular bone of animal models via increasing the expression of Parathyroid Hormone-related Proteins (PTHrP). However, the activities of both Gli2 and PTHrP were reduced by inhibiting Smad-independent pathways. This result suggests that the Smad-dependent pathway and Smad-independent pathways have significant roles in OSCC bone invasion (Cannonier *et al.*, 2016). The SIRT1 enzyme inhibits OSCC cells from migrating by increasing the level of epithelial markers such as E-cadherin and decreasing the level of mesenchymal markers *in vitro*. The targeted protein for SIRT1 was identified as Smad4. The increase of SIRT1 expression was correlated with the decrease level of Smad4 and inhibiting TGFβ signalling pathway (Chen *et al.*, 2014). *In vitro*, the increase of MiR-132 expression inhibits TGFβ1 signalling pathway specifically Smad dependent pathway, which regulates tumour growth, apoptosis and metastasis (Chen *et al.*, 2020).

1.16 Aims and Hypothesis:

1.16.1 Aims:

The aim of this study was to establish if the growth factor TGFβ1 has a role in oral cancer cell lines obtaining the ability to migrate by activating Smad-dependent or/and Smad-independent signalling pathways (**Figure 13**).

1.16.2 Objectives:

a) To investigate the morphological change and alteration in colony appearance of normal and oral cancer cell lines.

b) To investigate the effect of TGFβ1 on three cell lines and identify the mode of inducing cell migration in response to specific test conditions using different inhibitors for specific signalling pathways e.g. Smad, PI3K-Akt and MAKP Erk1/2.

c) To investigate the activation or phosphorylation status of specific signalling pathways in response to TGFβ1 in a normal cell line and oral cancer cell lines.

d) To investigate the expression and localisation of E-cadherin, N-cadherin, Claudin-1 and β-catenin linked with cell migration in normal and oral cancer cell lines.

1.16.3 Hypothesis:

TGFβ1 mediates oral cancer cell line migration via EMT by activating Smad, PI3K-Akt and Erk1/2/MAPK signalling pathways, inducing oral cancer cells to migrate singly or collectively.

1.16.4 Null Hypothesis:

TGFβ1 does not mediate oral cancer cell line migration via EMT by activating Smad, PI3K-Akt and Erk1/2/MAPK signalling pathways inducing oral cancer cells to migrate singly or collectively.

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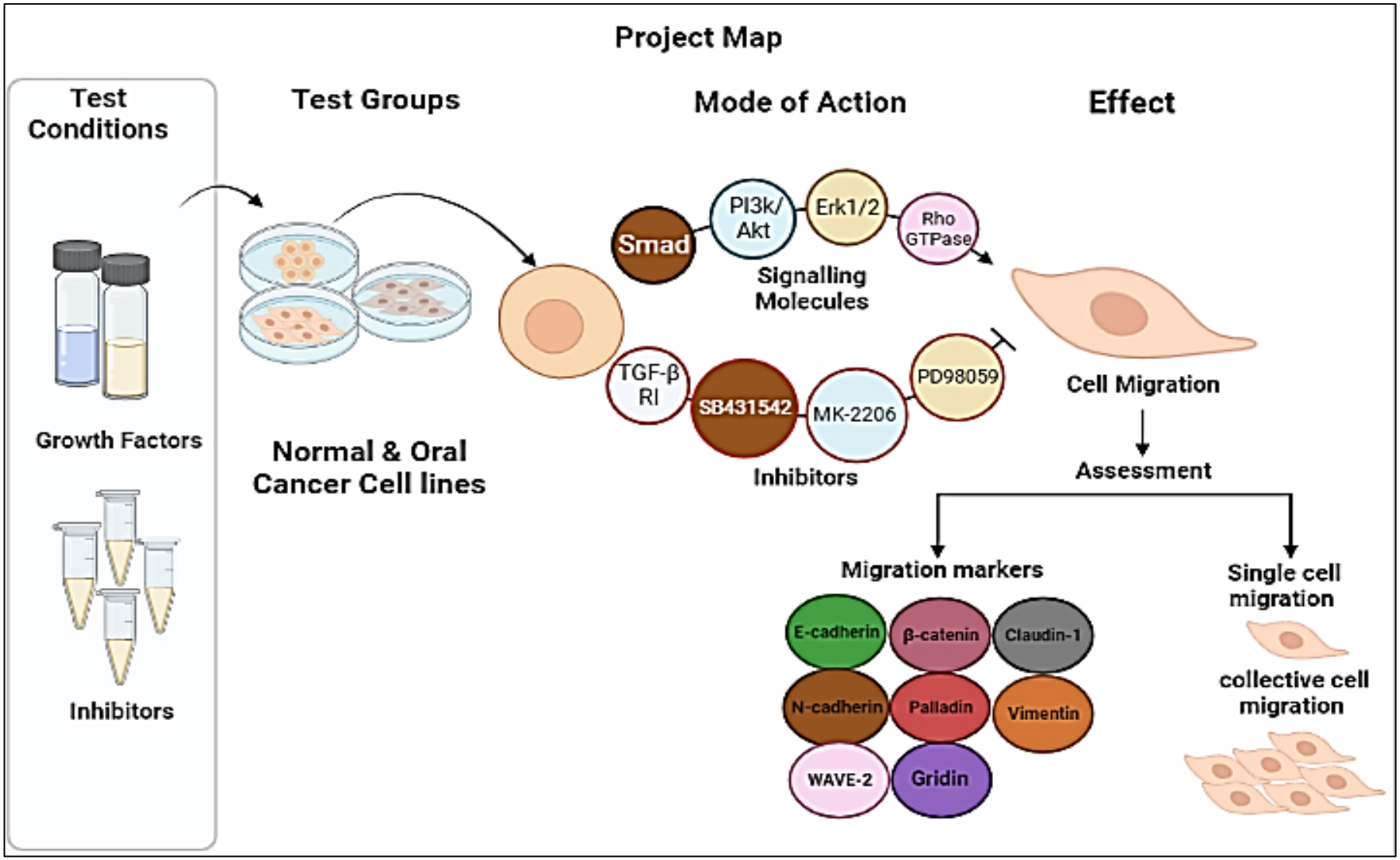

Figure 13: Outlines of the project plan.

The figure was created in BioRender.com.

Chapter 2: General Materials and Methods

General Materials and Methods:

This chapter illustrates the general methodology and the materials that were used for all experiments in this project (**Figure 13**). Any specific reagents that were used, modifications to certain protocols or the methods of data analysis will be detailed in their respective chapters. The protocols for the general procedures in this project, which included cell culture related procedures, Cell lysis, SDS-PAGE and Western blot are described below:

2.1 Cell Culture and Related Protocols:

This section contains all the materials and methods that were used in the cell culture protocols including cell sub-culturing, cell counting, cryopreservation, resuscitation and cell lysis (**Table 3**).

2.1.1 Cell Lines:

In this study, three cell lines were used to investigate the role of TGFβ1 in oral cancer cell migration.

Table 3: List of Cell lines

Cell line	Cell type	Derived from	Sources
HaCaT	Normal adult keratinocytes	Normal skin	Prof. S. L. Schor (Late), DDS
TYS	Oral adeno-squamous cell carcinoma	Minor salivary gland floor of the mouth	Dr. Koji Harada University of Tokushima, Japan
SAS-H1	Oral squamous cell carcinoma	Tongue	Japanese research bank

HaCaT Cell Line: Normal skin keratinocyte (squamous epithelial origin). The lack of an oral mucosal keratocyte cell line in the laboratory is the primary rationale for selecting the HaCaT cell line (Boukamp *et al.*, 1988). Despite being isolated from the squamous epithelium of the skin, their appearance and function are comparable to those of the oral mucosal squamous epithelial cells. This cell line was chosen as the control group in this project.

TYS Cell Line: Oral adenoid squamous cell carcinoma (an epithelial neoplastic cell line) was established from human oral mucosa-derived, well-differentiated squamous cell carcinoma (Yanagawa *et al.*, 1986). The sodium-butyrate-treated cell line was subsequently implanted into null mice. The resultant mass was

analysed histologically and determined to be a human adenoid squamous cell carcinoma originating from a small salivary gland located in the oral mucosa (Yanagawa *et al.*, 1986). This cell line was chosen to investigate their migratory behaviour, signalling pathway molecules and the status of migration marker expression in response to specific conditions.

SAS-H1 Cell Line: This cell line was derived from human tongue, a poorly differentiated squamous cell carcinoma. This cell line was obtained from the Japanese Research Resources Bank (JCRB) (Okumura *et al.*, 1996). SAS-H1 cells showed high invasive potential, when examined using a rat endothelial cell monolayer. The SAS-H1 cell line was selected for studying cell migration, signalling pathways and protein expression under controlled circumstances.

2.1.2 Equipment:

Table 4: List of equipment used in cell culture.

No.	Name of the equipment	Make
1	Class II biological safety cabinet	Medical Air Technology, Manchester, UK
2	Incubator	Thermo Scientific, Waltham, MA, USA
3	Water bath	Grant Instruments, Cambridge, UK
4	Centrifuge machine	Thermo Scientific, Waltham, MA, USA
5	Pipette	Thermo Scientific, Waltham, MA, USA
6	Pipette boy	Integra bioscience, Zizers, Switzerland
7	Nunclone cell culture dish	Thermo Fisher Scientific, Denmark
8	Automated cell counter, TC10	Bio-Rad, Hercules, CA, USA
9	Light Microscope, IX70	Olympus, Tokyo, Japan

2.1.3 Materials:

Table 5: List of reagents used in cell culture.

No.	Reagents	Company	Catalogue No.
1	FCS (Foetal Calf Serum)	Gibco-Thermo Fisher, Grand Island, New York, USA	10270-106
2	MEM (Minimum Essential Medium Eagle)	Sigma-Aldrich, St. Louis, MO, USA	M-5650
3	EGTA	Sigma-Aldrich, St. Louis, MO, USA	E-8145
4	Trypsin	Sigma-Aldrich, St. Louis, MO, USA	T-4549
5	L-Glutamine	Sigma-Aldrich, St. Louis, MO, USA	G-7513
6	Penicillin-Streptomycin	Sigma-Aldrich, St. Louis, MO, USA	P-4333
7	Sodium Pyruvate	Sigma-Aldrich, St. Louis, MO, USA	S-8636
8	PBS	Sigma-Aldrich, St. Louis, MO, USA	P-4417
9	Sodium bicarbonate	Merck, Darmstadt, Germany	S5761
10	DMSO for Freeze Mix	Sigma-Aldrich, St. Louis, MO, USA	D-5879
11	RIPA Buffer (50 mM Tris HCl, 150 mM NaCl, pH 7.4; 0.1% w/v SDS, 1% v/v Triton x-100, 1% w/v sodium deoxycholate and 5 mM EDTA)	NaCl : Sigma (S3014), Triton X100 Sigma (T8787), Sodium deoxy: Sigma (D6750), EDTA : Sigma (E0270), ,Protease inhibitor (Ref. A32955)/ Thermo Scientific, Pierce and Phosphatase inhibitor (04906845001)/ Roche Applied Science, Bavaria, Germany).	-
14	Human recombinant Transforming growth factor β1	Sigma-Aldrich, St. Louis, MO, USA	T7039
15	Human recombinant Epidermal growth factor	Gibco-Thermo Fisher, Grand Island, New York, USA	10450-013
16	TGF-β RI Kinase Inhibitor VII	Santa Cruz Biotechnology, Dallas, USA	SC-356133
17	MK-2206 2HCl	Selleck Chemicals LLC, Houston, USA	S1078
18	PD98059	Cell Signaling Technology Inc., Danvers, MA, USA	9900
19	SB431542	Selleck Chemicals LLC, Houston, USA	S1067
20	Freeze Mix	MEM (170 ml), FCS (20ml) and DMSO(10ml).	

2.1.4 Procedures for Cell Culture and Cell Lysis:

Cell Culture:

Similar growth medium was used for culturing the three cell lines in this study. The growth medium MEM was supplemented with 10% (v/v) Foetal Calf serum (FCS), 10mM sodium pyruvate, 200 mM L-glutamine and 5ml of penicillin-streptomycin. Cells were provided with the prepared growth medium and incubated at 37 ℃ and 5% CO_2. The procedures related to sub-culturing, cryopreservation and resuscitation, were carried out following the standard laboratory protocols of Cell and Molecular Biology unit at Dundee Dental Hospital and Research, UK. For regular maintenance, the growth medium was changed every 48 hours or 72 hours for the cell lines, which were grown in 90 mm Nunclon dishes.

For sub-culturing, cells were observed to have reached a confluence of around 80 - 90%, before passaging them into new dishes. The protocol started first with aspirating off the old growth medium, before adding 5 ml of PBS to wash the dish, this was repeated twice. This step was followed by addition 2 ml of TRYPSIN EGTA to the cells for five minutes at 37 ˚ C. The optimum temperature for the trypsin was 37 °C and the trypsin causes release of the cells from the dish due to proteolytic activity. 2 ml of 10% (v/v) FCS MEM was added to the dish to inactivate the trypsin. The contents of the dish were then collected in a universal tube and the suspension was spun for five minutes at 900 rpm, which allowed the formation of a cell pellet. The supernatant was carefully aspirated off and the cell pellet was re-suspended in fresh growth medium. Cells were put into new 90 mm dishes and placed in the incubator overnight.

For cryopreservation of the cells, the sub-culture protocol (above) was followed until the point where the supernatant was collected. Rather than adding growth medium to resuspend the cells, 1 ml of freeze mix was added to the cell pellet collected from a 90mm dish, loaded in cryovials and stored in - 80 ℃. Cell resuscitation was carried out by taking the cryovials from - 80 ℃, putting them in a water bath at 37 ℃ which allows them to defrost and adding them to new 90 mm dishes with fresh medium. Assessment for cell attachment was carried out after 4 - 6 hours.

Cell Lysis Protocol:

Cell lysis was carried out after the cells were treated with test conditions for specific incubation periods. The aim of the cell lysis procedure was to prepare samples (cell protein extracts) for the SDS-PAGE and Western blotting protocol. Initially, cells were washed twice with cold PBS at 4 ℃ before adding 500 µl of RIPA buffer and incubating for ten minutes. The RIPA buffer causes the cells to lyse and release their contents. Solubilised proteins were protected from proteolysis by addition of protease inhibitors and phosphatase inhibitors were also added to protect any phosphorylated residues. Finally, cells were scraped off the dish and the cell lysates were collected in Eppendorf tubes, which were then stored at -20 ℃.

2.2 SDS-PAGE and Western Blotting

2.2.1 Equipment:

Table 6: List of equipment used in the SDS-PAGE & WB protocol.

No.	Name of the equipment	Make
1	Pipette	Thermo Scientific, Waltham, MA, USA
2	Pipette boy	Integra bioscience, Zizers, Switzerland
3	Orbital shaker	Stuart Scientific, Staffordshire, UK
4	Magnetic stirrer	Stuart Scientific, Staffordshire, UK
5	Mini PROTEAN Tetra System	Bio-Rad, Hercules, CA, USA
6	Centrifuge (Sigma 1-13)	Sigma, Osterode am Harz, Germany
7	Power Pac 300	Bio-Rad, Hercules, CA, USA
8	ChemiDoc® MP imaging system	Bio-Rad, Hercules, CA, USA
9	Water bath	Grant Instruments, Cambridge, UK
10	Pierce G2 Fast Blotter	Thermo Scientific, Waltham, MA, USA
11	Gryo-rocker	Cole-Parmer Ltd, Staffordshire, UK
12	Rotamixer	Hook & Tucker Instruments Ltd, Croydon,UK
13	PURELAB OPTION	ELGA LabWater lane End industrial Park, UK
14	Sartorius CPA124S Balance	Sartorius Weighing Technology Gmbh, Göttingen, Germany

2.2.2 Materials:

Table 7: List of reagents used in the SDS-PAGE & WB protocol.

No.	Reagents	Company	Catalogue No.
1	Laemmli loading buffer	Bio-Rad, Hercules, CA, USA	161-0737
2	2-Mercaptoethanol	Sigma-Aldrich, St. Louis, MO, USA	M-3148
3	Running buffer (TGS)	Bio-Rad, Hercules, CA, USA	161-0772
4	Mini-PROTEAN Stain free precast gel 10-well	Bio-Rad, Hercules, CA, USA	456-8033
5	Mini-PROTEAN Stain free precast gel 15-well	Bio-Rad, Hercules, CA, USA	456-8126
6	Magic Mark XP western std.	Invitrogen, Carlsbad, CA, USA	LC 5602
7	Clarity Western ECL Substrate	Bio-Rad, Hercules, CA, USA	170-5061
8	Semi-skimmed milk powder	Marvel, London, UK	-
9	10xTri / Glycine/ SDS buffer	Bio-Rad, Hercules, CA, USA	1610772
10	Methanol	Fisher Scientific, Leicestershire, UK	10164633
11	Sodium Chloride	Sigma-Aldrich, St. Louis, MO, USA	S-3014
12	Hydrochloric acid	VWR BDH, PA, USA	20252335
13	Tween 20	Sigma-Aldrich, St. Louis, MO, USA	P1379
14	Immuno-Blot PVDF membrane for protein Blotting	Bio-Rad, Hercules, CA, USA	1620177
15	Extra thick filter paper	Bio-Rad, Hercules, CA, USA	1703960
16	Goat anti-rabbit IgG, HRP linked antibody	Cell Signaling Technology Inc., Danvers, MA, USA	7074

2.2.3 Protocol for SDS-PAGE and Western Blot:

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is utilised to sort proteins based on their molecular weight, which allows investigation of specific proteins after the blotting phase (Kielkopf, Bauer and Urbatsch, 2021). Western blotting is used following SDS-PAGE to transfer the fractioned proteins to a blotting membrane using an electric current. This step is followed by a blocking step, incubation with primary antibody, washing, incubation with secondary antibody (Kurien and Scofield, 2015).

This protocol involved four stages, which included sample preparation, electrophoresis, blotting and imaging. For the preparation stage, samples were

allowed to thaw and were then spun in microfuge for five minutes at 13,000 rpm. The soluble samples were then mixed with an equal volume of Laemmli loading buffer containing 5% (v/v) 2-mercaptoethanol and heated at 95℃ for five minutes in a water bath. This step causes the denaturation of the proteins in the sample and acquiring a negative charge for the movement in the electric field via SDS. The number of SDS molecules bound to the proteins is related to their size.

For the electrophoresis stage, samples were loaded onto 10% kD Biorad TGX stain-free precast gels with either 10 or 15 wells. Stain-free gels allows imaging for total protein prior to transfer step. Magicmarker XP Western standards were also loaded as a reference for protein molecular weight. The tank, which contained the gel, was connected to a power pack to apply the electrical current. The gel was run at 150V for 45 minutes. Once electrophoresis was complete, the gel was removed from the cassette, transferred to distilled water and assessed by recording the image using ChemiDoc imaging system. At this stage these are stain- free gels, where the total protein can be visualised under UV. Finally, the gel, still suitable for transfer by Western blotting, was transferred into transfer buffer for one minute before starting the blotting stage.

The aim of Western blotting is to transfer proteins from the gel after SDS-PAGE procedure to PVDF membrane. This procedure was followed by probing the bands on the membrane with specific primary antibodies for the target protein. The western blot protocol started with preparing two extra thick filter papers plus PVDF membrane (activated in MeOH) soaked in transfer buffer. To assemble the sandwich, the first filter paper was placed on the cathode of the blotter followed by the membrane, gel and the second filter paper on the anode side. A current with a constant voltage of 15V was applied for 42 minutes to transfer the proteins from the gel to the membrane. Once the transfer was completed, the PVDF membrane was incubated in blocking buffer, which was made with 1% (w/v) dried milk in 1 x TBST for 30 minutes. The reason behind the blocking step was to minimise the background noise and reduce non-specific binding on the PVDF membrane. The antibody for the targeted protein was diluted in the blocking buffer according to the manufacturer’s recommendation and incubated with the blot overnight at room temperature. The blot was then washed three times with 1 x TBST for 20 minutes each to remove excess of primary antibody and the blot

was then incubated with the secondary antibody (1:2000), diluted in 1 x TBST, for an hour at room temperature.

The aim of the visualisation stage was to develop the blot and detect the bands by adding the secondary antibody which is labelled with the enzyme horse radish peroxide (HRP). After incubating the blot with the secondary antibody, the blot was washed three times with 1 x TBST for 20 minutes each, followed by one time wash with 1 x TBS for five minutes. Finally, the blot was incubated with chemiluminescent substrate (Clarity Western ECL Substrate), which was prepared according to manufacturer recommendation (7ml for a blot), for five minutes at room temperature. The secondary antibody location was detected by introducing a substrate to HRP, the resultant chemiluminescence using the ChemiDoc imaging system (**Figure 14**).

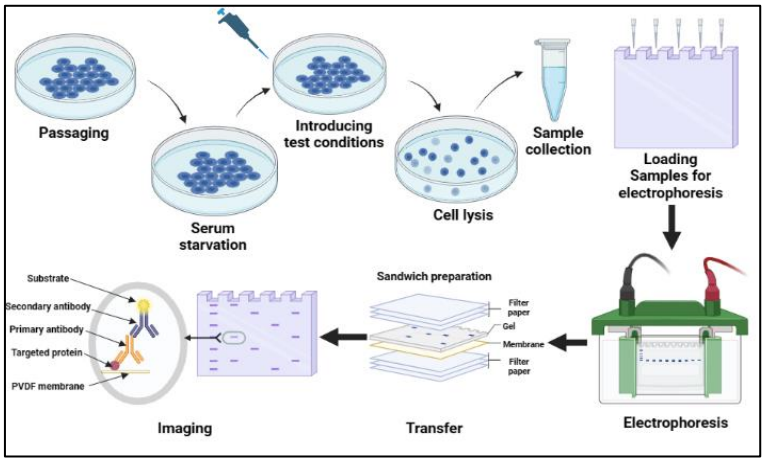

Figure 14: Cell culture, Cell lysis, SDS-PAGE and Western blotting protocols.

Figure, was created in Biorender.com, illustrates the protocol of SDS-PAGE & Western blotting.

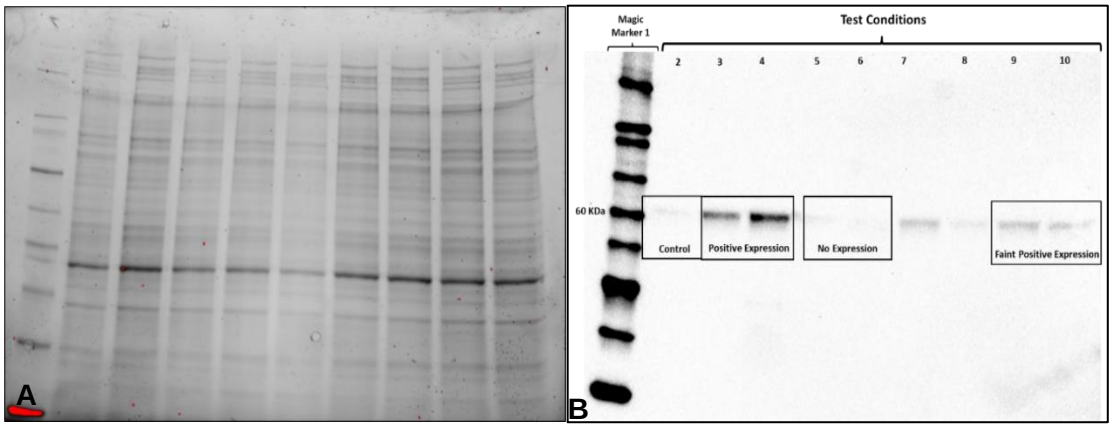

Figure 15: Illustration of ten well gel.

(A) The figure shows the ten wells gel after SDS protocol, which illustrates the separated protein bands. Magic Markers in the first lane indicates molecular weight of the proteins. The image was taken using the ChemiDoc imaging system before the blotting stage. **(B)** General design of the ten well immunoblots. The design consists of Magic Marker as reference for molecular weight, the location of the control sample in the blot, example of positive expression, example of faint expression and example of no expression. The image was taken using the ChemiDoc imaging system. The protocol for total protein normalisation can be found in appendix 8.

Chapter 3: Optimisation of the Growth Factors and Inhibitors

3.1 Optimisation of Materials:

Optimisation of some of the materials that were used in this project was required to ascertain their working concentrations. The same working concentrations were used throughout this study.

Transforming Growth Factor Beta 1:

To identify the optimum working concentration for the growth factor TGFβ1, an experiment with two phases was conducted to compare the effect of different concentrations and different incubation periods on the oral cancer cell lines. The first phase was based on the evidence that TGFβ1 activates the Smad-dependent pathway (Massagué, 2008). The SDS-PAGE and Western blot protocol was used to evaluate the phosphorylation of Smad in oral cancer cell lines. Different concentrations of the growth factor were used at a wide range of incubation periods.

This phase of evaluation had two stages, first stage was designed to use two working concentrations of TGFβ1 (10 ng/ml and 50 ng/ml) and to evaluate the phosphorylation of Smad molecules at different incubation periods (**Figure 16**).

Magic Marker	0 min	15 mins	30 mins	1 hour	2 hours	4 hours	24 hours	48 hours	Blank

Figure 16: Design of the blots for stage one TGFβ1 optimisation.

The second stage of phase I included different concentrations of the growth factor, which were used to evaluate Smad phosphorylation at specific incubation period (two hours) (**Figure 17**).

Magic Marker	SF-MEM	1pg/ml	10pg/ml	100pg/ml	1ng/ml	10ng/ml	20ng/ml	50ng/ml	100ng/ml

Figure 17: Design of the blots for stage two TGFβ1 optimisation.

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The second phase of the optimisation of TGFβ1 was based on the evaluation of the migratory effect of the growth factor on oral cancer cell lines by observing them at specific incubation periods i.e. 24 hours, 48 hours and 72 hours.

3.2 Materials and Methods:

Two oral cancer cell lines were used for the TGFβ1 optimisation experiment, TYS and SAS-H1. Reagents and protocols for cell culture, cell lysis, SDS-PAGE and Western blot were mentioned earlier (Pages 41 - 47). Reagents for these experiments are listed in (**Table 5** & **Table 8**). Both primary antibodies were prepared using the recommended dilutions in the manufacturer's datasheet for the Western blot protocol. For the phase I optimisation, the dilutions for both antibodies were 1:1000.

Table 8: Specific primary antibodies used for phase I TGFβ1 optimisation.

No.	Reagents	Company	Catalogue No.
1	Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) (D27F4) Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	8828
2	Phospho-Smad3 (Ser423/425) (C25A9) Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	9520

3.3 Protocol for Phase I (Time and Concentration Optimisation of TGFβ1):

The cell culture and cell lysis protocols used in this experiment are mentioned earlier in the general materials and methods. However, there were a few modifications to the standard procedure. Cells were plated in 60 mm dishes with a specific seeding density of 5 x 10^5 cells/dish and were incubated in 10% FCS MEM for overnight to attach and grow. Once cells had attached and formed colonies, SF-MEM was introduced to the experimental dishes for serum starvation. Finally, test conditions were prepared and applied to the cells to be subsequently evaluated at specific incubation periods.

3.4 Protocol for Phase II (Optimisation of the Effect TGFβ1 on Cell Migration):

Similar to phase I, a few adjustments were carried out to the standard cell culture protocol, including the seeding density which was 5 x 10^4 cells/dish. Also, only three concentrations of TGFβ1 were used to observe the migratory effect on the oral cancer cell lines, which were 1 ng/ml, 10 ng/ml and 50 ng/ml. The effect of these different concentrations of the growth factor was observed after 24 hours, 48 hours and 72 hours.

3.5 Results for Optimisation of Materials.

3.5.1 Optimisation of Growth Factor:

3.5.1.a TYS Cell Line

Phase I Time and Concentration Optimisation of TGFβ1:

The phosphorylation of Smad2/3 was detected in lysates treated with 10 ng/ml of TGFβ1 TYS cell line after 15 minutes, 30 minutes, one hour and two hours with (**Figure 18**). In lysates from cells treated with 50 ng/ml of TGFβ1, the TYS cell line showed phosphorylation of Smad2/3 after 30 minutes to one or two hours (**Figure 19**). For the blot with lysates from cells treated with different concentrations of TGFβ1 at two hours, TYS cells showed the phosphorylation of Smad3 in response to wide range of concentration of TGFβ1 including 1 ng/ml, 10 ng/ml, 20 ng/ml and 50 ng/ml of (**Figure 20**).

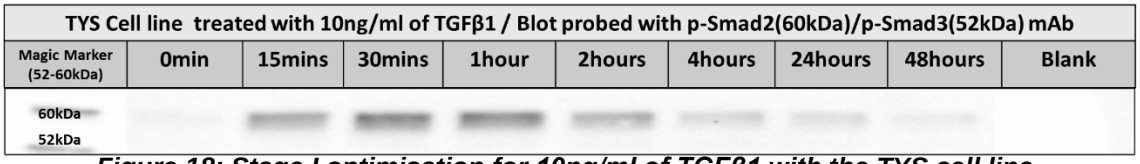


Figure 18: Stage I optimisation for 10ng/ml of TGFβ1 with the TYS cell line.

The TYS cells were incubated with 10ng/ml of TGFβ1 for: 0min, 15mins, 30mins, 1hr, 2hrs, 4hrs, 24hrs and 48 hrs. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Smad2/Smad3 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells, revealed that the phosphorylation of Smad2/Smad3 increases after 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours and 24 hours compared to time zero. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two.

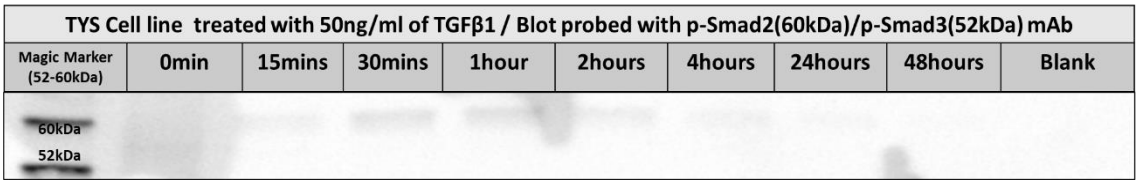


Figure 19: Stage I optimisation for 50ng/ml of TGFβ1 with the TYS cell line.

The TYS cells were incubated with 50ng/ml TGFβ1 for: 0min, 15mins, 30mins, 1hr, 2hrs, 4hrs, 24hrs and 48 hrs. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Smad2/Smad3 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells, revealed that the phosphorylation of Smad2/Smad3 increases after 30 minutes and 1 hour compared to time zero. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two.

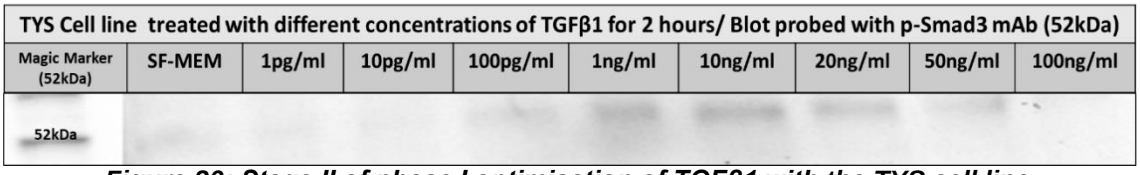


Figure 20: Stage II of phase I optimisation of TGFβ1 with the TYS cell line.

The TYS cells were incubated with different concentrations of TGFβ1 including 1pg/ml, 10pg/ml, 100pg/ml, 1ng/ml, 10ng/ml, 20ng/ml, 50ng/ml and 100ng/ml for 2 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Smad3 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells, revealed that the phosphorylation of Smad3 increases in lysates treated with 1ng/ml, 10ng/ml, 20ng/ml and 50ng/ml of TGFβ1 compared to negative control. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two.

Phase II Optimisation of the Effect of TGFβ1 on Cell Migration:

The SF-MEM test condition was used as the negative control group for this assessment. TYS cells incubated with SF-MEM for 24 and 48 hours showed epithelial cell characteristics including cuboid or oval shapes in closed compact colonies with no signs of morphological changes. However, after 72 hours with SF-MEM, TYS cells showed less compact colonies and a few cells that were separated from their cluster. TYS cells treated with different concentrations of TGFβ1, 1 ng/ml, 10 ng/ml and 50 ng/ml as separate test conditions, showed scattered cells with different morphological appearance including small round cells and some with a spindle shape (**Figure 21**).

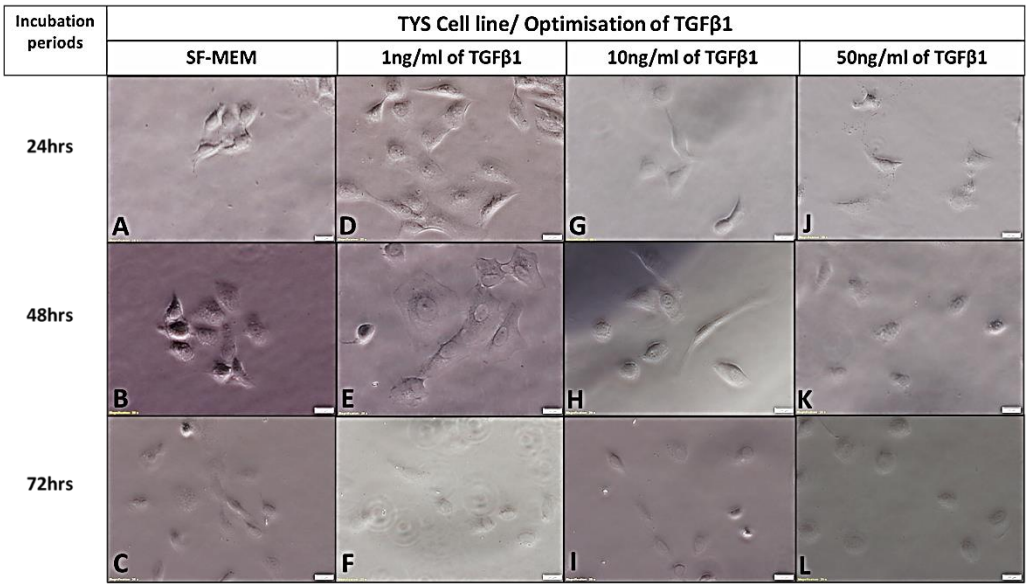


Figure 21: Phase II optimisation of TGFβ1 with the TYS cell line.

Images of the TYS cell line treated with different test conditions were taken after 24 hours, 48 hours and 72 hours. The test conditions included SF-MEM, 1 ng/ml of TGFβ1, 10 ng/ml of TGFβ1 and 50 ng/ml of TGFβ1. The observation was based on evaluating colony appearance, cell morphology and present of scattered cells, which were observed with all the test conditions. Images of culture dishes were taken with 200X magnification. Detailed methodology can be found in chapter two.

3.5.1.b SAS-H1 cell line:

Phase I Time and Concentration Optimisation of the Effect TGFβ1:

Smad 2/3 phosphorylation was observed in lysates from SAS-H1 cells treated with 10 ng/ml of TGFβ1 after 30 minutes (**Figure 22**). After 15 minutes incubation with 50 ng/ml of TGFβ1, lysates from SAS-H1 cell line showed an increase in p-Smad 2/3 compared to the negative control. This phosphorylation persisted for two hours before eventually becoming faint (**Figure 23**). The SAS-H1 cells showed Smad3 phosphorylation when treated with various concentrations of TGFβ1 after two hours (**Figure 24**).

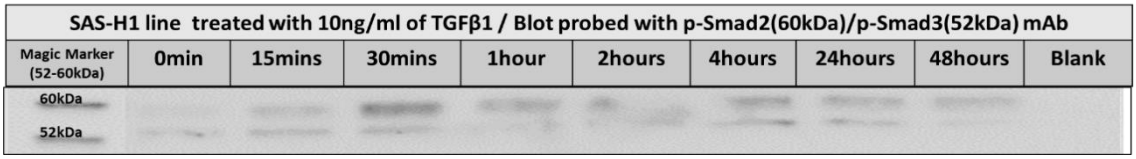


Figure 22: Stage I optimisation for 10 ng/ml of TGFβ1 with the SAS-H1 cell line.

The SAS-H1 cells were incubated with 10ng/ml of TGFβ1 for: 0min, 15mins, 30mins, 1hr, 2hrs, 4hrs, 24hrs and 48 hrs. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Smad2/Smad3 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells, revealed that the phosphorylation of Smad2/Smad3 increases after 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 24 hours 48 hours compared to time zero. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two.

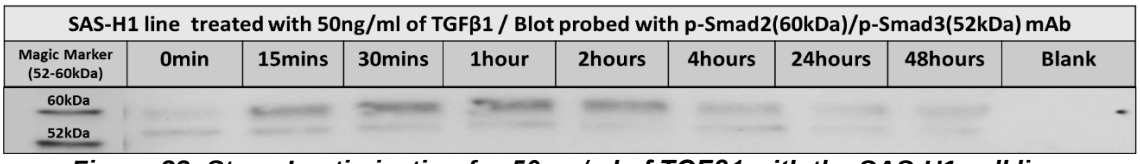


Figure 23: Stage I optimisation for 50 ng/ml of TGFβ1 with the SAS-H1 cell line.

The SAS-H1 cells were incubated with 50ng/ml TGFβ1 for: 0min, 15mins, 30mins, 1hr, 2hrs, 4hrs, 24hrs and 48 hrs. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Smad2/Smad3 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells, revealed that the phosphorylation of Smad2/Smad3 increases after 15 minutes, 30 minutes, 1 hour, 2 hours and 4 hours compared to time zero. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two.

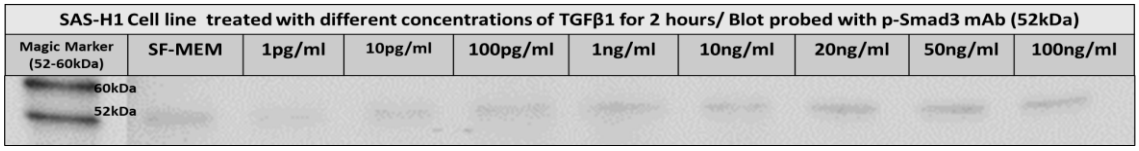


Figure 24: Stage II of phase I optimisation of TGFβ1 with the SAS-H1 cell line.

The SAS-H1 cells were incubated with different concentrations of TGFβ1 including 1pg/ml, 10pg/ml, 100pg/ml, 1ng/ml, 10ng/ml, 20ng/ml, 50ng/ml and 100ng/ml for 2 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Smad3 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells, revealed that the phosphorylation of Smad3 increases in lysates treated with all concentrations of TGFβ1 compared to negative control, except 1pg/ml and 10pg/ml. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two.

Phase II Optimisation of TGFβ1 Effect on Cell Migration:

This phase of assessment included a negative control group, which was the SF-MEM test condition, and three concentrations of TGFβ1. SAS-H1 cells incubated with SF-MEM displayed cuboid or oval morphologies in closed compact colonies without any evidence of morphological alterations after 24 and 48 hours. Similar to the TYS cell line, SAS-H1 cells treated with SF-MEM after 72 hours revealed that cells had less compact colonies and fewer cells that had broken away. Culture dishes treated separately with1 ng/ml, 10 ng/ml, and 50 ng/ml of TGFβ1 showed scattered cells with various morphological characteristics, including small round cells and others with spindle-shaped cells (**Figure 25**).

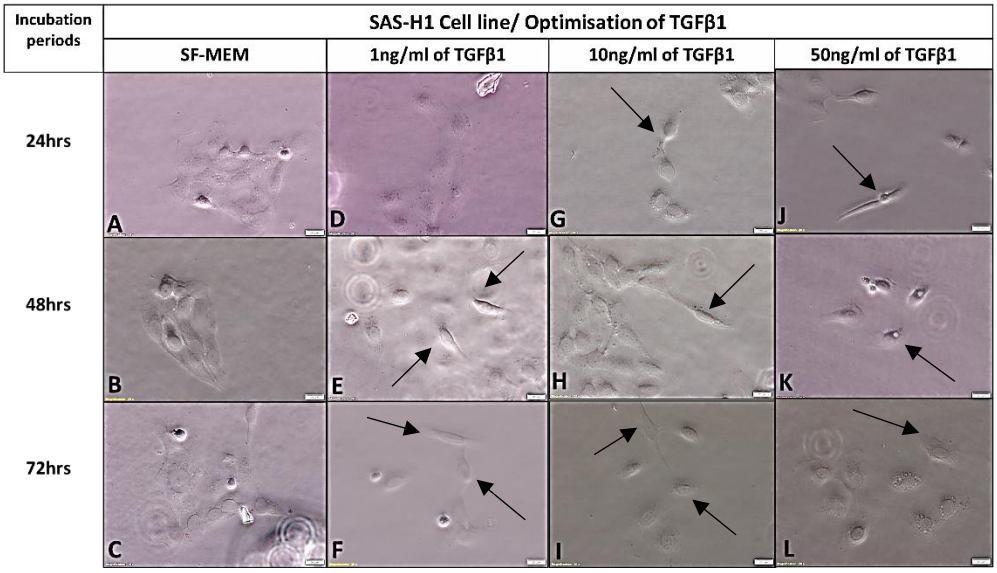


Figure 25:Phase II optimisation of TGFβ1 with the SAS-H1 cell line.

Images of the SAS-H1 cell line treated with different test conditions were taken after 24 hours, 48 hours and 72 hours. The test conditions included SF-MEM, 1 ng/ml of TGFβ1, 10 ng/ml of TGFβ1 and 50 ng/ml of TGFβ1. The observation was based on evaluating colony appearance, cell morphology and present of scattered cells, which were observed with all the test conditions. Images of culture dishes were taken with 200X magnification. Detailed methodology can be found in chapter two.

Findings:

Phase I optimisation of TGFβ1 showed the phosphorylation of Smad2/3 was more evident in culture dishes treated with 10 ng/ml of TGFβ1, especially after 30 minutes in both cell lines compared to the other concentrations. For phase II optimisation, all different concentrations of the growth factor showed evidence of morphological changes and scattered cells. These results suggested that oral cancer cells were induced to separate and migrate away from their colonies.

Based on those findings, it was found that 10 ng/ml of TGFβ1 phosphorylates Smad2/3 and induces the TYS and SAS-H1 to migrate.

3.5.2 Inhibitors Optimisation:

Inhibitors that were used in this project had to be optimised to identify the working dilutions. The aim of the optimisation was to establish the working concentration of each of the inhibitors that will cause no cells death. Most of the working concentrations of the inhibitors were prepared according to the recommended dilutions of the manufacturer. As it has been mentioned in the general materials chapter 2(**Table 5**), four different inhibitors were used in this project TGF-β RI Kinase Inhibitor VII, MK-2206, PD98059 and SB431542. The objective of this experiment was to find if there are significant differences between cells with SF-MEM and cells with inhibitors. All inhibitors in these experiments were prepared in DMSO (Dimethyl sulfoxide solvent).

TGF-β RI Kinase Inhibitor VII inhibits TGF-β RI Kinase in an ATP competitive manner, which showed a good inhibition of Smad2 phosphorylation by TGFβ in human lung cell line (Shimizu *et al.*, 2008).

MK-2206 is an Akt inhibitor, which is a highly potent and selective. This inhibitor is still under development for treating solid tumours. Also, it has been investigated in anticancer therapy in combination with other drugs such as docetaxel and carboplatin (Hirai *et al.*, 2010).

PD98059 is a highly selective inhibitor of MEK1 activation and the MAPK cascade. PD98059 binds to inactive MEK1 and blocks its activation by the upstream activators (Rosen *et al.*, 1994).

SB431542 is a specific and selective inhibitor of the TGFβ superfamily type I activin receptor-like kinase receptors (ALK). SB431542 is a selective inhibitor of Smad3 phosphorylation. Also, it has been reported that SB431542 has no inhibitory role against JNK, ERK or p38 MAPK pathways (Laping *et al.*, 2002).

All the inhibitors in this experiment were prepared to the recommended working dilutions according to their manufacturers.

Materials:

Table 9: List of reagents used for phase II optimisation of different inhibitors.

No.	Reagents	Company	Catalogue No.
1	Human recombinant Transforming growth factor β1 (10 ng/ml)	Sigma-Aldrich, St. Louis, MO, USA	T7039
2	DMSO	Sigma-Aldrich, St. Louis, MO, USA	D-5859
3	TGF-β RI Kinase Inhibitor VII (5 μM)	Santa Cruz Biotechnology, Dallas, USA	SC-356133
4	MK-2206 2HCI (1 μM)	Selleck Chemicals LLC, Houston, USA	S1078
5	PD98059 (1 μM, 5 μM, 10 μM & 20 μM)	Cell Signaling Technology Inc., Danvers, MA, USA	9900
6	SB431542 (5 μM)	Selleck Chemicals LLC, Houston, USA	S1067

Methods:

The general materials and techniques section describes the cell culture that was utilised in this study (**Table 3**). There are few additional requirements for this protocol. Cells were seeded at a density of 5 x 10^4 cells/dish in 60 mm dishes and allowed to adhere and grow overnight in 10% FCS MEM. Once colonies of cells were grown, SF-MEM was added to the plates to induce serum starvation. Finally, different inhibitors were prepared to specific concentrations, and the cells were subjected to them for specific periods of time.

HaCaT Cell Line:

The cell morphology and colony appearance of the HaCaT cell line in the different test conditions showed no significant difference to the control group (**Figure 26**). HaCaT cells showed all the epithelial cell characteristics, which are small rounded or cuboid cells in compact a colony. No signs of debris or floating cells were observed with all the inhibitors that were used in this experiment. The DMSO was used alone at same concentration with the HaCaT cell lines, which showed no difference to the control group (data not shown).

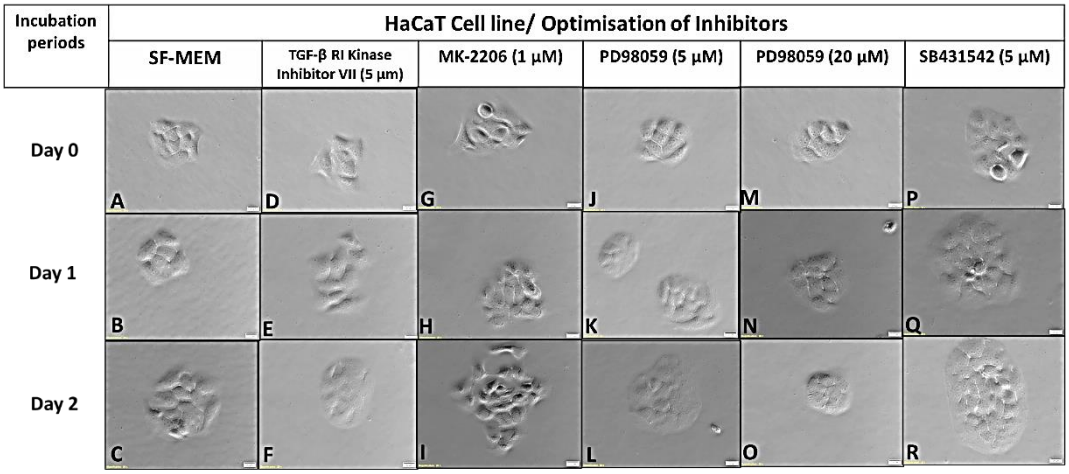


Figure 26: Optimisation of inhibitors using the HaCaT cell line.

Images of the HaCaT cell line with different inhibitors at time zero, 24 hours, and 48 hours. The test conditions included SF-MEM, 5 µM of TGF-β RI Kinase Inhibitor VII, 1 µM of MK-2206, 5 µM and 20 µM PD98059 and 5 µM of SB431542. The observation was based on evaluating colony appearance, cell morphology and cell death. Images of cells were taken at 200X magnification. No cell death was observed.

TYS Cell Line:

The cell shape and colony appearance of the TYS cell line, treated with test conditions including the four inhibitors, did not vary to that of SF-MEM group (**Figure 27**). TYS cells were observed attached and continued to grow with the test conditions similar to the HaCaT cell line. The DMSO was used alone at same concentration with the TYS cell lines, which showed no difference to the control group (data not shown).

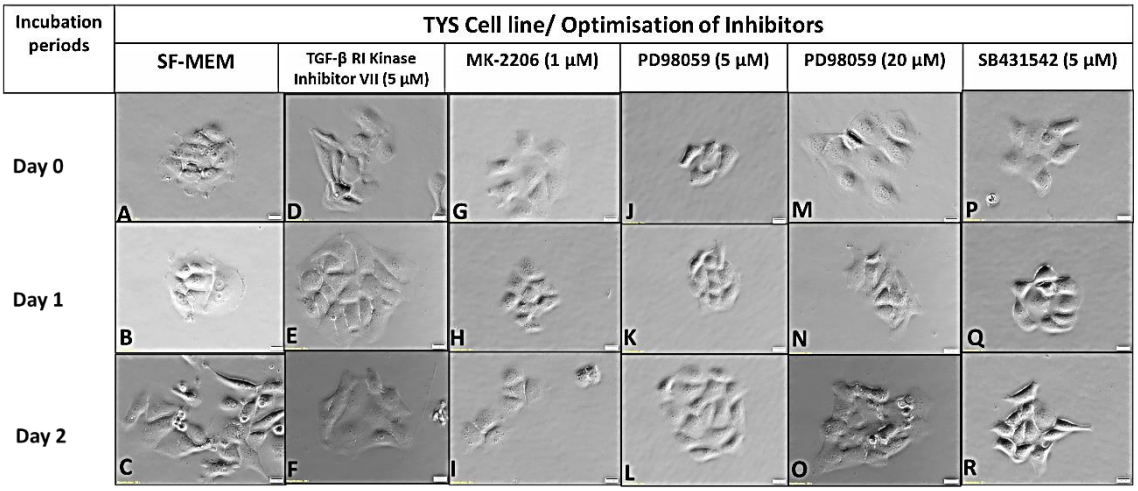


Figure 27: Optimisation of inhibitors using the TYS cell line.

Images of the TYS cell line with different inhibitors at time zero, 24 hours, and 48 hours. The test conditions included SF-MEM, 5 µM of TGF-β RI Kinase Inhibitor VII, 1 µM of MK-2206, 5 µM and 20 µM PD98059 and 5 µM of SB431542. The observation was based on evaluating colony appearance, cell morphology and cell death. Images of culture dishes were taken at 200X magnification. No cell death was observed.

SAS-H1 Cell Line:

With the SAS-H1 cell line, all test conditions with different inhibitors showed comparable results to the TYS and HaCaT cell lines (**Figure 28**) except samples with 20 µM of PD98059, where no attached cells were found (images not shown). Therefore, another experiment was carried out to evaluate the effect of different concentrations of PD98059 that did not cause cell death. SAS-H1 cells with 10 µM of PD98059 showed no signs of cells death or floating cells in the culture dish. The DMSO was used alone at same concentration with the SAS-H1 cell lines, which showed no difference to the control group (data not shown).

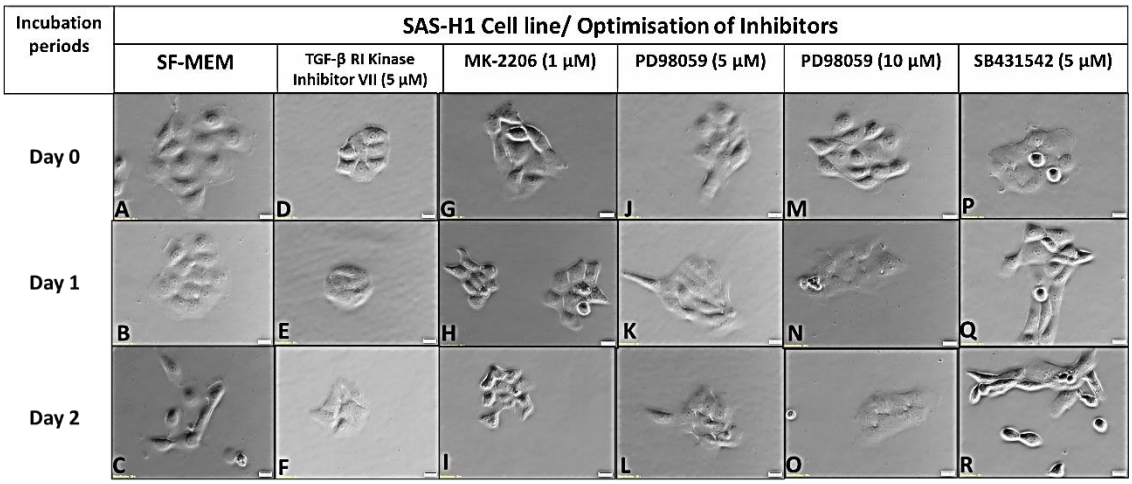


Figure 28: Optimisation of inhibitors using the SAS-H1 cell line.

Images of the SAS-H1 cell line with different inhibitors at time zero, 24 hours, and 48 hours. The test conditions included SF-MEM, 5 µM of TGF-β RI Kinase Inhibitor VII, 1 µM of MK-2206, 5 µM and 5 µM and 10 µM PD98059, and 5 µM of SB431542. The observation was based on evaluating colony appearance, cell morphology and cell death. Images of culture dishes were taken at 200X magnification. No cell death was observed.

To evaluate the inhibitory activity of PD98059, an experiment was designed with SAS-H1 cells. The object of this experiment was to investigate the ability of PD98059 to inhibit the migratory effect of TGFβ1. 5 µM and 10 µM of PD98059 equally blocked cells from scattering away from their colonies (**Figure 29**).

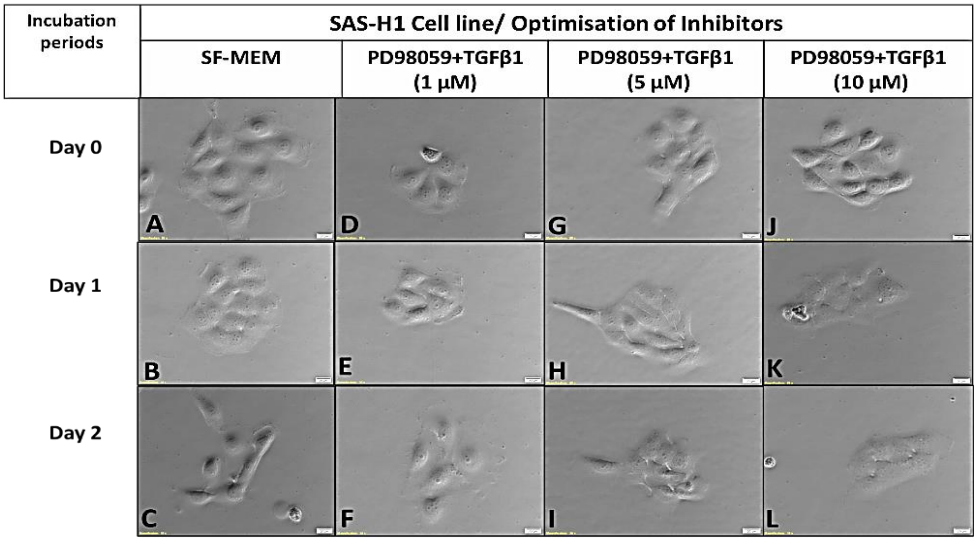


Figure 29: Optimisation of inhibitors using the SAS-H1 cell line for PD98059.

Images of the SAS-H1 cell line with different concentrations of PD98059 at time zero, 24 hours and 48 hours. The test conditions included SF-MEM, 10ng/ml of TGFβ + PD98059 (1 µM), 10ng/ml of TGFβ + PD98059 (5 µM) and 10ng/ml of TGFβ + PD98059 (20 µM) of PD98059. The observation was based on evaluating colony appearance, cell morphology and cell death. Images were taken at 200X magnification.

Findings:

The different dilutions of inhibitors caused no signs of cell death or alteration in cell morphology with the three cell lines except for SAS-H1 with 20 µM of PD98059. Culture dishes of SAS-H1 treated with the inhibitor showed no cells remained attached to the culture dish and a lot of debris was found, which suggested cell death. However, samples with 5 µM of PD98059 showed similarity to the control group regarding cell morphology and colony appearance.

Chapter 4: Investigation of Cell migration in Oral Cancer Cell Lines

4.1 Background

4.1.1 Cell Migration:

The ability of cells to migrate to new sites has been utilised in various physiological and pathological processes by different types of cells (Lauffenburger and Horwitz, 1996; Detchokul and Frauman, 2014). These processes include embryogenesis, inflammation, immune response, wound healing, angiogenesis and tumour metastasis. The fundamental requirement for cells to migrate is to gain a polarised morphology, which is represented by cells having a leading edge and trailing end (Lauffenburger and Horwitz, 1996; Hall, 2009). This change is followed by the formation of cell membrane extensions, mainly at the leading edge in the cell front, known as lamellipodia and filopodia. Both of these cell membrane extensions have distinct characteristics. Lamellipodia are described as sheet-like extensions, whereas filopodia are needle-like projections. Neither of these structures contain cytoplasmic organelles, but they are rich in actin and actin-associated proteins. During cell migration, cells are stabilised by forming new focal adhesions at the leading edge first, which continue to be formed toward the rear end of the cell. Two types of forces are needed to generate a locomoting cell, first, the generation of a protrusive force to produce cell membrane extensions, which depends upon actin polymerisation and structural organisation. The second force is needed to move the cell, which depends upon an active myosin-based motor, in a forwards direction, which is a called contractile force (Lauffenburger and Horwitz, 1996).

4.1.2 Modes of Cell Migration:

There are two modes of cell migration, single cell migration and collective cell migration (**Figure 30**) (Rørth, 2009).

1) **Single Cell Migration:**

Single cell migration depends upon a polarised morphology, creating cell membrane extensions by actin polymerisation and establishing focal adhesions to the stratum, which allows generation of protrusive and contractile forces (Rørth, 2009). The different types of single cell migration include blebby amoeboid migration, pseudopodal ameboid migration and mesenchymal migration (Friedl and Wolf, 2003; Lämmermann and Sixt, 2009).

2) Collective Cell Migration:

Collective cell migration describes cells moving together, while maintaining contact or being tightly connected. Therefore, an essential link has been established between cell movement and cell to cell contact in collective cell migration. The importance of collective cell migration can be observed in maintaining an intact tissue, while remodelling and the ability of motile cells to drag non-motile cells (Rørth, 2009). Studies report different types of epithelial collective cell migration such as sheet, sprouting or branching (Rørth, 2009) and collective migration of mesenchymal cells (Yilmaz and Christofori, 2010; Theveneau and Mayor, 2011).

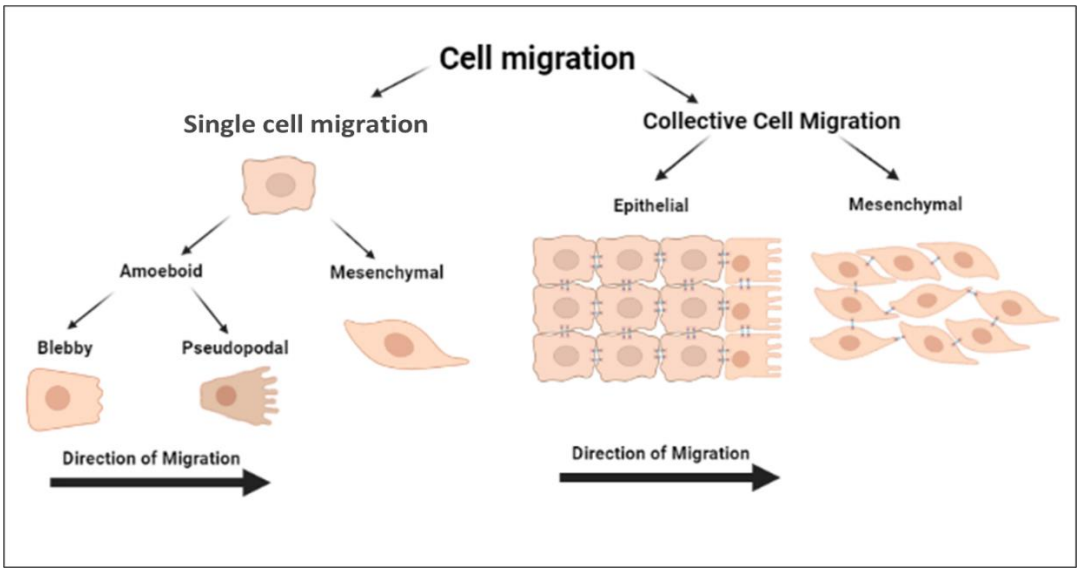


Figure 30: Different modes of cell migration.

This diagram shows an illustration of the different types and subtypes of modes of cell Migration *Created with BioRender.com (Thijssen – van Loosdregt* et al.*, 2021)***.**

4.2 Aims and Hypothesis

4.2.1 Aims:

The specific aim of the cell migration assays was to evaluate the morphological change and alteration in colony appearance related to cell migration of normal and oral cancer cell lines.

4.2.2 Objectives:

The assessment of cell migration was carried out to investigate the effect of TGFβ1 and EGF on normal and oral cancer lines. Also, the assessment included an identification of the mode of cell migration induced by the different test conditions. Finally, different inhibitors for specific signalling pathways e.g Smad, PI3K-Akt, MAKP Erk1/2 and Rho-GTPase were added to the assays, in addition to the growth factors, to explore their influence on the migration of normal and oral cancer cell lines.

4.2.3 Hypothesis:

It was hypothesised that TGFβ1 induces oral cancer cells to migrate via EMT either by single or individual cell migration or by collective cell migration. Also, that this process is regulated mainly by the Smad signalling pathway.

4.2.4 Null Hypothesis:

TGFβ1 does not induce cell migration via EMT in normal and oral cancer cell lines.

4.3 Materials and Methods

To assess the ability of cells to migrate under the effect of certain test conditions, three different assays were used. Each of the three assays gave insight into cell behaviour in terms of morphology and motility in response to the specific test conditions.

Cell Migration Assays:

Scatter assay, Gap closure assay and Scratch assay were used to observe the migratory effect of the test conditions on the normal and oral cancer cell lines.

4.3.1 Scatter Assay:

The Scatter assay is used to assess cell migration under different culture conditions, with the addition of exogenous growth factors and inhibitors. The basic premise of cell migration by epithelial cells is that cells lose adhesion to their neighbouring cells and change their cytoskeletal structure to allow them to move away from their colonies. Therefore, the assessment of cell migration using the Scatter assay is based upon four criteria, which included scattered cells (already migrated and separated from their colonies), changes in cell morphology (compared to the usual features of epithelial cells), colony appearance and the distance between neighbouring cells within the same colony (**Figure 32**).

Materials for Scatter Assay Protocol:

All materials used to culture cells in this assay were mentioned in the general materials (Pages 41 - 47). The test conditions included:

SF-MEM				
TGFβ1	TGFβ1 + TGF-β RI Kinase Inhibitor VII	TGFβ1 + MK-2206	TGFβ1 + PD98059	TGFβ1 + SB431542
EGF	EGF + TGF-β RI Kinase Inhibitor VII	EGF + MK-2206	EGF+ PD98059	EGF + SB431542

Scatter Assay Protocol:

Cells were seeded by the cell culture protocol that been mentioned in the general materials and methods (Pages 41 - 43). The plating density for cells in this assay was 5 x 10^4 per 60 mm dish, which allowed for the formation of small colonies. After passaging, cells were left overnight to grow before incubating them in serum free medium for 24 hours, which is known as serum starvation. Serum starvation is important step to eliminate the influence of the serum on the cells.Test conditions were prepared and added to the culture dishes, they were assessed at time zero, 24 hours or Day 1 and 48 hours or Day 2. Photomicrographs were taken using light microscope IX70 and camera XM10 (**Figure 31**).

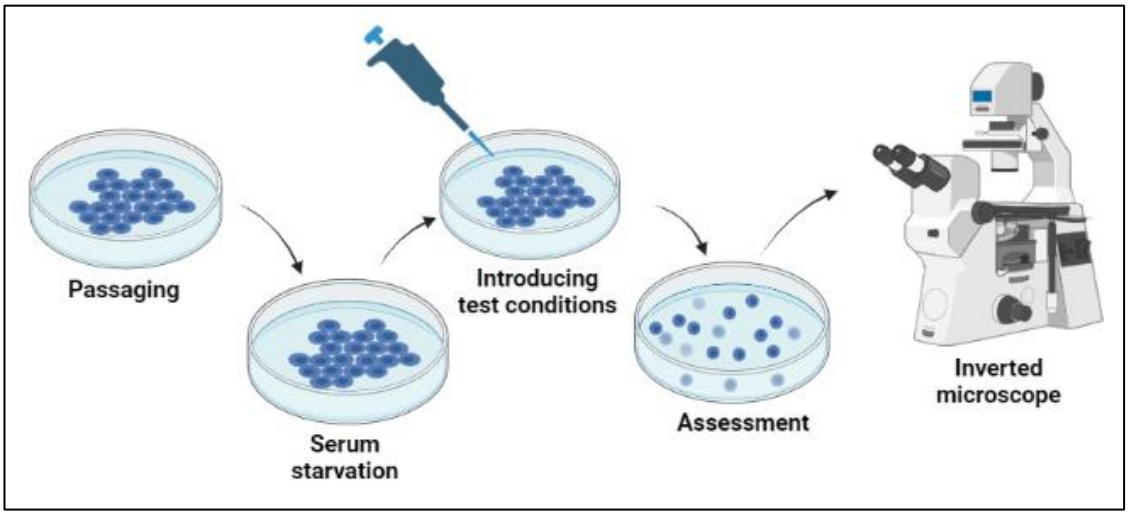


Figure 31: Scatter assay protocol and assessment.

The figure was created in Biorender.com.

Assessment of the Scatter Assay

As described earlier, four criteria were established to evaluate the status of cells regarding migration, after incubation with the test conditions. Photomicrographs were taken at 200x magnification at the assessment time. This magnification allowed capture of one or two colonies per frame with clear details of both colonies and cells (**Figure 32**).

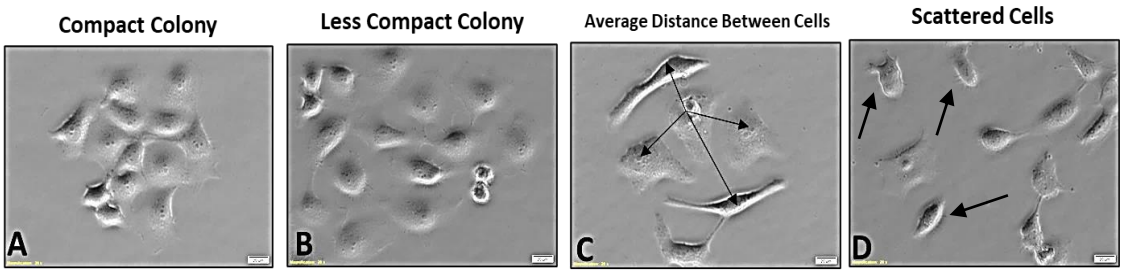


Figure 32: Scatter Assay observation and assessment protocol.

Four criteria were used to assess both cell migration and cell morphological changes. Picture (**A)** shows a compact colony of cells, picture (**B**) shows a less compact colony, picture (**C**) shows examples of the average distance between cells of the same colony using software called Image J and picture (**D**) shows scattered cells and changes in their morphology. All pictures for scatter assay experiments were taken at 200x magnification.

Protocol of Using ImageJ Software:

The assessment of the distance between neighbouring cells within the same colony, was carried out using the following protocol:

1- The ImageJ software was started followed by choosing File and Open to select an image.
2- To set the scale in µm, Analyze and Set Scale were selected, as a result a small new window came up. This window contained Distance in Pixels which must be 181.1667, known distance which must be 20, and unit of length which must be µm. The option Global was selected to save the setting for the whole session.
3- The Straight button (line shape) was selected from the measuring tools bar. By pressing line option, a line between the reference cell to the targeted cells was generated. The Analyze option was selected then Measure to show the measurements in a new window.
4- Data were saved in an Excel spreadsheet.

To evaluate the reproducibility of the measurements with this protocol, interclass and intraclass correlation for agreement were carried out using R software. Interclass correlation analysis showed an agreement at 0.986. In addition, intraclass correlation analysis showed an agreement at 0.94.

4.3.2 Gap Closure Assay:

The Gap closure assay is a wound healing assay that measures the cell-free area throughout different incubation times and different test conditions. This assay,

however, differs from the Scratch assay in two ways. Firstly, the cell-free zone is created by a barrier between the two chambers of the inserts, which causes no cell damage, whereas in the scratch assay, the gap is created with a scratch tool, which causes damage to cells. Secondly, the diameter of the cell-free area in the Gap closure assay is around 500 µm, which is suitable for the short incubation times with test conditions and reproducibility. It is difficult to reproduce the same diameter of the cell-free zone in the scratch assay (**Figure 33**). This was due to the use of a scratch tool to create the cell-free zone, which makes it hard to reproduce the size of cell-free area. Therefore, the Scratch assay is less accurate than the gap closure assay in terms of reproducibility. However, the scratch creates a larger cell-free zone, which could be utilised for longer incubation periods with the test conditions.

Materials:

Materials and methods used to culture the cells used in the Gap closure assay were previously described on pages 41 - 43 of the materials chapter (**Table 5**) except for the inserts. In this experiment, we used Culture-insert 2 wells, which were made of biocompatible silicone. The insert consists of two chambers separated by a wall which creates the cell-free zone. The width of the cell-free zone was around 500 µm. Materials were used in this assay mentioned in (

Table 10).

No.	Reagents	Company	Catalogue No.
1	Human recombinant Transforming growth factor β1 (10 ng/ml)	Sigma-Aldrich, St. Louis, MO, USA	T7039
2	Human recombinant Epidermal growth factor	Gibco-Thermo Fisher, Grand Island, New York, USA	10450-013
3	Culture-insert 2 wells	Ibidi cells in focus, Martinsried, Germany	81176

Table 10: Specific materials used for the Gap Closure Assay

Methods:

The experiment was designed to put five different inserts into one 90mm culture dish per test condition (**Figure 33**). Three inserts were randomly selected for assessment.

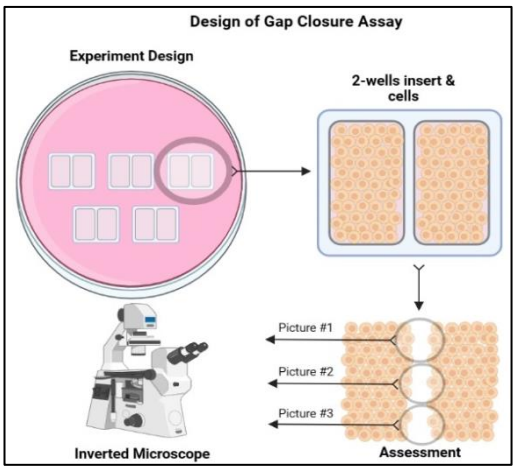


Figure 33: Design of experimental dishes with inserts for the Gap closure assay.

The diagram shows the design of the Gap closure assay experiment which includes placing and labelling five inserts in one 90mm dishes. The next step involved choosing three random inserts out of the five for assessment. The assessment include taking pictures from the three different areas of the insert top part: (#1,#2 and #3) at 40x Magnification.. Finally, assessment of the gap was completed by observation and use of Image J software to calculate how much of the gap remains.

Protocol for Gap Closure Assay

Cells were seeded into the inserts with at a cell density of 5 x 10^5 cells/ml in 90mm culture dishes. Once the cells reached 100% confluency within the chambers, the inserts were removed and the medium changed to SF-MEM for serum starvation. This step was followed by introducing the test conditions to the culture dishes and assessing them by taking photomicrographs at time zero, an hour, two hours, six hours and 24 hours. All photomicrographs were taken at 40X magnification (**Figure 34**).

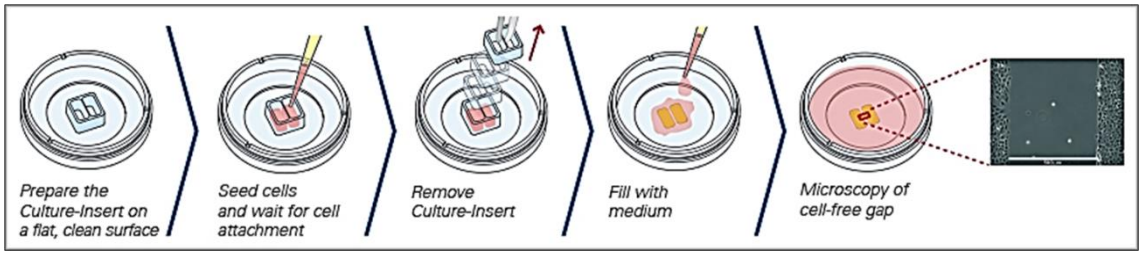


Figure 34: Gap closure assay protocol.

This figure shows the steps for the Gap closure assay protocol that was used to assess cell migration (ibidi, 2021). The permission to use this diagram has been granted by courtesy of ibidi.

Assessment of the Gap Closure Assay

The assessment of this assay was based on measuring the cell-free zone in multiple test conditions, at different incubations periods (**Figure 35**). ImageJ software was used to measure the cell-free zone by assessing three images of three different inserts. Therefore, the total number of samples was nine for each test condition, for each incubation period. Evaluating cell morphology and mode of migration was carried out by observing cells particularly those at the edges of the cell-free zone.

$$\boldsymbol{Cell\ free\ zone\ at\ specific\ time} = \left(\frac{\boldsymbol{Area\ of\ the\ gap\ at\ specific\ time}}{\boldsymbol{Area\ of\ gap\ at\ time\ zero}}\right) * \mathbf{100}$$

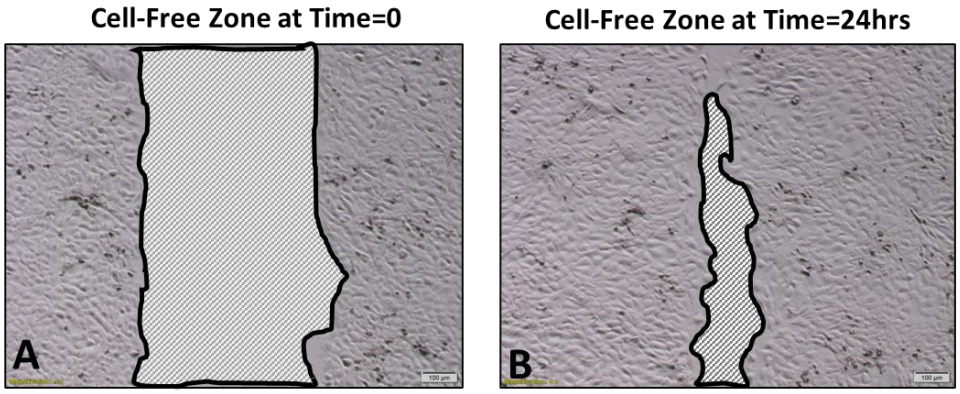


Figure 35: Demonstration of Cell-free assessment with the Gap closure assay.

This figure highlights the gap created by the **insert at time zero** before introducing the test conditions where the TYS cells were treated with Serum Free MEM only. **Picture (A)** shows the gap (highlighted area) at the start of the experiment, where time=0. **Picture (B)** illustrates how much of the gap remained (highlighted area) after the TYS cells were treated with 10ng/ml of TGFβ1 for 24 hours. This indicates that the growth factor induced the TYS cells to migrate into the cell free zone and resulted in a smaller gap compared to the start of experiment.

4.3.3 Scratch Assay:

Another method for evaluating cell migration, particularly the collective mode, is the Scratch assay (also known as the wound healing assay). The basic concept behind this assay, identical to the gap closure assay, is to monitor a cell-free zone over time, under various test conditions. The cell-free area was established in this assay using a scratch tool. The cell-free zone was measured at the beginning and end of the experiment (**Figure 37**).

Materials:

All materials were used to culture cells in Scratch assay were previously described on pages 41-43 of the materials chapter (**Table 5**). In this experiment,

cells were incubated with eleven different test conditions including inhibitors. The test conditions included:

SF-MEM				
TGFβ1	TGFβ1 + TGF-β RI Kinase Inhibitor VII	TGFβ1 + MK-2206	TGFβ1 + PD98059	TGFβ1 + SB431542
EGF	EGF + TGF-β RI Kinase Inhibitor VII	EGF + MK-2206	EGF+ PD98059	EGF + SB431542

Protocol for Scratch Assay

Cells were seeded into 60mm culture dishes with at a cell density of 5 x 10^5 cells/dish. Once cells reached 100% confluency, the medium was changed to SF-MEM for serum starvation for 24 hours. Afterwards, the scratch was created using a scratch tool. The culture dishes were washed twice with SF-MEM to remove all detached cells. The scratch was evaluated and a specific area within the scratch, which was marked for assessment. This step was followed by introducing the test conditions to the culture dishes and assessed by taking photomicrographs at time zero and 24 hours. All photomicrographs were taken at 40X magnification (**Figure 36**).

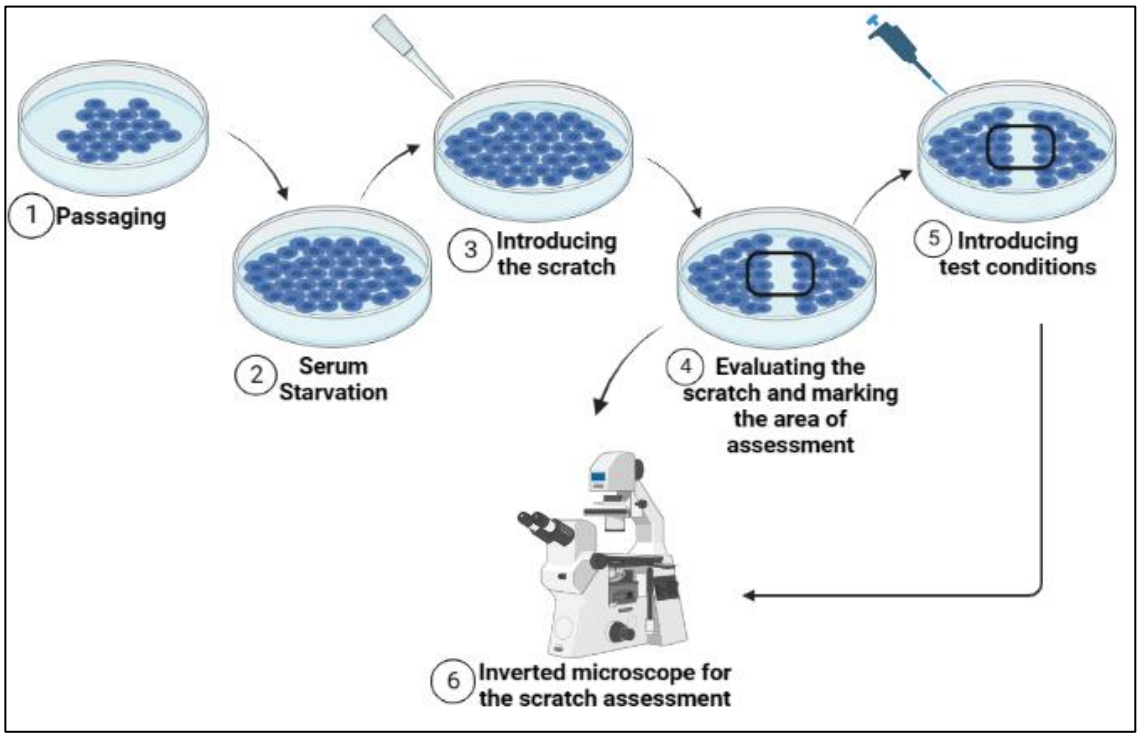


Figure 36: Scratch assay protocol and assessment.

Figure was created in Biorender.com.

Assessment of Scratch Assay

Throughout the experiment, measurement were made with ImageJ software by analysing photomicrographs taken from the specified areas of the cell culture dishes. Additionally, by analysing the cells in the gap, this assessment helped to determine the type of cell migration i.e. single cell migration or collective cell migration.

$$\boldsymbol{Cell\ free\ zone\ at\ specific\ time} = \left(\frac{\boldsymbol{Area\ of\ the\ gap\ at\ specific\ time}}{\boldsymbol{Area\ of\ gap\ at\ time\ zero}}\right) * \mathbf{100}$$

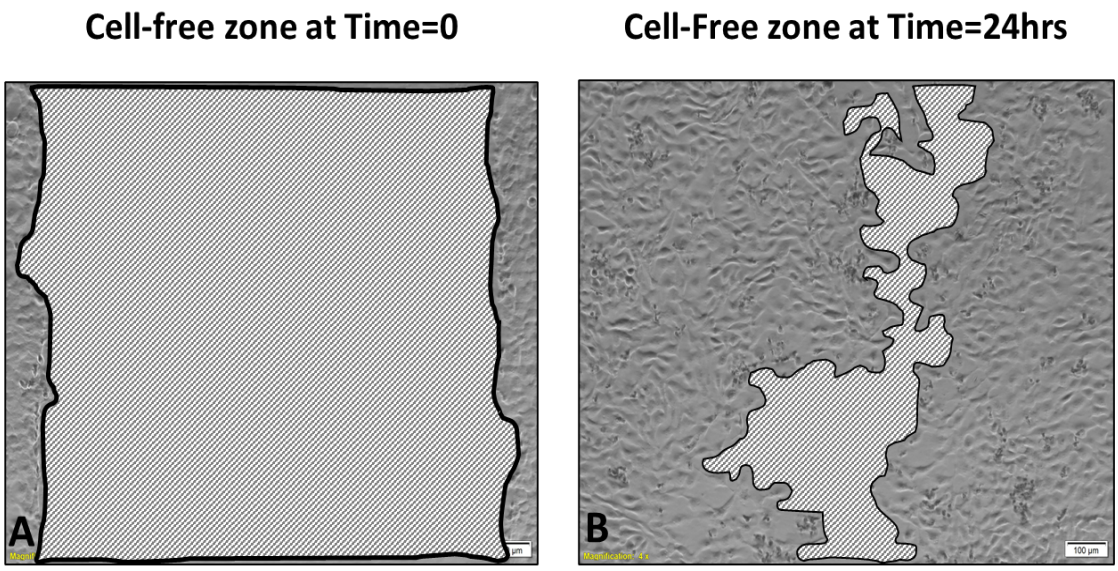


Figure 37: Demonstration of Cell-free assessment with the Scratch assay.

The figure shows a scratch assay experiment with the TYS cell line at two different incubation periods (At time = 0 and after 24 hours with test conditions). The cell free area (Highlighted in **Picture A**) was created with a 1000µl pipette tip after the cell were 100% confluent. **Picture B** shows cells migrated into the gap after 24 hours in the test conditions with a small cell-free area remaining. Both pictures were taken from the already marked area on the culture dish at **40X magnification**.

4.4 Results of Migration Assays:

To evaluate the migratory effect of the different test conditions on the normal and oral cancer cell lines, three types of assays were performed the Scatter assay, the Gap closure assay and the Scratch assay. These three distinct tests were carried out to assess the ability of cells to migrate as a result of the various test conditions applied. Each of the three migration assays provided insight to the behaviour of the cells in terms of morphology and motility.

4.4.1 HaCaT Cell Line

(i) **Scatter Assay:** The Scatter assay was used to investigate the effect of the test conditions on the HaCaT cell line. The assessment was carried out at two incubation periods, 24 hours and 48 hours, with eleven growth conditions (**Table 11**). The evaluation of the Scatter assay was based on four criteria: (1) the presence of dispersed cells, (2) differences in cell morphology compared to the typical characteristics of epithelial cells, (3) the shape of the colony and (4) the distance between neighbouring cells within the same colony (**Figure 38**).

(1) **Negative Control (Serum free MEM):** The HaCaT cells were tightly packed together in colonies, mimicking epithelial cell characteristics including a cobblestone appearance. No morphological changes in the cells or their colonies were observed at 24 hours and 48 hours (**Figure 38-A&B**). The average distance between the cells ranged from 22 µm to 27 µm (n=5).

(2) **10ng/ml of TGFβ1:** After 24 hours of exposure to the growth factor, cells began to display morphological changes, such as less-compact colonies and less cobblestone-like appearance. After 48 hours, these changes were more noticeable. In comparison to the negative control group, where cells were tightly packed, colonies of cells in the cultures treated with exogenous TGFβ1 had colonies less compact and larger cells, which had morphological changes without breaking cell-cell adhesions. As the cells were pulled in all directions, these morphological changes caused the cells to appear as large spherical cells (**Figure 38- C**). Single cells were

observed as a result of full dissociation from their colonies, especially after 48 hours incubation with the growth factor. Furthermore, when compared with results from 48 hours to the baseline measurements at time zero (**Figure 38- D**), the average distance between cells increased from 32 µm to 61.5 µm (5=n).

(3) **10ng/ml of EGF**: There was no evidence of any morphological changes or migrated cells after adding 10ng/ml of EGF to the HaCaT cell line, either at 24 hours or 48 hours. Cell morphology and colony appearance were almost identical to the negative control group at both incubation periods (**Figure 38- G&H**). Furthermore, the average cell-to-cell distance was around 35 µm (n=5).

(4) **10ng/ml of TGFβ1 plus 5µM of TGF-β RI Kinase Inhibitor VII:** The appearance of cells and colonies resembled the control group. Cells were in closely compact colonies with no morphological changes after 24 and 48 hours (**Figure 38- E&F**). Moreover, the measurements of the average cell-to-cell distance (27 µm to 32 µm) were similar to the average of cell-to-cell distance in control group (n=5). This indicates that the effect of the growth factor was blocked by the inhibitor, resulting in no changes in cell shape or appearance of the colonies at both incubation periods.

(5) **10ng/ml of EGF plus 5µM of TGF-β RI Kinase Inhibitor VII:** TGF-β RI Kinase Inhibitor VII with EGF when added to the cell cultures had no influence on cell morphology, colony appearance or the distance between cells (**Figure 38- I&J**). These results were comparable to addition of EGF alone and to the SF-MEM groups.

(6) **10ng/ml of TGFβ1 plus 1µM of MK-2206 (Akt Inhibitor):** Individual cells were observed after 48 hours of incubation with the Akt inhibitor (MK-2206) and TGFβ1 (**Figure 38- L**). After 24 hours in the test conditions, the average distance between cells increased from 23 µm, at the start of the experiment, to 39 µm (5=n). These findings suggested that an alteration in the colony pattern occurred at 24 hours and single cell migration occurred

after 48 hours. However, the morphological changes with these conditions differed from the changes that occurred to the cells in response to TGFβ1 alone. This result suggests an alternative migration mode or different mechanisms might be activated.

(7) **10ng/ml of EGF plus 1μM of MK-2206 (Akt Inhibitor):** There were no signs of migratory cells in the culture dishes with this test condition. However, cell shape and colony appearance seemed to be different to the negative control group or with EGF alone after 48 hours incubation (**Figure 38- Q&R**). Also, a slight increase in the average distance between cells was observed, although there was no individual cell migration.

(8) **10ng/ml of TGFβ1 plus 5μM of PD98059 (MAPK MEK1 Inhibitor):** In this condition, cells changed their shape to become large and round in appearance. These cells within the colonies were arranged differently compared to the control group, which resembled the TGFβ1 group (**Figure 38- M&N**). Moreover, the increase in the average distance between cells was significantly higher than in the control group and at time zero. This indicate that the MAPK pathway dose not regulate cell migration that is initiated by adding TGFβ1 to the HaCaT cells.

(9) **10ng/ml of EGF plus 5μM of PD98059 (MAPK MEK1 Inhibitor):** No signs of any morphological change were observed, the cells appeared similar to the SF-MEM and EGF alone groups (**Figure 38- S&T**).

(10) **10ng/ml of TGFβ1 plus 5μM of SB431542 (Smad Inhibitor):** With the Smad inhibitor, the effect of TGFβ1 was blocked and the samples appeared similar to those with TGFβ1 and TGFβ-RI Kinase Inhibitor VII (**Figure 38- O&P**). This result indicates that the Smad pathway has control over the morphological changes of HaCaT cell line as a result of adding TGFβ1.

(11) **10ng/ml of EGF plus 5μM of SB431542 (Smad Inhibitor):** No signs of any morphological change were observed in this test condition,

which was similar result to both SF-MEM and EGF alone groups (**Figure 38- U&V**).

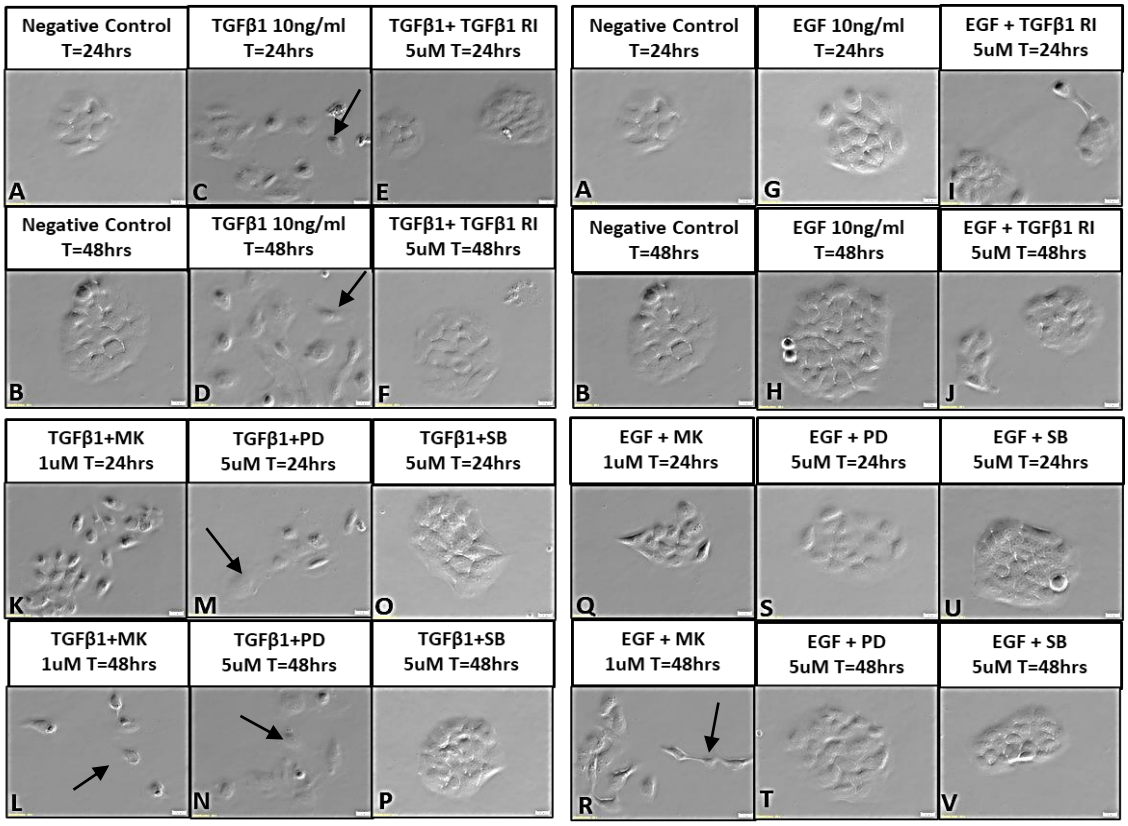


Figure 38: Observational assessment of the HaCaT cell line migration with the Scatter assay.

The assessment was carried out using the Scatter Assay protocol with different test conditions at 24 hours and 48 hours. The observation was based on evaluating colony appearance, cell morphology and the presence of scattered cells. Also, for this observation, pictures of all samples were taken using specific criteria, which includes 200X magnification and one colony or maximum of two small colonies. **Pictures (A)** and **(B)** represent the negative control samples at 24 hours and 48 hours respectively. **Pictures (C)** and **(D)** represent TGFβ1 treated cells which showed morphological changes and alteration in colony appearance. Also, these changes were seen with TGFβ1+PD98059 at 24 hours and 48 hours pictures **(M)** and **(N)**. In addition**,** TGFβ1+MK-2206 at 48 hours **(L)** and EGF+MK-2206 at 48hours **(R)** showed cell migration.

***Table 11: Summary of the Scatter assay results with the HaCaT cell line*.**

Pictures of each assay were taken at three different incubation periods (Time=zero, 24 hours and 48 hours). All pictures were taken at 200X magnification. Three criteria were used to assess the cell morphology and migration status. **(-)** refers to no significant change compared to time zero. Change in cell morphology or scattered cells referred to as (present), while alteration in colony appearance was referred to as (changed). Raw data can be found in appendix1 (page 298).

HaCaT Cell line	**Cell Morphology and Migration (Scattering)**			**Colony Appearance**			**Average of five Measurements Distance between Cells in µm**		
Test Conditions	**T=0**	**T=24hrs**	**T=48hrs**	**T=0**	**T=24hrs**	**T=48hrs**	**T=0**	**T=24hrs**	**T=48hrs**
Negative Control	_	_	_	_	_	_	22.3	23.2	26.1
TGFβ1	_	Present	Present	_	Changed	Changed	32.3	36.6	**61.5***
EGF	_	_	_	_	_	_	27.6	36.6	34.5
TGFβ1+5µM TGF-β RI Kinase Inhibitor VII	_	_	_	_	_	_	31.9	24.1	27.3
EGF+5µM TGF-β RI Kinase Inhibitor VII	_	_	_	_	_	_	22.2	25.7	22
TGFβ1+1µM MK2206	_	Present	Present	_	Changed	Changed	23.1	**39.7***	33.8
EGF+1µM MK2206	_	_	Present	_	_	Changed	20	23.1	**48.3***
TGFβ1+5µM PD98059	_	Present	Present	_	Changed	Changed	28.6	36.9	**67.68***
EGF+5µM PD98059	_	_	_	_	_	_	35.8	26.8	26.2
TGFβ1+5µM SB431542	_	_	_	_	_	_	34.3	30.9	24.3
EGF+5µM SB431542	_	_	_	_	_	_	34.4	23.8	17.2

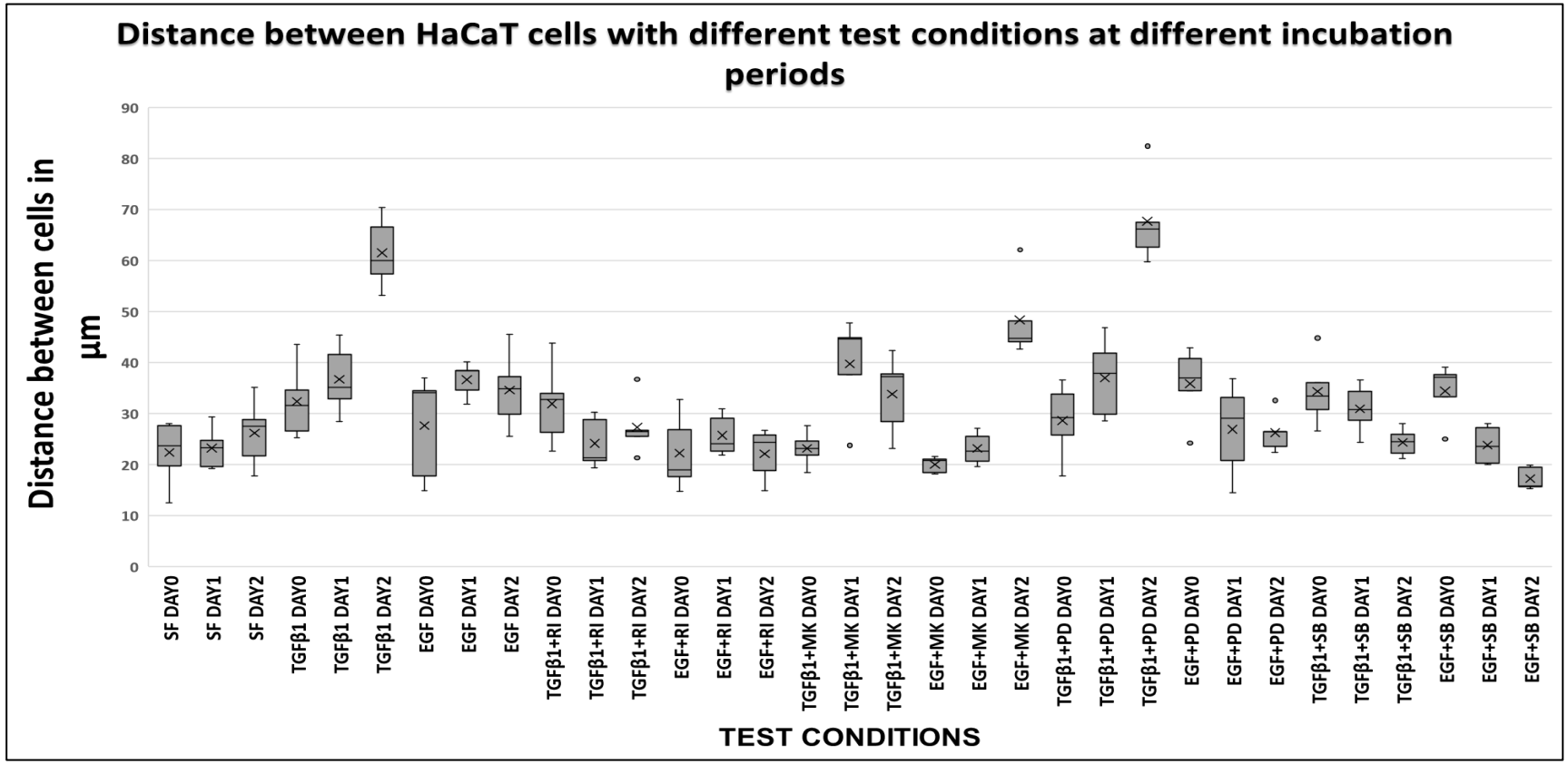


Figure 39: Scatter Assay assessment for the HaCaT cell line using the distance between cells.

The assessment was carried out by measuring the average cell-cell distance in µm. Each of the eleven test conditions was assessed at three different incubations periods Time=0, 24 hours and 48 hours. The assessments were based on pictures taken at 200X magnification. The protocol of measuring includes not less than three readings, but not more than five, using Image J software. Both mean and standard deviation of these samples were calculated to generate the graph. Only four test conditions showed an increase in the average distance between cells, which includes TGFβ1 at 48 hours, TGFβ1+MK2206 at 24 hours, EGF+MK2206 at 48 hours and TGFβ1+PD98059 at 48hours. Raw data can be found in appendix1 (page 298).

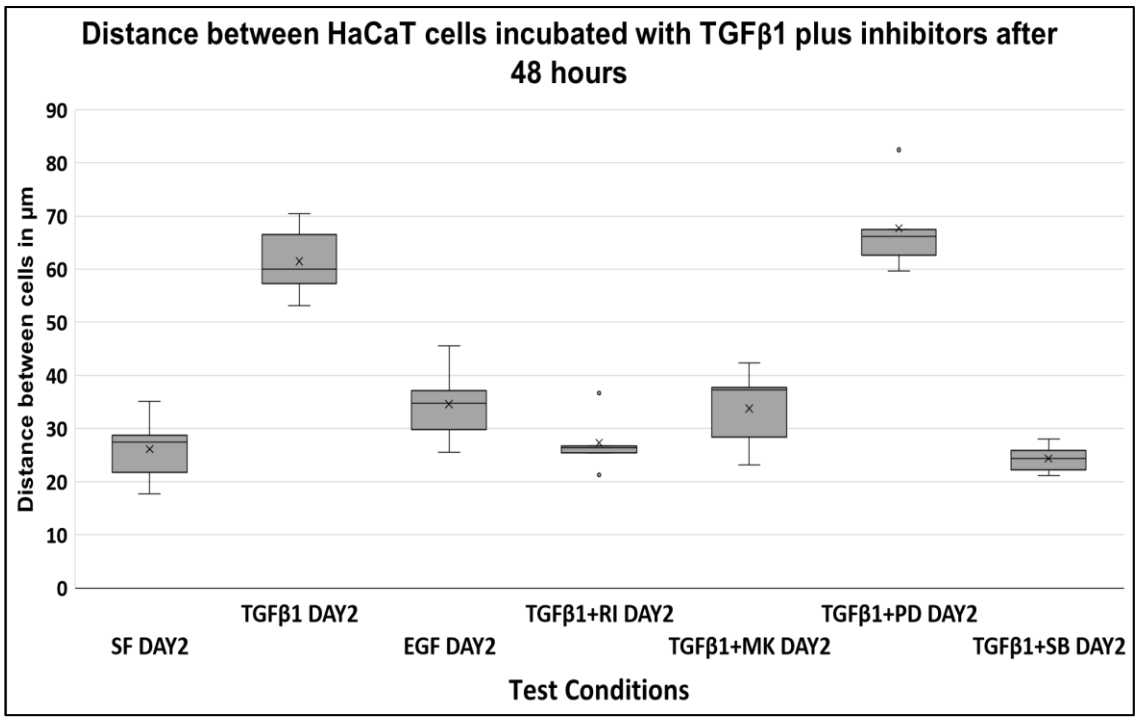


Figure 40: Scatter assay assessment for the HaCaT cell line after 48 hours with test conditions.

This assessment was carried out by measuring the average cell-cell distance in µm. Seven of test conditions were shown in this graph. These test conditions were SF-MEM after 48 hours, TGFβ1 after 48 hours, EGF after 48 hours, TGFβ1+ TGF-β RI Kinase Inhibitor VII after 48 hours, TGFβ1+MK-2206 after 48 hours, TGFβ1+PD98059 after 48 hours and TGFβ1+SB431542 after 48 hours. Raw data can be found in appendix1 (page 298).

(ii) **Gap Closure Assay:** The Gap closure assay was utilised to investigate the ability of TGFβ1 and EGF to induce cell migration. The cell migration was manifested by cells moving toward the gap over a specific period of time. This assay was also used to determine the mode of cell migration, either single or collective cell migration, by observing cells at the edge of the gap (**Figure 42**).

(1) **Negative Control (Serum-Free MEM):** The assessment of the cell-free area at specific incubation periods showed almost no significant change in size of the gap, which indicated no cell migration had occurred. After 24 hours, it was noticed that the size of the gap became less, compared to the previous incubation periods. However, most of the cell-free area was still present after 24 hours with serum-free MEM, which suggests that no cells had migrated into the gap. In addition, no individual cells or changes in cell morphology were observed at either edge of the gap (**Figure 42-E**).

(2) **10ng/ml of TGFβ1:** After specific incubation periods (an hour, two hours, and six hours), measurements of the cell-free area were similar to serum-Free MEM. However, most of the gap was covered by cells after 24 hours, which was not the case with the control, serum-Free MEM at the same incubation period. This suggested that the growth factor induced cells to migrate into the cell-free area at 24 hours. No single cells were observed at the gap or close to the leading front of sheets of cells, which indicates cells used a collective mode of migration to close the gap (**Figure 42-J**).

(3) **10ng/ml of EGF:** In contrary to other test conditions, the assessment of cultures incubated with 10ng/ml EGF, showed a slight increase in the cell-free area at an hour, two hours and six hours incubation periods compared to time zero (baseline). This increase in the size of the gap was not observed in both control and TGFβ1-treated culture. However, the measurements at 24 hours showed that the cell-free area was almost completely covered by cells, which was comparable to the readings from samples with TGFβ1. No individual cells were observed in the gap,

resembling the results with TGFβ1, implying that cells migrated collectively to the cell-free area after 6 hours (**Figure 42- O**).

Table 12: Summary of the Gap closure assay results for the HaCaT cell line.

The cells were incubated with the test conditions (SF-MEM, 10ng/ml TGFβ1 and 10ng/ml EGF) at different incubations periods (time zero, one hour, two hours, six hours and 24 hours). This observational assessment was based on three criteria used to describe the status of the gap which are open or (-) describe the gap at the beginning of the experiment, partially closed which indicates there is change in the size of the gap but still open and finally gap completely closed.

Test Conditions	T=0	T=1hr	T=2hrs	T=6hrs	T=24hrs
Serum free MEM	_	_	_	_	Partially closed
TGFβ1	_	_	_	_	Partially closed
EGF	_	_	_	_	Partially closed

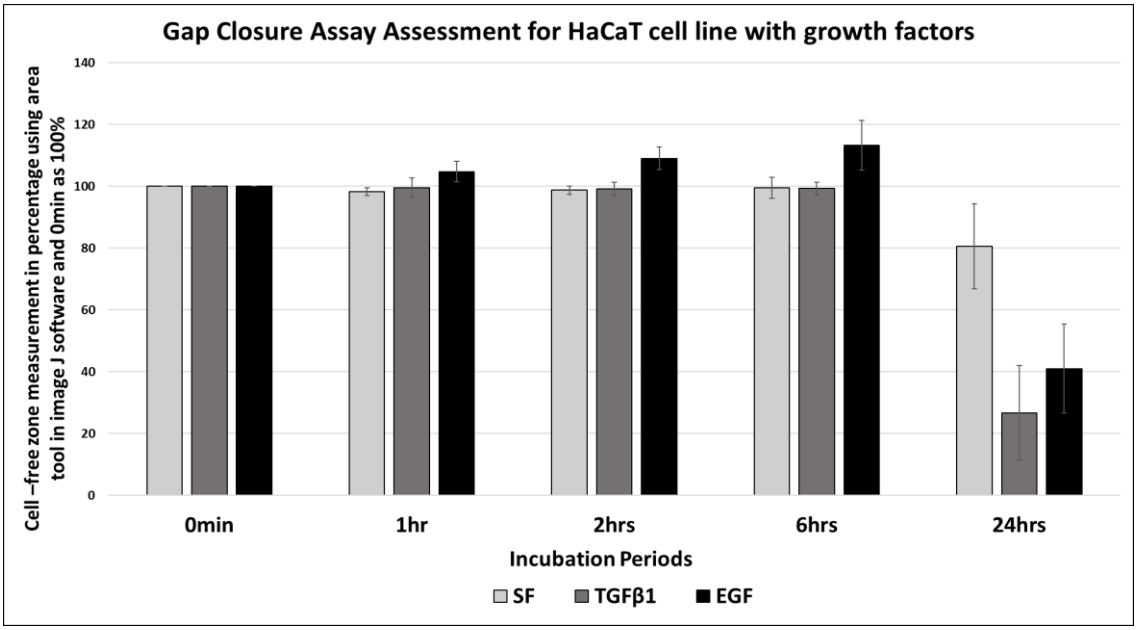


Figure 41: Assessment of the Gap closure assay with inserts for the HaCaT cell line.

Cells were incubated with either the negative control (SF-MEM alone) or with growth factors (10ng/ml TGFβ1 & 10ng/ml EGF) at incubations periods (time zero, one hour, two hours, six hours and 24 hours). The graph illustrates how much of the gap remains after the different incubation periods with the three test conditions. Measurements of the cell-free area were calculated using Image J software on images taken at 40X magnification. The formula for calculating the cell free zone was designed using a tool in the software called Area. The value of the Area was then transformed to a percentage, to compare the change in the size of the gap over the different incubation periods for the same sample. Data for the cell-free area at Time=zero was considered as 100%. The mean and standard deviation of nine measurements in total were calculated per test condition at the same incubation periods. Each test condition had five inserts, three of them were chosen randomly, before introducing the test conditions. The next step was three pictures were taken for each insert at specific incubation periods. This graph shows the significant decrease in the cell-free area with TGFβ1 and EGF at 24 hours. Raw data can be found in appendix 2 (page 303).

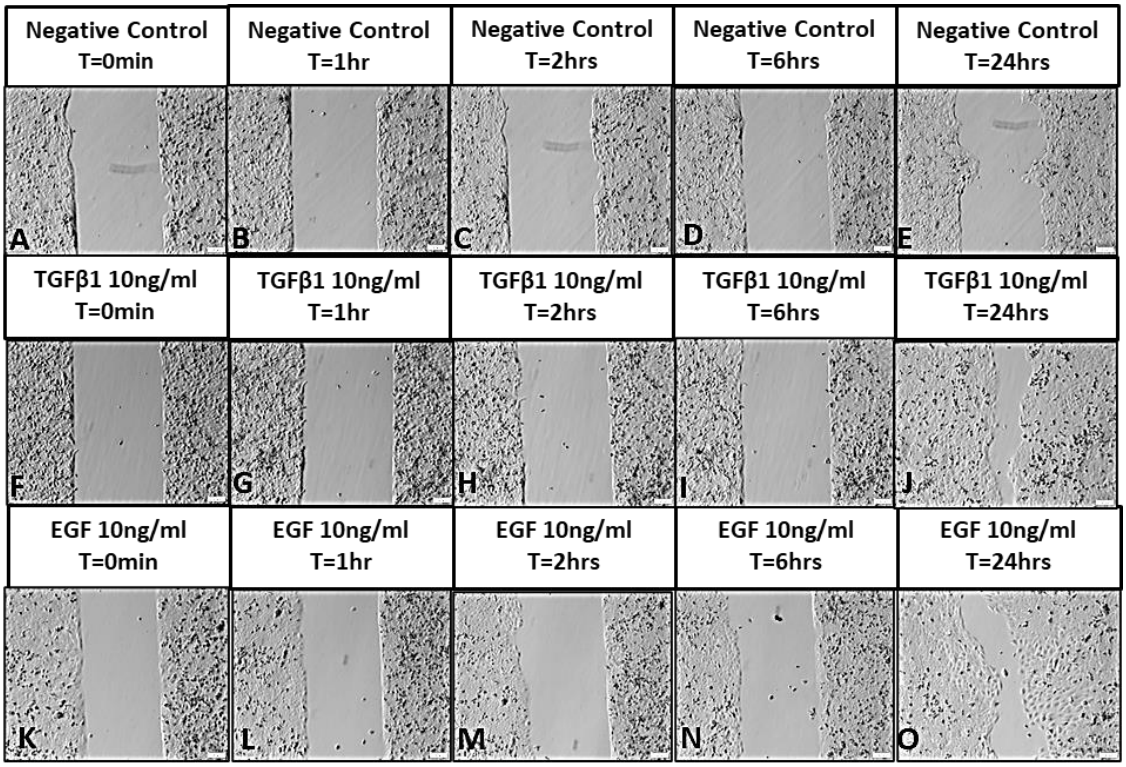


Figure 42: Gap closure assay with the HaCaT cell line.

Images of the Gap closure assay with inserts for the HaCaT cells at 40X magnification. The experiment contained different test conditions (SF-MEM, TGFβ1 and EGF) at different incubation periods (0 min, an hour, two hours, six hours and 24 hours). Three pictures were taken per insert for the observational assessment and three inserts were used per test condition. The total number of pictures per test condition was nine. At 24 hours, both TGFβ1 and EGF (pictures J and O respectively) showed reduced cell-free area compared to the negative control.

(iii) **Scratch Assay:** The Scratch assay is used to evaluate cell migration, particularly in the collective mode. The purpose of this assay was to keep track of a cell-free zone throughout the course of time, while subjecting it to a variety of test conditions. In this particular assay, the cell-free region was created with a scratch tool. The cell-free zone was measured at the beginning of the experiment as well as at the end for comparison.

(1) **Negative Control (Serum free MEM):** There were no signs of cell migration in SF-MEM. This was illustrated by the persistence of the gap that was created by the scratch tool. The gap was observed at 24 hours, which showed no evidence of collective cell migration (**Figure 44-B**).

(2) **10ng/ml of EGF:** With EGF, the HaCaT cell line showed a hint of closing the scratch by collective migration at 24 hours. This assessment was done by comparing the size of the gap using image J software and the protocol of measuring the area of interest at both Time = 0 and at 24 hours (**Figure 44-H**).

(3) **Rest of all Other Test Conditions:** The remainder of the assays with different the test conditions showed similar results to the Serum - Free group, which shows no significant cell migration (**Figure 44**).

Table 13: Summary of the Scratch assay findings for the HaCaT cell line.

The HaCaT cells' behaviour was assessed using the Scratch assay protocol regarding cell migration. Eleven test conditions were used in this protocol and images were taken at two different incubation periods (time=zero and 24 hours). The images were taken at 40X magnification from a specific area of the culture dish, which was marked before the beginning of the experiment.

Test Conditions to Assess Cell Migration using Scratch Assay for HaCaT Cell line		
Test Conditions	**T=0**	**T=24 hours**
Negative Control	_	_
TGFβ1	_	_
EGF	_	Gap partially close + Collective cell migration
TGFβ1 + 5μM TGF-β RI Kinase Inhibitor VII	_	_
EGF + 5μM TGF-β RI Kinase Inhibitor VII	_	_
TGFβ1 + 1μM MK-2206	_	_
EGF + 1μM MK-2206	_	_
TGFβ1 + 5μM PD98059	_	_
EGF + 5μM PD98059	_	_
TGFβ1 + 5μM SB431542	_	_
EGF + 5μM SB431542	_	_

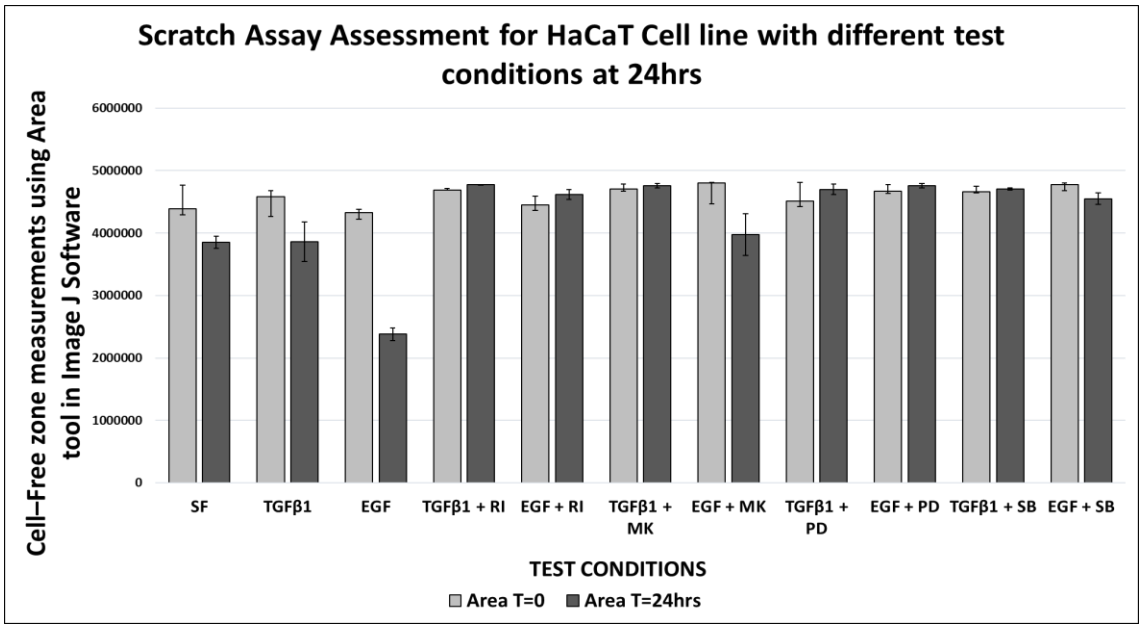


Figure 43: Scratch assay assessment of the HaCaT cell line with different test conditions.

The figure illustrates the difference in the cell-free area over different incubation periods with the test conditions. Data for the cell-free zone were calculated using Image J software, on images, taken at 40X magnification. The formula for calculating the cell free zone was done by using tool in the software called Area. Data for the cell-free area at Time=zero considered as the 100%. The mean and standard deviation of three measurements in total were calculated per test conditions at same incubation period. The most significant decrease in the size of the gap was recorded with the EGF-treated culture dishes at 24 hours, compared to both the negative control samples and the EGF culture dish at time = 0. Raw data can be found in appendix 3 (page 306).

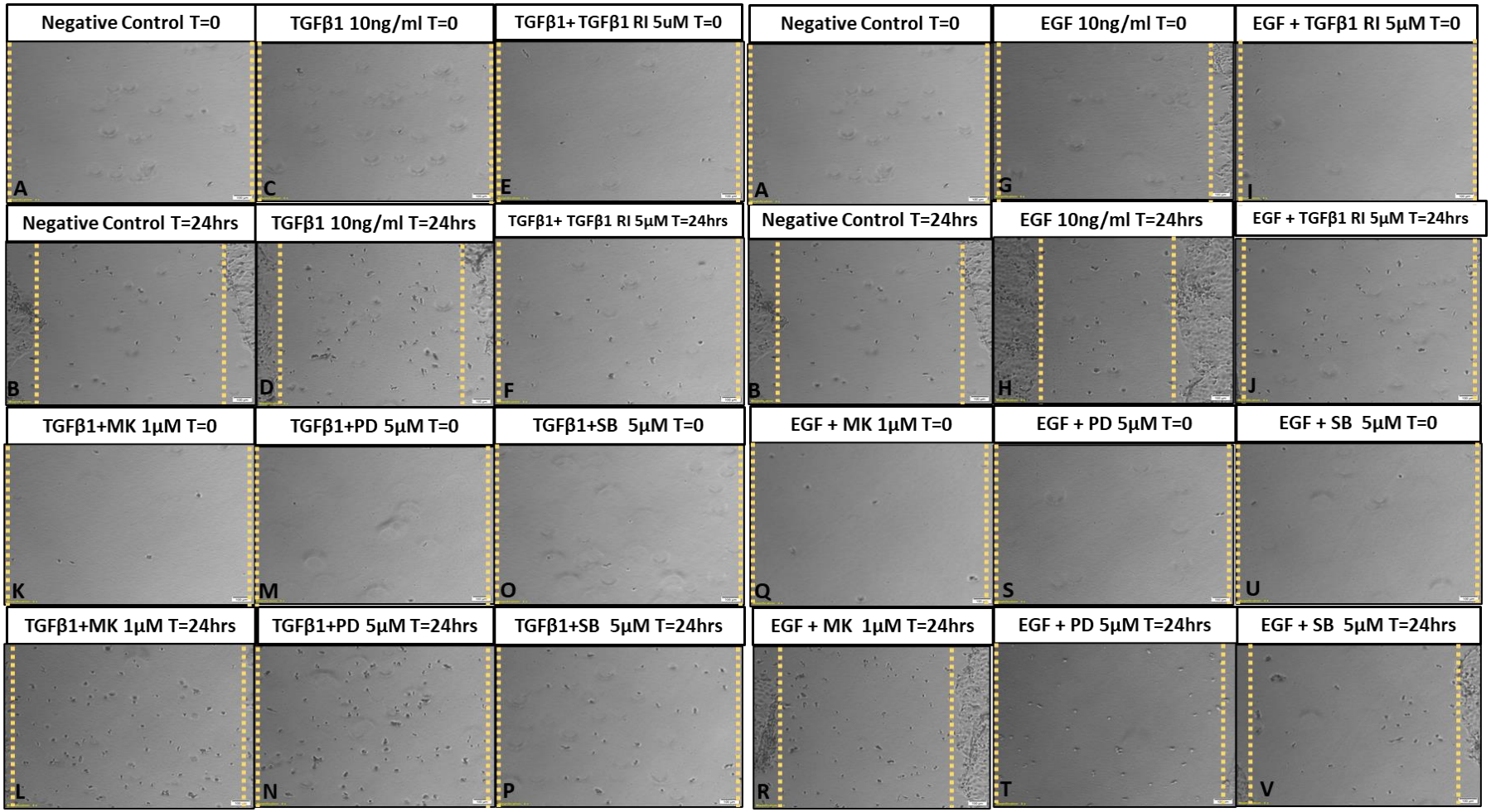


Figure 44: Scratch assay observation for the HaCaT cell line with different test conditions.

Images of the Scratch assay for the HaCaT cell line with the test conditions at Time = 0 and 24 hours were taken at 40X magnification. The yellow dotted lines represent the edges of the gap. Only one condition showed a smaller cell-free zone compared to the baseline, which was EGF group at 24 hours, picture (**H**).

Summary:

The migration assays demonstrated that the HaCaT cell line behaves differently under the effect of TGFβ1 compared to SF-MEM and EGF test conditions specially using the Scatter assay.

Cell morphological changes and cell migration were observed in the HaCaT cell line after 48 hours of incubation with TGFβ1, 10ng/ml TGFβ1 plus 1µM MK-2206 (Akt Inhibitor), PD98059 (MAKP Inhibitor), and 10ng/ml EGF plus 1µM MK-2206. This suggests that the migratory effect of TGFβ1 has been blocked by TGF-β RI Kinase Inhibitor VII (receptor inhibitor) and SB431542 (Smad Inhibitor). These findings indicates that the Smad signalling pathway regulates single and collective cell migration in the HaCaT cell line.

4.4.2 TYS Cell Line:

(i) **Scatter Assay:** The Scatter assay was used to determine the impact of the test conditions on the TYS cell line in terms of cell migration. The evaluation was conducted during two incubation periods (24 hours & 48 hours) for eleven growth conditions.

(1) **Negative Control (Serum free MEM):** After 24 hours and 48 hours with SF-MEM alone, the TYS cells were closely compact with no signs of morphological changes or migratory cells (**Figure 45-A&B**). The average distance between cells (five measurements) ranged between 22 µm to 34 µm.

(2) **10ng/ml of TGFβ1**: With the TGFβ1 group, few changes were observed. After 24 hours with the growth factor, few colonies showed less compact cells, when compared with the control group. Due to the change in colony architecture and becoming less compact, the TYS cells appeared large, flat and rounded in shape as an early sign of morphological change (**Figure 45-C**). After 48 hours with TGFβ1, cells migrated away from each other and dispersed, which resulted in changes to cell morphology and colony appearance. A few cells appeared to be elongated with complete separation from neighbouring

cells (**Figure 45-D**). The mean distance between cells increased to 56 µm, when compared to both time zero and 24 hours (n=5).

(3) **10ng/ml of EGF:** The assessment of the effect of EGF on the TYS cell line at 24 hours showed slight change in terms of cell morphology and colony appearance, compared to SF-MEM culture dishes. The change was observed as less-compacted colonies due to loss of close adhesion between the neighbouring cells, which led to morphological change in cells and colonies (**Figure 45-G**). These observations were supported by an increase in the average cell-to-cell distance (five measurements) compared to the one recorded at the beginning of the experiment. After 48 hours with the growth factor, cells displayed more morphological change i.e. they became elongated and had a spindle-like shape (**Figure 45-H**). Moreover, cells started to further move away from their colonies, which resulted in an increase in the average cell-to-cell distance compared time zero.

(4) **10ng/ml of TGFβ1 plus 5µM of TGF-β RI Kinase Inhibitor VII:** With the TGFβ1 receptor inhibitor, the migratory effect of TGFβ1 was blocked, no signs of morphological change, change in colony appearance (**Figure 45-E&F**) or increase in the average cell-to-cell distance were observed. This test condition showed comparable results to the negative control group and a contrasting outcome to the TGFβ1 only group.

(5) **10ng/ml of EGF plus 5µM of TGF-β RI Kinase Inhibitor VII:** Adding TGF-β RI Kinase Inhibitor VII with EGF to cultures of the TYS cell line did not block cell migration and morphological changes of the cells. Cells changed their shape and colonies dispersed at both 24 hours and 48 hours (**Figure 45-I&J**). Measuring the average distance between cells showed an increase, which suggested the inhibitor did not stop cells from migrating away from each other.

(6) **10ng/ml of TGFβ1 plus 1μM of MK-2206 (Akt Inhibitor):** The response of TYS to TGFβ1 plus Akt inhibitor showed contrasting results to the cultures incubated with TGFβ1 alone. At 24 hours, both scattered cells and alternations in cell morphology were absent (**Figure 45-K**) also, the distance between cells was within the same range (20 μm to 34 μm) as the negative control group and time zero. However, a few samples showed a range of results, starting with no scattered cells and cells in tight contact with each other, to colonies with cells completely separated, although the average cell-to-cell distance was similar to the control group.

(7) **10ng/ml of EGF plus 1μM of MK-2206 (Akt Inhibitor):** Culture dishes incubated with both EGF and Akt inhibitor (MK-2206) showed few migratory cells, changes in cell morphology, less compacted colonies (**Figure 45-Q**); and an increase in the average cell-to-cell distance at 24 hours. These findings were more evident after 48 hours, especially regarding colony appearance and cell morphology, where cells became elongated and more mesenchymal-like cells (**Figure 45-R**). This result indicates that the Akt inhibitor was not able to block EGF-induced migration of the TYS cell line.

(8) **10ng/ml of TGFβ1 plus 5μM of PD98059 (MAPK MEK1 Inhibitor):** The effect of TGFβ1 to induce colonies of TYS cells to become less compact with few changes in cell morphology, was not observed after 24 hours (**Figure 45-M**). This outcome suggests that this effect was blocked by the MAPK inhibitor. However, changes in cell shape, appearance of colonies, individual cell migration and an increase in cell-to-cell distance (around 58 μm on average) were recorded at 48 hours (**Figure 45-N**). The scatter effect was observed and cells completely separated and became small and rounded in shape. All of these findings suggested that the MAPK inhibitor had no effect on TGFβ1-induced cell migration after 48 hours.

(9) **10ng/ml of EGF plus 5µM of PD98059 (MAPK MEK1 Inhibitor):** The TYS cells incubated with EGF and a MAPK inhibitor showed morphological changes in the cells, alteration in colony appearance and a greater average distance between cells at both 24 and 48 hours (more evident at 48 hours) was observed (**Figure 45-S&T**).

(10) **10ng/ml of TGFβ1 plus 5µM of SB431542 (Smad Inhibitor):** Incubation of the TYS cells with TGFβ1 and a Smad inhibitor, blocked both changes in cell morphology and the ability of cells to migrate away from their colonies (**Figure 45-O&P**). In addition, the recording of the average cell-to-cell distance was less value than for the cells incubated with TGFβ1 alone. This indicates the Smad signalling pathway plays a role in cell migration induced by TGFβ1 in the TYS cell line.

(11) **10ng/ml of EGF plus 5µM of SB431542 (Smad Inhibitor):** Addition of a Smad inhibitor to the TYS cultures had no effect on EGF-induced migration. The cells started to migrate away from their colonies and changed their morphology to become small and round (**Figure 45-U&V**). All characteristics that normally accompanied cell migration were present I.e., change in cell morphology, change in colony appearance and increase in the average distance between neighbouring cells.

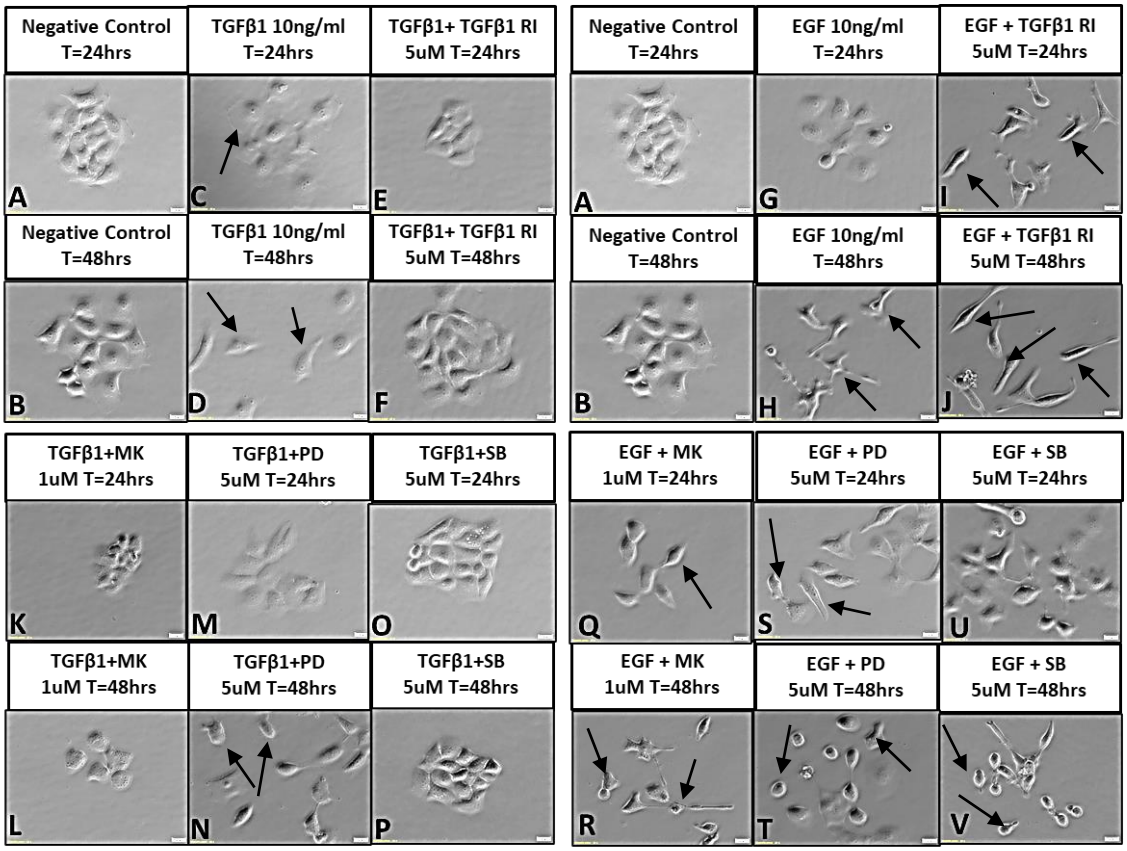


***Figure 45: Observational assessment of TYS cell line migration using the Scatter assay*.**

The images above show the Scatter assay for the TYS cell line with the test conditions, at two incubation periods: 24 hours and 48 hours. Images were taken at 200X magnification to evaluate both colony appearance, cell morphology and the presence of scattered cells. SF-MEM at 24 hours **(A)** and 48 hours **(B)** shows compact colonies with cell morphology consistent with epithelial cell characteristics. TGFβ1-treated culture dishes at 24 hours **(C)** and 48 hours **(D)** showed changes in colony appearance and in cell morphology, where individual cells were observed as a result of complete separation from their colony. The same findings were observed with other test conditions including EGF at 48 hours **(H**), EGF+ TGF-β RI Kinase Inhibitor VII at 24 hours **(I)** and 48 hours **(J),** TGFβ1+PD98059 at 48 hours **(N),** EGF+MK-2206 at 24 hours **(Q)** and 48 hours **(R),** EGF+PD98059 at 24 hours **(S),** 48 hours **(T),** EGF+SB431542 at 24 hours **(U)** and 48 hours **(V)**. However, the effect of TGFβ1 on cell morphology and colony appearance was blocked in culture dishes treated with TGFβ1+ TGF-β RI Kinase Inhibitor VII at 24 hours **(E)** at 48 hours **(F),** TGFβ1+SB431542 at 24 hours **(O)** and 48 hours **(P)**. samples with TGFβ1+MK-2206 at 24 hours **(K)** and 48 hours showed changes in colony appearance, but no scattered cells.

Table 14: Summary of the Scatter assay findings with the TYS cell line.

The samples were assessed by observing images of each test condition at three different incubation periods (Time=zero, 24 hours and 48 hours). All images were taken at 200X magnification. Three criteria were used to assess the cell morphology and migration status. **(-)** refers to no significant change compared to time zero. For morphological change or scattered cells, the status was referred to as (present), while alteration in colony appearance was referred to as (changed). The final criterion was measurement of the average distance between cells in different culture. Raw data can be found in appendix 1 page (page 299).

Test Conditions to Assess Cell Migration using the Scatter Assay for the TYS Cell line									
Test Conditions	**Cell Morphology and Migration (Scattering)**			**Colony Appearance**			**Average Distance of Five Measurements between Cells in µm**		
	T=0	**T=24hrs**	**T=48hrs**	**T=0**	**T=24hrs**	**T=48hrs**	**T=0**	**T=24hrs**	**T=48hrs**
Negative Control	_	_	_	_	_	_	30.8	23.5	32.7
TGFβ1	_	Present	Present	_	Changed	Changed	30.5	**48.9***	**56.4***
EGF	_	Present	Present	_	Changed	Changed	27.4	**53.8***	**58.4***
TGFβ1+5µM TGF-β RI Kinase Inhibitor VII	_	_	_	_	_	_	29.9	22.4	33.4
EGF+5µM TGF-β RI Kinase Inhibitor VII	_	Present	Present	_	Changed	Changed	24.6	**45***	**52.4***
TGFβ1+1µM MK2206	_	_	Present	_	_	Changed	34.1	19.5	23.8
EGF+1µM MK2206	_	Present	Present	_	Changed	Changed	33.4	**44.1***	**53.4***
TGFβ1+5µM PD98059	_	Present	Present	_	Changed	Changed	33.8	36.1	**58.5***
EGF+5µM PD98059	_	Present	Present	_	Changed	Changed	26.5	**59***	**50.2***
TGFβ1+5µM SB431542	_	_	_	_	_	_	26.4	23.2	26.1
EGF+5µM SB431542	_	Present	Present	_	Changed	Changed	26.5	**48.3***	**50.7***

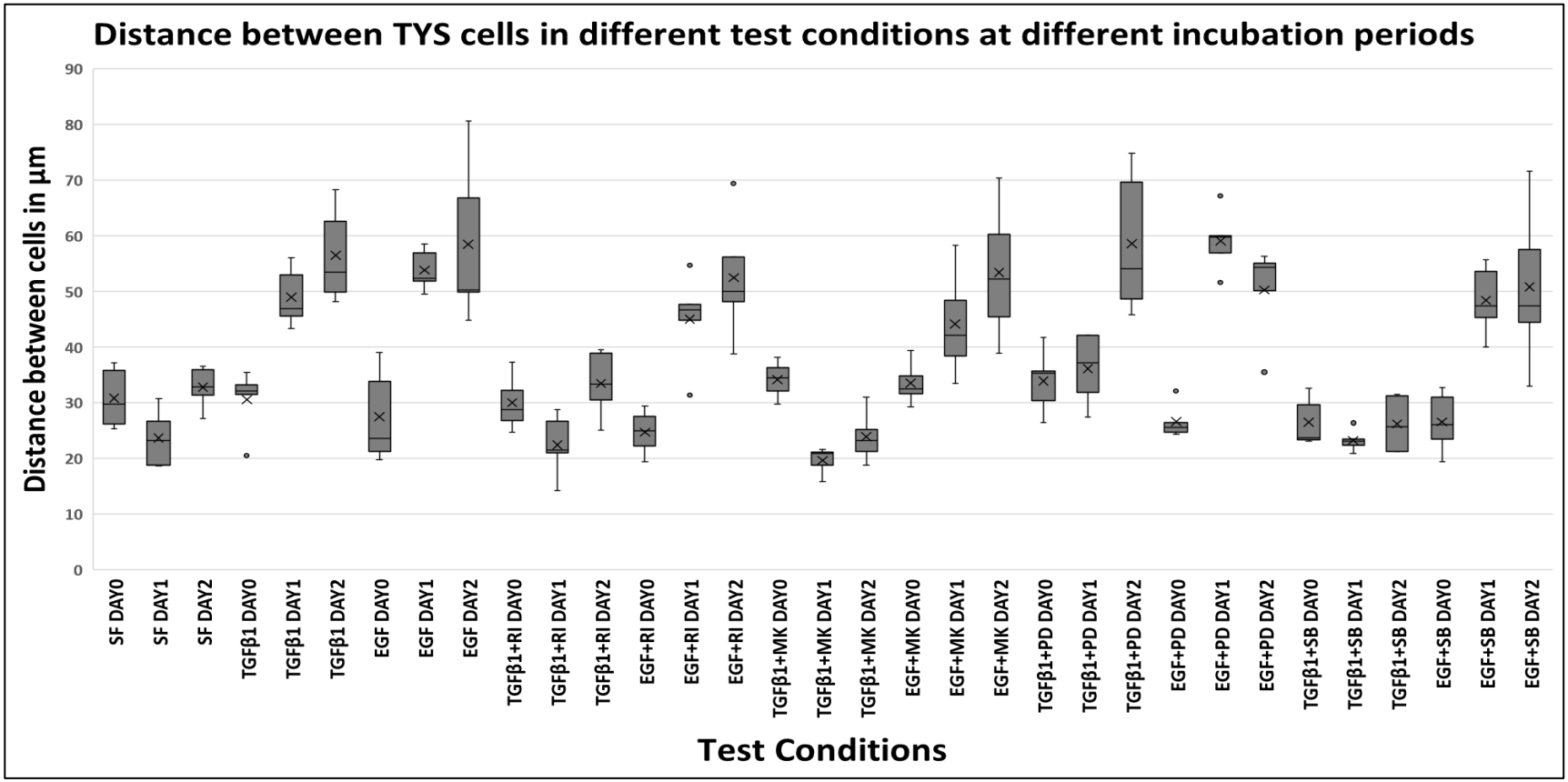


Figure 46: Scatter assay assessment for the TYS cell line using the distance between cells.

This graph shows assessment of the Scatter Assay for the TYS cell line using the measurements of cell-cell distance in µm with different test conditions at time zero, 24 hours and 48 hours. The assessment was based on specific criteria for image selection and the distance measurement protocol using Image J software. All images were taken at 200X magnification with clear details of cells and colonies. The graph shown the average distance between cells increased with both growth factors TGFβ1 & EGF at 24 hours and 48 hours. TGFβ1+TGF-β RI Kinase Inhibitor VII and TGFβ1+MK2206 at 24 hours & 48 hours plus TGFβ1+PD98059 and TGFβ1+SB431542 at 24 hours showed no significant change in the average distance between cells, compared to control group or time zero**.** Raw data can be found in appendix 1 (page 299).

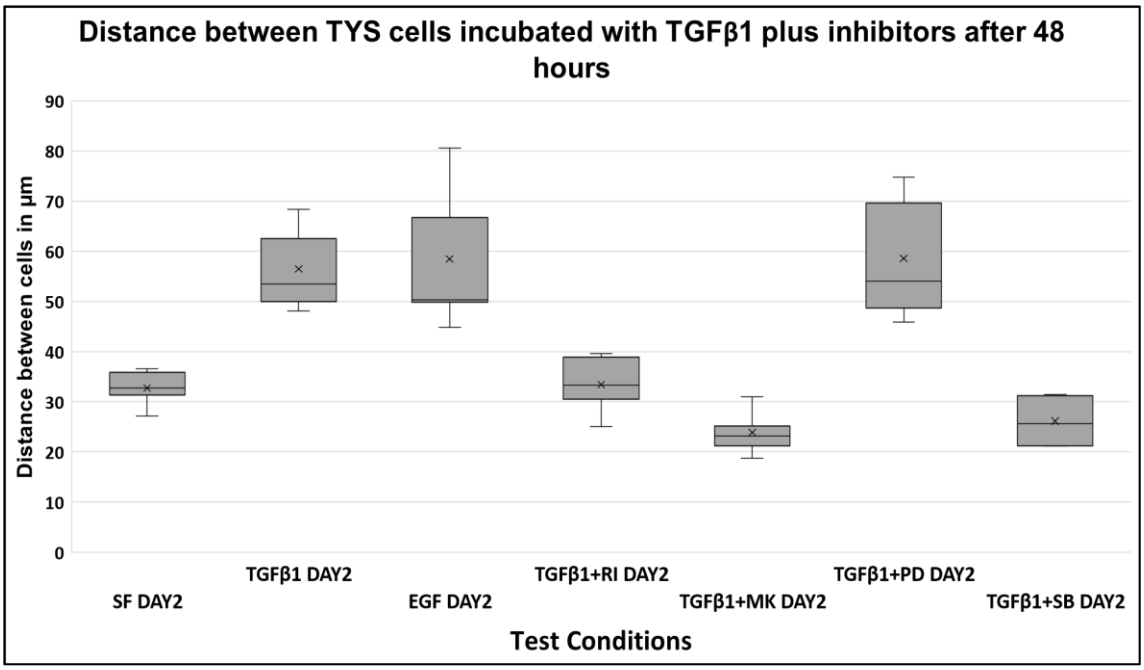


Figure 47: Scatter assay assessment for the TYS cell line after 48 hours with test conditions.

The assessment was carried out by measuring the average cell-cell distance in µm of seven test conditions after 48 hours. These test conditions were SF-MEM, TGFβ1, EGF, TGFβ1+ TGF-β RI Kinase Inhibitor VII, TGFβ1+MK-2206, TGFβ1+PD98059 and TGFβ1+SB431542. Raw data can be found in appendix 1 (page 299).

(ii) **Gap Closure Assay:** The role of growth factors, TGFβ1 and EGF, in the stimulation of the TYS cell line to migrate was evaluated using the Gap closure assay (**Figure 49**). The evaluation of this assay was based on how much the cells moved closer to the gap over a pre-determined period of time (**Figure 48**). In addition, by examining the cells at the edge of the gap, the mode of cell migration could be identified as single or collective, which was another benefit of this method**.**

(1) **Negative Control (Serum free MEM):** The evaluation of the cell-free area created by the barrier of the inserts with the TYS cell line in serum free-MEM medium, revealed that the gap gradually closed over the incubation period and was completely covered by cells after 24 hours (**Figure 49-E**). No changes in cell morphology or individual cells were observed. This suggests cells were able to migrate to the cell-free area without addition of exogenous growth factors.

(2) **10ng/ml of TGFβ1:** Adding exogenous TGFβ1 to the TYS assays showed no difference in effect to the serum Free-MEM group, where cells migrated and closed the gap after 24 hours (**Figure 49-J**). There were no signs of any single cells or cells with morphological change. Therefore, with this assay, it was difficult to determine the role of the growth factor in the migration of TYS cells to the cell-free area.

(3) **10ng/ml of EGF:** A similar pattern of cell migration was seen with EGF-treated cells to both the serum Free-MEM and TGFβ1 groups, where cells moved slowly toward the gap until it was completely closed after 24 hours. The noticeable difference with this group was that the majority of cells lost their adhesion and appeared as small round cells at 24 hours, which indicates single cell migration (**Figure 49-O**).

Table 15: Summary of the Gap closure assay results with the TYS cell line.

Cells were incubated with the test conditions (SF-MEM, 10ng/ml TGFβ1 and 10ng/ml EGF) at different incubations periods (time zero, one hour, two hours, six hours and 24 hours). This observational assessment was based on using the following three criteria to describe the status of the gap: open or (-) describes the gap at the beginning of the experiment, partially closed which indicates there is change in the size of the gap but still open and finally gap closed.

Test Conditions	T=0	T=1hr	T=2hrs	T=6hrs	T=24hrs
SF-MEM	_	_	_	Partially closed	Gap closed
TGFβ1	_	_	_	Partially closed	Gap closed
EGF	_	_	_	Partially closed	Gap closed

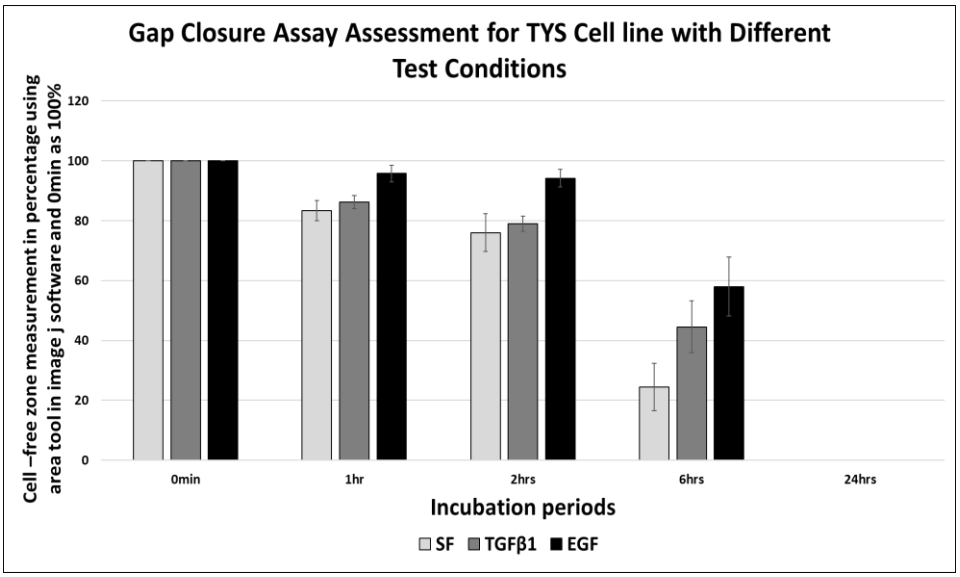


Figure 48: Assessment of the Gap closure assay for the TYS cell line.

Cells were incubated with either the negative control (SF-MEM alone) or with growth factors (10ng/ml TGFβ1 & 10ng/ml EGF) at incubation periods of time zero, one hour, two hours, six hours and 24 hours. The graph illustrates how much of the gap remains after the different incubation periods with the three test conditions. Measurements of the cell-free area were calculated using Image J software, on images taken at 40X magnification. The formula for calculating the cell free zone was derived using a tool in the software called Area. The value of the Area was then transformed to a percentage, to compare the change in the size of the gap over the different incubation periods for the same sample. Data for the cell-free area at Time = 0 was considered as 100%. The mean and standard deviation of six measurements in total were calculated per test condition, at the same incubation periods. Each test condition had five inserts, three of them were chosen randomly, before introducing the test conditions. Two pictures were taken for each insert at specific incubation periods. This graph shows the significant reduction in the cell-free area in all test conditions at 24 hours. Raw data can be found in appendix2 (page 304).

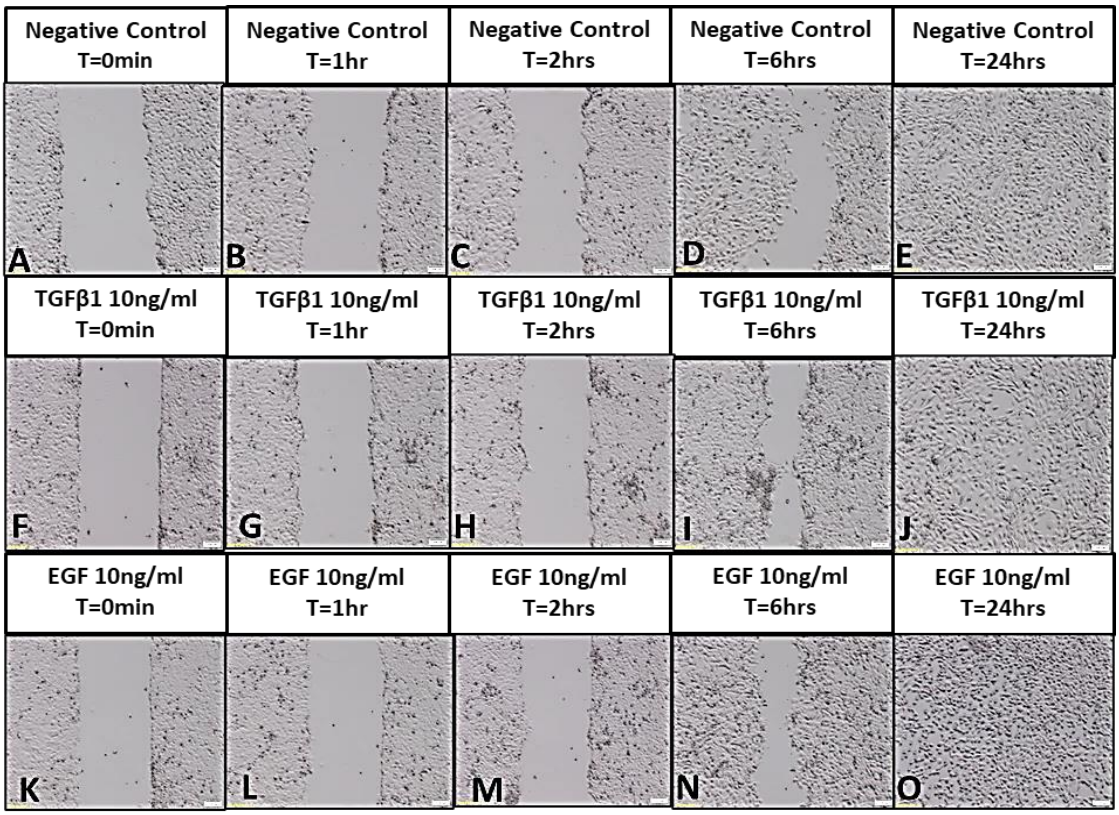


Figure 49: Gap closure assay with the TYS cell line.

Images from the Gap closure assay with inserts for the TYS cells, which were taken at 40X magnification. The experiment contained three test conditions (SF-MEM alone, TGFβ1 and EGF) at different incubation periods (0 min, an hour, two hours, six hours and 24 hours). Two images were taken per insert for the observational assessment and three inserts were used per test condition. The total number of images per test condition was six. All test groups showed a reduced cell-free area at six hours i.e. SF-MEM **(D),** TGFβ1 **(I)** and EGF **(N)**. After 24 hours the gap was completely covered by the TYS cells in the assays with SF-MEM **(E),** TGFβ1 **(J)** and EGF **(O)**. The only difference observed at 24 hours was with the EGF-treated culture dishes **(O),** where a high number of individual cells were seen in the observational area.

(iii) **Scratch Assay:** The Scratch assay was utilised to evaluate cell migration, in particular the collective mode of cell movement. The objective of this assay was to observe and record the behaviour of a cell-free zone over the course of time, while subjecting the TYS cells to a number of different growth conditions (**Figure 51**).

(1) **Negative Control (Serum free MEM):** With SF-MEM alone, the TYS cell line showed almost no change in the gap that had been created by the scratch tool after 24 hours. This Indicates that no cell migration occurred (**Figure 51-B**).

(2) **10ng/ml of TGFβ1:** The addition of exogenous TGFβ1 to culture of the TYS cell line resulted in decreasing the size of the gap after 24 hours (**Figure 51-D**). No single cells were observed at the edge of gap, which suggested that cells did not separate from the colonies to migrate into the gap. This suggests that the cells have started to migrate collectively, rather than individually.

(3) **10ng/ml of EGF:** Similar to the TGFβ1 group, the cell-free area had decreased when it was observed after 24 hours incubation in comparison to time zero (**Figure 51-H**). This suggests collective movement of the cells had occurred due to the effect of the exogenous EGF.

(4) **10ng/ml of TGFβ1 plus 5μM of TGF-β RI Kinase Inhibitor VII:** Cell migration at either edge of the gap, in response to the growth factor TGFβ1, was blocked by the effect of the inhibitor. The gap did not close after 24 hours in these growth conditions (**Figure 51-F**).

(5) **10ng/ml of EGF plus 5μM of TGF-β RI Kinase Inhibitor VII:** Using TGF-β RI Kinase inhibitor VII with EGF, blocked the ability of cells to migrate collectively and cells did not migrate into cell-free area (**Figure 51-J**).

(6) **10ng/ml of TGFβ1 plus 1μM of MK-2206 (Akt Inhibitor):** The combination of TGFβ1 and Akt inhibitor prevented the TYS cells from migrating and closing the gap (**Figure 51-L**). Therefore, the effect of the growth factor to cause cell migration was inhibited in the presence of MK-2206. This indicates the important role of PI3K/Akt pathway in the TYS cell migration.

(7) **10ng/ml of EGF plus 1μM of MK-2206 (Akt Inhibitor):** The ability of EGF to cause cell migration was also inhibited by MK-2206 and the cell-free gap persisted after 24 hours (**Figure 51-R**).

(8) **10ng/ml of TGFβ1 plus 5μM of PD98059 (MAPK MEK1 Inhibitor):** No signs of cell migration were observed after 24 hours, which suggested the inhibitor blocked the migratory effect of the growth factor (**Figure 51-N**). This result explained the important role of the MAPK signalling pathway in the collective cell migration process of the TYS cell line.

(9) **10ng/ml of EGF plus 5μM of PD98059 (MAPK MEK1 Inhibitor):** The migratory effect of EGF was inhibited by PD98059 (**Figure 51-T**). Therefore, the activation of the MAPK signalling pathway played a role in orchestrating the TYS cells to migrate into the gap.

(10) **10ng/ml of TGFβ1 plus 5μM of SB431542 (Smad Inhibitor):** The Smad inhibitor decreased the ability of TGFβ1 to induce cell migration subsequently, preventing gap closure (**Figure 51-P**). This suggests the Smad signalling pathway participated in regulating the cell migration of the TYS cell line into the gap.

(11) **10ng/ml of EGF plus 5μM of SB431542 (Smad Inhibitor):** Cell migration was observed in the presence of EGF and the Smad inhibitor, but was less than with EGF alone (**Figure 51-V**). These

results were evident, as a large part of the gap persisted and was not closed by the TYS cells.

Table 16: Summary of the Scratch assay results with the TYS cell line.

The observation of scratch assay for the TYS cell line regarding cell migration was carried out after 24 hours. Different test conditions were used in this protocol. Images were taken at two different incubation periods (time=zero and 24 hours) at 40X magnification from a specific area of the culture dish, which was marked before the beginning of the experiment.

Test Conditions	T= 0	T= 24hrs
Negative Control	_	_
TGFβ1	_	Gap partially closed + Collective cell migration
EGF	_	Gap partially closed + Collective cell migration
TGFβ1 + 5μM TGF-β RI Kinase Inhibitor VII	_	_
EGF + 5μM TGF-β RI Kinase Inhibitor VII	_	_
TGFβ1 + 1μM MK-2206	_	_
EGF + 1μM MK-2206	_	_
TGFβ1 + 5μM PD98059	_	_
EGF + 5μM PD98059	_	_
TGFβ1 + 5μM SB431542	_	_
EGF + 5μM SB431542	_	_

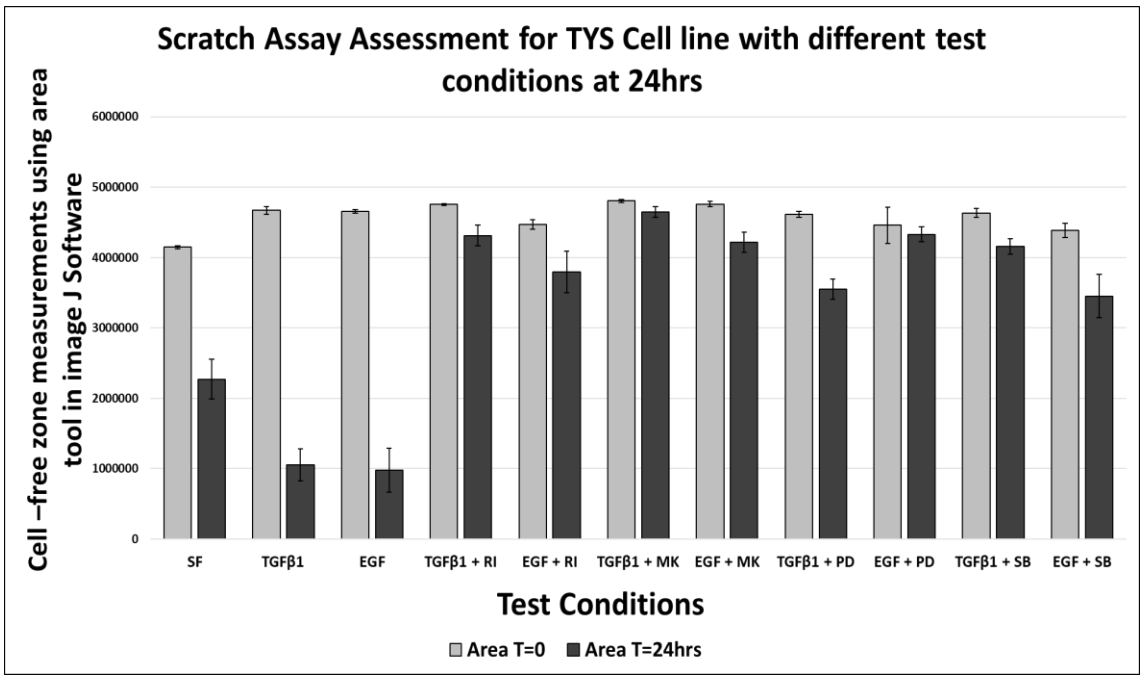


Figure 50: Scratch Assay assessment of the TYS cell line with different test conditions.

The figure illustrates the difference in the cell-free area over different incubation periods with the test conditions. Data for the cell-free zone were calculated using Image J software, on images, taken at 40X magnification. The formula for calculating the cell free zone was derived using a tool in the software called Area. Data for the cell-free area at Time=zero was considered to be 100%. The mean and standard deviation of three measurements in total were calculated per test conditions at the same incubation period. Raw data can be found in appendix 3 (page 307).

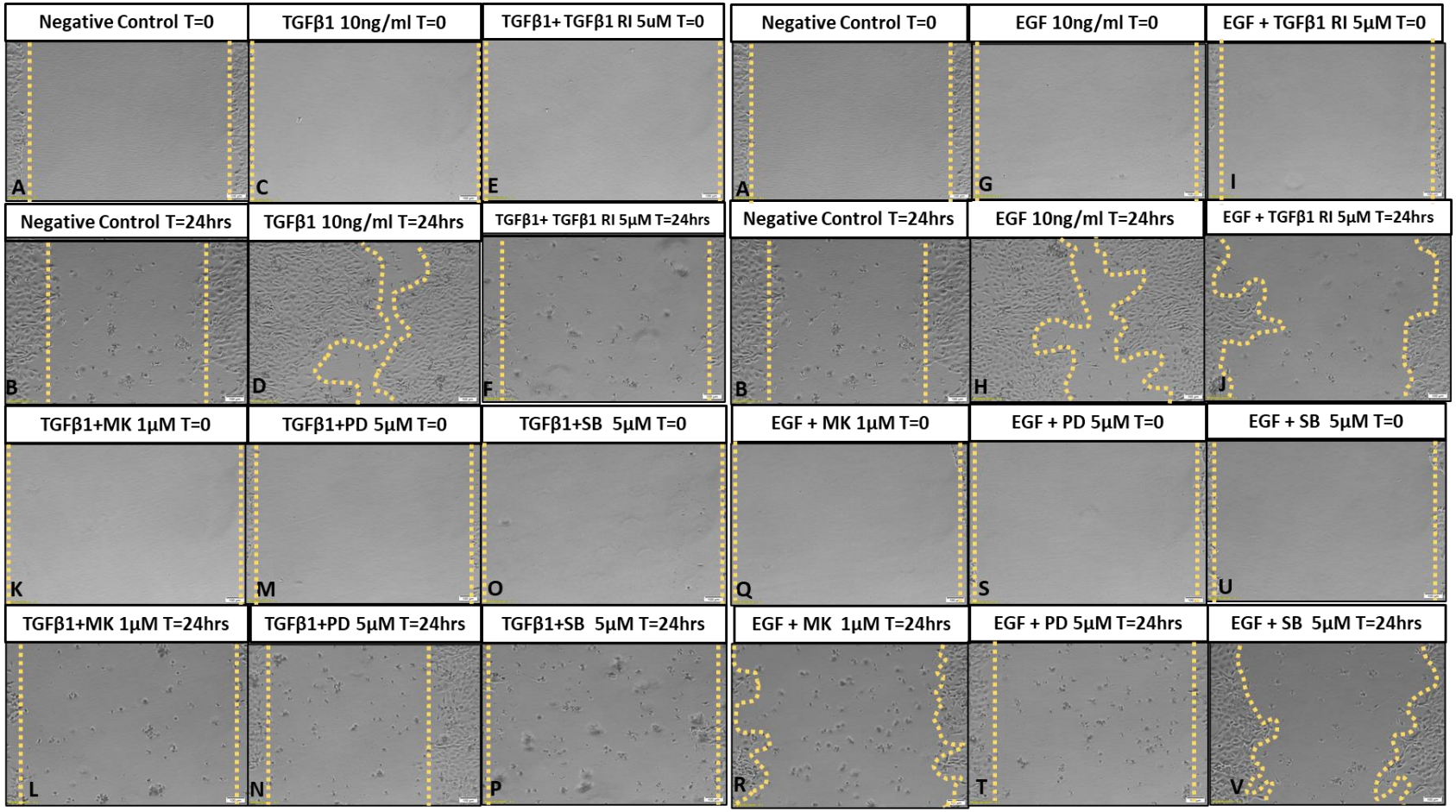


Figure 51: Scratch Assay observational assessment for the TYS cell line with different test conditions.

Images of the Scratch assay for the TYS cell line with the test conditions at Time = 0 and 24 hours were taken at 40X magnification. The dotted lines represent the edges of the gap. Assays containing TGFβ1 **(D)** and EGF **(H)** showed a significant decrease in the cell-free area as a result of the TYS cells migrating into it.

Summary:

The migration assays with the TYS cell line showed that the growth factors TGFβ1 and EGF induced cells to migrate, whether as single cell migration or collective cell migration.

The scatter assay was used to evaluate cell migration and morphological change of the TYS cell line. The TYS cells were found to be induced To migrate away from their colonies by the test conditions, except for the negative control, TGFβ1 plus 5 μM of TGFβ-RI Kinase Inhibitor VII and TGFβ1 plus 5 μM of SB431542. The remainder of the test conditions showed cell migration, especially at 48 hours.

For the Scratch assay, the TYS cell line showed collective cell migration with both of the growth factors, TGFβ1 and EGF, which was visualised by cells closing the scratch. All test conditions that included inhibitors and growth factors did not exhibit cell migration, except for one test condition, which was EGF with the Smad inhibitor. These findings suggest that more than one signalling pathway is involved in collective cell migration and any disruption of those signalling pathways preventing cells from migrating together.

4.4.3 SAS-H1 Cell Line:

(i) **Scatter Assay:** The SAS-H1 cell line was subjected to the Scatter assay protocol, so that the impact of the test conditions regarding cell migration could be investigated. The evaluation was conducted throughout the course of two separate incubation periods, 24 hours and 48 hours. A total of eleven growth conditions were used in this experiment (**Table 17**).

(1) **Negative Control (SF-MEM):** The SAS-H1 cell line showed the usual characteristics of epithelial cell in SF-MEM after 24 hours and 48 hours (**Figure 52-A&B**). These features included small round cells in close-packed colonies with an average distance between neighbouring cells ranging from 31 μm to 38 μm (n=5).

(2) **10ng/ml of TGFβ1:** The migratory effect of the addition of exogenous TGFβ1 on the SAS-H1 cell line was mainly observed after 48 hours. The morphology of the SAS-H1 cells changed from small and round cells adherent to neighbouring cells to large oval-shape cells that were completely detached from their colonies (**Figure 52-D**). These changes led to an alteration in colony appearance and an increase in the average distance between cells, which was over 60 µm (n=5).

(3) **10ng/ml of EGF:** The SAS-H1 cell line showed morphological changes when exogenous EGF was added to the culture dishes. After 24 hours and 48 hours, cells had lost their cell adhesion, becoming small round single cells, which were completely scattered (**Figure 52-G&H**). The average distance between neighbouring cells that were incubated with EGF increased to reach 44 µm and 52 µm at 24 hours and 48 hours respectively in comparison to the baseline (n=5).

(4) **10ng/ml of TGFβ1 plus 5µM of TGF-β RI Kinase Inhibitor VII:** Adding TGF-β RI Kinase Inhibitor VII with TGFβ1 to the culture dishes prevented all of the changes in cell morphology and colony appearance that were observed with TGFβ1 alone (**Figure 52-E&F**). Also, the distance between neighbouring cells stayed within a similar range to the baseline measurements i.e. 20 µm to 25 µm (n=5). This indicates that the receptor inhibitor blocked the migratory effect of the growth factor on the SAS-H1 cell line, especially after 48 hours.

(5) **10ng/ml of EGF plus 5µM of TGF-β RI Kinase Inhibitor VII:** The combination of the TGFβ-RI Kinase Inhibitor VII and EGF did not inhibit the migratory effect of the growth factor on the SAS-H1 cells. These cells showed changes in cell morphology, colony appearance and an increase in the average cell-to-cell distance (**Figure 52-I&J**), which were similar findings to the EGF alone group. Therefore, the inhibitor had no influence on EGF stimulated migration of the SAS-H1 cell line.

(6) **10ng/ml of TGFβ1 plus 1μM of MK-2206 (Akt Inhibitor):** To investigate the role of the Akt signalling pathway in cell migration, an Akt inhibitor, MK-2206, was added with TGFβ1 to the SAS-H1 cell line. Cell morphology and colony appearance changed in response to these test conditions. Also, scattered cells were observed as a result of individual cell migration at both 24 and 48 hours (**Figure 52-K&L**). In addition, the average distance between neighbouring cells was higher than in the serum free - MEM group. In contrast to the TGFβ1 group, only single cell migration was observed, as early as 24 hours. This was also supported by the increase in the average cell-to-cell distance at 24 hours, compared to the TGFβ1 alone group.

(7) **10ng/ml of EGF plus 1μM of MK-2206 (Akt Inhibitor):** The response of SAS-H1 cells to the EGF plus Akt inhibitor showed different outcomes to the EGF-alone test condition. Although changes in both cell morphology and colony appearance were observed, few single migratory cells were observed, especially at 24 hours (**Figure 52-Q&R**). The average distance between cells slightly increased at 24 hours to 39 μm (n=5), but this average decreased to its baseline level at time zero. Moreover, colonies in these growth conditions had a different appearance to the control samples, with no individual cells.

(8) **10ng/ml of TGFβ1 plus 5μM of PD98059 (MAPK Inhibitor):** The migratory effect of the growth factor was blocked by the inhibitor. There were no signs of any morphological changes among the SAS-H1 cells and their colonies (**Figure 52-M&N**). Also, the average distance between cells was close to the average at time zero and the control group.

(9) **10ng/ml of EGF plus 5μM of PD98059 (MAPK Inhibitor):** Adding PD98059 with EGF to cultures of SAS-H1 had no effect on preventing cells from migrating or changing their morphology. These findings

resembled the effect of EGF alone (**Figure 52-S&T**). Also, the SAS-H1 cells had scattered from their colonies and the average distance between cells increased to 60 µm compared to the baseline, which was 24 µm (n=5).

(10) **10ng/ml of TGFβ1 plus 5µM of SB431542 (Smad Inhibitor):** When a Smad inhibitor was added with the TGFβ1, the results varied depending upon the incubation period. After 24 hours of incubation, the morphological appearance of the SAS-H1 cell line resembled that of the negative control group with small round cells in compact colonies. No signs of any cell migration were observed after 24 hours (**Figure 52-O**). However, all assessment criteria included changes in cell shape, colony appearance, and the distance between cells, which indicated cell migration had occurred after 48 hours (**Figure 52-P**). Also, importantly, the changes in cell morphology and colony appearance and the increase in average distance between cells at 48 hours were different compared to the cells grown in TGFβ1 alone.

(11) **10ng/ml of EGF plus 5µM of SB431542 (Smad Inhibitor):** The SAS-H1 cells, when incubated with a combination of EGF and the Smad inhibitor, showed no changes in cell or colony appearance compared to the negative control at 24 hours (**Figure 52-U**). However, changes in cell morphology, alteration in colony shape and an increase in the distance between cells were observed at 48 hours (**Figure 52-V**). These findings suggested that cell migration had occurred. The main feature of the cells in this test condition was the morphology of cell, which had a spindle-like shape with few cells maintaining cellular adhesion, whereas the cells from the EGF alone group were completely separated completely from their colonies. Therefore, the Smad pathway might have an influence on the mode of migration of SAS-H1.

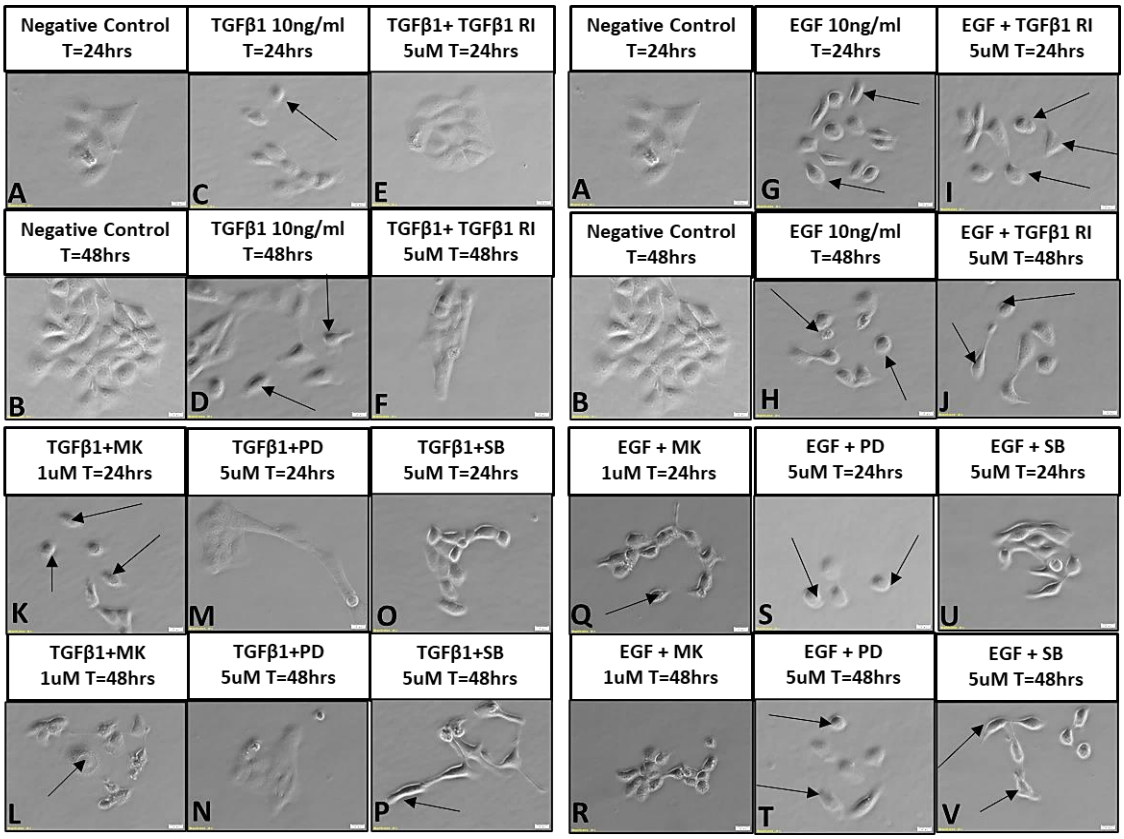


***Figure 52: Observational assessment of the SAS-H1 cell line migration using the Scatter assay*.**

The figure shows the Scatter assay for the SAS-H1 cell line with different test conditions at two incubation periods, 24 hours and 48 hours. Each image was taken at 200X magnification to assess colony appearance, cell morphology and cell scattering. SF-MEM (Negative Control) at 24 hours **(A)** and 48 hours **(B)** shows a compact colony with cell morphology consistent with epithelial characteristics. TGFβ1-treated cells at 48 hours **(D)** show a change in colony appearance and in cell morphology where single cells were observed because of complete separation from the colony. The findings from other test conditions showed changes in cell morphology and colony appearance including EGF at 24 hours **(G)** and 48 hours **(H**), EGF+ TGF-β RI Kinase Inhibitor VII at 24 hours **(I)** and 48 hours **(J),** TGFβ1+MK-2206 at 24 hours **(K)** and 48 hours **(L),** EGF+PD98059 at 24 hours **(S)** and 48 hours **(T),** and EGF+SB431542 at 48 hours **(V)**. However, the effect of TGFβ1 on cell morphology and colony appearance was blocked in culture dishes treated with TGFβ1+ TGF-β RI Kinase Inhibitor VII at 24 hours **(E)** and at 48 hours **(F),** and TGFβ1+PD98059 at 24 hours **(M)** and 48 hours **(N)**. Samples with TGFβ1+ SB431542 at 24 hours **(O)** and 48 hours **(P),** EGF+MK-2206 at 24 hours **(Q)** and 48 hours **(R),** and EGF+SB431542 at 24 hours **(U)** showed changes in colony appearance but no scattered cells.

Table 17: Summary of the Scatter assay findings with the SAS-H1 cell line.

The cultures were assessed based on images of cells in each test condition, at three different incubation periods (Time=zero, 24 hours and 48 hours). All pictures were taken at 200X magnification. Three criteria were used to assess the morphology and migration status of the cells. Changes in cell morphology or scattered cells are referred to as present or (-) which refers to no significant changes compared to time zero. An alteration in colony appearance compared to time zero was referred to as (changed). The final criterion indicates the average distance between cells in the growth conditions, which include five measurements per test condition (n=5). Raw data can be found in appendix 1 (page 301).

Test Conditions to Assess Cell Migration using the Scatter Assay for the SAS-H1 Cell line									
Test Conditions	**Changes in Cell Morphology and Migration (Scattering)**			**Colony Appearance**			**Average Distance between Cells of Five Measurements in µm**		
	T=0	**T=24hrs**	**T=48hrs**	**T=0**	**T=24hrs**	**T=48hrs**	**T=0**	**T=24hrs**	**T=48hrs**
Negative Control	_	_	_	_	_	_	30.6	26.3	22.8
TGFβ1	_	Present	Present	_	Changed	Changed	29.5	30.8	**64.1***
EGF	_	Present	Present	_	Changed	Changed	29.9	**44***	**52.6***
TGFβ1+5µM TGF-β RI Kinase Inhibitor VII	_	_	_	_	_	_	25.5	20	24.4
EGF+5µM TGF-β RI Kinase Inhibitor VII	_	Present	Present	_	Changed	Changed	30.1	**49.3***	**60.1***
TGFβ1+1µM MK2206	_	Present	Present	_	Changed	Changed	35.2	**50.8***	**45.7***
EGF+1µM MK2206	_	Present	_	_	Changed	Changed	22.7	39.3	29.2
TGFβ1+5µM PD98059	_	_	_	_	_	_	26.8	38.5	29.7
EGF+5µM PD98059	_	Present	Present	_	Changed	Changed	23.1	**55.5***	**60.5***
TGFβ1+5µM SB431542	_	Present	Present	_	Changed	Changed	24.4	23.4	**40***
EGF+5µM SB431542	_	Present	Present	_	Changed	Changed	34.5	21.8	29.5

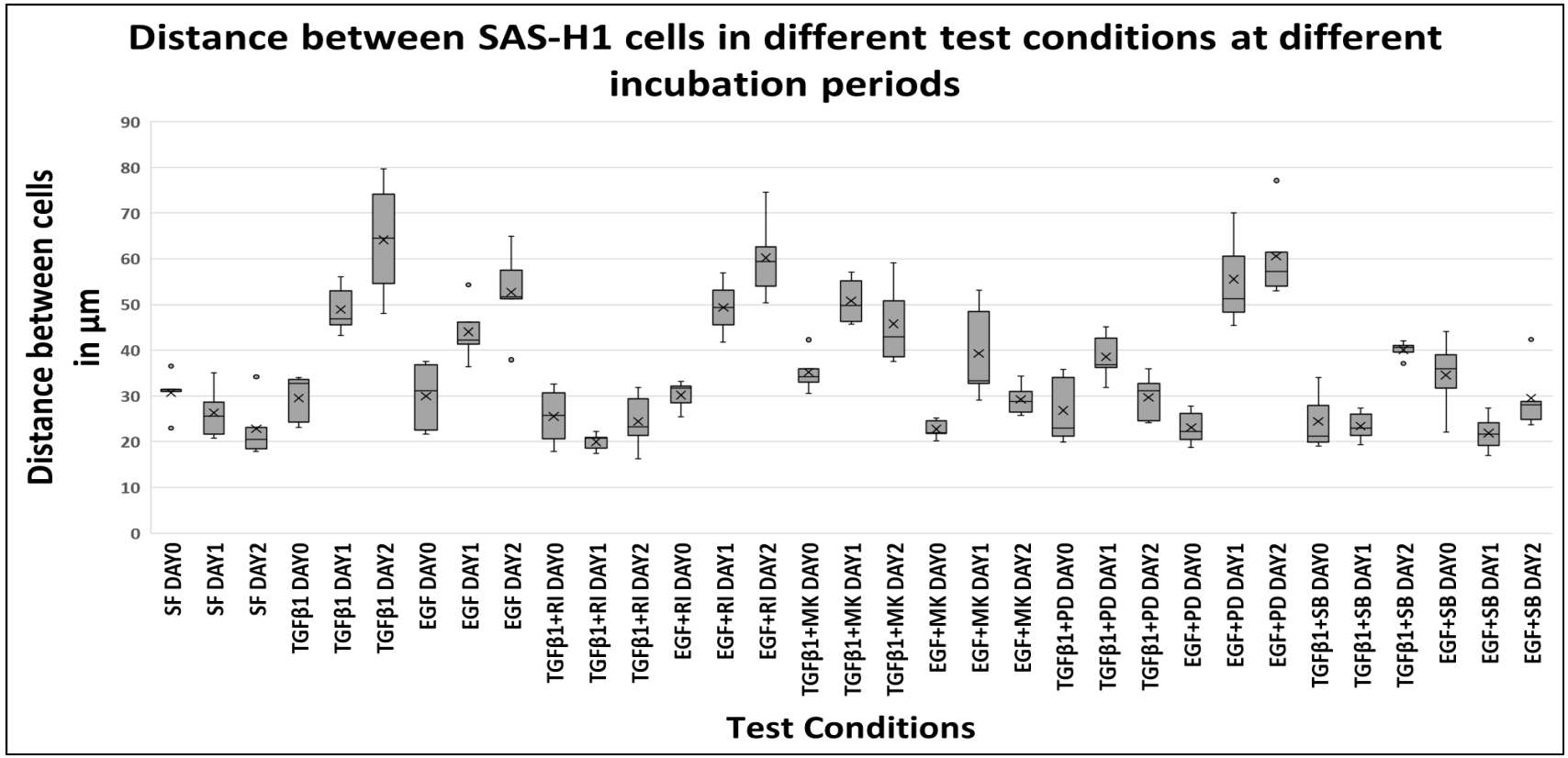


Figure 53: Scatter Assay assessment for the SAS-H1 cell line using distance between cells.

The graph illustrates the assessment of the Scatter Assay for the SAS-H1 cell line using the average cell-cell distance in µm with test conditions at time zero, 24 hours and 48 hours. The assessment was based on specific criteria for image selection and the measurement protocol. All images were taken at 200X magnification. The graph shows that the average distance between cells increased with both growth factors TGFβ1 & EGF, mainly at 48 hours. TGFβ1+TGF-β RI Kinase Inhibitor VII and TGFβ1+PD98059 at 24 hours & 48 hours, and TGFβ1+SB431542 at 24 hours showed no increase in the average distance between cells compared to TGFβ1-treated culture. Also, TGFβ1+MK-2206, EGF+TGF-β RI Kinase Inhibitor VII and EGF+ PD98059 at 24 hours & 48 hours showed a high average cell to cell distance. Raw data can be found in appendix 1 (page 301).

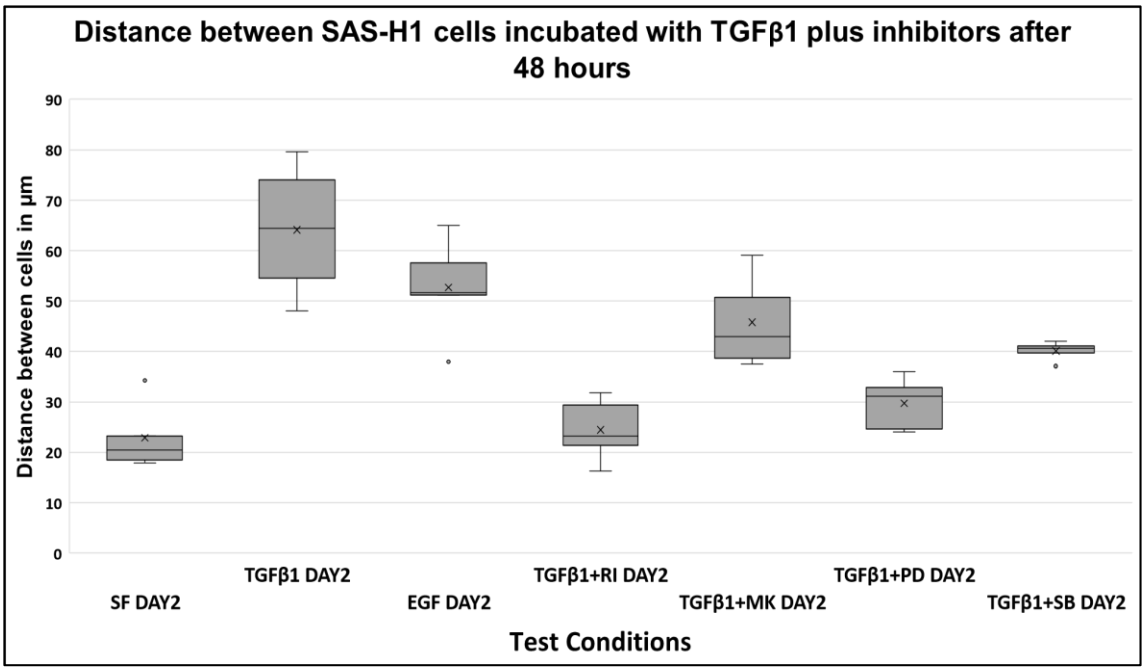


Figure 54: Scatter Assay assessment for the SAS-H1 cell line after 48 hours with test conditions.

The assessment was carried out by measuring the average cell-cell distance in µm for the seven test conditions. These test conditions were SF-MEM, TGFβ1, EGF, TGFβ1+ TGF-β RI Kinase Inhibitor VII, TGFβ1+MK-2206, TGFβ1+PD98059 and TGFβ1+SB431542. Raw data can be found in appendix 1 (page 301).

(ii) **Gap Closure Assay:** The Gap closure experiment was used to investigate the effect of growth factors TGFβ1 and EGF to induce the SAS-H1 cell line to migrate. The assessment of cell migration was indicated by the movement of cells toward the cell-free area after specific time periods with the test conditions (**Table 18**). Furthermore, this assay was used to identify the mode of cell migration, single or collective cell migration, by examining cells at either edge (**Figure 56**).

(1) **Negative Control (Serum Free MEM):** The assessment of the cell-free area showed that there was no significant change after short incubation periods including an hour, two hours and six hours compared to time zero. The gap persisted at 24 hours in serum-free MEM with a large part of it not being invaded by the SAS-H1 cells (**Figure 56-E**). There were no signs of morphological change to the cells, individual cells at the border of the gap or in the cell-free zone.

(2) **10ng/ml of TGFβ1:** The cell-free area in the TGFβ1-treated culture dishes resembled the negative control after one hour, two hours and six hours. However, after 24 hours of observation, the SAS-H1 cells had partially closed the gap (**Figure 56-J**). Also, there was no evidence of individual cells in the remaining gap. These results suggested that TGFβ1 induce the SAS-H1 cells to migrate and invade the cell-free zone.

(3) **10ng/ml of EGF:** The culture dishes that had been incubated with exogenous EGF showed the same response as those incubated with 10ng/ml of TGFβ1. The gap had closed after 24 hours (**Figure 56-O**). However, with EGF, it was observed that cells had also changed their morphology and migrated into the cell-free area individually, which was not observed with the cells incubated with TGFβ1-treated assays.

Table 18: Summary of the Gap closure assay with the SAS-H1 cell line.

Cells were incubated with the test conditions (SF-MEM, 10ng/ml TGFβ1 and 10ng/ml EGF) for different incubations periods (time zero, one hour, two hours, six hours and 24 hours). This observational assessment was based on the three criteria describing the status of the gap: which was open or (-) describing the gap at the beginning of the experiment, partially closed which indicates there was a change in the size of the gap but still open and finally, gap closed.

Test Conditions	T=0	T=1hr	T=2hrs	T=6hrs	T=24hrs
SF-MEM	_	_	_	_	_
TGFβ1	_	_	_	_	Gap partially closed
EGF	_	_	_	_	Gap closed

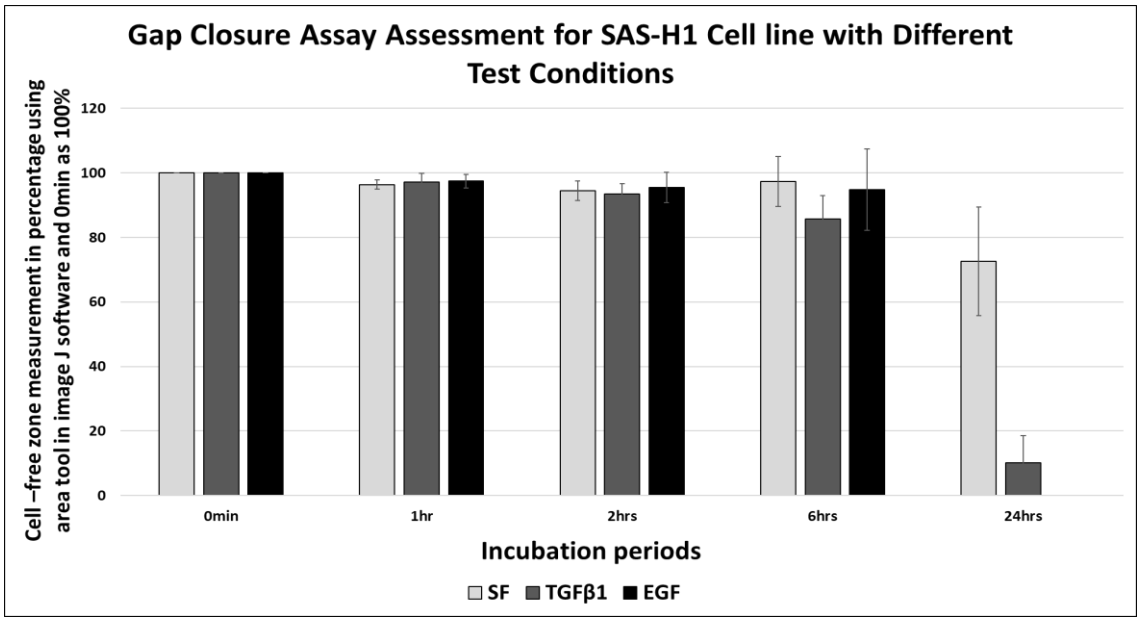


Figure 55: Assessment of the Gap closure assay with inserts for the SAS-H1 cell line.

Cells were incubated with either the negative control (SF-MEM alone) or with growth factors (10ng/ml TGFβ1 & 10ng/ml EGF) at incubations periods: time zero, one hour, two hours, six hours and 24 hours. The graph illustrates how much of the gap remains after the different incubation periods with the three test conditions. Measurements of the cell-free area were calculated using Image J software, on images taken at 40X magnification. The formula for calculating the cell free zone was derived using a tool in the software called Area. The value of the Area was then transformed to a percentage, to compare the change in the size of the gap over the different incubation periods for the same sample. The cell-free area at Time = 0 was considered as the 100%. The mean and standard deviation of nine measurements in total were calculated per test condition at the same incubation periods. Each test condition had five inserts, three of them were chosen randomly, before introducing the test conditions. Three pictures were then taken for each insert at specific incubation periods. The graph shows a significant reduction in the cell-free area in all test conditions at 24 hours. Raw data can be found in appendix 2 (page 305).

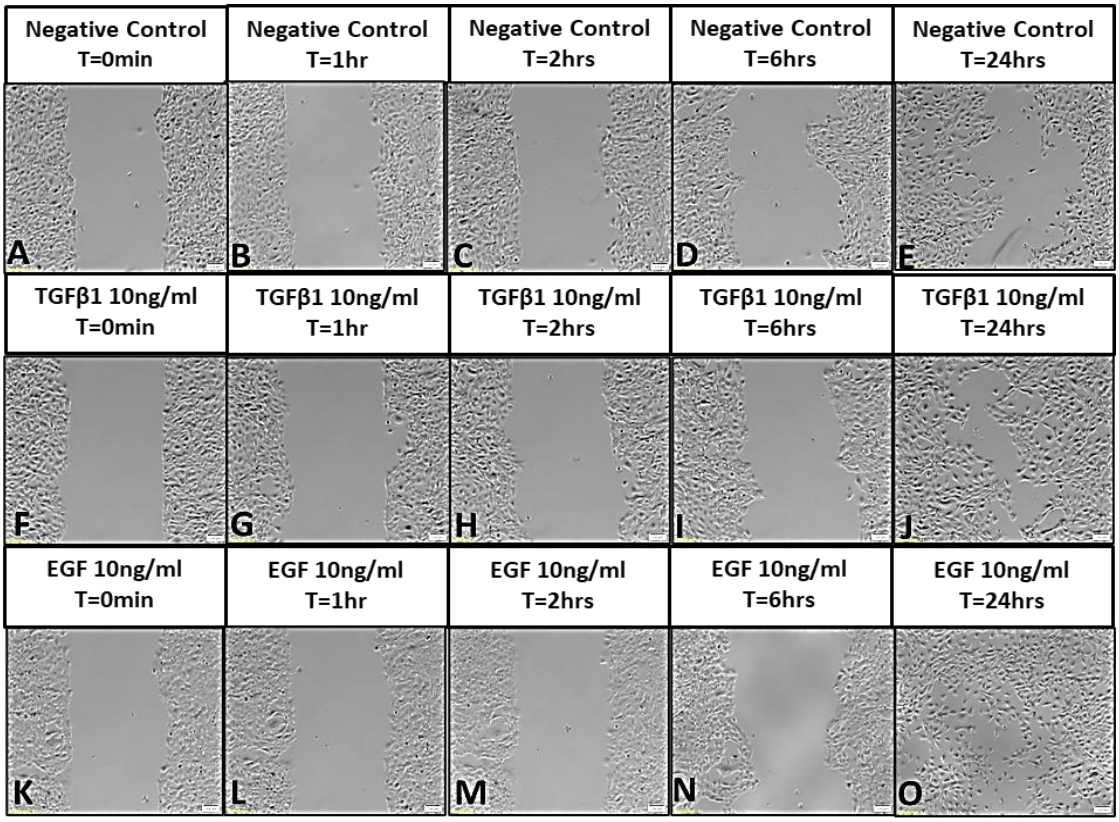


Figure 56: Gap closure Assay with the SAS-H1 cell line.

Images of the Gap closure assay with inserts for the SAS-H1 cells at 40X magnification. There were three different test conditions (SF-MEM, TGFβ1 and EGF) at different incubation periods (0 min, an hour, two hours, six hours and 24 hours). Three images were taken per insert for the observational assessment and three inserts were used per test condition. The total number of images per test condition was nine. All test conditions showed persistence of the gap at six hours. The gap remained open with cultures incubated with SF-MEM **(D)** at 24 hours incubation. TGFβ1 **(J)** and EGF **(O)** showed complete gap closure at 24 hours. The only difference was observed at 24 hours with the EGF-treated assays **(O),** where individual cells were observed in the gap.

(iii) Scratch Assay: The Scratch assay was used to assess cell migration, especially the collective mode of movement. The aim of this experiment was to record the size of the cell-free zone over time, while incubating SAS-H1 with a range of growth conditions. In this test, the cell-free area was created by using an instrument to make the scratch. At the start and the end of the scratch experiment, measurements of the cell-free zone were taken (**Table 19**).

(1) **Negative Control (SF-MEM):** In SAS-H1 showed almost no cell migration towards the gap after 24 hours, when incubated in SF-MEM alone. (**Figure 58-B**).

(2) **10ng/ml of TGFβ1:** After 24 hours, the SAS-H1 cells migrated into the gap, when incubated with exogenous TGFβ1. No individual cells were observed in the gap. This suggested that the cells used only collective cell migration to move towards the cell-free area (**Figure 58-D**).

(3) **10ng/ml of EGF:** When the SAS-H1 cell line was incubated with exogenous EGF, cells migrated and closed the gap after 24 hours. The cell-free area was completely covered with cells. It was difficult to determine which mode of cell migration was used by the SAS-H1 cell line (**Figure 58-H**).

(4) **10ng/ml of TGFβ1 plus 5μM of TGF-β RI Kinase Inhibitor VII:** After 24 hours incubation with TGFβ1 and TGF-β RI Kinase Inhibitor, the cell-free area remained almost the same compared to the baseline (time zero) (**Figure 58-F**). This indicated that the TGFβ-RI Kinase Inhibitor VII prevented SAS-H1 cells from migrating and closing the gap.

(5) **10ng/ml of EGF plus 5μM of TGF-β RI Kinase Inhibitor VII:** After 24 hours, cells incubated with a mixture of EGF and TGFβ-RI Kinase

Inhibitor VII showed less gap closure compared to the EGF-only samples, but there was evidence of migratory cells (**Figure 58-J**). It appeared that the inhibitor stopped some of the cells from moving towards the gap.

(6) **10ng/ml of TGFβ1 plus 1μM of MK-2206 (Akt Inhibitor):** Adding an Akt inhibitor with TGFβ1 to the culture dishes prevented cells from migrating toward the gap. However, cell migration was observed compared to time zero samples. Both individual migration and collective cell migration were observed (**Figure 58-L**). As a result of using an Akt inhibitor, the gap was partially closed suggesting that the Akt pathway had a role in cell migration of the SAS-H1 cell line.

(7) **10ng/ml of EGF plus 1μM of MK-2206 (Akt Inhibitor):** After 24 hours, the SAS-H1 cells migrated toward the cell-free zone in medium contained both EGF and MK-2206. Signs of cells migrating individually and collectively were also observed (**Figure 58-R**). In addition, small gaps continued to persist, although some areas showed contact between the cells from both edges of the scratch. These results suggest there was an alteration in the mode of cell migration compared to the EGF-alone group.

(8) **10ng/ml of TGFβ1 plus 5μM of PD98059 (MAPK Inhibitor):** The assessment of cell migration in culture dishes incubated with both TGFβ1 and the MAPK inhibitor revealed that the gap persisted after 24 hours (**Figure 58-N**). This indicated the PD98059 inhibited cell migration, which was activated by TGFβ1.

(9) **10ng/ml of EGF plus 5μM of PD98059 (MAPK Inhibitor):** The results of adding the MAPK inhibitor with EGF to the SAS-H1 cell line showed that the inhibitor prevented the induction of cell migration by the growth factor and the gap still existed after 24 hours (**Figure 58-T**).

(10) **10ng/ml of TGFβ1 plus 5μM of SB431542 (Smad Inhibitor):** 24 hours incubation with TGFβ1 and the Smad inhibitor, SB431542 prevented the SAS-H1 cells from migrating toward the cell-free area (**Figure 58-P**). These findings indicate that the Smad inhibitor blocked the activation of cell migration by TGFβ1.

(11) **10ng/ml of EGF plus 5μM of SB431542 (Smad Inhibitor):** Using the Scratch assay to assess cell migration in culture conditions containing both EGF and a Smad inhibitor revealed two findings: The gap still existed after 24 hours with the growth factor and inhibitor and secondly, many individual cells were found in the gap, which suggested that single cell migration had occurred (**Figure 58-V**).

Table 19: Summary of the Scratch assay assessment with the SAS-H1 cells

The table shows the observation of the SAS-H cell line regarding cell migration using the scratch assay protocol. Images were taken at two incubation periods (time=zero and 24 hours) at 40X magnification from a specific area of the culture dishes, which was marked before the beginning of the experiment. The assessment of this assay was built on two criteria: the status of the gap and the type of cell migration in terms of single or collective. The status of the gap has three categories whether it is open (-) at the start of the experiment, partially closed or closed.

Test Conditions to Assess Cell Migration (Scratch Assay) SAS-H1 Cell line		
Test Conditions	**T= 0**	**T= 24hrs**
Negative Control	_	_
TGFβ1	_	Gap partially closed + Collective cell migration
EGF	_	Gap partially closed + Collective cell migration
TGFβ1 + 5μM TGF-β RI Kinase Inhibitor VII	_	_
EGF + 5μM TGF-β RI Kinase Inhibitor VII	_	_
TGFβ1 + 1μM MK2206	_	_
EGF + 1μM MK2206	_	Gap partially closed + Collective cell migration + Single cell migration
TGFβ1 + 5μM PD98059	_	_
EGF + 5μM PD98059	_	_
TGFβ1 + 5μM SB431542	_	_
EGF + 5μM SB431542	_	Single cell migration

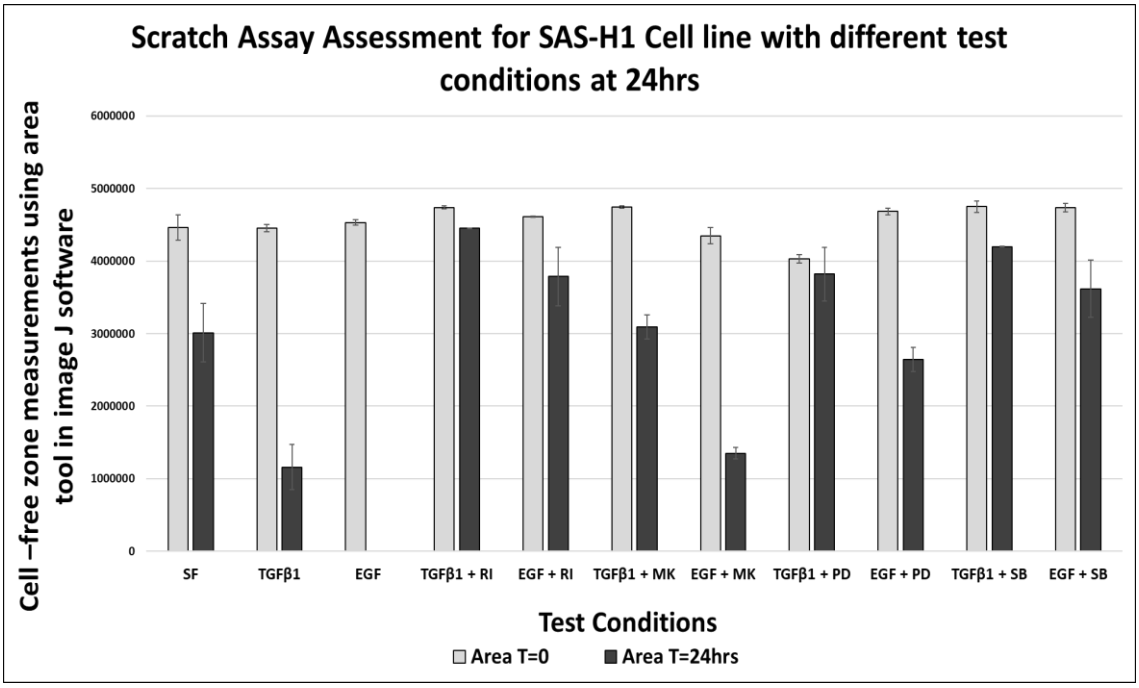


Figure 57: Scratch Assay assessment of the SAS-H1 cell line with different test conditions

The figure illustrates the difference in the cell-free area over different incubation periods with the test conditions. Data for the cell-free zone were calculated using Image J software, on images, taken at 40X magnification. The formula for calculating the cell free zone was derived using a tool in the software called Area. Data for the cell-free area at Time=zero was considered as 100%. The mean and standard deviation of three measurements in total were calculated per test conditions at the same incubation period. Raw data can be found in appendix 3 (page 308).

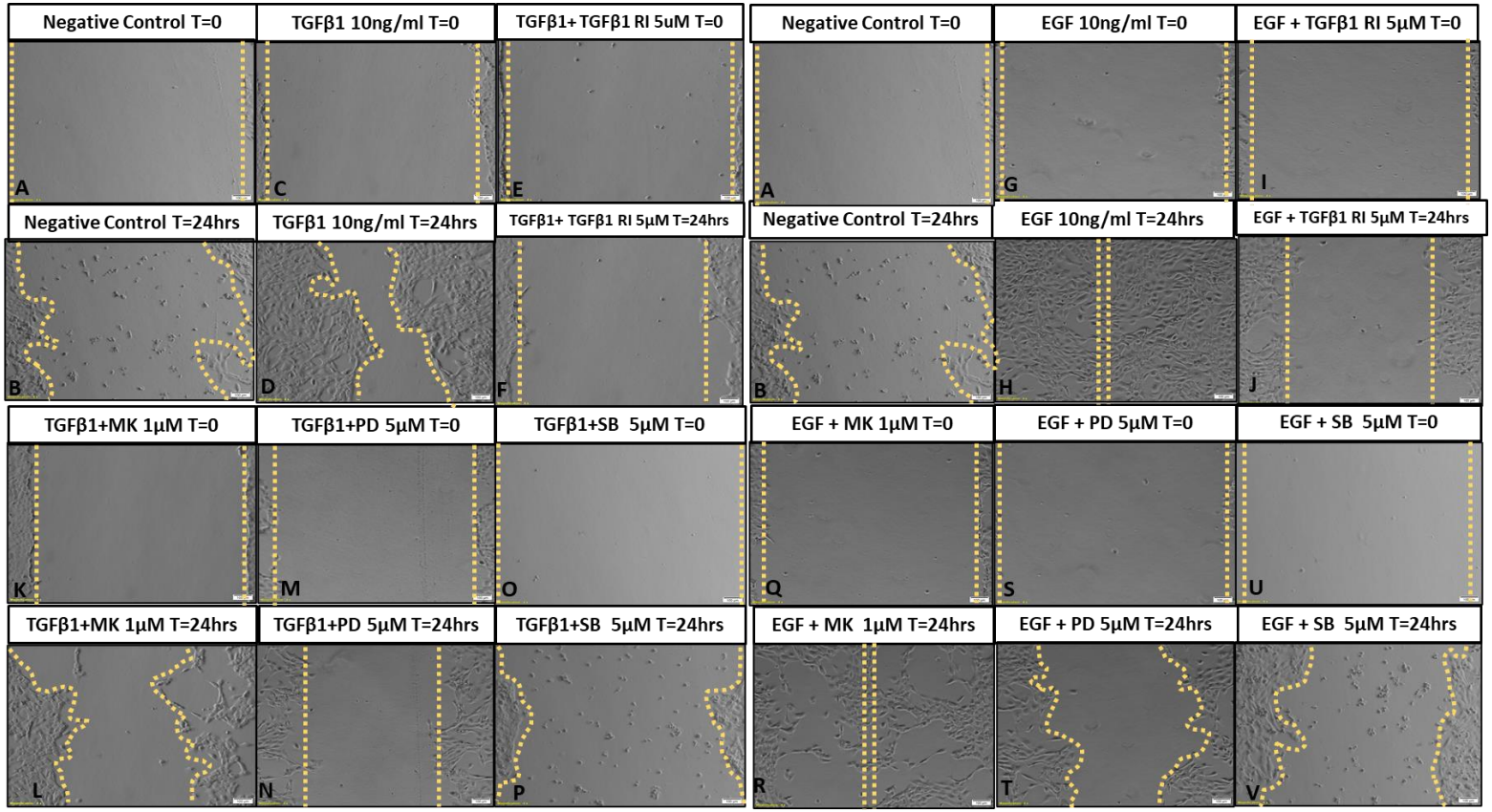


Figure 58: Scratch Assay assessment for the SAS-H1 cell line with different test conditions

Images of the Scratch Assay for the SAS-H1 Cell Time = Zero and 24 hours at 40X magnification. Images were taken from specific areas, which were marked at the beginning of the experiment as a reference. The total number of pictures taken for each test condition was three per each incubation period. The yellow dotted lines represent the edges of the gap. By observing the test groups at two incubation periods, culture dishes with TGFβ1 **(D)**, EGF **(H)** and EGF+MK-2206 cultures dishes **(R)** showed a significant decrease in the gap because of SAS-H1 cells migrating into it. In addition, the EGF+SB431542 culture dish **(V)** showed single cells in the gap.

Summary:

Data collected from the Scatter assay for the SAS-H1 showed change in cell morphology, alteration in colony appearance and scattered cells in response to both growth factors. These effects generated by TGFβ1 were blocked only by the MAPK inhibitor, however, the migratory effect of EGF was not stopped completely by any of the inhibitors used in this experiment.

The assessment of the wound healing assays revealed that both TGFβ1 and EGF stimulated the SAS-H1 cells to migrate and close the gap. In the case of TGFβ1, all inhibitors (TGF-β RI Kinase Inhibitor VII, Akt inhibitor, MAPK inhibitor, and Smad inhibitor) prevented cells from closing the gap and migrating collectively. However, EGF with inhibitors produced two distinct results. Two of the inhibitors, TGF-β RI Kinase Inhibitor VII and MAPK inhibitor, prevented cell migration and the gap from closing, while the other two, the Akt inhibitor and Smad inhibitor, did not stop migration.

4.5 Summary of Migration Assays Results:

In the Scatter assay, the test conditions included growth factors alone or growth factors with inhibitors, induced some form of change in the cell morphology and colony appearance, except in three conditions. These growth conditions were SF-MEM group (the control group), TGFβ1 plus TGF-RI Kinase Inhibitor VII (Receptor inhibitor) and TGFβ1 plus PD98059 (MAKP Inhibitor) only with the SAS-H1 cell line. These findings indicate that the MAPK signalling pathway has a major role in the cell migration of the SAS-H1 cell line. Moreover, the morphological changes of the cells with the Smad inhibitor plus growth factors and Akt inhibitor plus growth factors were different from the changes that happened to the SAS-H1 cells when exogenous TGFβ1 and exogenous EGF were added alone. These results suggesting the possibility of different mechanisms for cell migration induction. For the wound healing assays, data suggested the possibility that more than one signalling pathway is involved in collective cell migration.

4.6 Discussion:

Three migration assays were used to investigate the migratory effect of TGFβ1 on normal and oral cancer cell lines. In addition, different inhibitors for specific signalling pathways were used to establish a link between the growth factor and cell migration. The typical appearance of the epithelial cells varies from sheet, tubes or vesicles. With these forms of epithelial cells, maintaining an apical to basal polarity is crucial. Also, epithelial cells are closely associated with each other by specialised adhesion structures used to maintain their close proximity (Xu, Lamouille and Derynck, 2009). When epithelial cells lose their polarity (apical-basal polarity), disconnect from their neighbouring cells and change their cytoplasmic organisation, they acquire the ability to migrate (Kalluri and Weinberg, 2009; Bu and Chen, 2017). These characteristics were reported in the early phase of EMT, which starts with disassembly of tight junctions, disruption of cellular polarity and promoting cytoskeleton rearrangement (Xu, Lamouille and Derynck, 2009). The change in epithelial cell morphology from cuboidal to a spindle-like shape was observed after treatment with TGFβ (Miettinen *et al.*, 1994). This evidence was supported by the morphological assessment of oral cancer cell lines, which included measuring the distance between cells at time zero and after the treatment with the test conditions *in vitro*. The principle of measuring the distance between cells utilised changes in cell morphology including a loss of polarity, loss of cell adhesion and cytoskeletal rearrangement. This protocol added numerical support to the findings from the observational assessment.

The Scatter assay and wound healing assays provided the evidence for cell migration with the normal and oral cancer cell lines in response to TGFβ1. These results are in agreement with studies investigating the role of TGFβ signalling pathways in both physiological and pathological conditions (Massagué, 2008). In a pathological context, especially cancer, the TGFβ signalling pathway favours tumour growth, evading immune surveillance and invasion of the underlying tissues and metastasis (Massagué, 2008). Each of the cell lines used in this project showed a distinct pattern of response to the growth conditions. These patterns ranged from the presence or not of cell migration and the mode of cell migration i.e. whether single or collective. These data support the evidence that epithelial cells have to change their morphology and characteristics to be able to move away from their colonies (Xie *et al.*, 2018).

Each of the cell lines showed distinct morphological features as result of cell migration. For the HaCaT cell line, TGFβ1 induced cells to become large and round compared to the control group, while EGF did not promote any cells to migrate. These findings are supported by reports of the role of the TGFβ signalling pathway during wound healing, where the growth factor induces epithelial cells to migrate via undergoing EMT. However, the observation of epithelial cells highlights their transformation to fibroblast like cells (**Figure 59**) (Morikawa, Derynck and Miyazono, 2016).

The treatment of the TYS cell line with TGFβ1 resulted in the epithelial cells changing to large, rounded shapes within the colony or small rounded cells, after being separated from it. Addition of EGF to the TYS cells resulted in a change in morphology to a spindle shape and also caused them to scatter (**Figure 60**). Regarding the SAS-H1 cells, TGFβ1 induced cells to become large and round in shape, similar to the changes observed with the HaCaT and TYS cell lines. The treatment with exogenous EGF induced the SAS-H1 cells to separate from their colonies and become small and rounded in shape (**Figure 61**). These findings are in agreement with the fact that TGFβ signalling also has the ability to induce differentiation of epithelial cells by acquiring some oncogenic mutations (Xie *et al.*, 2018).

As a result of treatment with exogenous TGFβ1, cancer cells gain fibroblast-like characteristics, such as losing cell polarity and cell to cell adhesion. Both breast and prostate cancer, especially bone metastases, showed a high level of TGFβ signalling (Xie *et al.*, 2018). However, Grünert (2003), suggested that there are a few differences between the concept of scattering and EMT. The two main points reported by Grünert were, firstly EMT needs around four to six days for completion and the second a 3D culture model is needed to confirm the EMT process, which depends on the cell type. Also, further confirmation needs to be established by investigating expression of epithelial and mesenchymal markers. Epithelial cells can be induced to become fibroblastoid in shape after being treated with different growth factors, including TGFβ. Subsequently, these changes lead to the activation of a wide range of signalling pathways, which have been linked to cell scattering or EMT including Smad, ErK/ MAPK, PI3K/Akt and GTPase (Grünert, Jechlinger and Beug, 2003).

In the case of the HaCaT and TYS cell lines, the migratory effect of TGFβ1 was blocked by both inhibitors: TGF-β RI Kinase Inhibitor VII and SB431542 (**Figure 59 & Figure 60**). This indicates that the Smad signalling pathway has a role in migration of the HaCaT and TYS cell lines. In the case of SAS-H1, the effect of the growth factor was also inhibited by TGF-β RI Kinase Inhibitor VII, but not with SB431542 (**Figure 61**). However, using the MAPK inhibitor (PD98059) with TGFβ1 led to the SAS-H1 cells to show less evidence of cell migration.

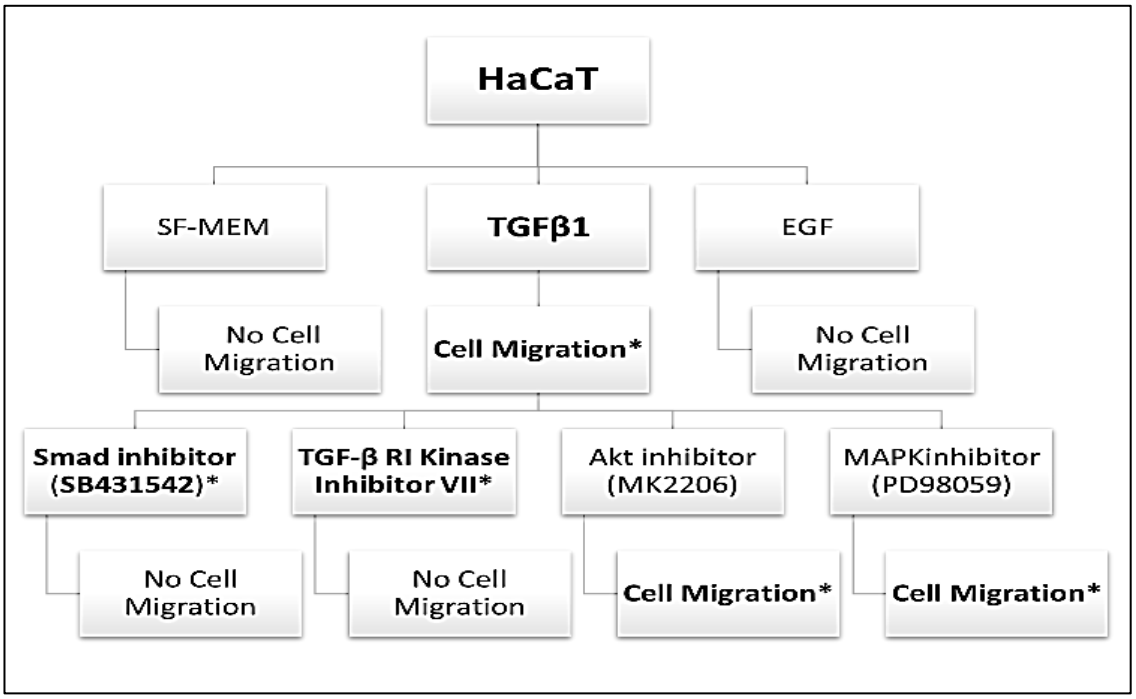


Figure 59: Summary of the Scatter assay results on the HaCaT cell line using different signalling pathway inhibitors.

The diagram shows the summary of the interactions between growth factors and signalling pathways in terms of cell migration for the HaCaT cell line. The migratory response of the HaCaT cell line to TGFβ1 was blocked only by using SB431542 and TGF-β RI Kinase Inhibitor VII.

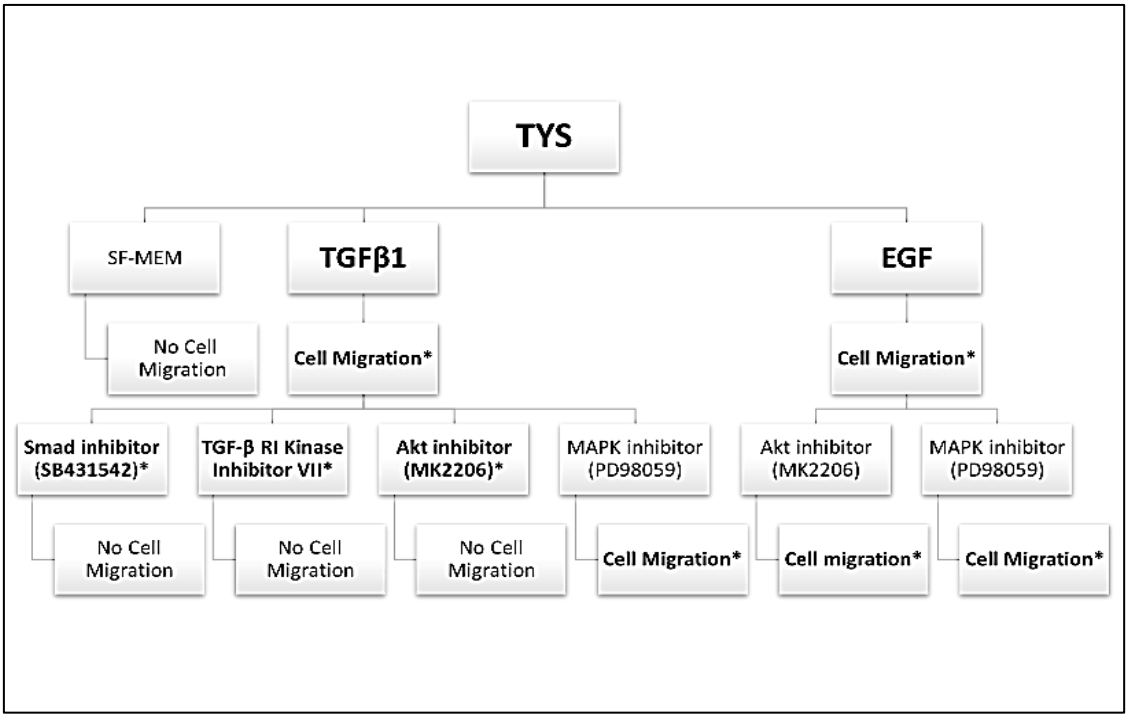


Figure 60: Summary of the Scatter assay results on the TYS cell line using different signalling pathway inhibitors.

The diagram shows the summary of the interactions between growth factors and signalling pathways in terms of cell migration for the TYS cell line. The migratory response of the TYS cell line to TGFβ1 was blocked by using SB431542, TGF-β RI Kinase Inhibitor VII and MK-2206. In response to EGF, TYS cells continued to migrate even after addition of MK-2206 and PD98059.

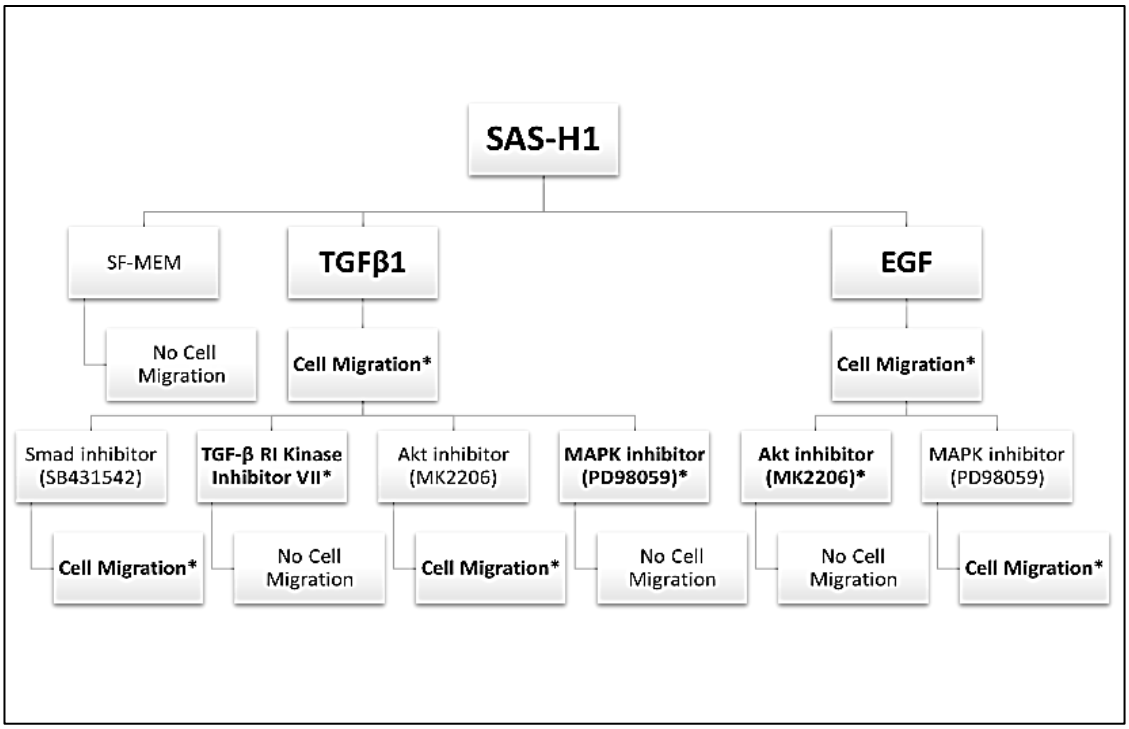


Figure 61: Summary of the Scatter assay results on the SAS-H1 cell line using different signalling pathway inhibitors.

This diagram shows the summary of the interactions between growth factors and signalling pathways in terms of cell migration for the SAS-H1 cell line. The migratory response of SAS-H1 cell line to TGFβ1 was blocked only by using TGF-β RI Kinase Inhibitor VII and PD98059. In response to EGF, SAS-H1 cells continued to migrate after introducing PD98059, but it was blocked by MK-2206.

Furthermore, the investigation regarding the ability of TGFβ1 to induce the single or collective mode of cell migration in normal and oral cancer cell lines was carried out. By including inhibitors for different signalling pathways, the assessment was based on establishing the link between cell migration and specific signalling pathways. The criteria used to identify collective cell migration includes cells moving together, cells maintain their contact during migration, or the ability for cells to affect each other while migrating either by promoting movement or impeding it (Rørth, 2009).

In the cell migration assessment, two types of wound healing assays were used to evaluate collective cell migration including the Gap closure assay and the Scratch assay. Even though those assays are similar, there is a fundamental difference between them. With the Gap closure assay, a barrier was used to create a free-cell zone by using inserts, while the protocol of Scratch assay included using a scratch tool to remove part of the monolayer of cells to create a cell-free zone. This process might add other variables to the experiment such as ligands released as result of cells damage during creating of the wound. Also, cells at the edge of the scratch, might lose their tight adhesion to neighbouring cells due to the tension during creating the scratch.

Both assays have advantages and disadvantages regarding experiment design and outcomes. The Gap closure assay has the advantage of recreating a cell-free zone with a linear distance around 500 µm, which represents the thickness of the barrier of the insert. This feature allowed generation of cell-free zones with similar dimensions. However, due to the small size of the gap, cells might be able to close it even in the negative control due to proliferation or cell migration. To overcome this issue, we used the Scratch assay to create a large cell-free zone area to allow the assessment to be carried out for a longer period. However, the major disadvantage of this assay is the difficulty in producing a cell-free area with similar dimensions for each of the assay dishes.

For the Scratch assay with the HaCaT cell line, only samples that were treated with EGF showed a reduced cell free zone compared to time zero and the negative control group. However, the Gap closure assay showed that both TGFβ1 and EGF induced cells to migrate toward the gap. The TYS cell line showed a collective mode of cell migration with both growth factors, in both of the wound healing assays. However, the TYS cells managed to migrate into the cell-

free zone in the Gap closure assay even with SF-MEM only. The cell-free area persisted with the different inhibitors used in the Scratch assay, suggesting more than one signalling pathway is involved in collective cell migration. The response of SAS-H1 to the test conditions was different to TYS. First, in the Gap closure assay, both TGFβ1 and EGF induced the SAS-H1 cells to migrate into the gap, but not with SF-MEM alone. There were similar results to the TYS cells with the Scratch assay, both growth factors promoted the SAS-H1 cells to migrate into the cell free area and this effect was blocked with different inhibitors except EGF and MK2206. As Friedl reported, different types of cancer demonstrated collective cell migration using *in vitro* models such as oral squamous cell carcinoma, breast cancer and colorectal carcinoma. Also, it was suggested there is a link between TGFβ and collective cell migration during cancer cell movement (Friedl and Gilmour, 2009).

Chapter 5: Investigating the Activation of Signalling Pathway Molecules Following TGFβ1 Induction.

5.1 Background:

The transforming growth factor β superfamily including TGFβ and BMPs, regulate a range of physiological processes such as cell proliferation, differentiation and cell migration (Derynck and Zhang, 2003). In cancer, it was reported that TGFβ has two distinct roles during carcinogenesis, first acting as a tumour suppressor at the early stage of cancer development and then at the late stage, it enhances tumour progression (Derynck and Zhang, 2003; Bierie and Moses, 2006; Xie *et al.*, 2018). The activation of TGFβ signalling pathways leads to signal transport by two types of pathways (Derynck and Zhang, 2003; Xie *et al.*, 2018). First the Smad-dependent pathway, which is a linear signalling pathway starting with an interaction between the ligand and type II to type I receptor kinase receptors, resulting in Smad protein phosphorylation and ligand-induced transcription. Secondly, the Smad-independent pathways including PI3K/Akt, MAPK and Rho signalling pathways (Derynck and Zhang, 2003; Xie *et al.*, 2018). Among these downstream molecules only Smads are specifically activated by TGFβ family members (Bierie and Moses, 2006).

TGFβ family members, TGFβ receptors and downstream molecules have all been investigated for their roles in cancer development and progression (Xie *et al.*, 2003; Bierie and Moses, 2006). The general consensus is that TGFβ has multiple roles in human cancer. These roles range from inhibition of cell growth to inducing a change in cell morphology and migration (Bierie and Moses, 2006). The activation of the Smad-dependent signalling pathway, in many types of cells, regulates the inhibition of cell growth, apoptosis and epithelial cell differentiation (Xie *et al.*, 2018). Also, in tumour cells, the Smad-dependent pathway is linked to cancer cell metastasis via mediating EMT, when cells acquiring motile properties and lose cell polarity and cell-to-cell adhesion (Xie *et al.*, 2018).

The PI3K-Akt signalling pathway has a crucial role in human development and cancer progression. The regulation of these processes is controlled by PIP3, which orchestrates the functions of a wide range of proteins, including proteins such as kinase B or Akt (Alzawi *et al.*, 2022). Akt regulates a wide range of cellular activities including cell migration (Ahmed *et al.*, 2020; Alzawi *et al.*, 2022). The PI3K-Akt pathway induces cell migration by regulating cytoskeletal re-arrangement (Alzawi *et al.*, 2022). To produce a high level of protein kinase B

(PKBα) activity, phosphorylation of both residues Thr308 and Ser473 in response to growth factor is required (Alessi *et al.*, 1996).

As a major signalling pathway involved in many cellular processes, the mitogen-activated protein kinases (MAPK) can be activated through a wide range of external stimuli including TGFβ1 (Santibáñez *et al.*, 2002) and EGF (Berridge, 2014). One of the MAPK pathways is the Erk1/2 or p44/42, which is activated by MEK1 and MEK2. The Erk1/2 signalling pathway has been investigated and targeted in cancer diagnosis and treatment (Roberts and Der, 2007). In tumour cells, studies reported that the presence of TGFβ1 was correlated with an increase in invasiveness and the production of uro-kinase-like plasminogen activator. This process requires the activation of the Ras-ErK1/2 signalling pathway (Santibáñez *et al.*, 2002).

Rho-family proteins regulate various cellular activities including cell migration (Etienne-Manneville and Hall, 2002; Wennerberg and Der, 2004; Vega and Ridley, 2008). RhoA, Rac1 and Cdc42 have a role in reorganising actin in the cytoskeleton, yet different outcomes in terms of cell shape and movement (Wennerberg and Der, 2004; Hall, 2009; Algayadh, Dronamraju and Sylvester, 2016). Rac1 induces actin polymerisation and lamellipodia formation, which are curtain like extension at the leading edge. Lamellipodia contain protrusive sheets of actin. Filopodia are thin finger-like extensions from the cytoplasm, which contain tight actin bundles. The formation of filopodia is induced by Cdc42 (Wennerberg and Der, 2004).

Four different signalling pathways that have been linked to cell migration were investigated, including the Smad pathway, the PI3K-Akt pathway, the MAPK pathway and the Rho-GTPase pathway. Experiments were designed to investigate the upregulation and downregulation of each of these signalling pathways in response to the test conditions at different incubation periods.

5.2 Aims and Hypothesis:

5.2.1 Aims:

The purpose of this study was to investigate the status of specific signalling molecules regarding their activation or phosphorylation in response to TGFβ1 in normal and oral cancer cell lines.

5.2.2 Objectives:

(**a**) To identify at which incubation periods the signalling pathways were activated, after the cells were treated with the growth factors only.

(**b**) To evaluate the expression and/or activation of the signalling molecules in the presence of specific inhibitors in addition to TGFβ1, at specific incubation periods.

(**c**) To identify the similarities and the differences between the response of normal and oral cancer cell lines to the test conditions in this study.

5.2.3 Hypothesis:

It was hypothesised that TGFβ1 upregulates the Smad pathway, the PI3K-Akt pathway, the MAPK pathway and the Rho-GTPase pathway in normal and oral cancer cell lines. The activation of these signalling pathways occurs prior or around the occurrence of cell migration.

5.2.4 Null Hypothesis:

TGFβ1 fails to activate the targeted signalling pathways.

5.3 Materials and Methods:

Reagents and the protocol for the SDS-PAGE and Western blotting were described in the general materials and methods chapter two (Pages 44 & 45). However, specific antibodies were used in this protocol to investigate the specific signalling molecules and are described below (**Table 20**).

Table 20: Specific antibodies used for the SDS-PAGE & Western Blot protocol.

No.	Reagents/Antibodies	Company	Catalogue No.
1	Phospho-Smad2 (Ser465/467) (138D4) Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	3108
2	Phospho-Akt (Thr308) (D25E6) Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	13038
3	Phospho-Akt (Ser473) (D9E) Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	4060
4	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	4370
5	Phospho-Rac1/Cdc42 (Ser71) Antibody	Cell Signaling Technology Inc., Danvers, MA, USA	2461
6	Anti-rabbit IgG, HRP linked Antibody	Cell Signaling Technology Inc., Danvers, MA, USA	7074

Assessment:

Image Lab software was used to assess and analyse protein expression or phosphorylation in the test conditions at specific incubation periods. The assessment was carried out at two stages:

1- **First Stage or Stage I**: The activation of the signalling molecules was analysed at different incubation periods includes 0 min, an hour, two hours, four hours, six hours, 24 hours and 48 hours with only two growth factors: TGFβ1 and EGF (**Figure 62**). The aim of this stage was to evaluate the activation of the targeted proteins and identify the best incubation periods for that activation.

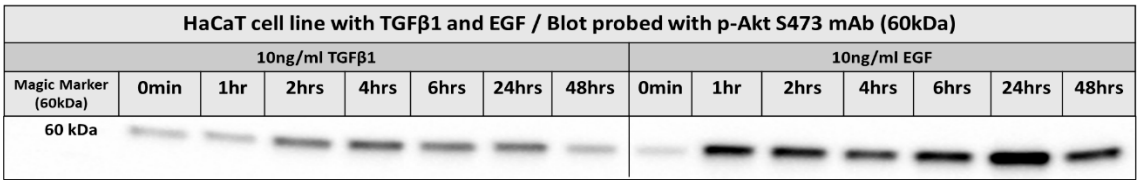


Figure 62: An example of the design for Stage I assessment.

The HaCaT cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Akt S473 monoclonal antibody at a 1:2000 dilution, followed by goat anti-rabbit secondary.

2- **Second Stage or Stage II:** At this stage of assessment, eight different test conditions were used including control group (SF-MEM) at 48 hours, TGFβ1 at 48 hours, EGF at 48 hours, TGFβ1+ TGF-β RI Kinase Inhibitor VII at 48 hours, TGFβ1+MK-2206 at 48 hours, TGFβ1+PD98059 at 24 hours and 48 hours, and TGFβ1+SB431542 at 24 hours and 48 hours (**Figure 63**). The selection of these incubation periods was based on the findings from previous experiments such as migration assays and the first stage of analysis of protein expression using the SDS-PAGE and Western Blot protocol. Data from this stage allowed determination of the effects of the test conditions on the status of signalling molecules at specific incubation periods.

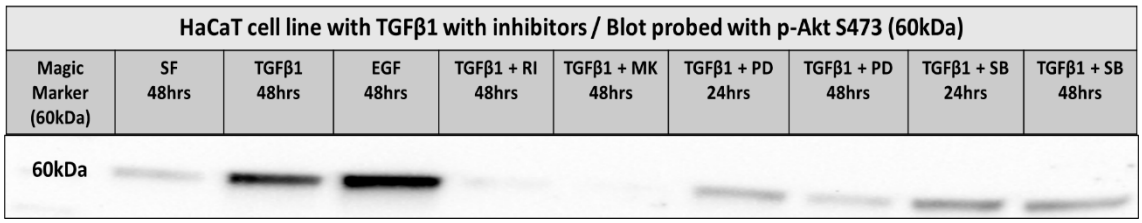


Figure 63: An example of the design for Stage II assessment.

The HaCaT cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Akt S473 monoclonal antibody at a 1:2000 dilution, followed by goat anti-rabbit secondary.

5.4 Results of Activation of Signalling Pathways in Oral Cancer Cell Lines:

5.4.1 HaCaT Cell Line:

Before the stage II assessment, observational assessment for the HaCaT cell line was carried out by taking photomicrographs of the samples prior to the cell lysis protocol (**Figure 64**). The aim of this observation was to evaluate the status of the cells in terms of cell morphology or colony appearance.

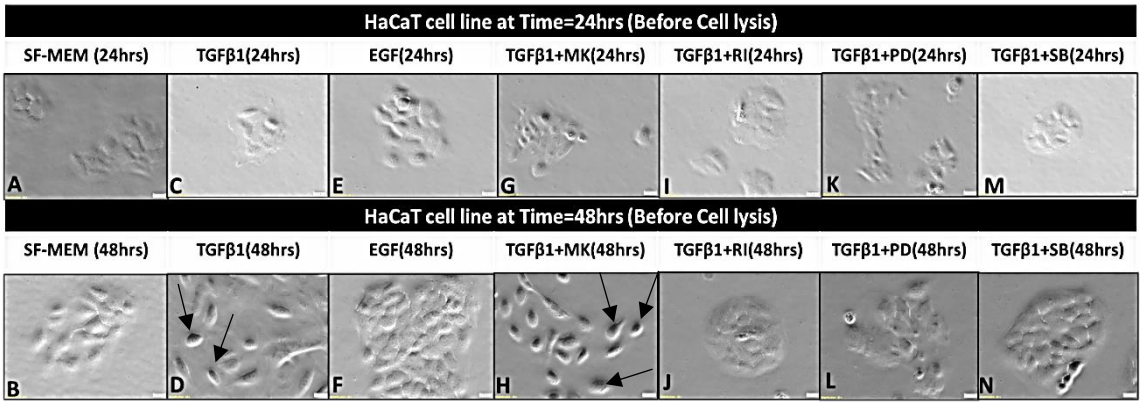


Figure 64: Observational assessment for the HaCaT cell line.

Images for the HaCaT cell line with SF-MEM at 48 hours, TGFβ1 at 48hours, EGF at 48 hours, TGFβ1+ TGF-β RI Kinase Inhibitor VII at 48 hours, TGFβ1+MK2206 at 48 hours, TGFβ1+PD98059 at 24 hours and 48 hours, and TGFβ1+SB431542 at 24 hours and 48 hours before cell lysis. All images were taken at 200x magnification to observe both cell morphology and colony appearance. (↑) scattered cells.

Culture dishes that were treated with TGFβ1 at 48 hours (**Figure 64-D**), TGFβ1 with MK-2206 at 48 hours (**Figure 64-H**) and TGFβ1 with PD98059 at 48 hours (**Figure 64-L**) showed changes in both cell morphology and colony appearance. Cells appeared to be large and rounded in shape, not in compact colonies and with no evidence of scattered cells. These changes in cell morphology were linked to specific incubation periods, which indicated the occurrence of cell migration before starting the cell lysis protocol.

Smad Pathway Assessment for the HaCaT Cell Line:

(a) <u>Stage I Assessment of Smad Pathway:</u>

To investigate the activation of the Smad signalling pathway in response to both growth factors, TGFβ1 and EGF, p-Smad2 was evaluated at different incubation periods. An upregulation in Smad2 phosphorylation was detected in response to TGFβ1 compared to time zero (**Figure 65**). The phosphorylation of Smad2 started after an hour incubation period with

TGFβ1 and continued until 24 hours. This upregulation in p-Smad2 level decreased significantly after 48 hours (**Figure 66**). There was no phosphorylation of Smad2 in response to EGF throughout the incubation periods (**Figure 65**).

Table 21: The format of stage I assessment for Smad pathway in the HaCaT cell line.

(1) Magic Marker						
(2) TGFβ1 (0min)	**(3)** TGFβ1 (1 hour)	**(4)** TGFβ1 (2 hours)	**(5)** TGFβ1 (4 hours)	**(6)** TGFβ1 (6 hours)	**(7)** TGFβ1 (24 hours)	**(8)** TGFβ1 (48 hours)
(9) EGF (0min)	**(10)** EGF (1 hour)	**(11)** EGF (2 hours)	**(12)** EGF (4 hours)	**(13)** EGF (6 hours)	**(14)** EGF (24 hours)	**(15)** EGF (48 hours)

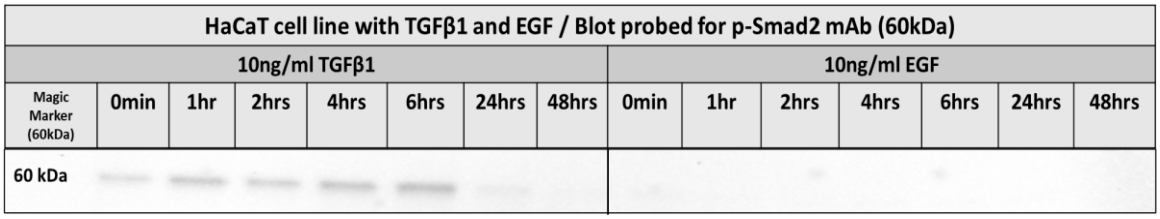


Figure 65: Stage I assessment for p-Smad2 in the HaCaT cell line.

The HaCaT cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Smad2 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells, revealed that the phosphorylation of Smad2 increases after 1 hour, 2 hours, 4 hours, 6 hours and 24 hours compared to time zero. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 4 (page 309).

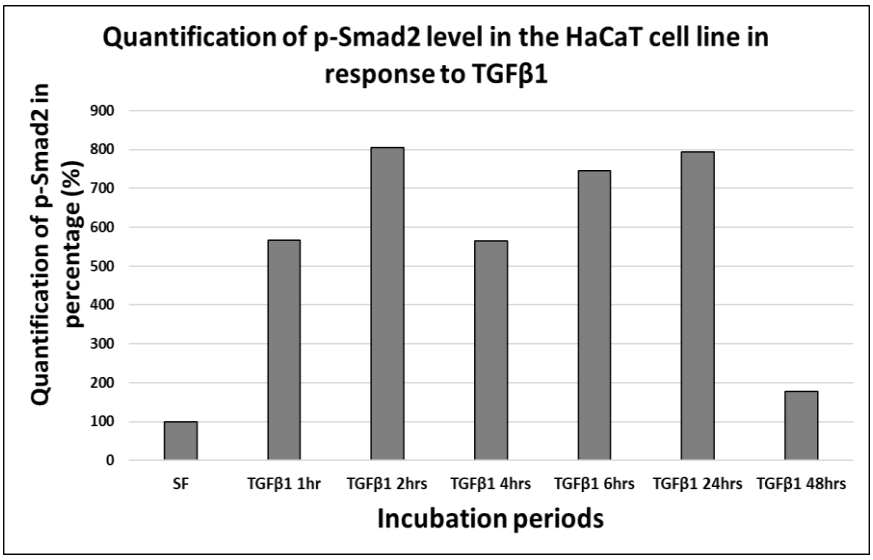


Figure 66: Quantification of p-Smad2 level in the HaCaT cell line.

This bar chart shows the quantification of p-Smad2 in the HaCaT cell line for stage I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). Time zero lane was considered as 100% for protein quantification. Lysates from HaCaT cells treated with TGFβ1 (10ng/ml) after 1 hour, 2 hours, 4 hours, 6 hours and 24 hours showed an increase in Smad2 phosphorylation compared to negative control.

Table 22: Quantification of p-Smad2 in the HaCaT cell line with TGFβ1 at different incubation periods.

HaCaT	0 minute	1 hour	2 hours	4 hours	6 hours	24 hours	48 hours
TGFβ1	100 %	565.9 %	804.6 %	565.1 %	745.1 %	793.1 %	177.4 %

(b) Stage II Assessment of Smad Pathway:

The aim of this stage was to assess the phosphorylation of Smad2 in the HaCaT cell line after treatment with the test conditions for stage II assessment. From the assessment of stage I (**Table 21**), lysates treated with TGFβ1 showed phosphorylation of Smad2 after an hour, two hours, four hours, six hours, and 24 hours. The level of p-Smad2 in the cells after 48 hours incubation with TGFβ1 returned to the same level as the SF MEM samples (baseline). Based on the migration assay results, where the HaCaT cell line was treated with TGFβ1, cells started to migrate significantly after 48 hours. Also, it was established from the stage I assessment, that p-Smad2 was not detect in the HaCaT cell line after 48 hours with any test conditions in the stage II assessment (**Table 23**). In addition, only two test conditions were investigated at 24 hours, which were TGFβ1+PD98059 and TGFβ1+SB431542 which showed no detectable level of p-Smad2.

Table 23: The format of stage II assessment for Smad pathway in the HaCaT cell line.

(1) Magic marker	**(2)** SF-MEM (48 hours)	**(3)** TGFβ1 (48 hours)	**(4)** EGF (48 hours)	**(5)** TGFβ1+ TGF-β RI Kinase Inhibitor VII (48 hours)
(6) TGFβ1+MK2206 (48 hours)	**(7)** TGFβ1+PD98059 (24 hours)	**(8)** TGFβ1+PD98059 (48 hours)	**(9)** TGFβ1+SB431542 (24 hours)	**(10)** TGFβ1+SB431542 (48 hours)

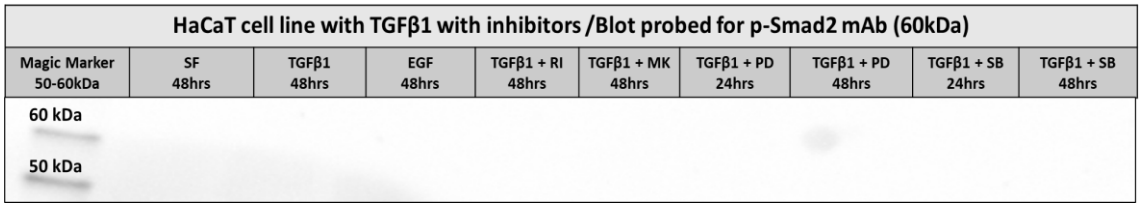


Figure 67: Stage II Assessment for p-Smad2 in the HaCaT cell line.

The HaCaT cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Smad2 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from HaCaT cells treated with the different test conditions showed no phosphorylation of Smad2. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 5 (page 316).

The findings from the stage I assessment suggested that p-Smad2 was detected only in lysates from cells treated with TGFβ1 at early incubation periods. The stage II assessment of p-Smad2 was not detected, even at 24 hours incubation period with TGFβ1+PD98059 and TGFβ1+SB431542 test conditions and with all other test conditions at 48 hours incubation period. This suggests that the activation of the Smad-dependent pathway was an early event.

PI3K-Akt Pathway Assessment for the HaCaT Cell Line:

(a) **<u>Stage I Assessment of PI3K-Akt Pathway:</u>**

The assessment of PI3k-Akt pathway activation was based on investigating the phosphorylation of two key proteins in the pathway. On activation of PI3K by the growth factors, a sequence of reactions leads to phosphorylation of Akt at two amino acid residues: Thr308 and Ser473.

Table 24: The format of stage I assessment for PI3K-Akt Pathway in the TYS cell line.

(1) Magic Marker						
(2) TGFβ1 (0min)	**(3)** TGFβ1 (1 hour)	**(4)** TGFβ1 (2 hours)	**(5)** TGFβ1 (4 hours)	**(6)** TGFβ1 (6 hours)	**(7)** TGFβ1 (24 hours)	**(8)** TGFβ1 (48 hours)
(9) EGF (0min)	**(10)** EGF (1 hour)	**(11)** EGF (2 hours)	**(12)** EGF (4 hours)	**(13)** EGF (6 hours)	**(14)** EGF (24 hours)	**(15)** EGF (48 hours)

1- **<u>Thr308</u>:** Samples with TGFβ1, showed an upregulation in phosphorylation of Akt T308 at different incubation periods, including

an hour, two hours, four hours, six hours and 24 hours. The highest levels of phosphorylation for Akt T308 were observed after 24 hours with TGFβ1, compared to time zero. In the case of the HaCaT cell line with EGF, the highest level of Akt T308 phosphorylation was observed after an hour's incubation with the growth factor, then the expression started to decrease towards four hours incubation, before it increased again at both six hours and 24 hours (**Figure 68**). With both growth factors, the phosphorylation of Akt T308 was similar at 48 hours to the zero time (**Figure 69**).

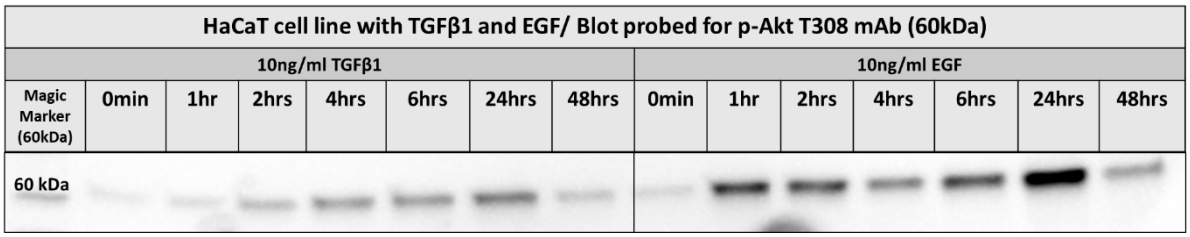


Figure 68: Stage I assessment for p-Akt-T308 in the HaCaT cell line.

The HaCaT cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Akt T308 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells, revealed that the phosphorylation of Akt T308 increases after 2, 4, 6 and 24 hours, while lysates from EGF-treated cells increases the level of p-Akt T308 after 1, 2, 4, 6 and 24 hours compared to time zero. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 4 (page 310).

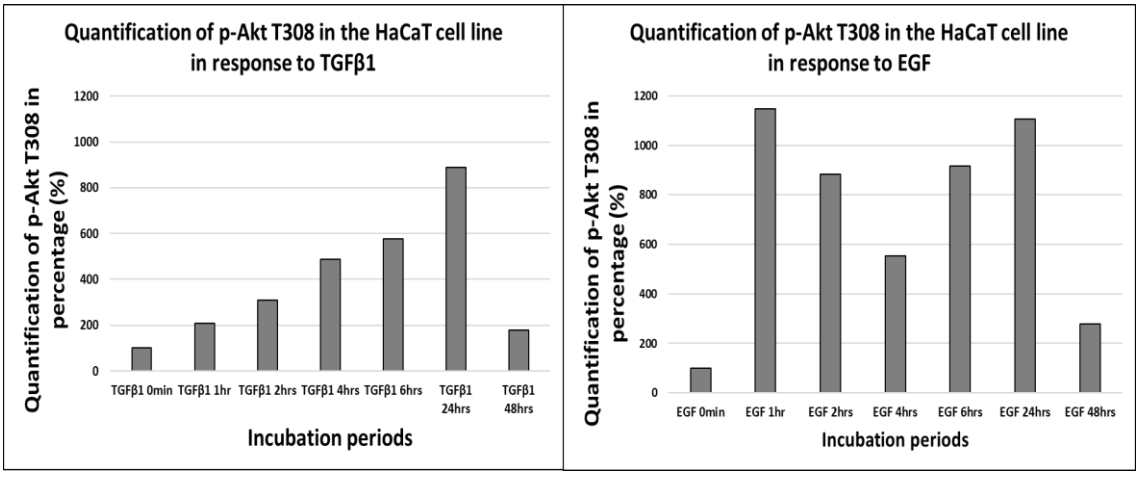


Figure 69: Quantification of p-Akt T308 in the HaCaT cells.

This bar chart shows the quantification of p-Akt T308 in the HaCaT cell line for stage I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from HaCaT cells treated with TGFβ1 (10ng/ml) after 1, 2, 4, 6 and 24 hours showed an increase in Akt T308 phosphorylation compared to negative control. Also, lysates from EGF treated cells showed high p-Akt T308 at all incubation periods compared to the baseline.

Table 25: Quantification of p-Akt T308 in the HaCaT cell line with TGFβ1 and EGF at different incubation periods.

HaCaT	0 minute	an hour	2 hours	4 hours	6 hours	24 hours	48 hours
TGFβ1	100%	207.5%	310.2%	488.5%	576.9%	887.2%	178.1%
EGF	100%	1146.3%	883.9%	552.9%	915.2%	1105.3%	278.3%

2- **<u>Ser473</u>:** The phosphorylation of Akt S473 was significantly higher in cells treated with EGF compared to TGFβ1, at all incubation times. With the TGFβ1 samples, p-Akt S473 was upregulated at two hours, four hours, six hours, and 24 hours, while at 48 hours it decreased. EGF increased the phosphorylation of Akt S473 significantly at different incubation periods especially after an hour in the HaCaT cells (**Figure 70**). Overall, the level of p-Akt S473 remained relatively high at all incubation periods, even at 48 hours when the EGF-treated group compared with the baseline (**Figure 71**).

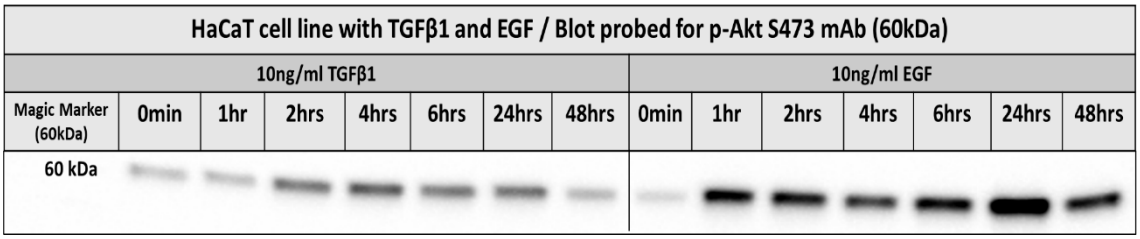


Figure 70: Stage I assessment for p-Akt-S473 in the HaCaT cell line.

The HaCaT cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Akt S473 monoclonal antibody at a 1:2000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells, revealed that the phosphorylation of Akt S473 increases after 2, 4, 6 and 24 hours, while lysates from EGF-treated cells increases the level of p-Akt S473 after 1, 2, 4, 6 and 24 hours compared to time zero. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 4 (page 310).

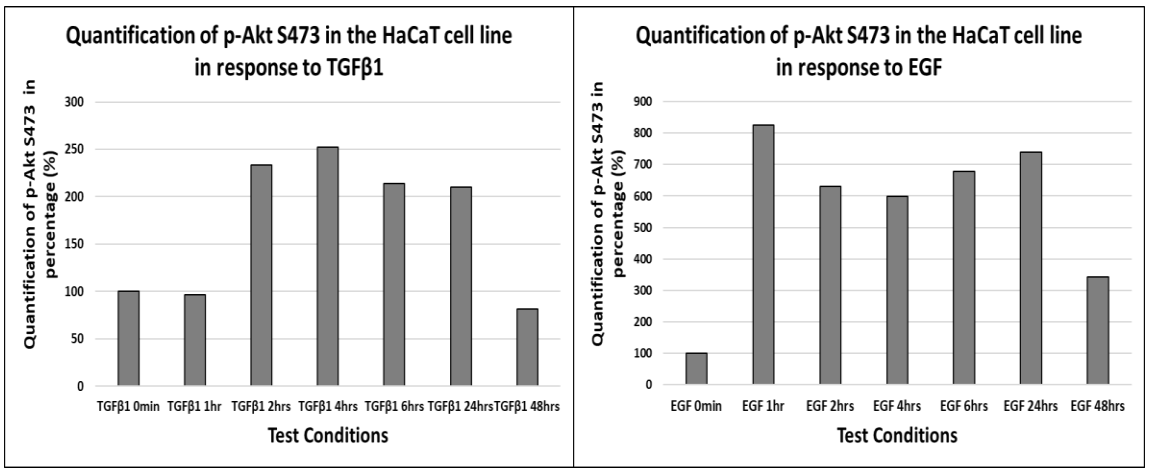


Figure 71: Quantification of p-Akt S473 in the HaCaT cell line.

This bar chart shows the quantification of p-Akt S473 in the HaCaT cell line for stage I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from HaCaT cells treated with TGFβ1 (10ng/ml) after 1, 2, 4, 6 and 24 hours showed an increase in Akt S473 phosphorylation compared to negative control. Also, lysates from EGF treated cells showed high p-Akt S473 at all incubation periods compared to the baseline.

Table 26: Quantification of p-Akt S473 in the HaCaT cell line with TGFβ1 and EGF at different incubation periods.

HaCaT	0 minute	1 hour	2 hours	4 hours	6 hours	24 hours	48 hours
TGFβ1	100%	96.8%	233.2%	252.1%	213.9%	209.8%	81.7%
EGF	100%	825.6%	629.6%	598.6%	677.4%	739.9%	342.9%

(b) **<u>Stage II Assessment of PI3K-Akt Pathway:</u>**

The assessment of Akt residues S473 and T308 for phosphorylation was investigated in response to different test conditions are described below:

Table 27: The format of stage II assessment for PI3K-Akt pathway in the HaCaT cell line.

(1) Magic marker	**(2)** SF-MEM (48 hours)	**(3)** TGFβ1 (48 hours)	**(4)** EGF (48 hours)	**(5)** TGFβ1+ TGF-β RI Kinase Inhibitor VII (48 hours)
(6) TGFβ1+MK2 206 (48 hours)	**(7)** TGFβ1+PD98 059 (24 hours)	**(8)** TGFβ1+PD98059 (48 hours)	**(9)** TGFβ1+SB431542 (24 hours)	**(10)** TGFβ1+SB431542 (48 hours)

1- **<u>Thr308</u>:** The phosphorylation of Akt T308 was upregulated by both TGFβ1 and EGF at 48 hours. With the inhibitors of stage II assessment (**Table 27**), Akt T308 phosphorylation was downregulated to the level of the negative control (**Figure 72**). These findings suggested that all the inhibitors in this stage of assessment decreased the phosphorylation of Akt T308 level in the HaCaT cell line.

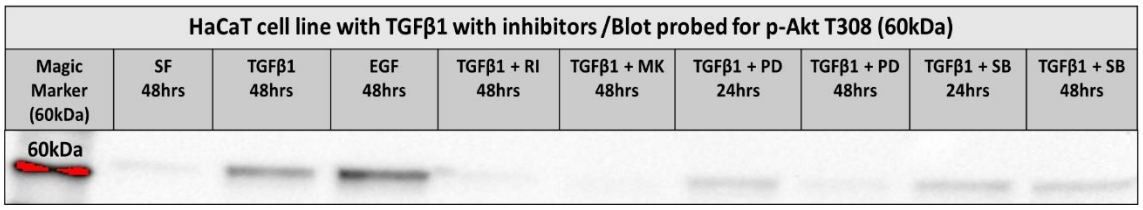


Figure 72: Stage II Assessment for p-Akt T308 in the HaCaT cell line.

The HaCaT cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Akt T308 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Lysates from HaCaT cells treated with TGFβ1 (10ng/ml) after 48 hours EGF (10ng/ml) after 48 hours and showed an increase in phosphorylation of Akt T308 compared to the negative control. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 5 (page 317).

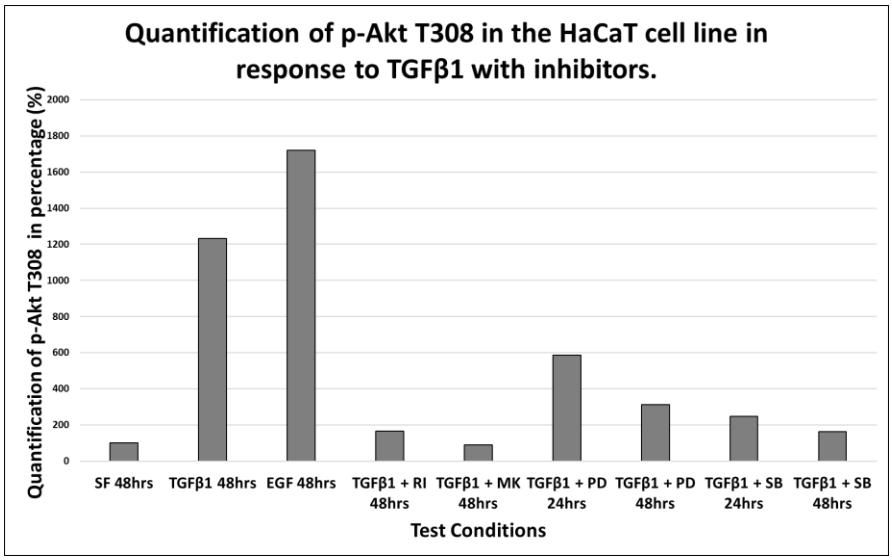


Figure 73: Quantification of stage II assessment for p-Akt-T308 in the HaCaT cell line.

The bar chart shows the quantification of p-Akt T308 in the HaCaT cell line for phase II assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from HaCaT cells treated with TGFβ1 (10ng/ml) for 48 hours and EGF (10ng/ml) after 48 hours showed an increase in Akt T308 phosphorylation compared to negative control. Also, lysates from cells treated with TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) for 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) after 24 and 48 hours and TGFβ1 (10ng/ml)+SB431542 (5μM) after 24 hours showed less level of p-Akt T308 compared to the negative control.

Table 28: Quantification of p-Akt T308 in the HaCaT cell line with the stage II format.

HaCaT Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	586.2%
10ng/ml TGFβ1 48 hours	1233%	**TGFβ1+PD98059 48 hours**	312.9%
10ng/ml EGF 48 hrs	1721.2%	**TGFβ1+SB431542 24 hours**	247.2%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	165.6%	**TGFβ1+SB431542 48 hours**	163.8%
TGFβ1+MK2206 48 hours	89.1%		

2- **<u>Ser473</u>:** The phosphorylation of Akt S473 residue in this stage of the assessment (**Table 27**) resembled the pattern observed with phosphorylation of the Akt T308 residue, where both TGFβ1 and EGF induced an increase in Akt S473 phosphorylation. However, the level of Akt protein phosphorylation was decreased with all the different inhibitors and TGFβ1 (**Figure 74**).

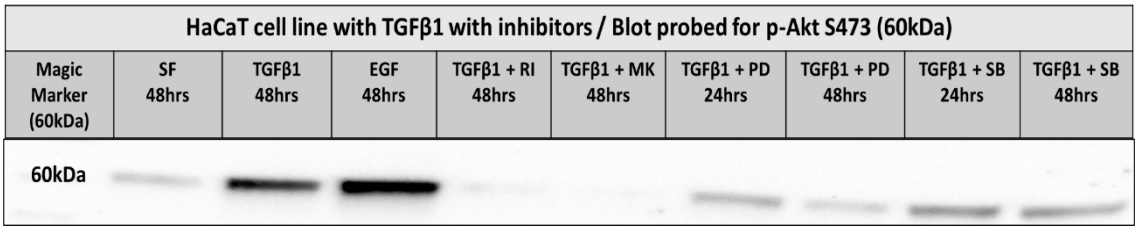


Figure 74: Stage II Assessment for p-Akt S473 in the HaCaT cell line.

The HaCaT cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Akt S473 monoclonal antibody at a 1:2000 dilution, followed by goat anti-rabbit secondary. Lysates from HaCaT cells treated with TGFβ1 (10ng/ml) after 48 hours and EGF (10ng/ml) after 48 hours showed an increase in phosphorylation of Akt S473 compared to the negative control. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 5 (page 317).

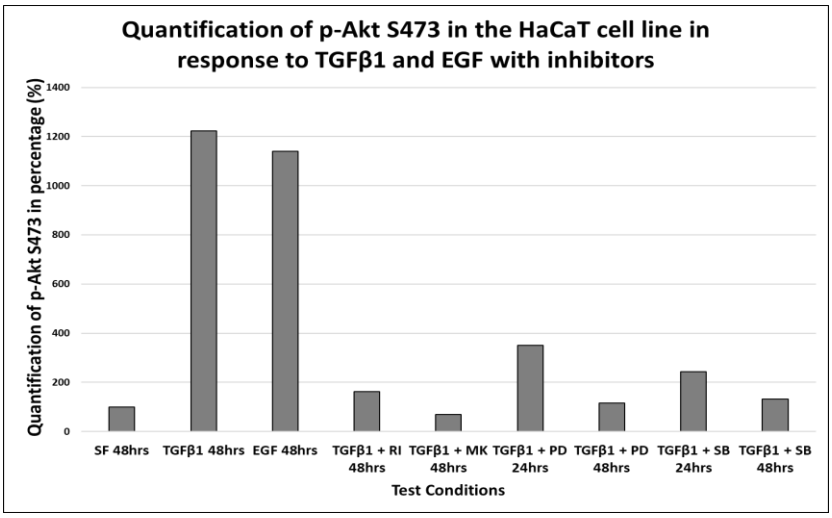


Figure 75: Quantification of stage II assessment for p-Akt S473 of the HaCaT.

The bar chart shows the quantification of p-Akt S473 in the HaCaT cell line for phase II assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from HaCaT cells treated with TGFβ1 (10ng/ml) for 48 hours and EGF (10ng/ml) after 48 hours showed an increase in Akt S473 phosphorylation compared to negative control. Also, lysates from cells treated with TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) for 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) after 24 and 48 hours and TGFβ1 (10ng/ml)+SB431542 (5μM) after 24 hours showed a lower level of p-Akt S473 compared to the negative control.

Table 29: Quantification of p-Akt S473 in the HaCaT cell line with stage II format.

HaCaT Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	351.4%
10ng/ml TGFβ1 48 hours	1222.4%	**TGFβ1+PD98059 48 hours**	115.8%
10ng/ml EGF 48 hrs	1139.6%	**TGFβ1+SB431542 24 hours**	244.1%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	162.8%	**TGFβ1+SB431542 48 hours**	132.5%
TGFβ1+MK2206 48 hours	69.8%		

The phosphorylation of both Akt residues T308 and S473 was upregulated by both growth factors and showed a similar pattern. Also, the inhibitors, which were used with the growth factors, downregulated phosphorylation of both residues.

MAPK Pathway Assessment for the HaCaT Cell Line:

(a) **<u>Stage I Assessment of MAPK Pathway:</u>**

With TGFβ1, the phosphorylation of p44/42 increased significantly after 24 hours compared to time zero or the other incubation periods (**Figure 76**). The highest level of phospho-p44/p42 was recorded after one hour with EGF (**Figure 76**). p44/42 phosphorylation remained significantly high at

the rest of the incubation periods, except 48 hours, compared to time zero. These findings illustrated two distinctive patterns of p44/p42 phosphorylation with two different growth factors (**Figure 77**).

Table 30: The format of stage I assessment for phospho-p44/42 pathways in the HaCaT cell line.

(1) Magic Marker						
(2) TGFβ1 (0min)	**(3)** TGFβ1 (1 hour)	**(4)** TGFβ1 (2 hours)	**(5)** TGFβ1 (4 hour)	**(6)** TGFβ1 (6 hours)	**(7)** TGFβ1 (24 hours)	**(8)** TGFβ1 (48 hours)
(9) EGF (0min)	**(10)** EGF (1 hour)	**(11)** EGF (2 hours)	**(12)** EGF (4 hour)	**(13)** EGF (6 hours)	**(14)** EGF (24 hours)	**(15)** EGF (48 hours)

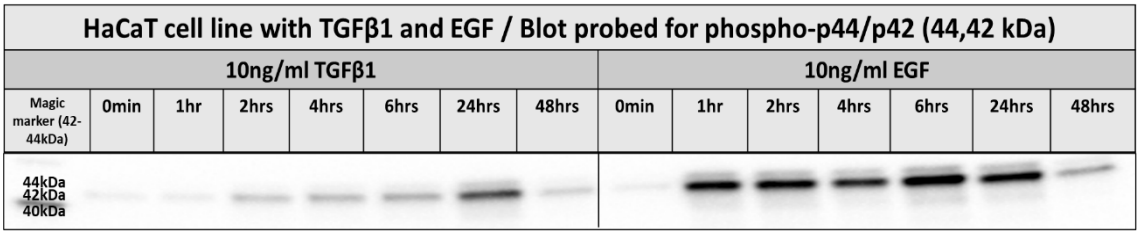


Figure 76: Stage I assessment for phospho-p44/42 in the HaCaT cell line.

The HaCaT cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a phospho-p44/42 monoclonal antibody at a 1:2000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells, revealed that the phosphorylation of phospho-p44/42 increases after 24 hours, while lysates from EGF-treated cells showed an increased level of phospho-p44/42 after 1, 2, 4, 6 and 24 hours compared to time zero. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 4 (page 310).

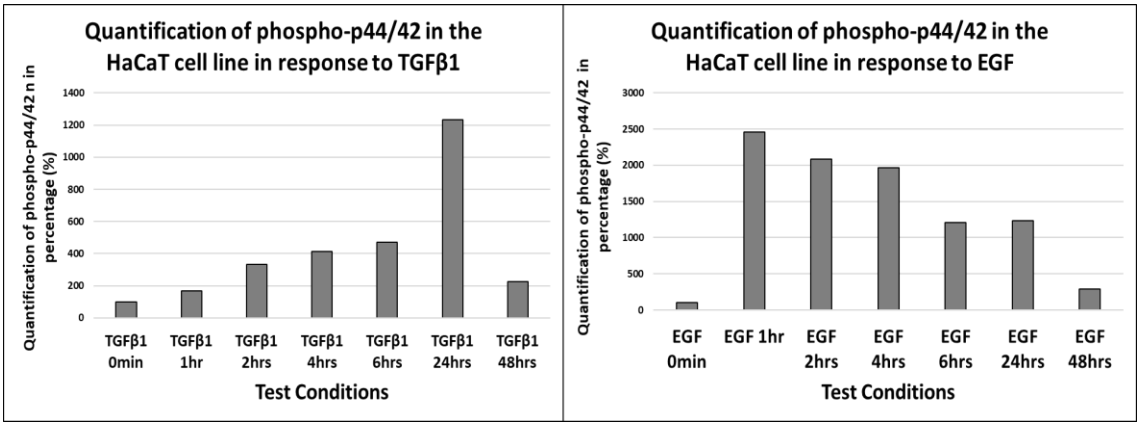


Figure 77: Quantification of phospho-p44/42 in the HaCaT cell line.

This bar chart shows the quantification of phospho-p44/p42 in the HaCaT cell line for stage I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from HaCaT cells treated with TGFβ1 (10ng/ml) after 24 hours showed an increase in p44/p42 phosphorylation compared to the negative control. Also, lysates from EGF treated cells showed high phospho-p44/p42 at all incubation periods compared to the baseline.

Table 31: Quantification of phospho-p44/42 in the HaCaT cell line with TGFβ1 and EGF at different incubation periods.

HaCaT	0 minute	1 hour	2 hours	4 hours	6 hours	24 hours	48 hours
TGFβ1	100%	166.6%	333.9%	413.5%	471.7%	1231.4%	224.1%
EGF	100%	2456.4%	2084.6%	1968.1%	1205.3%	1235.5%	285.1%

(b) Stage II Assessment of MAPK Pathway:

The investigation of phospho-p44/42 level at this stage of assessment, where different inhibitors were used, showed two outcomes. First, an increase in the level of phospho-p44/42 was detected with TGFβ1 samples and EGF samples after 48 hours compared to SF-MEM at 48 hours. This was also confirmed by the findings from stage I assessment. In addition, the phosphorylation of p44/42 increased in the samples contained TGFβ1 with MK-2206, suggesting there was a link between the PI3K/Akt and MAPK pathways (**Figure 78**). The second showed a low level of phospho-p44/42, which included SF-MEM at 48 hours, TGFβ1 with TGF-β RI Kinase Inhibitor VII, TGFβ1 with PD98059 and TGFβ1 with SB431542 (**Figure 79**).

Table 32: The format of stage II assessment for ErK1/2-MAPK pathway in the HaCaT cell line.

(1) Magic marker	**(2)** SF-MEM (48 hours)	**(3)** TGFβ1 (48 hours)	**(4)** EGF (48 hours)	**(5)** TGFβ1+ TGF-β RI Kinase Inhibitor VII (48 hours)
(6) TGFβ1+MK2206 (48 hours)	**(7)** TGFβ1+PD98059 (24 hours)	**(8)** TGFβ1+PD98059 (48 hours)	**(9)** TGFβ1+SB431542 (24 hours)	**(10)** TGFβ1+SB431542 (48 hours)

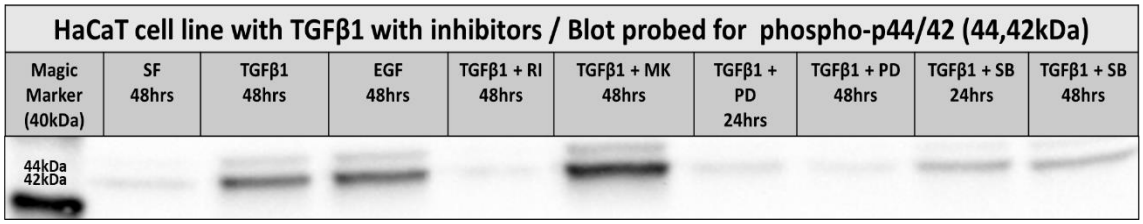


Figure 78: Stage II Assessment for phospho-p44/42 in the HaCaT cell line.

The HaCaT cell line was incubated with nine different test conditions (**Table 32**). Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a phospho-p44/42 monoclonal antibody at a 1:2000 dilution, followed by goat anti-rabbit secondary. Lysates from HaCaT cells treated with TGFβ1 (10ng/ml) after 48 hours, EGF (10ng/ml) after 48 hours and TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours showed an increase in phosphorylation of phospho-p44/42 compared to the negative control. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 5 (page 317).

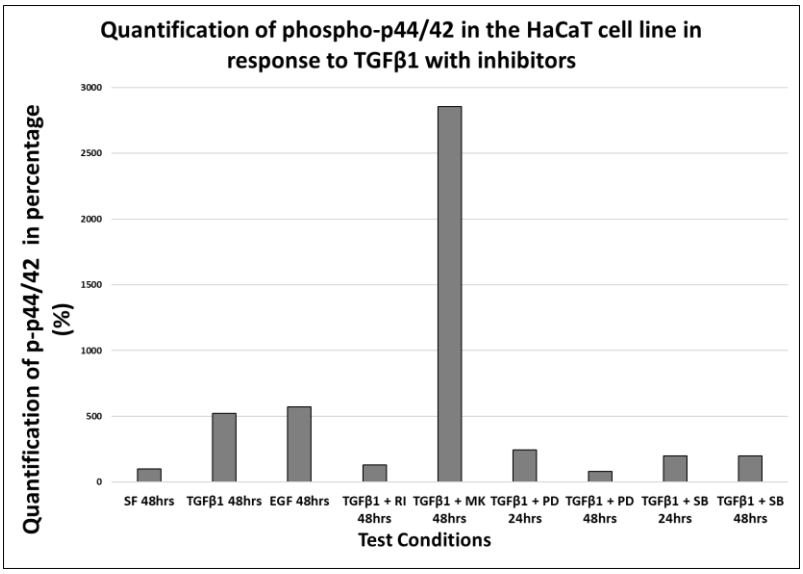


Figure 79: Quantification of phospho-p44/42 in the HaCaT cell line.

The bar chart shows the quantification of phospho-p44/42 in the HaCaT cell line for phase II assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from HaCaT cells treated with TGFβ1 (10ng/ml) +MK-2206 (1μM) after 48 hours and TGFβ1 (10ng/ml) for 48 hours and EGF (10ng/ml) after 48 hours showed an increase in phospho-p44/42 phosphorylation compared to the negative control.

Table 33: Quantification of phospho-p44/42 in the HaCaT cell line with stage II format.

HaCaT Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	244.4%
10ng/ml TGFβ1 48 hours	522.9%	**TGFβ1+PD98059 48 hours**	80.8%
10ng/ml EGF 48 hrs	570.8%	**TGFβ1+SB431542 24 hours**	196.9%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	130.3%	**TGFβ1+SB431542 48 hours**	197.7%
TGFβ1+MK-2206 48 hours	2856.9%		

In summary of the stage I assessment of the HaCaT cell line, both growth factors upregulated the phosphorylation of p44/42 at different incubation periods. With TGFβ1, phosho-p44/42 was detected after 24 hours, whereas with EGF, the phosphorylation was higher compared to the control sample (SF-MEM) after an hour and continued to be high until 24 hours. For the second stage of assessment, a significant increase in phospho-p44/42 levels was noted in lysates from cells incubated with TGFβ1 and MK-2206 in comparison to the rest of the test conditions. These findings suggest a link between the PI3K/Akt and MAPK signalling pathways, when they were activated by TGFβ1.

Rho-GTPase Pathway Assessment for HaCaT Cell line:

(a) **Stage I Assessment of Rho-GTPase Pathway:**

Addition of exogenous TGFβ1 to cultures of HaCaT cells for different incubation periods did not result in phosphorylation of Rac1/Cdc42. However, when the same incubation periods were used, cultures/assays with exogenous EGF added, showed phosphorylation of Rac1/Cdc42, mainly between an hour incubation and 24 hours, as visualised by SDS PAGE and Western blotting (**Figure 80**).

Table 34: The format of stage I assessment for Rac1/Cdc42 in the HaCaT cell line.

(1) Magic Marker						
(2) TGFβ1 (0min)	**(3)** TGFβ1 (1 hour)	**(4)** TGFβ1 (2 hours)	**(5)** TGFβ1 (4 hours)	**(6)** TGFβ1 (6 hours)	**(7)** TGFβ1 (24 hours)	**(8)** TGFβ1 (48 hours)
(9) EGF (0min)	**(10)** EGF (1 hour)	**(11)** EGF (2 hours)	**(12)** EGF (4 hours)	**(13)** EGF (6 hours)	**(14)** EGF (24 hours)	**(15)** EGF (48 hours)

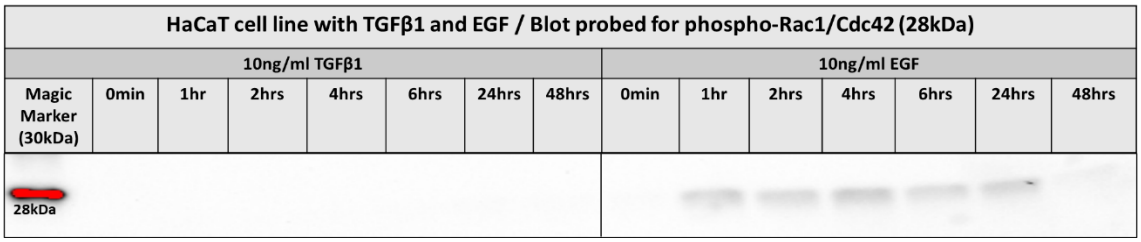


Figure 80: Stage I assessment for phosphorylation of Rac1/Cdc42 in the HaCaT cell line.

The HaCaT cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Rac1/Cdc42 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from EGF-treated cells, revealed that the phosphorylation of Rac1/Cdc42 increases after 1, 2, 4, 6 and 24 hours. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 4 (page 311).

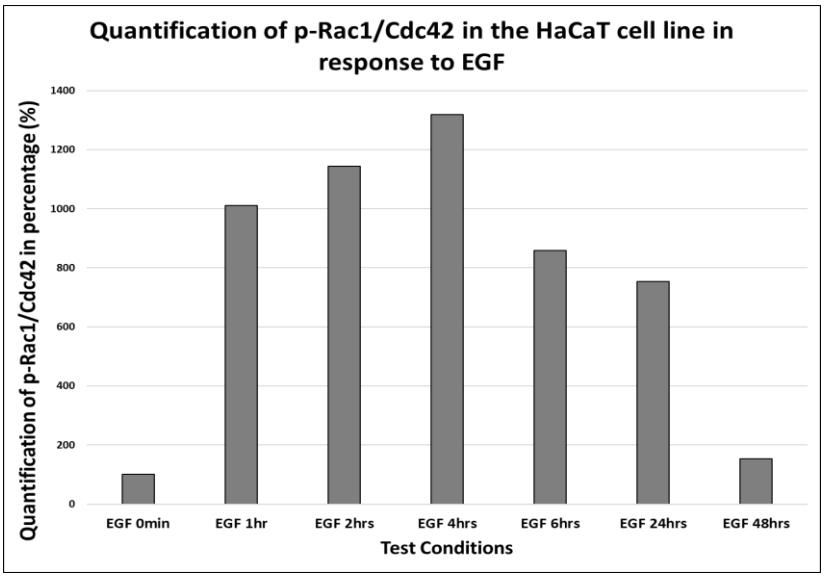


Figure 81: Quantification of p-Rac1/Cdc42 in the HaCaT cell line.

This bar chart shows the quantification of p-Rac1/Cdc42 in the HaCaT cell line for stage II assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). Time zero lane was considered as 100% for protein quantification. Lysates from HaCaT cells treated with EGF1 (10ng/ml) after 1 hour, 2hours, 4 hours, 6 hours and 24 hours showed an increase in Rac1/Cdc42 phosphorylation compared to negative control.

Table 35: Quantification of p-Rac1/Cdc42 in the HaCaT cell line with EGF at different incubation periods.

HaCaT	0 minute	1 hour	2 hours	4 hours	6 hours	24 hours	48 hours
EGF	100%	1010.2%	1143.7%	1318.7%	858.8%	753.1%	153.6%

(b) **<u>Stage II Assessment of Rho-GTPase Pathway:</u>**

Using different test conditions including inhibitors, p-Rac1/Cdc42 was not detected in the HaCaT cells after 24 or 48 hours (**Figure 82**). The test conditions that were used in this assessment are described below:

Table 36: The format of stage II assessment for p-Rac1/Cdc42 in the HaCaT cell line.

(1) Magic marker	**(2)** SF-MEM (48 hours)	**(3)** TGFβ1 (48 hours)	**(4)** EGF (48 hours)	**(5)** TGFβ1+ TGF-β RI Kinase Inhibitor VII (48 hours)
(6) TGFβ1+MK2206 (48 hours)	**(7)** TGFβ1+PD98059 (24 hours)	**(8)** TGFβ1+PD98059 (48 hours)	**(9)** TGFβ1+SB431542 (24 hours)	**(10)** TGFβ1+SB431542 (48 hours)

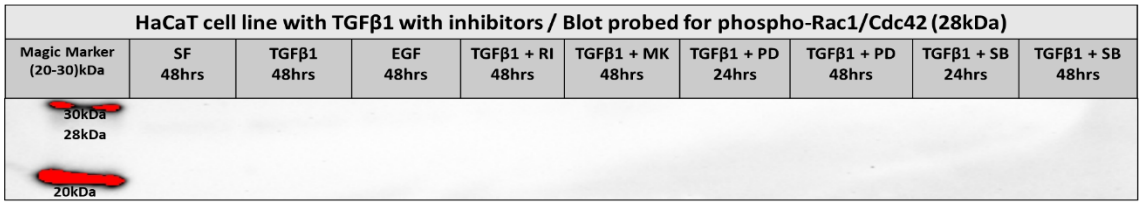


Figure 82: Stage II Assessment of p-Rac1/Cdc42 in the HaCaT cell line.

The HaCaT cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Rac1/Cdc42 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Lysates from HaCaT cells did not show phosphorylation of Rac1/Cdc42 after been treated with the test conditions of stage II assessment (**Table 36**). The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 5 (page 318).

These findings suggested that only EGF induced activation of the Rho-GTPase signalling pathway in the HaCaT cell line, while TGFβ1 has no effect on this pathway.

5.4.2 TYS Cell line:

After adding test conditions according to the stage II assessment format, photomicrographs from each sample were taken to assess the cell morphology, colony appearance and cell scattering. All photomicrographs were taken at 200X magnification, before cells were lysed according to the standard method described in general material methods in chapter 2 (page 44).

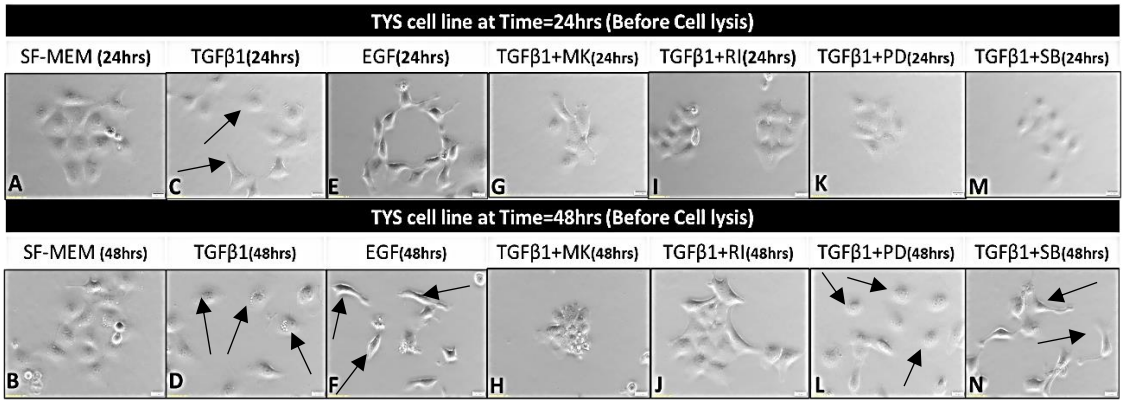


Figure 83: Observational assessment of the TYS cell line.

Images for the TYS cell line incubated with the test conditions. Cells grown in 60mm dishes with SF-MEM at 48 hours, TGFβ1 at 48hours, EGF at 48 hours, TGFβ1+ TGF-β RI Kinase Inhibitor VII at 48 hours, TGFβ1+MK-2206 at 48 hours, TGFβ1+PD98059 at 24 hours and 48 hours, and TGFβ1+SB431542 at 24 hours and 48 hours before cell lysis. All images were taken at 200x magnification to observe both cell morphology and colony appearance. (↑) scattered cells.

Cells cultured with the addition of TGFβ1 (**Figure 83-C&D**), EGF (**Figure 83-E&F**), TGFβ1 with PD98059 (**Figure 83-L**), and TGFβ1 with SB431542 (**Figure 83-N**), showed substantial changes in cell shape and colony appearance of the TYS cell line. When cells were incubated with the addition of TGFβ1 and TGFβ1 + PD98059, they were found to be dispersed and had a large, rounded shape, whereas cells in samples incubated in EGF alone and TGFβ1 + SB431542 had a long spindle shape. TYS cells incubated in SF-MEM, where cells are in a colony and did not undergo morphological changes, contrasted with cells incubated in TGFβ1. The TYS cells incubated in TGF-RI Kinase Inhibitor VII plus TGFβ1 and TGFβ1 plus MK-2206 showed a similar morphological appearance to cells with SF-MEM.

Smad Pathway Assessment for TYS Cell Line:

(a) <u>Stage I Assessment of Smad Pathway</u>:

Phosphorylation of Smad2 in response to TGFβ1 showed high levels after one hour, two hours, four hours, six hours and 24 hours compared to time zero (**Figure 84**). This phosphorylation decreased after 48 hours in cells treated with exogenous TGFβ1. However, EGF had little effect on Smad2 phosphorylation in the TYS cells, the level of which was not significant throughout the incubation periods (**Figure 85**).

Table 37: The format of stage I assessment for p-Smad2 in the TYS cell line.

(1) Magic Marker						
(2) TGFβ1 (0min)	**(3)** TGFβ1 (1 hour)	**(4)** TGFβ1 (2 hours)	**(5)** TGFβ1 (4 hours)	**(6)** TGFβ1 (6 hours)	**(7)** TGFβ1 (24 hours)	**(8)** TGFβ1 (48 hours)
(9) EGF (0min)	**(10)** EGF (1 hour)	**(11)** EGF (2 hours)	**(12)** EGF (4 hours)	**(13)** EGF (6 hours)	**(14)** EGF (24 hours)	**(15)** EGF (48 hours)

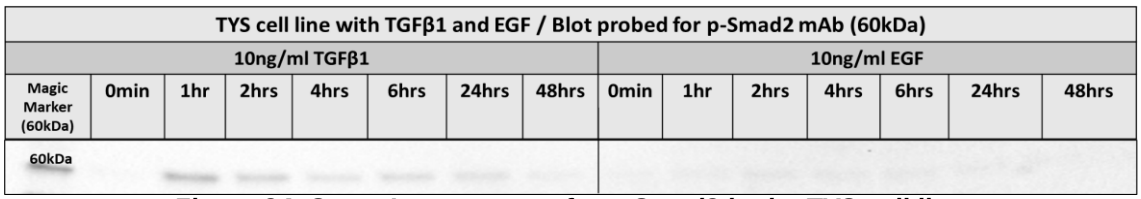


Figure 84: Stage I assessment for p-Smad2 in the TYS cell line.

The TYS cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Smad2 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells, revealed that the phosphorylation of Smad2 increases after 1 hour, 2 hours, 4 hours, 6 hours and 24 hours compared to time zero. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 4 (page 312).

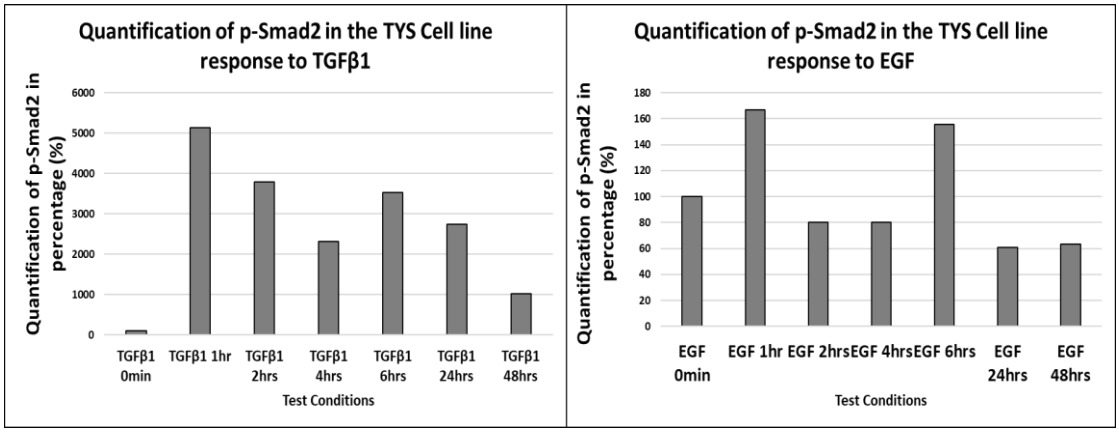


Figure 85: Quantification of p-Smad2 in the TYS cell line.

This bar chart shows the quantification of p-Smad2 in the TYS cell line for stage I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The time zero lane was considered as 100% for protein quantification. Lysates from TYS cells treated with TGFβ1 (10ng/ml) after 1 hour, 2 hours, 4 hours, 6 hours, 24 hours and 48 hours showed an increase in Smad2 phosphorylation compared to the negative control.

Table 38: Quantification of p-Smad2 in the TYS cell line with TGFβ1 and EGF at different incubation periods.

TYS	0 minute	1 hour	2 hours	4 hours	6 hours	24 hours	48 hours
TGFβ1	100%	5128.9%	3787.6%	2313.9%	3520.4%	2740.8%	1012.9%
EGF	100%	166.8%	80.3%	80.3%	155.6%	60.7%	63.3%

(b) <u>Stage II assessment of Smad Pathway</u>:

At this stage of assessment, there was no phosphorylation of Smad2 with any of the test conditions, which included different inhibitors at both 24 hours and 48 hours (**Figure 86**).

Table 39: The format of stage II assessment for p-Smad2 in the TYS cell line.

(1) Magic marker	**(2)** SF-MEM (48 hours)	**(3)** TGFβ1 (48 hours)	**(4)** EGF (48 hours)	**(5)** TGFβ1+ TGF-β RI Kinase Inhibitor VII (48 hours)
(6) TGFβ1+MK2206 (48 hours)	**(7)** TGFβ1+PD98059 (24 hours)	**(8)** TGFβ1+PD98059 (48 hours)	**(9)** TGFβ1+SB431542 (24 hours)	**(10)** TGFβ1+SB431542 (48 hours)

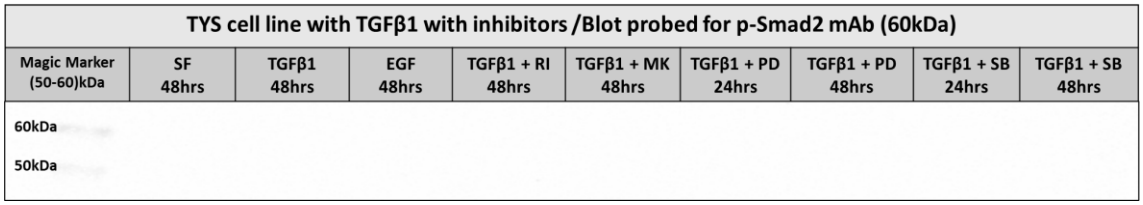


Figure 86: Stage II Assessment of p-Smad2 in the TYS cell line.

The TYS cells were incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Smad2 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from TYS cells treated with the different test conditions showed no phosphorylation of Smad2. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 5 (page 319).

The phosphorylation of Smad2 occurred in TYS cells treated with TGFβ1 as early as an hour, however, it was not detected after 24 and 48 hours.

PI3K/Akt Pathway Assessment for TYS Cell line

(a) Stage I assessment of PI3k-Akt Pathway:

Table 40: The format of stage I assessment for PI3k-Akt pathway in the TYS cell line.

(1) Magic Marker						
(2) TGFβ1 (0min)	**(3)** TGFβ1 (1 hour)	**(4)** TGFβ1 (2 hours)	**(5)** TGFβ1 (4 hours)	**(6)** TGFβ1 (6 hours)	**(7)** TGFβ1 (24 hours)	**(8)** TGFβ1 (48 hours)
(9) EGF (0min)	**(10)** EGF (1 hour)	**(11)** EGF (2 hours)	**(12)** EGF (4 hours)	**(13)** EGF (6 hours)	**(14)** EGF (24 hours)	**(15)** EGF (48 hours)

1- **Thr308**: Using SDS-PAGE and Western blotting, the phosphorylation of Akt T308 was detected with all of the test conditions at all incubation periods (**Figure 87**). For TGFβ1 samples, there was a slight increase in the level of p-Akt T308 at 24 hours compared to the rest of the incubation periods, including time zero. In the case of EGF, the peak of Akt T308 phosphorylation was detected after two hours incubation of the cells with the growth factor, the level then started to decrease to the baseline level after 24 and 48 hours (**Figure 88**).

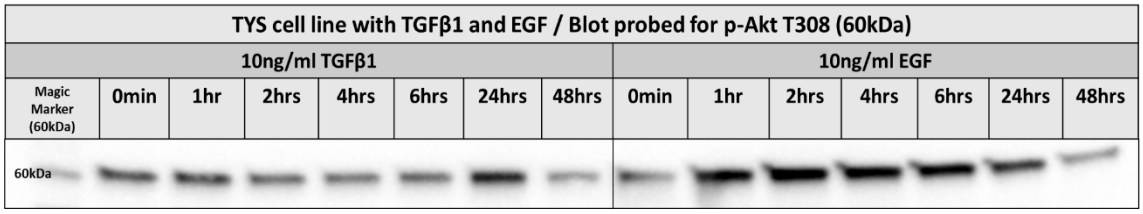


Figure 87: Stage I assessment for p-Akt T308 in the TYS cell line

The TYS cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Akt T308 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells and EGF-treated cells, revealed that the phosphorylation of Akt T308 increases at all incubation periods. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 4 (page 312).

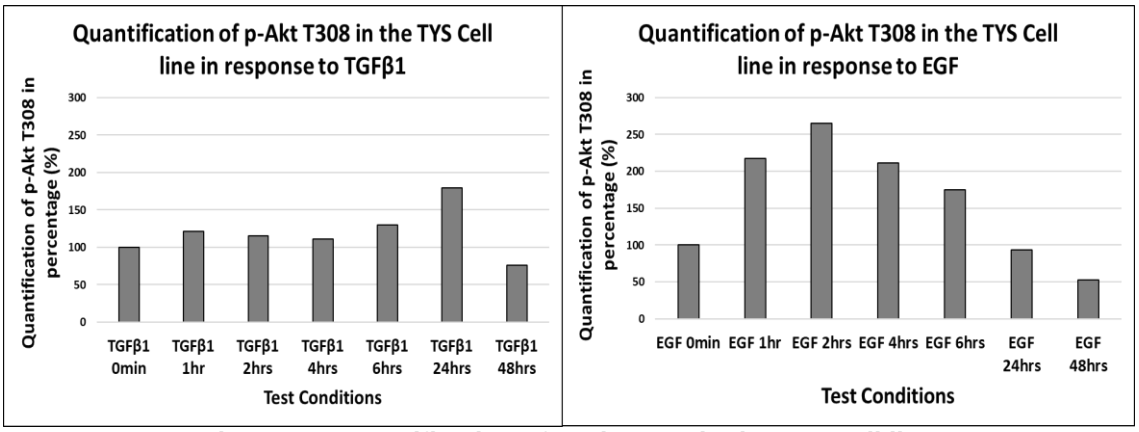


Figure 88: Quantification of p-Akt T308 in the TYS cell line.

This bar chart shows the quantification of p-Akt T308 in the TYS cell line for stage I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from TYS cells treated with TGFβ1 (10ng/ml) after 24 hours showed an increase in Akt T308 phosphorylation compared to the negative control. Also, lysates from EGF treated cells showed high p-Akt T308 after 1, 2, 4 and 6 hours compared to the baseline.

Table 41: Quantification of p-Akt T308 in the TYS cell line with TGFβ1 and EGF at different incubation periods.

TYS	0 minute	1 hour	2 hours	4 hours	6 hours	24 hours	48 hours
TGFβ1	100%	120.9%	115%	110.7%	129.7%	179.6%	76.2%
EGF	100%	217.5%	165.3%	211.8%	175.3%	93.4%	52.8%

2- **Ser473:** Generally, the amount of p-Akt S473 with both growth factors showed a similarity to the level of p-Akt T308 (**Figure 89**), where the highest level of phosphorylation of the Akt protein in lysates from

TGFβ1 treated cells was detected after 24 hours and after two hours with the lysates from cells treated with EGF (**Figure 90**).

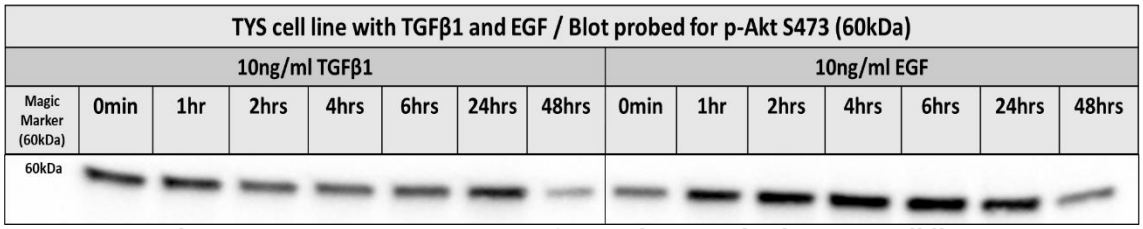


Figure 89: Stage I assessment for p-Akt-S473 in the TYS cell line.

The TYS cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Akt S473 monoclonal antibody at a 1:2000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells and EGF-treated cells, revealed that the phosphorylation of Akt S473 increases at all incubation periods. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 4 (page 312).

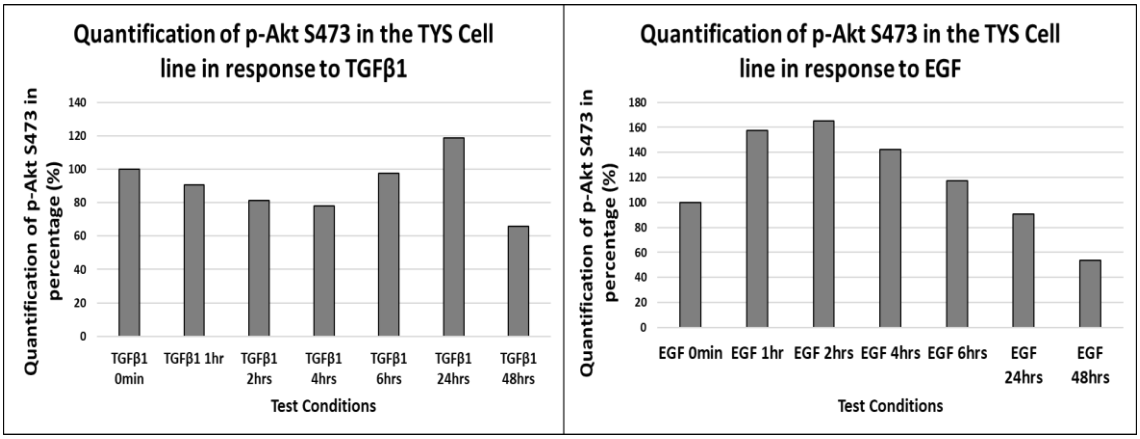


Figure 90: Quantification of p-Akt S473 in the TYS cell line.

This bar chart shows the quantification of p-Akt S473 in the TYS cell line for stage I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from TYS cells treated with TGFβ1 (10ng/ml) after 24 hours showed an increase in Akt S473 phosphorylation compared to the negative control. Also, lysates from EGF treated cells showed high p-Akt S473 after 1, 2, 4 and 6 hours compared to the baseline.

Table 42: Quantification of p-Akt S473 in the TYS cell line with TGFβ1 and EGF at different incubation periods.

TYS	0 minute	1 hour	2 hours	4 hours	6 hours	24 hours	48 hours
TGFβ1	100%	90.5%	81.1%	78.1%	97.6%	118.7%	65.9%
EGF	100%	157.4%	165.2%	142.4%	117.1%	90.8%	53.6%

(b) Stage II assessment of PI3K-Akt Pathway:

Table 43: The format of stage II assessment for PI3K-Akt pathway for in the TYS cell line.

(1) Magic marker	**(2)** SF-MEM (48 hours)	**(3)** TGFβ1 (48 hours)	**(4)** EGF (48 hours)	**(5)** TGFβ1+ TGF-β RI Kinase Inhibitor VII (48 hours)
(6) TGFβ1+MK-2206 (48 hours)	**(7)** TGFβ1+PD98059 (24 hours)	**(8)** TGFβ1+PD98059 (48 hours)	**(9)** TGFβ1+SB431542 (24 hours)	**(10)** TGFβ1+SB431542 (48 hours)

1- **Thr308:** The format of this stage of the assessment was designed to evaluate the activation of Akt by phosphorylation of T308 in the test conditions including growth factor plus inhibitors (**Figure 91**). Firstly, lysates of cells treated with TGFβ1 for 48 hours, showed the highest level of p-Akt T308 compared to SF-MEM at 48 hours. Second, TGFβ1 with MK-2206 (Akt inhibitor) showed the lowest level of p-Akt T308, which suggests an effective block of the PI3K/Akt pathway (**Table 44**). Finally, the remainder of the test conditions showed a similar level of phosphorylation of Akt T308 to SF-MEM at 48 hours (**Figure 92**).

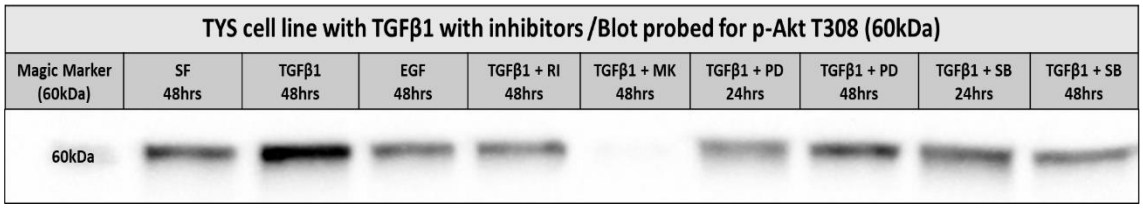


Figure 91: Stage II Assessment for p-Akt T308 in the TYS cell line.

The TYS cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Akt T308 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from TYS cells treated the test conditions of phase II assessment showed an phosphorylation of Akt T308 compared to the negative control, except lysates from cells treated with TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 5 (page 319).

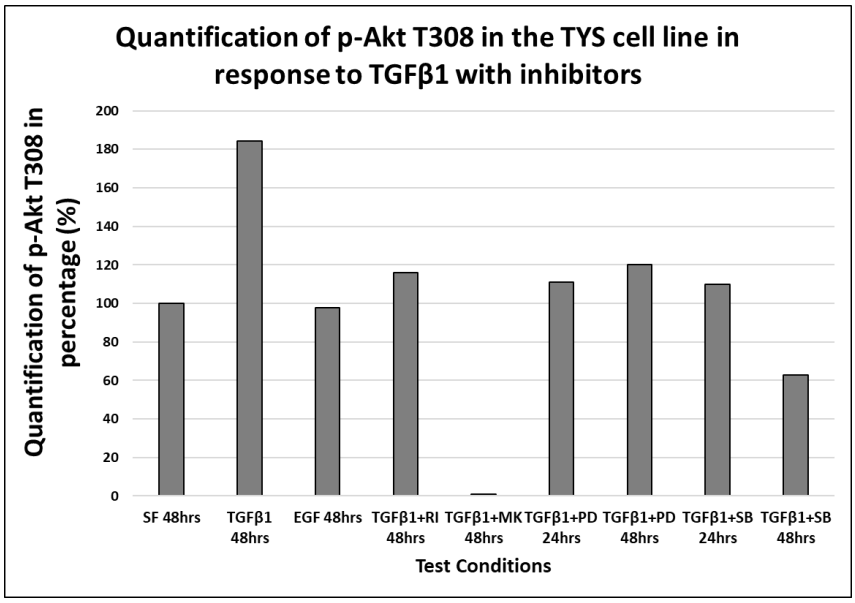


Figure 92: Quantification of p-Akt-T308 in the TYS cell line.

The bar chart shows the quantification of p-Akt T308 in the TYS cell line for phase II assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from TYS cells treated with TGFβ1 (10ng/ml) for 48 hours showed an increase in Akt T308 phosphorylation compared to negative control. Also, lysates from cells treated with TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours showed less level of p-Akt T308 compared to the negative control.

Table 44: Quantification of p-Akt T308 in the TYS cell line with stage II format.

TYS Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	111%
10ng/ml TGFβ1 48 hours	184.1%	**TGFβ1+PD98059 48 hours**	119.9%
10ng/ml EGF 48 hrs	97.9%	**TGFβ1+SB431542 24 hours**	109.7%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	115.8%	**TGFβ1+SB431542 48 hours**	62.9%
TGFβ1+MK-2206 48 hours	0.96%		

2- **<u>Ser473</u>**: The phosphorylation of the Akt S473 residue decreased in all test conditions compared to SF-MEM at 48 hours, even with both growth factors TGFβ1 and EGF (**Figure 93**). These findings were consistent with the results from the stage I assessment, which suggests the phosphorylation of this residue happened early on (**Table 45**). The lowest level of p-Akt S473 was detected in lysates from cells treated with both TGFβ1 and the inhibitor MK-2206, which was similar to Akt T308 phosphorylation with the same test conditions (**Figure 94**).

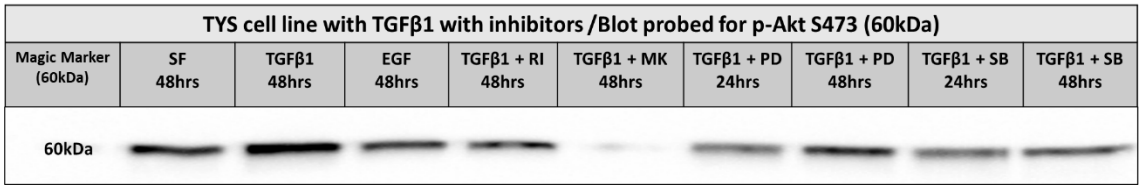


Figure 93: Stage II Assessment for p-Akt S473 in the TYS cell line.

The TYS cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Akt S473 monoclonal antibody at a 1:2000 dilution, followed by goat anti-rabbit secondary. All lysates from TYS cells treated the test conditions of phase II assessment showed an phosphorylation of Akt S473 compared to the negative control, except lysates from cells treated with TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 5 (page 319).

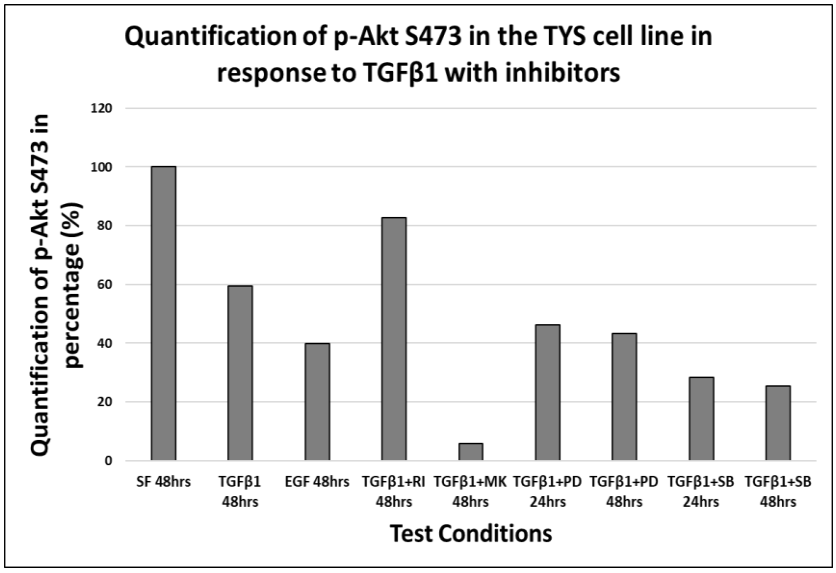


Figure 94: Quantification of p-Akt-S473 in the TYS cell line.

The bar chart shows the quantification of p-Akt S743 in the TYS cell line for phase II assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. All lysates from TYS cells treated with the test conditions from phase II assessment showed a decrease in Akt S743 phosphorylation compared to negative control, especially, lysates from cells treated with TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours.

Table 45: Quantification of p-Akt S473 in the TYS cell line with stage II format.

TYS Cell line with conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	46.3%
10ng/ml TGFβ1 48 hours	59.5%	**TGFβ1+PD98059 48 hours**	43.2%
10ng/ml EGF 48 hrs	39.7%	**TGFβ1+SB431542 24 hours**	28.3%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	82.8%	**TGFβ1+SB431542 48 hours**	25.4%
TGFβ1+MK-2206 48 hours	5.8%		

At stage I of the assessment, Akt residues T308 and S473 were phosphorylated in the TYS cell line in response to TGFβ1 and EGF. Lysates from TYS cells that were treated with TGFβ1 showed Akt T308 and Akt S473 phosphorylation after 24 hours, while lysates treated with EGF showed an earlier phosphorylation between an hour to six hours. In addition, the inhibitor MK2206 was able to block the phosphorylation of both proteins in the presence of the growth factor, TGFβ1. Cells incubated with the other inhibitors in the stage II assessment, TGFβ1+ TGF-β RI Kinase Inhibitor VII, TGFβ1+MK-2206, TGFβ1+PD98059 and TGFβ1+SB431542, showed similar levels of Akt T308 and Akt S473 phosphorylation to the negative control.

MAPK Pathway Assessment for TYS Cell line

(a) <u>Stage I Assessment of MAPK Pathway</u>:

In response to TGFβ1, the phosphorylation of p44/42 in the TYS cells increased after 24 hours and 48 hours when compared to time zero (**Figure 95**). Lysates from cells treated with EGF showed a high level phospho-p44/42, which was detected at early incubation periods, especially after an hour, two hours and four hours (**Figure 96**). The phosphorylation of p44/42 was higher than the baseline at all the incubation periods in lysates from TYS cells that were treated with EGF. Both growth factors-initiated phosphorylation of p44/42 in two different patterns.

Table 46: The format of stage I assessment for phospho-p44/42 in the TYS cell line.

(1) Magic Marker						
(2) TGFβ1 (0min)	**(3)** TGFβ1 (1 hour)	**(4)** TGFβ1 (2 hours)	**(5)** TGFβ1 (4 hours)	**(6)** TGFβ1 (6 hours)	**(7)** TGFβ1 (24 hours)	**(8)** TGFβ1 (48 hours)
(9) EGF (0min)	**(10)** EGF (1 hour)	**(11)** EGF (2 hours)	**(12)** EGF (4 hours)	**(13)** EGF (6 hours)	**(14)** EGF (24 hours)	**(15)** EGF (48 hours)

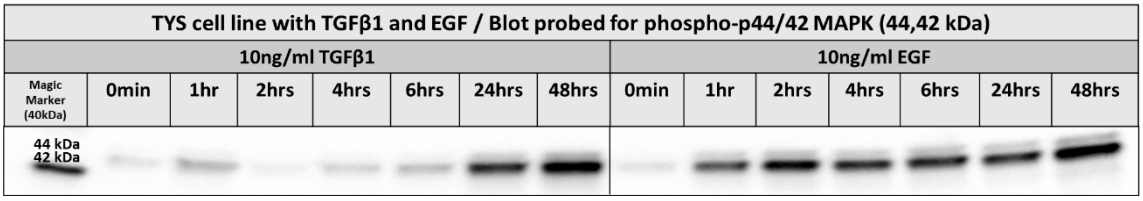


Figure 95: Stage I assessment for phospho-p44/42 in the TYS cell line.

The TYS cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a phospho-p44/42 monoclonal antibody at a 1:2000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells, revealed that the phosphorylation of phospho-p44/42 increases after 24 hours and 48 hours, while lysates from EGF-treated cells increases the level of phospho-p44/42 after 1, 2, 4, 6, 24 hours and 48 hours compared to time zero. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 4 (page 313).

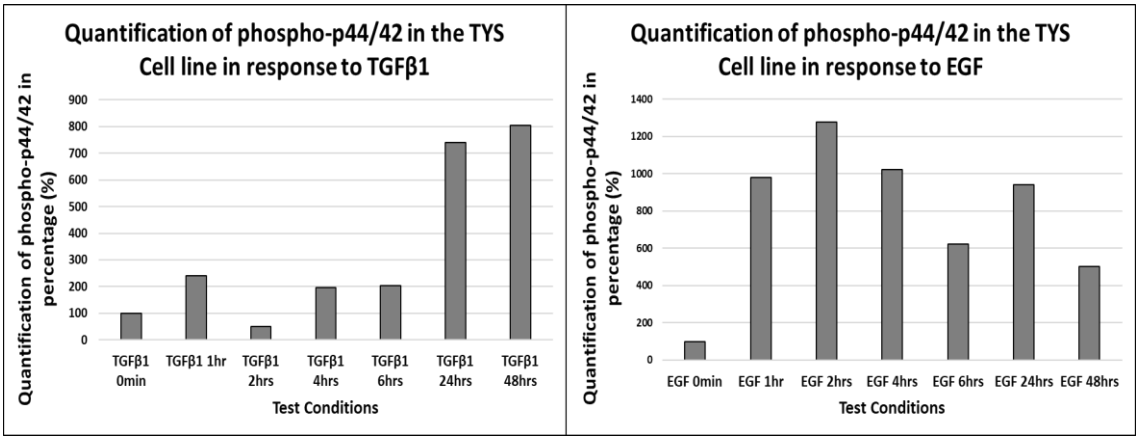


Figure 96: Quantification of phospho-p44/42 in the TYS cell line.

This bar chart shows the quantification of phospho-p44/p42 in the TYS cell line for stage I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from TYS cells treated with TGFβ1 (10ng/ml) after 24 hours and 48 hours showed an increase in p44/p42 phosphorylation compared to negative control. Also, lysates from EGF treated cells showed high phospho-p44/p42 at all incubation periods compared to the baseline.

Table 47: Quantification of phospho-p44/42 in the TYS cell line with TGFβ1 and EGF at different incubation periods.

TYS	**0 minute**	**1 hour**	**2 hours**	**4 hours**	**6 hours**	**24 hours**	**48 hours**
TGFβ1	100%	239.9%	50.1%	196.3%	203.8%	738.9%	804.4%
EGF	100%	980.1%	1276.1%	1023.3%	624.1%	939.9%	500.9%

(b) <u>Stage II Assessment of MAPK Pathway</u>:

This stage of the assessment was designed to investigate the effects of different inhibitors with TGFβ1 on the phosphorylation of p44/42. TGFβ1 + TGF-β RI Kinase Inhibitor VII after 48 hours, TGFβ1 + MK-2206 after 48 hours and TGFβ1 + SB431542 after 24 hours showed similar levels of phospho-p44/42 to TGFβ1-alone at 48 hours (**Figure 97**). Lysates from TYS cells that were treated with EGF alone after 48 hours, TGFβ1 + PD98059 after 24 and 48 hours, and TGFβ1 + SB431542 after 48 hours showed a reduction to the level of SF-MEM after 48 hours (**Figure 98**).

Table 48: The format of stage II assessment for phospho-p44/42 in the TYS cell line.

(1) Magic marker	**(2)** SF-MEM (48 hours)	**(3)** TGFβ1 (48 hours)	**(4)** EGF (48 hours)	**(5)** TGFβ1+ TGF-β RI Kinase Inhibitor VII (48 hours)
(6) TGFβ1+MK2206 (48 hours)	**(7)** TGFβ1+PD98059 (24 hours)	**(8)** TGFβ1+PD98059 (48 hours)	**(9)** TGFβ1+SB431542 (24 hours)	**(10)** TGFβ1+SB431542 (48 hours)

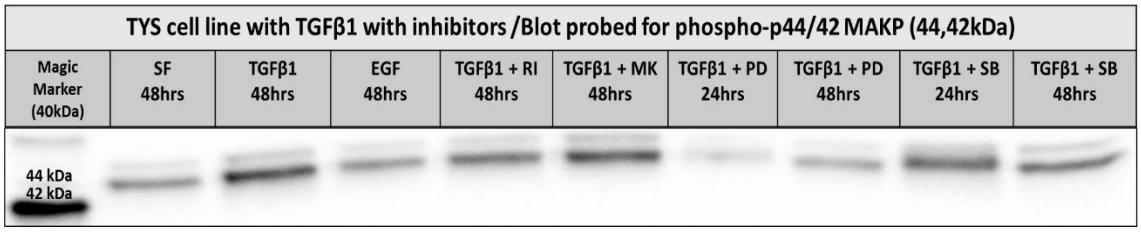


Figure 97: Stage II Assessment for phospho-p44/42 in the TYS cell line.

The TYS cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a phospho-p44/42 monoclonal antibody at a 1:2000 dilution, followed by goat anti-rabbit secondary. Lysates from TYS cells treated with TGFβ1 (10ng/ml) after 48 hours, EGF (10ng/ml) after 48 hours and TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours showed an increase in phosphorylation of phospho-p44/42 compared to the negative control. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 5 (page 320).

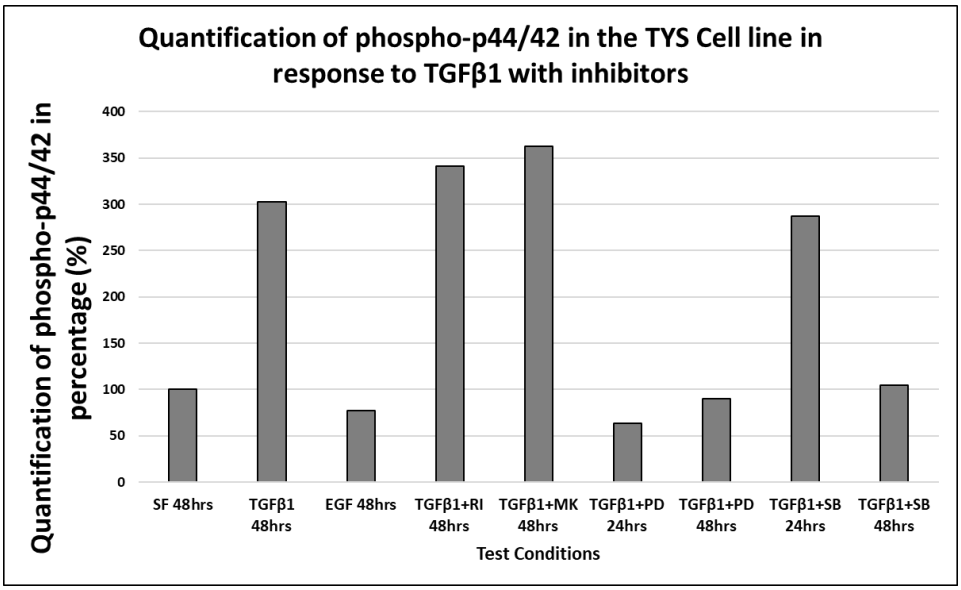


Figure 98: Quantification of phospho-p44/42 in the TYS cell line.

The bar chart shows the quantification of phospho-p44/42 in the TYS cell line for phase II assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from TYS cells treated with TGFβ1 (10ng/ml) for 48 hours, EGF (10ng/ml) after 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) after 48 hours and TGFβ1 (10ng/ml)+SB431542 (5μM) after 24 hours showed an increase in phospho-p44/42 phosphorylation compared to negative control.

Table 49: Quantification of p-p44/42 in the TYS cell line with stage II format.

TYS Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	63.4%
10ng/ml TGFβ1 48 hours	302.7%	**TGFβ1+PD98059 48 hours**	89.9%
10ng/ml EGF 48 hrs	77.6%	**TGFβ1+SB431542 24 hours**	287.1%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	340.9%	**TGFβ1+SB431542 48 hours**	104.3%
TGFβ1+MK-2206 48 hours	362.1%		

The phosphorylation of p44/42 was detected in the TYS cells incubated with TGFβ1 for 24 and 48 hours, while lysates of the cells treated with EGF showed the phosphorylation of p44/42 occurred as early as an hour and continued until 48 hours. PD98059 inhibited the upregulation of p44/42 phosphorylation in the TYS cells (**Table 49**).

Rho-GTPase Pathway Assessment for TYS Cell line

(a) Stage I Assessment for Rho-GTPase Pathway:

SDS-PAGE and Western blotting were used to investigate the effect of TGFβ1-alone and EGF-alone on the phosphorylation of Rac1/Cdc42. With both growth factors, the protein bands on the blots were faint, which suggested low levels of p-Rac1/Cdc42 in response to the test conditions (**Figure 99**). According to the normalisation of the blot, the test conditions with TGFβ1 showed slightly higher levels of p-Rac1/Cdc42 than with EGF, especially after 24 hours and 48 hours (**Figure 100**).

Table 50: The format of stage I assessment for p-Rac1/Cdc42 in the TYS cell line.

(1) Magic Marker						
(2) TGFβ1 (0min)	**(3)** TGFβ1 (1 hour)	**(4)** TGFβ1 (2 hours)	**(5)** TGFβ1 (4 hours)	**(6)** TGFβ1 (6 hours)	**(7)** TGFβ1 (24 hours)	**(8)** TGFβ1 (48 hours)
(9) EGF (0min)	**(10)** EGF (1 hour)	**(11)** EGF (2 hours)	**(12)** EGF (4 hours)	**(13)** EGF (6 hours)	**(14)** EGF (24 hours)	**(15)** EGF (48 hours)

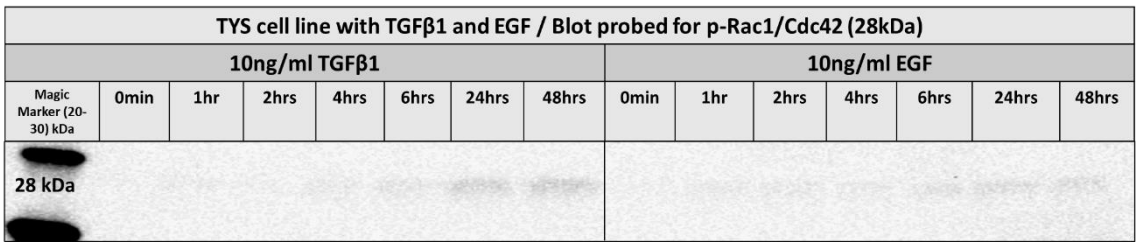


Figure 99: Stage I assessment for p-Rac1/cdc42 in the TYS cell line.

The TYS cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Rac1/Cdc42 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1 -treated cells, revealed that the phosphorylation of Rac1/Cdc42 increases after 24 hours and 48 hours. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 4 (page 313).

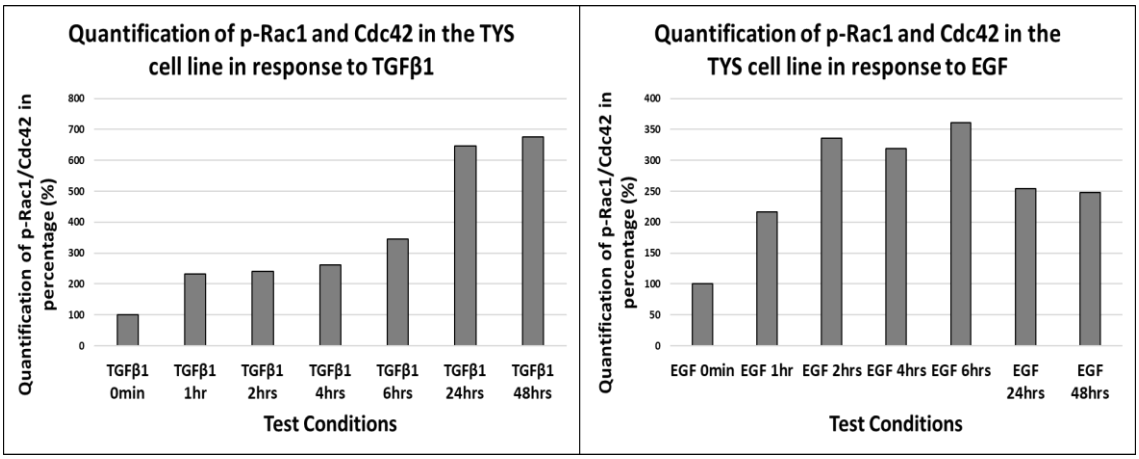


Figure 100: Quantification of p-Rac1/Cdc42 in the TYS cell line.

This bar chart shows the quantification of p-Rac1/Cdc42 in the TYS cell line for stage I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). Time zero lane was considered as 100% for protein quantification. Lysates from TYS cells treated with TGFβ1 for 24 hours and 48 hours. Also, lysates from cells treated with EGF1 (10ng/ml) after 1 hour, 2 hours, 4 hours, 6 hours, 24 hours and 48 hours showed an increase in Rac1/Cdc42 phosphorylation compared to the negative control.

Table 51: Quantification of p-Rac1/cdc42 in the TYS cell line with TGFβ1 and EGF at different incubation periods.

TYS	0 minute	1 hour	2 hours	4 hours	6 hours	24 hours	48 hours
TGFβ1	100%	232.2%	241.2%	261.4%	345.8%	646.5%	676.3%
EGF	100%	216.1%	335.8%	319.4%	360.9%	254.5%	247.6%

(b) <u>Stage II assessment for Rho-GTPase Pathway:</u>

At this stage of the assessment, the p-Rac1/Cdc42 level was noticeably higher in the TYS cells that were incubated in TGFβ1 with the MAPK inhibitor at 24 hours compared to the rest of test conditions (**Figure 101**). A similar level of phosphorylated Rac1/Cdc42 was detected in the rest of test the conditions including SF-MEM at 48 hours (**Figure 102**).

Table 52: The format of stage II assessment for p-Rac1/Cdc42 in the TYS cell line.

(1) Magic marker	**(2)** SF-MEM (48 hours)	**(3)** TGFβ1 (48 hours)	**(4)** EGF (48 hours)	**(5)** TGFβ1+ TGF-β RI Kinase Inhibitor VII (48 hours)
(6) TGFβ1+MK2206 (48 hours)	**(7)** TGFβ1+PD98059 (24 hours)	**(8)** TGFβ1+PD98059 (48 hours)	**(9)** TGFβ1+SB431542 (24 hours)	**(10)** TGFβ1+SB431542 (48 hours)

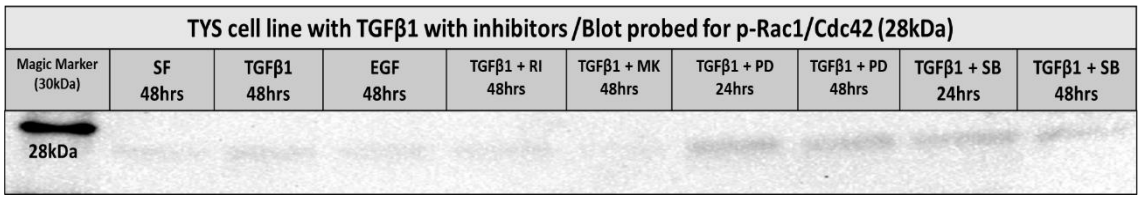


Figure 101: Stage II Assessment for p-Rac1/Cdc42 in the TYS cell line.

The TYS cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Rac1/Cdc42 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Lysates from TYS cells treated TGFβ1 (10ng/ml)+PD98059 (5μM) after 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) after 24 and 48 hours showed phosphorylation of Rac1/Cdc42. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 5 (page 320).

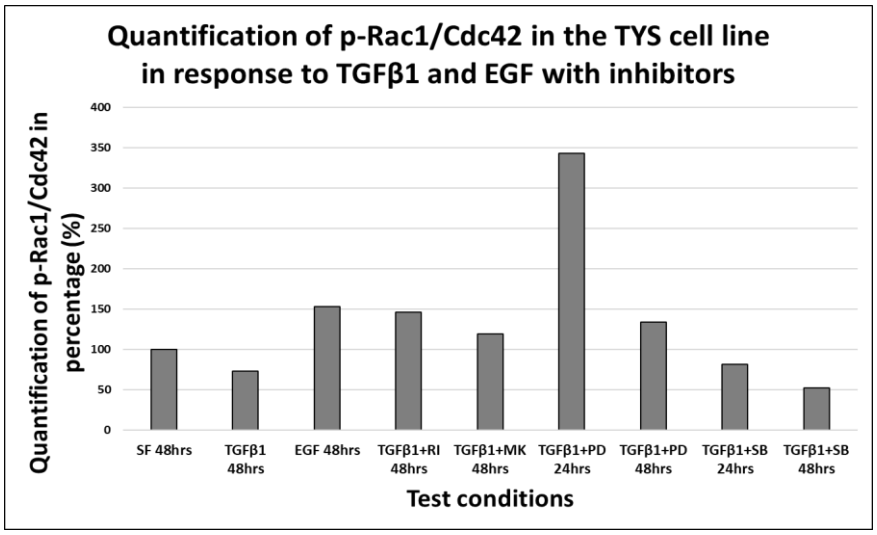


Figure 102: Quantification of p-Rac1/Cdc42 in the TYS cell line.

The bar chart shows the quantification of p-Rac1/Cdc42 in the TYS cell line for phase II assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from TYS cells treated with TGFβ1 (10ng/ml)+PD98059 (5μM) after 24 hours showed an increase in p-Rac1/Cdc42 phosphorylation compared to negative control.

Table 53: Quantification of p-Rac1/Cdc42 in the TYS cell line with stage II format.

TYS Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	343.1%
10ng/ml TGFβ1 48 hours	72.8%	**TGFβ1+PD98059 48 hours**	133.6%
10ng/ml EGF 48 hrs	153.4%	**TGFβ1+SB431542 24 hours**	81.7%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	146.3%	**TGFβ1+SB431542 48 hours**	52.1%
TGFβ1+MK-2206 48 hours	119.6%		

The phosphorylation of Rac1/Cdc42 was detected in the TYS cells with TGFβ1 alone and EGF alone. This phosphorylation level did not change at stage II assessment, except with lysates from TYS cells that were treated TGFβ1 + PD98059 after 24 hours. These samples showed an increase in Rac1/Cdc42 phosphorylation level compared to the negative control (SF-MEM after 48 hours).

5.4.3 SAS-H1 Cell Line:

Observation of the SAS-H1 cell line was carried out after 24 and 48 hours incubation with the test conditions according to stage II assessment format, before starting the protocol for cell lysis. Images of the cell cultures were taken at 200X magnification to evaluate cell morphology, colony appearance and scattered cells.

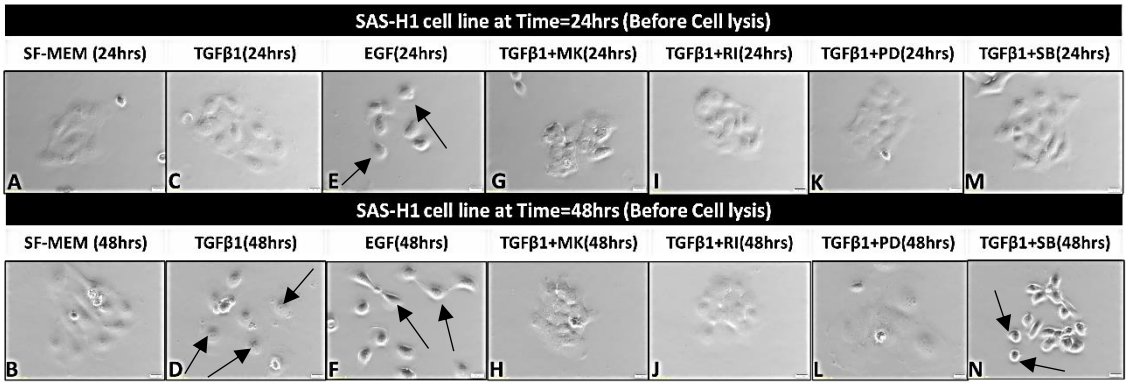


Figure 103: Observational assessment for the SAS-H1 cell lines.

Images of the SAS-H1 cell line with SF-MEM at 48 hours, TGFβ1 at 48hours, EGF at 48 hours, TGFβ1+ TGF-β RI Kinase Inhibitor VII at 48 hours, TGFβ1+MK2206 at 48 hours, TGFβ1+PD98059 at 24 hours and 48 hours and TGFβ1+SB431542 at 24 hours and 48 hours before cell lysis. All images were taken at 200x magnification to observe cell morphology, colony appearance and scattered cells. (↑) scattered cells.

The SF-MEM control group displayed a compact colony with small round or cuboid cells and no signs of cell movement (**Figure 103-A&B**). Four of the test conditions TGFβ1 (**Figure 103-D**), EGF (**Figure 103-E&F**), TGFβ1 + PD98059 (**Figure 103-L**), and TGFβ1 + SB431542 (**Figure 103-N**) induced cells to migrate away from the colony. When cells were incubated with the EGF and TGFβ1 + SB431542, especially at 48 hours, the SAS-H1 cells appeared small, round, and separated from the colony. In addition, cultures treated with TGFβ1 alone and TGFβ1 + PD98059 revealed large round cells, separated from the colony or in a less compact colony.

Smad Pathway Assessment for SAS-H1 Cell Line:

(a) <u>Stage I assessment of Smad Pathway</u>:

The SAS-H1 cell line showed no Smad2 phosphorylation with both growth factors TGFβ1 and EGF after all the incubations periods, which included time zero, an hour, two hours, four hours, six hours, 24 hours and 48 hours (**Figure 104**).

Table 54: The format of stage I assessment for p-Smad2 in the SAS-H1 cell line.

(1) Magic Marker						
(2) TGFβ1 (0min)	**(3)** TGFβ1 (1 hour)	**(4)** TGFβ1 (2 hours)	**(5)** TGFβ1 (4 hours)	**(6)** TGFβ1 (6 hours)	**(7)** TGFβ1 (24 hours)	**(8)** TGFβ1 (48 hours)
(9) EGF (0min)	**(10)** EGF (1 hour)	**(11)** EGF (2 hours)	**(12)** EGF (4 hours)	**(13)** EGF (6 hours)	**(14)** EGF (24 hours)	**(15)** EGF (48 hours)

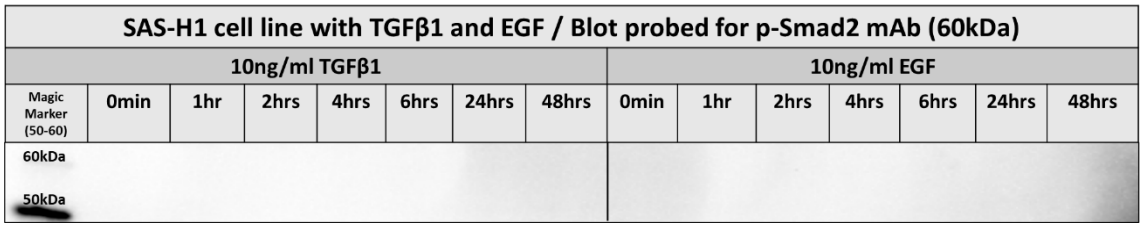


Figure 104: Stage I assessment for p-Smad2 in the SAS-H1 cell line.

The SAS-H1 cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Smad2 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells and EGF-treated cells, revealed no phosphorylation of Smad2 at all incubation periods. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 4 (page 314).

(b) <u>Stage II Assessment of Smad Pathway</u>:

Similar to the stage I assessment of the SAS-H1 cell line, the phosphorylation of Smad2 was not detected with the test conditions including inhibitors at both incubation periods, 24 and 48 hours (**Figure 105**).

Table 55: The format of stage II assessment for p-Smad2 in the SAS-H1 cell line.

(1) Magic marker	**(2)** SF-MEM (48 hours)	**(3)** TGFβ1 (48 hours)	**(4)** EGF (48 hours)	**(5)** TGFβ1+ TGF-β RI Kinase Inhibitor VII (48 hours)
(6) TGFβ1+MK2206 (48 hours)	**(7)** TGFβ1+PD98059 (24 hours)	**(8)** TGFβ1+PD98059 (48 hours)	**(9)** TGFβ1+SB431542 (24 hours)	**(10)** TGFβ1+SB431542 (48 hours)

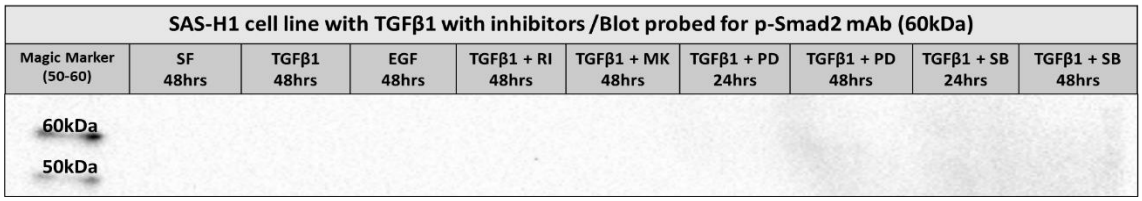


Figure 105: Stage II Assessment for p-Smad2 in the SAS-H1 cells.

The SAS-H1 cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Smad2 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from SAS-H1 cells treated with the different test conditions showed no phosphorylation of Smad2. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 5 (page 321).

PI3K-Akt Pathway Assessment for SAS-H1 Cell line

Table 56: The format of stage I assessment for PI3K-Akt pathway in the SAS-H1 cell line.

(1) Magic Marker						
(2) TGFβ1 (0min)	**(3)** TGFβ1 (1 hour)	**(4)** TGFβ1 (2 hours)	**(5)** TGFβ1 (4 hours)	**(6)** TGFβ1 (6 hours)	**(7)** TGFβ1 (24 hours)	**(8)** TGFβ1 (48 hours)
(9) EGF (0min)	**(10)** EGF (1 hour)	**(11)** EGF (2 hours)	**(12)** EGF (4 hours)	**(13)** EGF (6 hours)	**(14)** EGF (24 hours)	**(15)** EGF (48 hours)

(a) <u>Stage I assessment of PI3k-Akt Pathway</u>:

1- **<u>Thr308</u>**: Cells incubated with EGF-alone and TGFβ1-alone both showed upregulation of Akt T308 phosphorylation or more pAkt T308 compared to the baseline, which was time zero. These samples showed an increase in p-Akt T308 levels after two hours, four hours, and six hours incubation periods, before it returned to the time zero level at both 24 hours and 48 hours (**Figure 106**). Also, EGF treated SAS-H1 cells showed an upregulation in Akt T308 phosphorylation or more p-Akt T308 at the different incubation periods. The highest level of p-Akt T308 was detected after two hours, four hours and six hours (**Figure 107**).

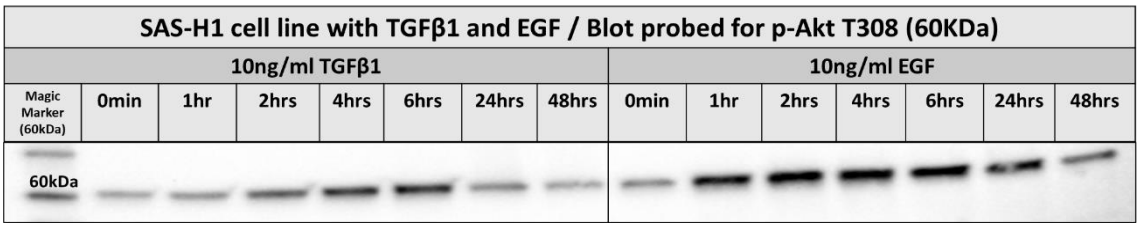


Figure 106: Stage I assessment for p-Akt T308 in the SAS-H1 cell line.

The SAS-H1 cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Akt T308 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells and EGF-treated cells, revealed that the phosphorylation of Akt T308 increases at all incubation periods. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 4 (page 314).

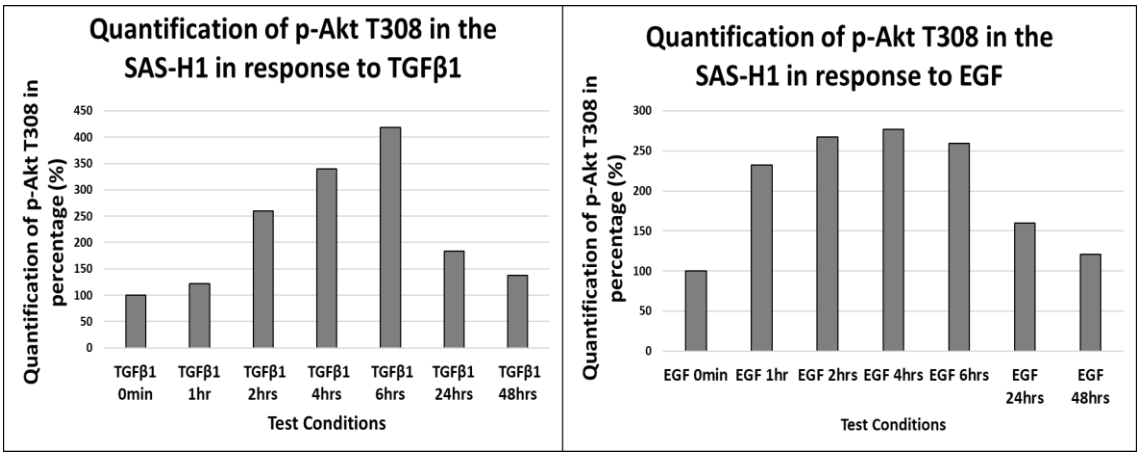


Figure 107: Quantification of p-Akt T308 in the SAS-H1 cells.

This bar chart shows the quantification of p-Akt T308 in the SAS-H1 cell line for stage I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from SAS-H1 cells treated with TGFβ1 (10ng/ml) after 2, 4 and 6 hours showed an increase in Akt T308 phosphorylation compared to negative control. Also, lysates from EGF treated cells showed high p-Akt T308 after 1, 2, 4, 6 and 24 hours compared to the baseline.

Table 57: Quantification of p-Akt T308 expression in the SAS-H1 cell line with TGFβ1 and EGF at different incubation periods.

SAS-H1	0 minute	1 hour	2 hours	4 hours	6 hours	24 hours	48 hours
TGFβ1	100%	121.4%	259.6%	339.7%	418.1%	184.2%	137.8%
EGF	100%	232.6%	267.5%	276.5%	259.2%	159.8%	120.8%

2- **<u>Ser473:</u>** The upregulation of p-Akt S473 levels were observed in lysates from SAS-H1 cell treated with TGFβ1-alone and EGF-alone (**Figure 108**). The highest level of phosphorylation of Akt S473 was

after two hours to six hours with TGFβ1 and after an hour to six hours with EGF compared to time zero (**Figure 109**).

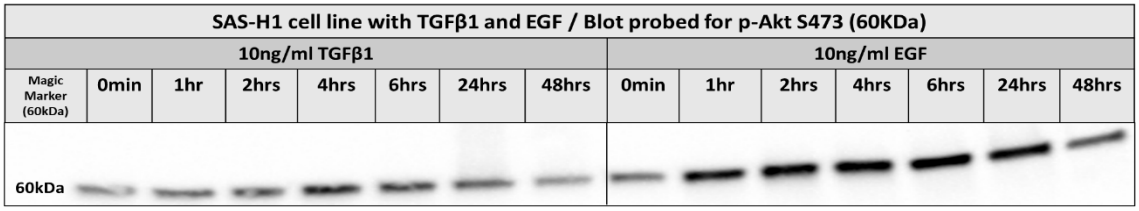


Figure 108: Stage I assessment for p-Akt-S473 in the SAS-H1 cell line.

The SAS-H1 cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Akt S473 monoclonal antibody at a 1:2000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells and EGF-treated cells, revealed that the phosphorylation of Akt S473 increases at all incubation periods. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 4 (page 314).

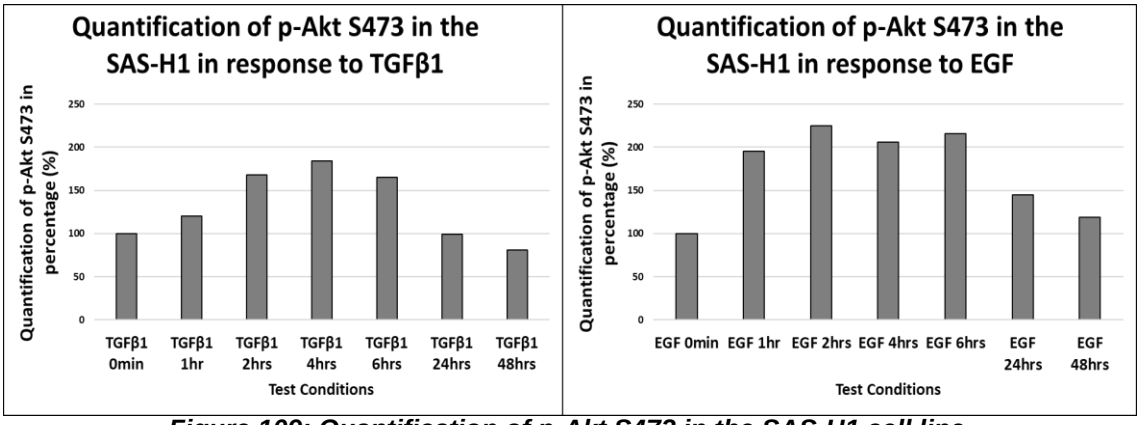


Figure 109: Quantification of p-Akt S473 in the SAS-H1 cell line.

This bar chart shows the quantification of p-Akt S473 in the SAS-H1 cell line for stage I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from SAS-H1 cells treated with TGFβ1 (10ng/ml) after 2, 4 and 6 hours showed an increase in Akt S473 phosphorylation compared to negative control. Also, lysates from EGF treated cells showed high p-Akt S473 after 1, 2, 4, 6 and 24 hours compared to the baseline.

Table 58: Quantification of p-Akt S473 in the SAS-H1 cell line with TGFβ1 and EGF at different incubation periods.

SAS-H1	0 minute	1 hour	2 hours	4 hours	6 hours	24 hours	48 hours
TGFβ1	100%	120.1%	168.1%	183.9%	164.9%	99.4%	80.8%
EGF	100%	195.7%	225.2%	205.6%	215.9%	145.1%	118.8%

(b) Stage II assessment of PI3k-Akt Pathway:

Table 59: The format of stage II assessment for PI3K-Akt pathway in the SAS-H1 cell line.

(1) Magic marker	**(2)** SF-MEM (48 hours)	**(3)** TGFβ1 (48 hours)	**(4)** EGF (48 hours)	**(5)** TGFβ1+ TGF-β RI Kinase Inhibitor VII (48 hours)
(6) TGFβ1+MK2206 (48 hours)	**(7)** TGFβ1+PD98059 (24 hours)	**(8)** TGFβ1+PD98059 (48 hours)	**(9)** TGFβ1+SB431542 (24 hours)	**(10)** TGFβ1+SB431542 (48 hours)

1- **Thr308:** Three distinctive levels of Akt T308 phosphorylation were observed with the different test conditions compared to SF-MEM at 48 hours as the baseline (**Figure 110**). First, the upregulation of Akt T308 levels compared to the baseline was observed in lysates from cells treated with TGFβ1 after 48 hours, EGF after 48 hours, TGFβ1 + PD98059 after 24 and 48 hours and TGFβ1 + SB431542 after 24 hours. Second, the phosphorylation level of Akt T308 was lower than SF-MEM after 48 hours in response to TGFβ1 + TGF-β RI Kinase Inhibitor VII after 48 hours and TGFβ1 + SB431542 after 48 hours. Finally, lysates from cells that were treated TGFβ1 + MK-2206 showed a significant reduction in the amount of p-Akt T308 (**Figure 111**). These findings suggested effective inhibition of Akt T308 phosphorylation had occurred, even in the presence of the growth factor.

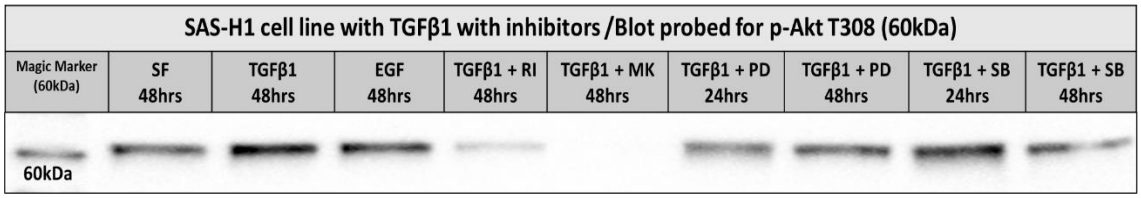


Figure 110: Stage II Assessment for p-Akt T308 in the SAS-H1 cell line.

The SAS-H1 cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Akt T308 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from SAS-H1 cells treated the test conditions of phase II assessment showed an phosphorylation of Akt T308 compared to the negative control, except lysates from cells treated with TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) after 48 hours and TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 5 (page 321).

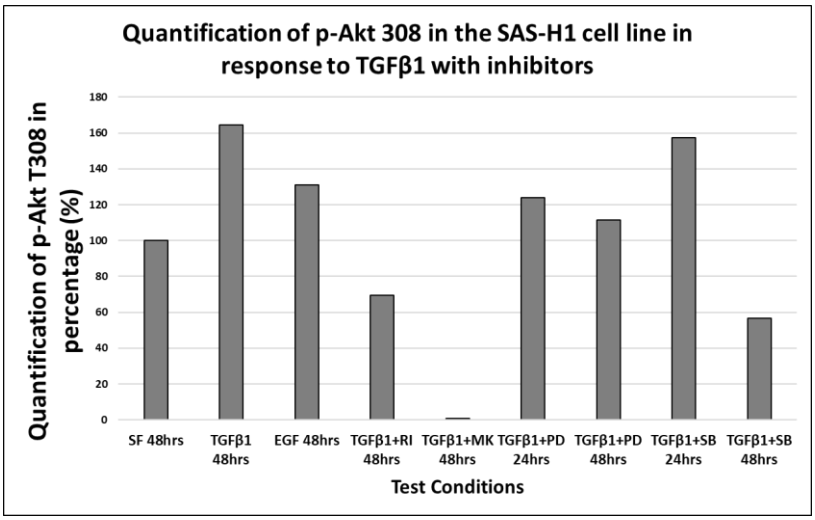


Figure 111: Quantification of p-Akt T308 in the SAS-H1 cell line.

The bar chart shows the quantification of p-Akt T308 in the SAS-H1 cell line for phase II assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from SAS-H1 cells treated with TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) after 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours and TGFβ1 (10ng/ml)+SB431542 (5μM) after 48 hours showed less level of p-Akt T308 compared to the negative control.

Table 60: Quantification of p-Akt T308 in the SAS-1 cell line with stage II format.

SAS-H1 Cell line test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	123.8%
10ng/ml TGFβ1 48 hours	164.7%	**TGFβ1+PD98059 48 hours**	111.6%
10ng/ml EGF 48 hrs	131%	**TGFβ1+SB431542 24 hours**	157.5%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	69.4%	**TGFβ1+SB431542 48 hours**	56.8%
TGFβ1+MK-2206 48 hours	0.92%		

2- **Ser473:** The phosphorylation of Akt S473 in the SAS-H1 cells showed an upregulation and downregulation with the test conditions (**Figure 112**). The highest level of p-Akt S473 was observed in lysates prepared from cells which were incubated with TGFβ1 plus PD98059 for 24 hours, in comparison to the negative control (SF-MEM). Secondly, the level of Akt S473 phosphorylation, at a level similar or close to SF-MEM samples, was observed in lysates of cells incubated with TGFβ1 for 48 hours, EGF for 48 hours, TGFβ1 + PD98059 at 24 hours and TGFβ1 + SB431542 at 24 hours. Thirdly, the phosphorylation of Akt T308 was seen with TGFβ1 plus TGF-β RI Kinase Inhibitor VII at 48 hours and TGFβ1 with SB431542 at 48 hours which was lower than the SF-MEM.

Finally, no p-Akt S473 was observed in lysates prepared from cells treated with TGFβ1 plus MK-2206 for 48 hours (**Figure 113**).

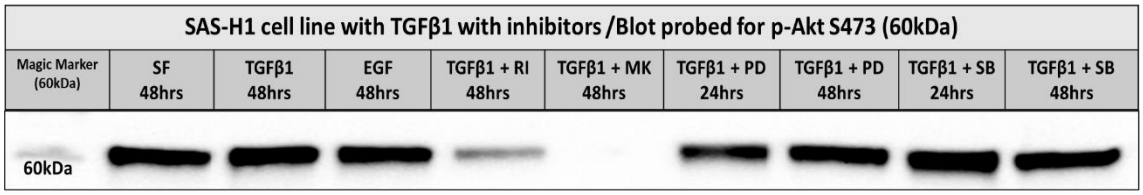


Figure 112: Stage II Assessment of p-Akt S473 in the SAS-H1 cell line.

The SAS-H1 cell line was incubated with nine different test conditions (**Table 59**). Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Akt S473 monoclonal antibody at a 1:2000 dilution, followed by goat anti-rabbit secondary. All lysates from SAS-H1 cells treated the test conditions of phase II assessment showed an phosphorylation of Akt S473 compared to the negative control, except lysates from cells treated with TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 5 (page 321).

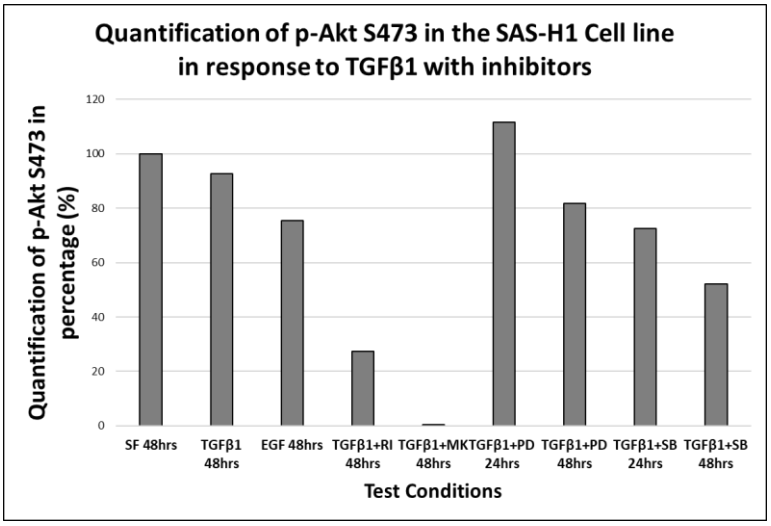


Figure 113: Quantification of p-Akt-S473 in the SAS-H1 cell line.

The bar chart shows the quantification of p-Akt S473 in the SAS-H1 cell line for phase II assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from SAS-H1 cells treated with TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) after 48 hours and TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours showed less level of p-Akt S473 compared to the negative control.

Table 61: Quantification of p-Akt S473 in the SAS-H1 cell line with stage II format.

SAS-H1 Cell line test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	111.6%
10ng/ml TGFβ1 48 hours	92.7%	**TGFβ1+PD98059 48 hours**	81.7%
10ng/ml EGF 48 hrs	75.5%	**TGFβ1+SB431542 24 hours**	72.5%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	27.4%	**TGFβ1+SB431542 48 hours**	52.1%
TGFβ1+MK-2206 48 hours	0.37%		

The phosphorylation of both Akt residues T308 and S473 showed an upregulation in lysates from cells treated with TGFβ1-alone and EGF-alone, especially after four and six hours. TGF-β RI Kinase Inhibitor VII and MK-2206 inhibited the phosphorylation at both sites.

MAPK Pathway Assessment for SAS-H1 Cell line

(a) Stage I assessment of MAPK (phospho-p44/42):

By comparing the effect of TGFβ1 and EGF on phospho-p44/42 in the SAS-H1 cell line, it was observed that there were two different patterns of phosphorylation in response to each growth factor. In the case of cells cultured with TGFβ1, phospho-p44/42 was upregulated in the SAS-H1 cell line at 24 hours and 48 hours (**Figure 114**). Cells cultured with EGF showed the presence of phospho-p44/42 from an hour's incubation and this continued up to 24 hours.

Table 62: The format of stage I assessment for phospho-p44/42 in the SAS-H1 cell line.

(1) Magic Marker						
(2) TGFβ1 (0min)	**(3)** TGFβ1 (1 hour)	**(4)** TGFβ1 (2 hours)	**(5)** TGFβ1 (4 hours)	**(6)** TGFβ1 (6 hours)	**(7)** TGFβ1 (24 hours)	**(8)** TGFβ1 (48 hours)
(9) EGF (0min)	**(10)** EGF (1 hour)	**(11)** EGF (2 hours)	**(12)** EGF (4 hours)	**(13)** EGF (6 hours)	**(14)** EGF (24 hours)	**(15)** EGF (48 hours)

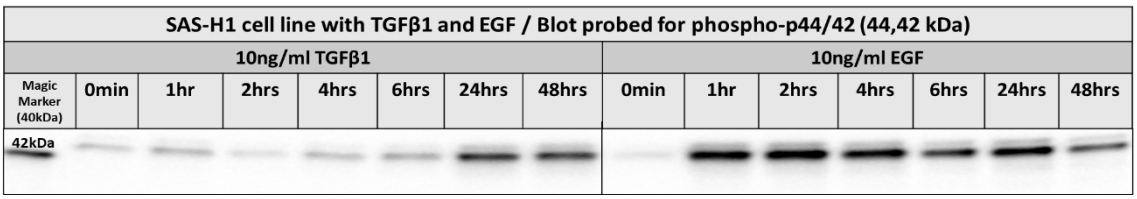


Figure 114: Stage I assessment of phospho-p44/42 in the SAS-H1 cell line.

The SAS-H1 cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a phospho-p44/42 monoclonal antibody at a 1:2000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells, revealed that the phosphorylation of phospho-p44/42 increases after 24 hours and 48 hours, while lysates from EGF-treated cells increases the level of phospho-p44/42 after 1, 2, 4, 6, 24 hours and 48 hours compared to time zero. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 4 (page 315).

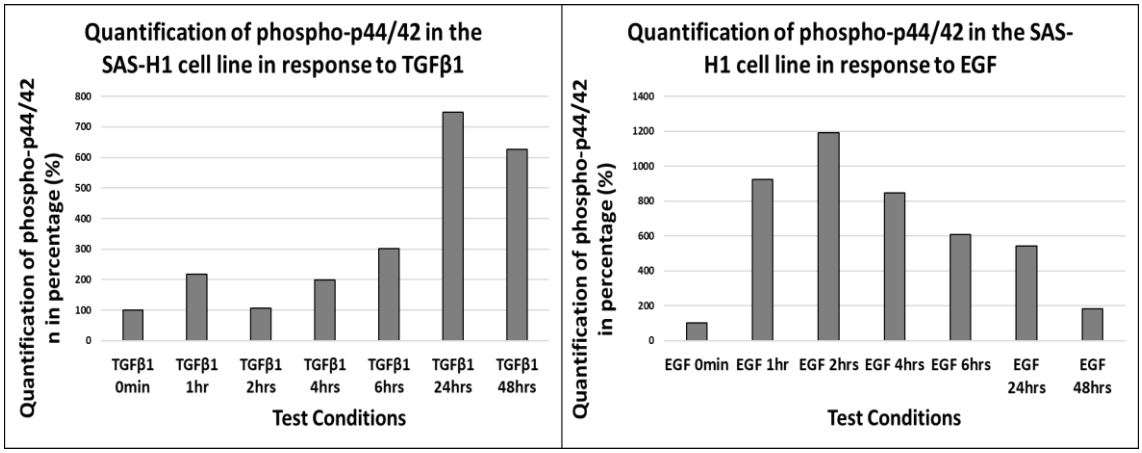


Figure 115: Quantification of phospho-p44/42 in the SAS-H1 cell line.

This bar chart shows the quantification of phospho-p44/p42 in the SAS-H1 cell line for stage I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from SAS-H1 cells treated with TGFβ1 (10ng/ml) after 24 hours and 48 hours showed an increase in p44/p42 phosphorylation compared to the negative control. Also, lysates from EGF treated cells showed high phospho-p44/p42 at all incubation periods compared to the baseline.

Table 63: Quantification of phospho-p44/42 in the SAS-H1 cell line with TGFβ1 and EGF at different incubation periods

SAS-H1	0 minute	1 hour	2 hours	4 hours	6 hours	24 hours	48 hours
TGFβ1	100%	218.3%	106.3%	198.4%	301.9 %	747.5%	625.7%
EGF	100%	924.2%	1189.8%	846.9%	608.5%	541%	182.1%

(b) <u>Stage II assessment of MAPK (phospho-p44/42)</u>:

The effect of different of inhibitors on p44/p42 phosphorylation was compared to the control, SF-MEM at 48 hours (**Figure 116**). Firstly, lysates from cells treated with TGFβ1-alone and EGF-alone after 48 hours showed high levels of phosphorylated -p44/42 compared to the SF-MEM control. Secondly, three test conditions including TGFβ1 + TGF-β RI Kinase Inhibitor VII, TGFβ1 + MK-2206 and TGFβ1 + PD98059 showed lower levels of phospho-p44/42 than TGFβ1 alone after 48 hours, however, it was higher than SF-MEM. Finally, the highest level of phospho-p44/42 was recorded with cells cultured with TGFβ1 + SB431542 at 24 hours before it decreased to the level observed with the TGFβ1-treated cells (**Figure 117**).

Table 64: The format of stage II assessment for phospho-p44/42 in the SAS-H1 cell line.

(1) Magic marker	**(2)** SF-MEM (48 hours)	**(3)** TGFβ1 (48 hours)	**(4)** EGF (48 hours)	**(5)** TGFβ1+ TGF-β RI Kinase Inhibitor VII (48 hours)
(6) TGFβ1+MK2206 (48 hours)	**(7)** TGFβ1+PD98059 (24 hours)	**(8)** TGFβ1+PD98059 (48 hours)	**(9)** TGFβ1+SB431542 (24 hours)	**(10)** TGFβ1+SB431542 (48 hours)

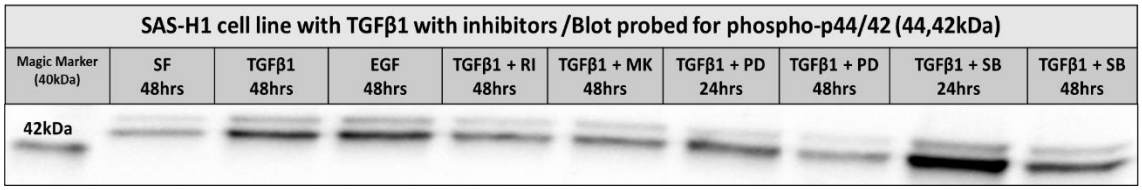


Figure 116: Stage II Assessment of phospho-p44/42 in the SAS-H1 cell line.

The SAS-H1 cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a phospho-p44/42 monoclonal antibody at a 1:2000 dilution, followed by goat anti-rabbit secondary. Lysates from SAS-H1 cells treated with the test conditions of phase II assessment showed an increase in phosphorylation of phospho-p44/42 compared to the negative control. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 5 (page 322).

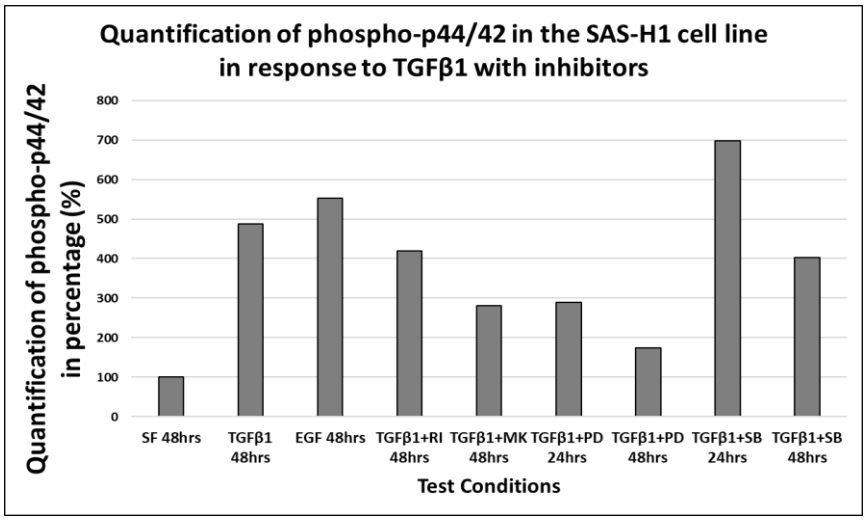


Figure 117: Quantification of phospho-p44/42 in the SAS-H1 cell line.

The bar chart shows the quantification of phospho-p44/42 in the SAS-H1 cell line for phase II assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from SAS-H1 cells treated with the test conditions of phase II assessment showed an increase in phospho-p44/42 phosphorylation compared to the negative control.

Table 65: Quantification of phospho-p44/42 in the SAS-H1 cell line with stage II format.

SAS-H1 Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	289.5%
10ng/ml TGFβ1 48 hours	487.4%	**TGFβ1+PD98059 48 hours**	173.7%
10ng/ml EGF 48 hrs	552.6%	**TGFβ1+SB431542 24 hours**	698.3%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	419.3%	**TGFβ1+SB431542 48 hours**	402.8%
TGFβ1+MK2206 48 hours	281.1%		

The presence of phospho-p44/42 was detected by SDS PAGE and Western blotting of cell lysates prepared from SAS-H1 cells that had been incubated with TGFβ1 for 24 hours and 48 hours, while lysates fromSAS-H1 cells treated with EGF showed the presence of phospho-p42/44 after an hour's incubation and this continued until 48 hours. Only samples from cells incubated with PD98059 plus TGFβ1 showed a significant reduction of p44/42 phosphorylation compared to the rest of the test conditions (**Table 65**).

Rho-GTPase Pathway Assessment for SAS-H1 Cell line

(a) <u>Stage I assessment for Rho-GTPase</u>:

Western blotting of lysates from SAS-H1 cells incubated with TGFβ1 or EGF did not reveal any p-Rac1/cdc42at any of the time points indicated. These incubation periods included time zero as baseline, an hour, two hours, four hours, six hours, 24 hours and 48 hours (**Figure 118**).

Table 66: The format of stage I assessment for p-Rac1/Cdc42 in the SAS-H1 cell line.

(1) Magic Marker						
(2) TGFβ1 (0min)	**(3)** TGFβ1 (1 hour)	**(4)** TGFβ1 (2 hours)	**(5)** TGFβ1 (4 hours)	**(6)** TGFβ1 (6 hours)	**(7)** TGFβ1 (24 hours)	**(8)** TGFβ1 (48 hours)
(9) EGF (0min)	**(10)** EGF (1 hour)	**(11)** EGF (2 hours)	**(12)** EGF (4 hours)	**(13)** EGF (6 hours)	**(14)** EGF (24 hours)	**(15)** EGF (48 hours)

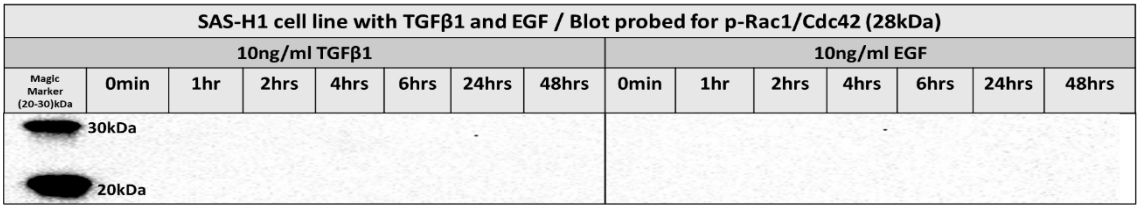


Figure 118: Stage I assessment of p-Rac1/Cdc42 in the SAS-H1 cell line.

The SAS-H1 cells were incubated with TGFβ1 or EGF for: 0, 1, 2, 4, 6, 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Rac1/Cdc42 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Western blotting of lysates from TGFβ1-treated cells and EGF-treated cells, revealed that the phosphorylation of Rac1/Cdc42 did not occur. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 4 (page 315).

(b) Stage II assessment for Rho-GTPase:

Overall, the phosphorylation of Rac1/Cdc42 with the test conditions of stage II assessment was low, appearing as a faint band on the blot (**Figure 119**). However, using Image Lab normalisation, EGF alone after 48 hours and TGFβ1 + PD98059 after 24 hours showed an increase in p-Rac1/Cdc42 levels when compared to the control sample SF- MEM at 48 hours (**Figure 120**).

Table 67: The format of stage II assessment for p-Rac1/Cdc42 in the SAS-H1 cell line.

(1) Magic marker	**(2)** SF-MEM (48 hours)	**(3)** TGFβ1 (48 hours)	**(4)** EGF (48 hours)	**(5)** TGFβ1+ TGF-β RI Kinase Inhibitor VII (48 hours)
(6) TGFβ1+MK2206 (48 hours)	**(7)** TGFβ1+PD98059 (24 hours)	**(8)** TGFβ1+PD98059 (48 hours)	**(9)** TGFβ1+SB431542 (24 hours)	**(10)** TGFβ1+SB431542 (48 hours)

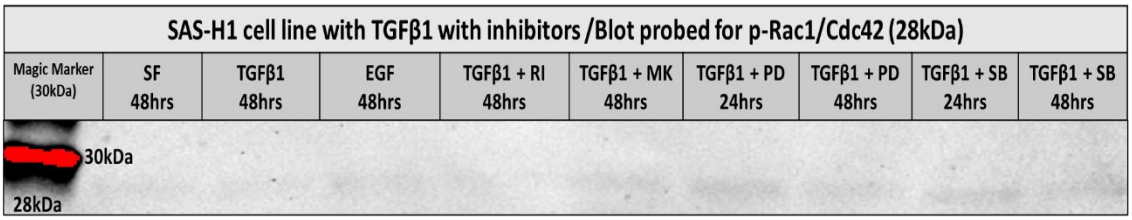


Figure 119: Stage II Assessment of p-Rac1/Cdc42 in the SAS-H1 cell line.

The SAS-H1 cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a p-Rac1/Cdc42 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 5 (page 322).

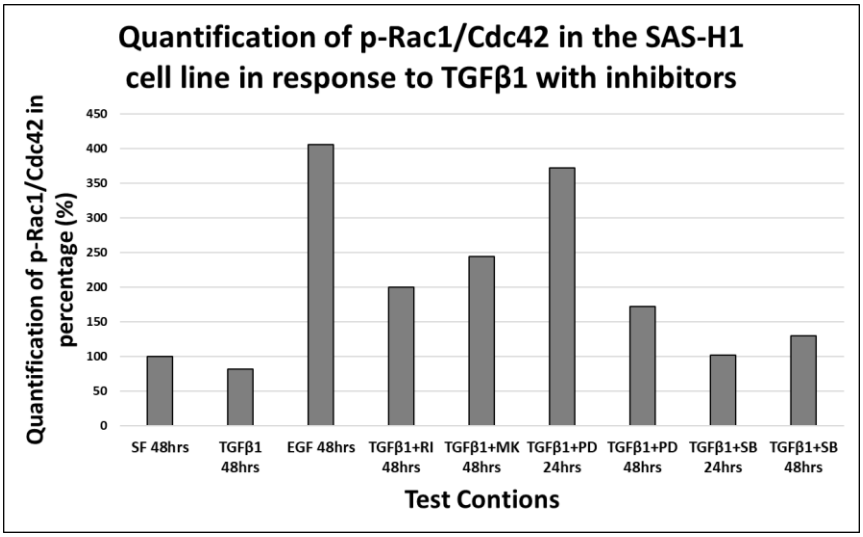


Figure 120: Quantification Stage II Assessment of p-Rac1/Cdc42 in the SAS-H1 cells.

The bar chart shows the quantification of p-Rac1/Cdc42 in the SAS-H1 cell line for phase II assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from SAS-H1 cells treated with EGF (10ng/ml) after 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) after 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours TGFβ1 and (10ng/ml)+PD98059 (5μM) after 24 and 48 hours showed an increase in p-Rac1/Cdc42 phosphorylation compared to the negative control.

Table 68: Quantification of p-Rac1/Cdc42 in the SAS-H1 cell line with stage II format.

SAS-H1 Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	372.5%
10ng/ml TGFβ1 48 hours	82.2%	**TGFβ1+PD98059 48 hours**	172%
10ng/ml EGF 48 hrs	406.2%	**TGFβ1+SB431542 24 hours**	101.9%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	200.4%	**TGFβ1+SB431542 48 hours**	129.4%
TGFβ1+MK-2206 48 hours	244.4%		

5.5 Summary of the Activation of Signalling Pathways:

Based on the findings from the migration assays, cells from different cell lines started to migrate or showed signs of morphological change after incubation with TGFβ1.

The HaCaT cell line showed a different response to the conditions in this experiment. TGFβ1 was found to activate all the signalling molecules that were analysed expect the Rho-GTPase signalling pathway, while EGF activated all of them except the Smad-dependent pathway before or around 24 hours evaluation.

In the case of the TYS cell line both growth factors activated all the targeted signalling pathways in this study, except for EGF which did not show consistent phosphorylation of Smad2. Activation of the signalling pathways occurred prior to or around 24 hours after addition of the exogenous factors.

The SAS-H1 cell line showed no phosphorylation of Smad2 and Rac1/Cdc42 in response to TGFβ1 and only EGF activated Rac1/Cdc42 by phosphorylation after 48 hours. The rest of the signalling molecules were observed to be upregulated in the SAS-H1 in response to both growth factors.

Table 69: Summary of Stage I assessment for Signalling pathways.

Cell line	Signalling pathways	Summary of Stage I assessment (Incubation periods)	
		TGFβ1	**EGF**
HaCaT	**p-Smad2**	+ (1-24hrs)	–
	p-Akt T308	+ (1-24hrs)	+ (1-48hrs)
	p-Akt S473	+ (2-24hrs)	+ (1-48hrs)
	phospho-p44/42	+ 24hrs	+ (2-24hrs)
	p-Rac1/Cdc42	–	+ (1-24hrs)
TYS	**p-Smad2**	+ (1-48hrs)	–
	p-Akt T308	+ (0-48hrs)	+ (0-48hrs)
	p-Akt S473	+ (0-48hrs)	+ (0-48hrs)
	phospho-p44/42	+ (24-48hrs)	+ (1-24hrs)
	p-Rac1/Cdc42	+ (24-48hrs)	+ (1-48hrs)
SAS-H1	**p-Smad2**	–	–
	p-Akt T308	+ (2-6hrs)	+ (1-24hrs)
	p-Akt S473	+ (2-6hrs)	+ (1-24hrs)
	phospho-p44/42	+ (24-48hrs)	+ (1-24hrs)
	p-Rac1/Cdc42	–	–

Table 70: Summary of Stage II assessment for Signalling Pathways.

Cell line	Signalling pathways	Summary of Stage II assessment (TGFβ1 with inhibitors)						
		SF-MEM 48hrs	TGFβ1 48hrs	EGF 48hrs	TGFβ1+TGF-β RI Kinase Inhibitor VII/ 48hrs	TGFβ1+MK-2206 48hrs	TGFβ1+PD98059 24hrs & 48hrs	TGFβ1+SB431542 24hrs & 48hrs
HaCaT	p-Smad2	–	–	–	–	–	–	–
	p-Akt T308	–	+	+	–	–	+ (24hrs)	+ (24hrs)
	p-Akt S473	–	+	+	–	–	+ (24hrs)	+ (24hrs)
	phospho-p44/42	–	+	+	–	+	–	–
	p-Rac1/Cdc42	–	–	–	–	–	–	–
TYS	p-Smad2	–	–	–	–	–	–	–
	p-Akt T308	+	+	+	+	–	+	+
	p-Akt S473	+	+	+	+	–	+	+
	phospho-p44/42	–	+	–	+	+	–	+ (24hrs)
	p-Rac1/Cdc42	–	–	+	+	+	+ (24hrs)	–
SAS-H1	p-Smad2	–	–	–	–	–	–	–
	p-Akt T308	+	+	+	–	–	+	+ (24hrs)
	p-Akt S473	+	+	+	–	–	+	+ (24hrs)
	phospho-p44/42	–	+	+	+	+	+ (24hrs)	+
	p-Rac1/Cdc42	–	–	+	+	+	+ (24hrs)	–

5.6 Discussion:

Previously, it has been well documented that TGFβ1 activates or phosphorylates different signalling pathways including Smad, ErK-MAPK (Massagué, 1998), PI3K-Akt (Bakin *et al.*, 2000; Liu *et al.*, 2019) and Rho-GTPase (Edlund *et al.*, 2002). We have investigated the activation of those signalling pathways in normal and oral cancer cell lines in response to different test conditions, including addition of exogenous TGFβ1. Also, in an attempt to establish a link between the signalling pathways and cell migration, the experiments were carried out using specific inhibitors for each of the signalling pathways.

To our knowledge from the previous experiments on cell migration, TGFβ1 induces cell migration of normal and oral cancer cell lines, however, it appeared that this process was regulated by different signalling pathways dependent upon the cell type. In the case of HaCaT, cell migration was blocked only by SB431542 and TGF-β RI Kinase Inhibitor VII. With the TYS cell line, both of the growth factors that were used in the migration assays (Scatter assay, Gap closure assay and Scratch assay), induced cell migration. SB431542, TGF-β RI Kinase Inhibitor VII and MK-2206 inhibited the migratory effect of TGFβ1. These findings suggest that both the Smad and PI3K-Akt pathway have a role in cell migration. In the case of EGF, TYS cells continued to show cell migration in the presence of both MK-2206 and PD98059, which indicates EGF induces cell motility via other than the PI3/Akt and MAPK signalling pathway. The assessment of cell migration for the SAS-H1 showed no migratory cells in the presence of TGFβ1 with two of the inhibitors, TGF-β RI Kinase Inhibitor VII and PD98059. This led to the conclusion that TGFβ1 promotes cell migration in SAS-H1 via the ErK/MAPK signalling pathway. However, cells migration induced by EGF in the SAS-H1 cell line was blocked by MK-2206, which indicates the role of the PI3K-Akt pathway in the migration process. Only TGF-β RI Kinase Inhibitor VII inhibited the migratory effect of TGFβ with all the lines used in this study.

Each one of the signalling pathways investigated in this study have been linked with cell migration. The Smad pathway mainly regulates EMT induced by TGFβ (Massagué, 2008; Yu *et al.*, 2011; Dey *et al.*, 2022). The PI3K-Akt signalling pathway regulates cell migration via EMT in breast cancer (Bakin *et al.*, 2000) and oral squamous cell carcinoma cell lines (Islam, 2018; Wang *et al.*, 2020; Dey *et al.*, 2022). Studies showed the activation of the ErK/MAPK signalling pathways

promotes cell migration (Sulzmaier and Ramos, 2013; Kim *et al.*, 2019) and progression of OSCC, via activation of MMP-2 (Pramanik and Mishra, 2022). A study on the effect of TGFβ on the actin cytoskeleton reported that it was required for lamellipodia formation by the activation of small GTPases Cdc42 and RhoA (Edlund *et al.*, 2002). The combination of activation of the Smad-dependent pathway and the effect on the cell junction complex by the Smad-independent pathways resulted in cells undergoing EMT (Grünert, Jechlinger and Beug, 2003; Massagué, 2008).

The analysis of the timing of the activation of signalling pathways in response to two growth factors TGFβ and EGF were carried out. From previous migration experiments on both normal and oral cancer cell lines (Chapter 4), it was known that the process of cell migration occurred around 24 hours to 48 hours after adding the growth factors. Therefore, the activation of the signalling pathways has to happen prior to or around the time of cell migration. In addition, the ability of specific inhibitors to block the activation of those signalling pathways, including Smad inhibitors (SB431542 and TGF-β RI Kinase Inhibitor VII), Akt inhibitor (MK-2206) and ErK/MAPK inhibitor (PD98059), was investigated.

Stage one assessment of the HaCaT cell line showed that Smad2 phosphorylation occurred after one hour with TGFβ1 and continued until 24 hours. Also, the phosphorylation of both Akt residues, T308 and S473, was observed around the same incubation periods in response to TGFβ1, which was between 2 to 24 hours. The level of phospho-p44/42 also increased in response to TGFβ1 especially after 24 hours, however, the phosphorylation of Rac1/Cdc42 was not detected.

At stage two of assessment for the HaCaT cell lines, the phosphorylation of Smad2 and Rac1/Cdc42 was not detected. This might be because the assessment was carried out after 48 hours and the phosphorylation occurred as early event. Regarding phosphorylation of the Akt residues, S473 and T308, SB431542, TGF-β RI Kinase Inhibitor VII, MK-2206 and PD98059 decreased the amounts of phosphorylated Akt T308 and Akt S473 compared to the levels in the HaCaT cells treated with TGFβ1 after 48 hours. Interestingly, the level of phospho-p44/42 increased significantly when cells were treated with TGFβ1 + MK-2206 compared to cells treated with TGFβ1 alone or the negative control. These findings indicate that the Smad-dependent pathway has a role in the

migration of HaCaT cells. However, there are two possibilities regarding PI3k-Akt and Erk/MAPK signalling pathways. First, both signalling pathways have no role in cell migration or one of the pathways acts as alternative route for the signal to be transduced when the other signalling pathway is blocked.

Stage one assessment for the TYS cell line showed that TGFβ1 caused an increase in p-Smad2 levels between an hour and 24 hours, p-Akt T308 and p-Akt S473 after 24 hours, phospho-p44/42 after 24 and 48 hours and p-Rac1/Cdc42 after 24 and 48 hours. However, lysates from TYS cells treated with EGF showed few differences compared to those treated with TGFβ1. Firstly, the level of p-Smad2 remained similar to the negative control. Secondly, Akt T308 and Akt S473 showed a high level of phosphorylation compared to the levels seen with TGFβ1. Finally, EGF induced the phosphorylation of p44/42 and Rac1/Cdc42 earlier than TGFβ1 in the TYS cell line.

Stage two assessment for the TYS cell line showed no phosphorylation of Smad2 at 48 hours, which supports the findings from stage one that Smad phosphorylation was an early event in TYS cells after treatment with TGFβ1. MK-2206 effectively blocked the phosphorylation of both Akt residues. Also, PD98059 kept the level of phospho-p44/42 low, similar to the levels observed with SF-MEM (negative control). From previous work related to cell migration assessment, it was reported that SB431542, TGF-β RI Kinase Inhibitor VII and MK-2206 prevented TYS cells from migrating, but PD98059 did not. Data from the assessment of activation of the signalling pathways showed an upregulation in terms of activity of the Smad-dependent pathway and PI3K-Akt pathway in response to TGFβ1. These findings indicate both signalling pathways have a role in migration of the TYS cell line. The migration of the TYS cells, induced by EGF, was not blocked by MK-2206 or PD98059, which indicates another signalling pathway might mediate the migration process.

With the SAS-H1 cell line, the stage one assessment of the Smad pathway showed no phosphorylation of Smad2 after cells were treated with TGFβ1. Although, phosphorylation of Smad2/3 was found at the optimisation stage, p-Smad2 was not detected at stage one (assessing the effect growth factors at different incubation periods) or stage two assessments (assessing the effect TGFβ1 with different inhibitors). The reason might be linked to using the p-Smad2/3 antibody at the optimisation stage or the phosphorylation of Smad2

occurred earlier than the incubation periods that were chosen i.e. 1 hour. For the activation of the other signalling pathways in this study, the SAS-H1 cells showed both similarities and differences to the TYS cell line. Both Akt T308 and Akt S473 phosphorylation were increased after cells were treated with TGFβ1 as well as EGF at the stage one assessment. This increase in phosphorylation happened mainly between two hours and six hours in the case of lysates of cells treated with TGFβ1 and from one hour to 24 hours for those treated with EGF. Similar levels of phospho-p44/42 were found in SAS-H1 to that observed with the TYS cells, where samples showed high levels of phosphorylated p44/42 after 24 hours and 48 hours treatment with TGFβ1. Also, EGF increased the level (amount) of phospho-p44/42 in the SAS-H1 cells between an hour and 24 hours.

MK-2206 inhibited the phosphorylation of both Akt residues in the SAS-H1 cells, however, it was observed that MK-2206 did not block cells from migrating in the migration assays assessment (Scatter assay, Gap closure assay and Scratch assay). Moreover, TGF-β RI Kinase Inhibitor VII and PD98059 prevented the SAS-H1 cells from migrating. PD98059, inhibitor of the MAPK cascade, managed to decrease the level of phospho-p44/42 close to the level in SF-MEM (negative control), which indicates the role of Erk/MAPK signalling pathway in the SAS-H1 migration. This agrees with studies that suggest that the role of the MAPK signalling pathway in OSCC metastasis is via EMT (Zhao *et al.*, 2019; Hsu *et al.*, 2022). A high level of p-Rac1/Cdc42 was found in the SAS-H1 cells that were treated with EGF for 48 hours, which suggests the possibility that GTPase signalling might mediate cell migration in the SAS-H1 cell line.

Chapter 6: Assessment of Migration Markers in Oral Cancer Cell Lines.

6.1 Background:

In this chapter, the expression or activation of proteins that were linked to the cell migration process either by up- or downregulation was investigated. This investigation added support to the findings from the cell migration assays by linking the changes in cell morphology and colony appearance with the expression of epithelial markers such as E-cadherin, β-Catenin and Claudin-1 and mesenchymal markers such as N-Cadherin in normal and oral cancer cell lines. Also, the assessment included proteins associated with the creation of cytoplasmic extensions such as WAVE-2 and Palladin.

E-cadherin, also known as CDH1, is a member of the cadherin superfamily that is involved in cell-cell adhesion. Well-known characteristics of the cadherins is that they are cell adhesion molecules and calcium-dependent. The different members of the cadherin family were originally named according to the tissue that they were prominently expressed in. The E in E-cadherin stand for epithelial, while N in N-cadherin for neural tissues and R for retinal tissues in R-cadherin. In epithelial tissues, E-cadherin is mainly associated with other proteins in the epithelial junctional complex called zonula adherens. These proteins help epithelial cells to keep their form and polarity, which leads to maintenance of transport and barrier functions (Gumbiner, 2005; van Roy, 2014). E-cadherin is also recognised to suppress both tumorigenicity and tumour dissemination (Gumbiner, 2005; Martin, 2014; van Roy, 2014) and is considered as a prognostic biomarker in OSCC (Bagutti, Speight and Watt, 1998; Bánkfalvi *et al.*, 2002; Rivera *et al.*, 2017).

As mentioned earlier, N-cadherin is also a member of the cadherin superfamily and the N stands for neuronal cadherin, which is known as CDH2. Although there are similarities between E-cadherin and N-cadherin, each one of them has several distinct functions. An example of a specific function is that N-cadherin induces the activity of fibroblast growth factor receptor 1, while E-cadherin does not. The majority of N-cadherin functions occur normally in non-epithelial tissues such as the brain and neural tissues. In carcinoma, N-cadherin is known as mesenchymal cadherin (van Roy, 2014). One of the processes that it is thought to facilitate in the progression of oral cancer metastasis is an epithelial to mesenchymal transition (EMT) (van Roy, 2014; Li *et al.*, 2017, p. 1). During EMT, the N-cadherin replaces E-cadherin, which is known as the cadherin switch,

which helps cancer cells in both local invasion and metastatic dissemination (Derycke and Bracke, 2004; van Roy, 2014). Using OSCC cell lines, it was found that TGFβ1 upregulates N-cadherin expression, which leads to EMT progression (Hirano *et al.*, 2020).

β-catenin is considered as a multifunctional molecule, which participates mainly in two distinct processes of cell-to-cell adhesion and signal transduction. In normal epithelial cells, β-catenin is expressed in the cell membrane, which has a role in regulating E-cadherin function regarding cell adhesion. β-catenin binds to E-cadherin to create a complex that helps to establish cell to cell contact (Zargaran, 2020). Any disturbance in the stability of this complex by dissociation between β-catenin and E-cadherin leads to the loss of cell adhesion and cells become migratory (Zargaran, 2020). These features were found in oral squamous cell carcinoma samples (Liu *et al.*, 2010). The main feature of the dissociation of β-catenin and E-cadherin is that the presence of the protein becomes less at the cell membrane and results in free β-catenin in the cytoplasm and the nucleus. In normal cells, free β-catenin is phosphorylated in the cytoplasm by binding to a degradation complex (Zargaran, 2020). Evidence suggests that the change in the expression of β-catenin is linked to differentiation and the acquisition of the ability to invade by epithelial cancer cells (Zargaran, 2020) including epithelial cells of oral cancer (Lo Muzio *et al.*, 1999). β-catenin could be considered as prognostic biomarker for OSCC (Ramos-García and González-Moles, 2022).

Claudin family members act as a backbone for tight junctions in cells by being involved in two main processes, first acting as barriers and the second is to control the permeability of the cells to molecules and ions. The family of claudins includes about 27 members, which play an essential role in cell permeability by mediating the paracellular permeability of the epithelial cells to small molecules. However, any modification at the post-translation phase of claudins has the potential to change the paracellular functions, which includes the transportation of substances through the intracellular space. In cancer, claudin family members have been linked to involvement in growth and progression of tumours by losing their role in cell adhesion, which leads to migration and invasion of cancer cells (Bhat *et al.*, 2020). Also, studies showed the significant role of claudins in EMT (Bhat *et al.*, 2020; Chang *et al.*, 2022). Moreover, the loss of claudins' role in tight

junctions leads to changes in cell polarity and defects in epithelial integrity. However, different reports have suggested two types of expression for claudin-1. It has been found that claudin-1 expression was decreased in breast and oesophageal cancers and upregulated in oral squamous cell and nasopharyngeal cancers (Bhat *et al.*, 2020).

The control of cell morphology and cell motility is maintained by actin dynamics in cells, which is mediated by different molecules including members of the Rho-GTPase family and Arp2/3 complex as they have been activated by extracellular signals. On activation, especially Arp2/3, promotes the growth of actin filaments, which create a protruding force (Bompard and Caron, 2004). This activation occurs through Wiskott-Aldrich syndrome proteins (WASPs) (Bompard and Caron, 2004; Algayadh, Dronamraju and Sylvester, 2016). There are five members of WASPs family found in mammals, which include WASP, N-WASP and three WASP family verprolin homologous (WAVE) proteins, also known as SCAR (Bompard and Caron, 2004). WAVE proteins have a major function in the Rac-induced actin polymerisation (Miki, Suetsugu and Takenawa, 1998). Studies report that WAVE-2 is involved in filopodia formation (Bompard and Caron, 2004; Kedrin *et al.*, 2007).

Palladin was discovered recently as an actin- associated protein, which has an important role in co-localising with actin-rich structures such as filopodia, lamellipodia and invadopodia/podosomes (Najm and El-Sibai, 2014). Palladin has a role in the assembly and maintenance of actin filaments in different types of cells (Goicoechea, Arneman and Otey, 2008; Najm and El-Sibai, 2014). One of the Palladin isoforms, 90 kDa, was expressed in vertebrates and has three Ig-domains. Myopalladin and myotilin are close relatives of Palladin. Also, Palladin has significant role in mediating cytoskeletal organisation by interacting with actin-binding proteins. Studies reported the expression of Palladin in different types of cancers including breast, pancreatic and colorectal cancers. In cancer cell lines, it was suggested that Palladin might have a role in invasive ability by enhancing podosome formation and localisation. Moreover, researchers have linked the role of Rho-GTPases in cytoskeletal remodelling to Palladin, which results in the formation of invadopodia and podosomes. Eps8 and Ezrin are Palladin-binding partners that are associated with the metastatic abilities of cancer cells (Najm and El-Sibai, 2014).

Gridin/GIV is a multidomain protein, which regulates the PI3k/Akt signalling pathway downstream. Studies suggested different abilities of GIV, which includes providing a link from G protein signalling to trafficking events at the golgi, participating in growth factor endocytosis and enhancing PI3K-Akt signalling for actin remodelling in migratory cells. The ability of GIV to enhance Akt signalling for cell migration is not limited to a specific signal, which includes different classes of receptors such as G protein-coupled receptors and growth factor receptors. This suggests Gridin acts as a large platform for different sources of stimuli from different receptors at the leading edge in the migrating cells (Ghosh, Garcia-Marcos and Farquhar, 2011). It was reported that Gridin was highly expressed in some cancer cells with high metastatic potential (Ghosh, Garcia-Marcos and Farquhar, 2011; Yamamura *et al.*, 2015).

The three major cytoskeletal proteins in the cell include microfilaments, intermediate filaments and microtubules. The intermediate filament has six different classes. Vimentin is a type III intermediate filament, which can be found in mesenchymal cells. In adults, vimentin expression becomes limited to the central nervous system, muscle, connective tissue and mesenchymal cells (Satelli and Li, 2011). Recently, studies have linked the expression of Vimentin to different types of cancers such as gastrointestinal tumours, breast cancer, prostate cancer (Satelli and Li, 2011) and oral cancer (Chaw *et al.*, 2012; Zhou *et al.*, 2015). This has been supported by finding vimentin expression in epithelial cells, which indicates cells have undergone EMT (Satelli and Li, 2011).

6.2 Aims and Hypothesis:

6.2.1 Aims:

The aim of this chapter was to analyse the expression of specific proteins that were linked with cell migration in normal and oral cancer cell lines.

6.2.2 Objectives:

(**a**) To assess the links between expression of migration markers and cell migration.

(**b**) To investigate the expression of mesenchymal markers in the epithelial cells and acquisition of any fibroblast-like properties i.e. EMT.

(**c**) To identify the expression and localisation of E-cadherin, N-cadherin, Claudin-1 and β-catenin, which would give an insight about the migratory status of the different cell lines in response to the test conditions.

6.2.3 Hypothesis:

TGFβ1 mediates oral cancer cell line migration via EMT because of the activation of signalling pathways such as Smad, PI3K-Akt and MAPK.

6.2.4 Null Hypothesis:

TGFβ1 does not mediate oral cancer cell line migration via EMT.

6.3 Materials and Methods:

Two types of methods were utilised to investigate the status of the migration markers in this experiment. SDS-PAGE and Western blotting gave insight about the expression of the migration markers in the whole samples, while Immunocytochemistry indicated the level of expression and localisation of those proteins within cells.

6.3.1 SDS-PAGE and Western Blotting:

Materials and Methods:

Details of the reagents and methodology for SDS-PAGE and Western blotting can be found In Chapter two, “General Materials and Methods” (page 44) (**Table 6**). Nevertheless, certain antibodies were utilised in this experiment to evaluate the expression of a variety of migration markers (**Table 71**). These antibodies are described below:

Table 71: Specific primary antibodies used in SDS-PAGE & Western Blot Protocol.

No.	Reagents/Antibodies	Type of marker	Company	Catalogue No.
1	E-Cadherin (24E10) Rabbit mAb	Epithelial Marker	Cell Signaling Technology Inc, USA	3195
2	N-Cadherin (D4R1H) Rabbit mAb	Mesenchymal Marker	Cell Signaling Technology Inc, USA	13116
3	β-Catenin (D10A8) Rabbit mAb	Epithelial Marker	Cell Signaling Technology Inc, USA	8480
4	Claudin-1 (D5H1D) Rabbit mAb	Epithelial Marker	Cell Signaling Technology Inc, USA	13255
5	WAVE-2 (D2C8) Rabbit mAb	Migration marker	Cell Signaling Technology Inc, USA	3659
6	Palladin (D9H2) Rabbit mAb	Migration marker	Cell Signaling Technology Inc, USA	8518
7	Gridin antibody	Migration marker	Cell Signaling Technology Inc, USA	14200
8	Vimentin (R28) Antibody	Mesenchymal Marker	Cell Signaling Technology Inc, USA	3932

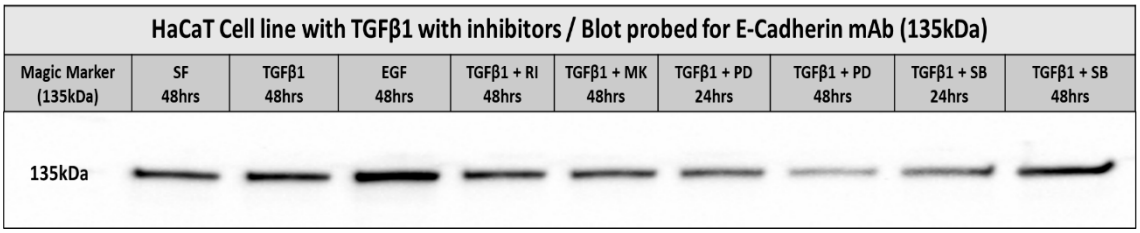


Figure 121: An example of immunoblot design for Phase I assessment of migration markers.

The HaCaT cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a E-cadherin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 323).

6.3.2 Immunocytochemistry (ICC):

This technique was used to investigate the expression and localisation of specific migration markers within cells. For the level of the protein expression, this assay was used as the second phase to confirm the results from phase I (SDS-PAGE & Western Blot), plus it gave insight about the location of certain markers within the cells (e.g. cell membrane, cytoplasm or nucleus). Also, ICC provided information about the migration status of the cells by observation of cell morphology, cell adhesion and colony appearance. Specific migration markers were chosen for this technique and this selection was based on the results from Phase I. The same set of test conditions used in phase I were used here.

Equipment:

Table 72: Specific equipment were used in Immunocytochemistry protocol.

No.	Name of the equipment	Make
1	Light and Fluorescence Microscope, IX70	Olympus, Tokyo, Japan
2	Digital Camera XM10	Olympus, Tokyo, Japan

Materials:

Table 73: Specific reagents were used in Immunocytochemistry protocol.

No.	Reagents	Company	Catalogue No.
1	Methanol	VWR BDH, PA, USA	101586B
2	PBS	Sigma-Aldrich, St. Louis, MO, USA	P-4417
3	Tween 20	Sigma-Aldrich, St. Louis, MO, USA	P1379

4	Triton X100	Sigma-Aldrich, St. Louis, MO, USA	T8787
5	Normal goat serum	Vactor laboratories, Burlingame,CA, USA	S1000
6	ImmEdge Pen	Vactor laboratories, Burlingame,CA, USA	H-4000
7	E-Cadherin (24E10) Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	3195
8	N-Cadherin (D4R1H) Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	13116
9	Claudin-1 (D5H1D) Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	13255
10	β-Catenin (D10A8) Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	8480
11	Anti-Rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate)	Cell Signaling Technology Inc., Danvers, MA, USA	4412
12	DAPI	Cell Signaling Technology Inc., Danvers, MA, USA	4083
13	Aqueous mounting media	Sigma-Aldrich, St. Louis, MO, USA	M-1289

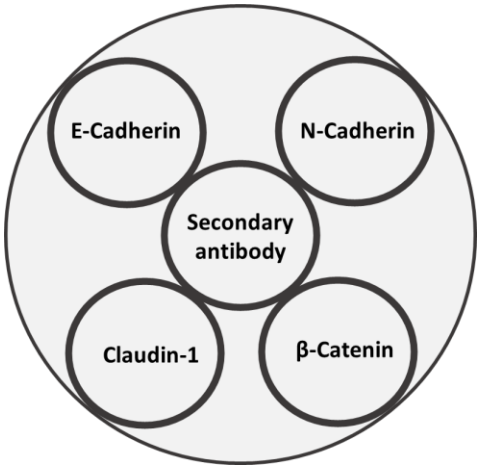


Figure 122: Design of 60 mm culture used during Immunocytochemistry protocol.

This design was used to evaluate the expression and localisation of the four proteins.

Immunocytochemistry Protocol:

This protocol started with preparing 60 mm culture dishes by labelling them with rings for each of the targeted proteins for the staining phase. Similar to the Scatter assay protocol (page 65), cells were seeded at 5 x 10^4 cells/dish and incubated overnight at 37°C and 5 % CO_2. Next, cells were serum starved for 24 hours in SF-MEM, before introducing the test conditions. Samples were fixed at specific

incubation periods to be stained and assessed. For cell fixation, culture dishes were washed twice with cold PBS to remove the medium, before adding cold methanol for 20 minutes. Samples were washed three times for five minutes each with PBS, then treated with 0.2% (v/v) Triton X-100 in PBS for five minutes. This step was also followed by three washes with PBS for five minutes each. Samples were treated with 5% (v/v) normal goat serum (NGS) in PBS with 0.1% (v/v) Tween (PBS-T), which was used as blocking agent, for an hour at room temperature. The primary antibodies for the four targeted proteins were prepared and diluted in 5% (v/v) NGS-PBST at the manufacturer's recommended dilutions. Samples were incubated with the primary antibodies overnight at 4°C in a humidified chamber. Samples were equilibrated at room temperature for an hour before washing them with PBS three times, for five minutes each wash. The dishes were then incubated with the secondary antibody conjugated with Alexa Fluor 488, which was diluted in 5% (v/v) NGS-PBST as per the manufacturer's recommendation, for an hour in a humidified chamber. Samples were then washed three times with PBS, before incubating them with 1µg/ml of DAPI in PBS for five minutes. Dishes were then rinsed with PBS and covered by slips using aqueous mounting medium (**Figure 123**).

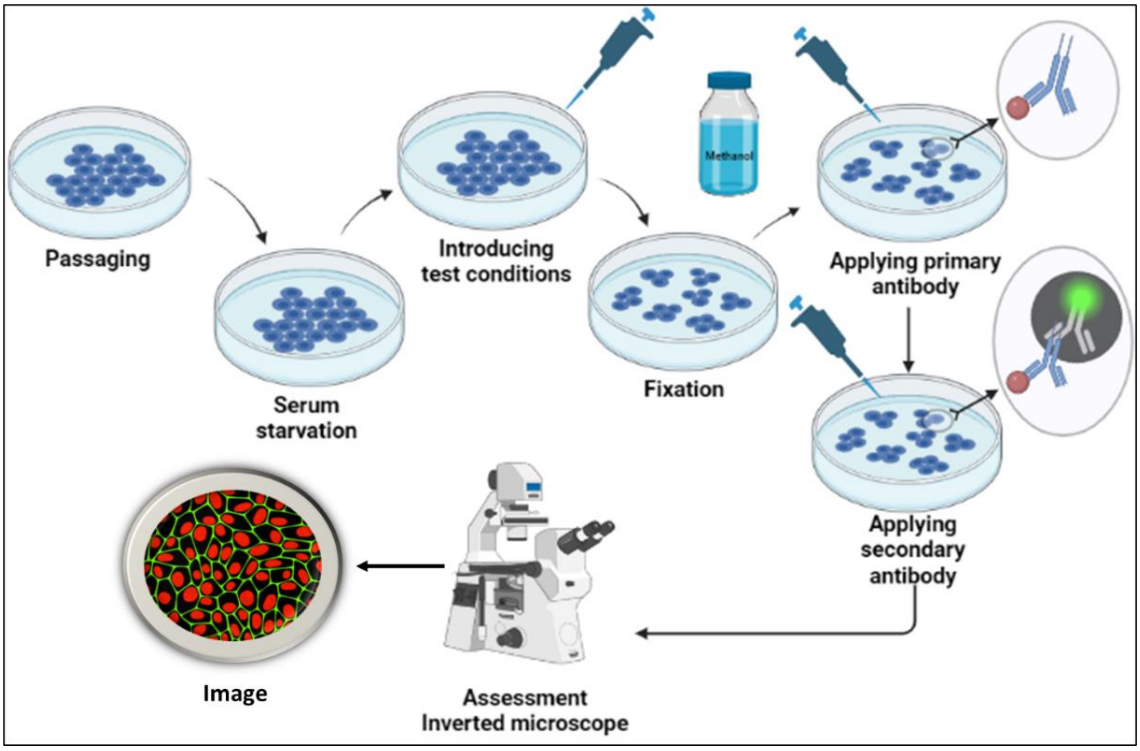


Figure 123: Illustration of different steps of cell culture, cell fixation and Immunocytochemistry protocol.

Figure was created in Biorender.com.

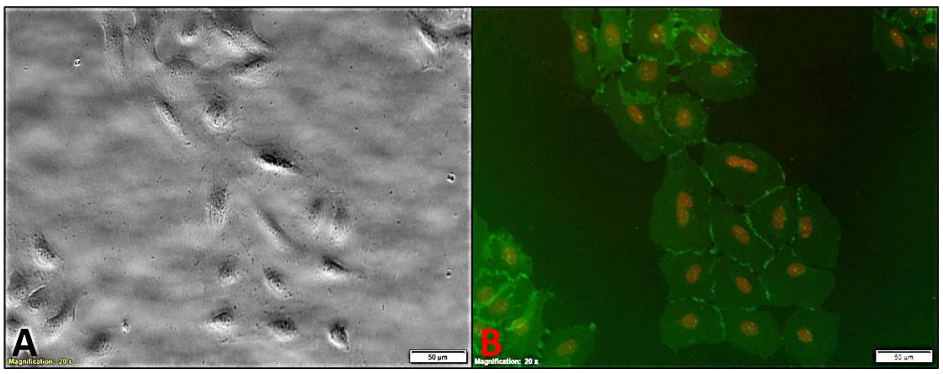


Figure 124: Example of phase II assessment for migration markers by Immunocytochemistry assay.

Images of HaCaT cells after treatment with the test conditions. These samples were then prepared before staining treatment with Triton. The samples were then probed with a E-cadherin monoclonal antibody at 1:200 dilution, followed by secondary anti rabbit IgG (labelled with fluorescent dye) then DAPI. Picture **(A)** is a bright field image which was taken at 200X magnification. Picture **(B)** was made by merging two different images of the same field at the same magnification 200X. The first image shows the expression of E-cadherin with fluorescence (**green colour**), while the second image shows the DAPI stain (**red colour**) of the nucleus. Detailed methodology can be found in chapter two.

Assessment Format for the Migration Markers:

Two methods of assessing the migration markers were used in this experiment. SDS-PADE and Western blotting was utilised as a phase I assessment to evaluate the expression or change in the expression of eight different migration markers, compared to the control samples. The phase II assessment included an immunocytochemistry protocol to evaluate expression of the markers and their localisation within normal and oral cancer cell lines. Eight migration markers were investigated in the phase I assessment (**Table 71**), while only four of them were used in phase II (**Table 73**).

1) SDS-PAGE and Western Blot:

To investigate the expression of the proteins associated with cell migration, SDS-PADE and Western blotting were used in phase I of the evaluation. A specific format of blot design was used to assess the expression of eight different proteins. The markers were E-cadherin, N-cadherin, β-catenin, Claudin-1, WAVE-2, Palladin, Gridin and vimentin. The expression of these markers was investigated by preparing lysates of the cells after treatment with SF-MEM after 48 hours as the control group, TGFβ1 after 48 hours, EGF after 48 hours, TGFβ1 + TGF-β RI Kinase Inhibitor VII after 48 hours, TGFβ1 + MK-2206 after 48 hours, TGFβ1 + PD98059 after 24 hours and 48 hours and TGFβ1 + SB431542 after 24

hours and 48 hours. Image lab software (BioRad) was used to analyse the expression of each protein in response to these test conditions.

2) Immunocytochemistry (ICC) Assay:

ICC was used to investigate the expression and the localisation of specific migration markers within the cells. For protein expression, this assay was used as a second phase to confirm the results from phase I assessment (SDS-PAGE and Western blotting) in addition to giving an insight about the location of certain markers within the cells (e.g. cell membrane, cytoplasm or nucleus). Also, ICC provided information about the migration status of the cells by observing cell morphology, cell adhesion and colony appearance. Specific migration markers were chosen for this technique and the selection was based on the results from the phase I assessment. The test conditions used were the same as those in phase I, with the addition of each test condition being investigated after 24 hours and 48 hours. Assessment was carried out by capturing images of the samples with the fluorescence microscope IX70 and digital camera XM10. The analysis was based on observation of the expression and localisation of the proteins. This was supported by using Image J software to calculate the correct total cell fluorescence protocol (CTCF) values (El-Sharkawey, 2016). CTCF was utilised to quantify the expression of these proteins in an average of five to ten cells, in the same photomicrograph.

6.4 Results of Expression of Migration Markers in Oral Cancer-derived Cell lines:

6.4.1 HaCaT Cell Line:

(A) Phase I Assessment of the Migration Markers:

Observation of colony appearance, cell morphology and cell migration were carried out before cell lysis. This observation was established by taking photomicrographs at 200X magnification with each of the different test conditions after 24 hours and 48 hours.

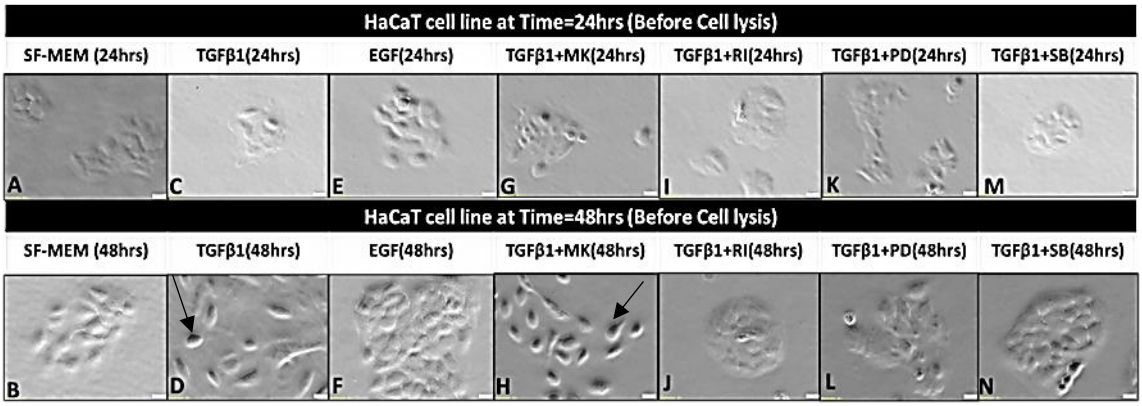


Figure 125: HaCaT cell line morphological assessment.

Images for the HaCaT cell line incubated with SF-MEM at 48 hours, TGFβ1 at 48hours, EGF at 48 hours, TGFβ1+ TGF-β RI Kinase Inhibitor VII at 48 hours, TGFβ1+MK2206 at 48 hours, TGFβ1+PD98059 at 24 hours and 48 hours, and TGFβ1+SB431542 at 24 hours and 48 hours before cell lysis. All images were taken at 200x magnification to observe both cell morphology and colony appearance. (↑) scattered cells.

HaCaT cells treated with TGFβ1 after 48 hours (**Figure 125-D**), TGFβ1 + MK-2206 after 48 hours (**Figure 125-H**) and TGFβ1 + PD98059 after 48 hours (**Figure 125-L**) showed changes in both cell morphology and colony appearance. In these growth conditions, cells appeared to be large, round and not in compact colonies with evidence of a few scattered cells compared to the negative control (SF-MEM) culture dishes after 48 hours.

1) E-cadherin:

The expression of E-cadherin was assessed in the HaCaT cell line after treatment with the different test conditions (**Figure 126**). The analysis of blots using image lab software (BioRad) and SF-MEM lane as the control sample, showed different levels of E-cadherin expression. For example, the expression of the protein increased in lysates from cells treated with

EGF for 48 hours and TGFβ1 + TGF-β RI Kinase Inhibitor VII for 48 hours compared to SF-MEM after 48 hours. In contrast, E-cadherin expression was less than the negative control in lysates of cells treated with TGFβ1 + PD98059 after 24 hours and 48 hours, and TGFβ1 + SB431542 after 24 hours (**Figure 127**).

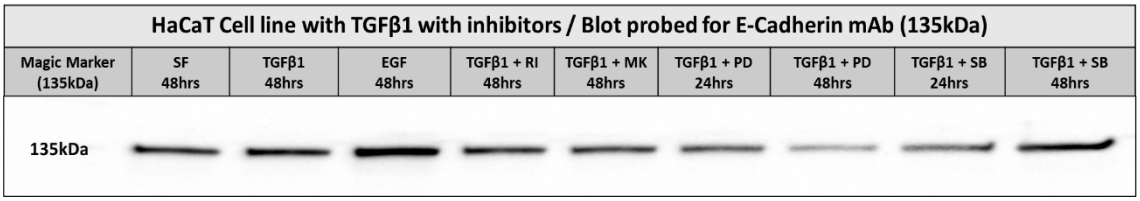


Figure 126: E-cadherin expression in the HaCaT cell line using WB.

The HaCaT cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a E-cadherin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from HaCaT cells treated with the different test conditions showed E-cadherin expression. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 323).

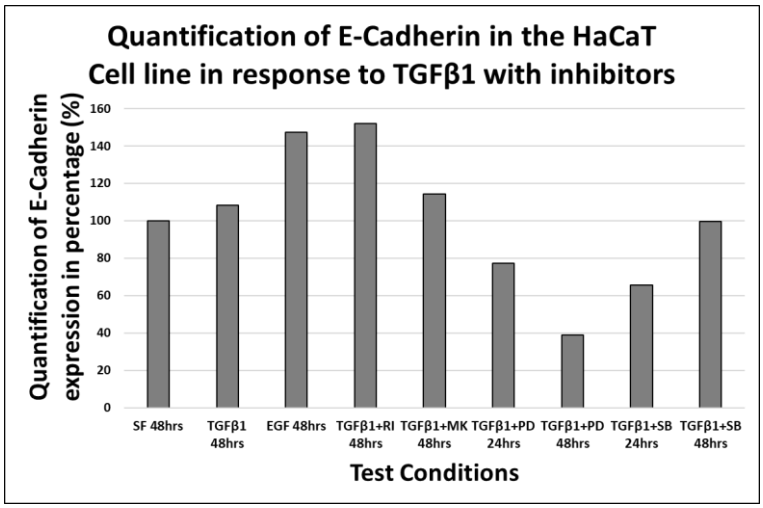


Figure 127: Quantification of E-Cadherin expression in the HaCaT cell line.

The bar chart shows the quantification of E-cadherin expression in the HaCaT cell line for phase I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from HaCaT cells treated with EGF (10ng/ml) after 48 hours and TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) after 48 hours showed an increase in E-cadherin expression compared to negative control. Also, lysates from cells treated with TGFβ1 (10ng/ml)+PD98059 (5μM) after 24 and 48 hours and TGFβ1 (10ng/ml)+SB431542 (5μM) after 24 hours showed less expression of E-cadherin compared to the baseline.

Table 74: Quantification of E-Cadherin expression in the HaCaT cell line with phase I assessment.

HaCaT Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	77.4%
10ng/ml TGFβ1 48 hours	108.2%	**TGFβ1+PD98059 48 hours**	39%
10ng/ml EGF 48 hrs	147.4%	**TGFβ1+SB431542 24 hours**	65.7%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	152%	**TGFβ1+SB431542 48 hours**	99.7%
TGFβ1+MK2206 48 hours	114.3%		

2) N-cadherin:

Using SF-MEM at 48 hours as a negative control, N-cadherin expression increased in lysates from cells treated with TGFβ1 after 48 hours, TGFβ1 + MK-2206 after 48 hours and TGFβ1 + PD98059 after 24 hours and after 48 hours compared. Lysates from EGF-treated cells after 48 hours showed N-cadherin expression similar to SF-MEM at 48 hours. However, those treated with TGFβ1 + TGF-β RI Kinase Inhibitor VII samples after 48 hours and TGFβ1 + SB431542 after 24 hours and after 48 hours showed less N-cadherin expression compared to the negative control (**Figure 128**). This suggests the expression of N-cadherin increased due to the presence of TGFβ1, however, it was blocked by TGF-β RI Kinase Inhibitor VII and SB431542. The expression of N-cadherin in the HaCaT cell line is consistent with the findings from the observation of the cells before cell lysis, where changes in cell morphology and colony appearance have occurred (**Figure 129**).

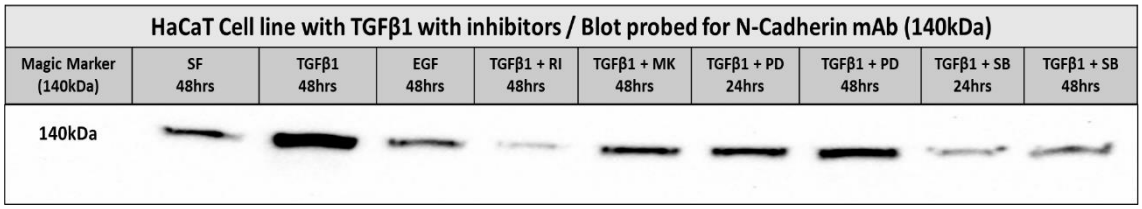


Figure 128: N-cadherin expression in the HaCaT cell line using WB.

The HaCaT cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a N-cadherin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Lysates from HaCaT cells treated with TGFβ1 (10ng/ml) after 48 hours, TGFβ1 (10ng/ml)+MK2206 (1μM) after 48 hours and TGFβ1 (10ng/ml)+PD98059 (5μM) after 24 and 48 hours showed an increase in N-cadherin expression to the negative control. Also, lysates from cells treated with TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) after 48 hours and TGFβ1 (10ng/ml)+SB431542 (5μM) after 24 and 48 hours showed less N-cadherin expression compared to the negative control. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 324).

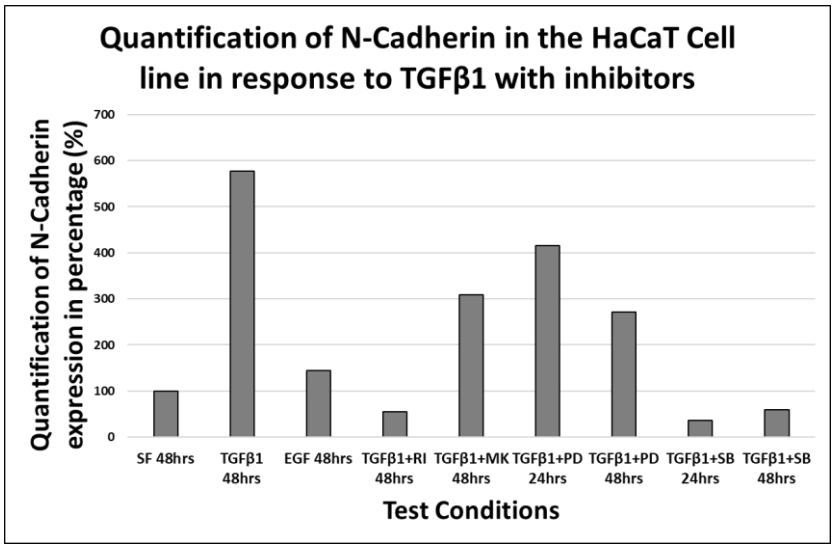


Figure 129: Quantification of N-Cadherin expression in the HaCaT cell line.

The bar chart shows the quantification of N-cadherin expression in the HaCaT cell line for phase I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from HaCaT cells treated with TGFβ1 (10ng/ml) after 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours and TGFβ1 (10ng/ml)+PD98059 (5μM) after 24 and 48 hours showed an increase in N-cadherin expression compared to the negative control, while lysates from cells treated with TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) after 48 hours and TGFβ1 (10ng/ml)+SB431542 (5μM) after 24 and 48 hours showed less N-cadherin expression compared to the negative control.

Table 75: Quantification of N-Cadherin expression in the HaCaT cell line with phase I assessment.

HaCaT Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	415%
10ng/ml TGFβ1 48 hours	576.4%	**TGFβ1+PD98059 48 hours**	271%
10ng/ml EGF 48 hrs	143.8%	**TGFβ1+SB431542 24 hours**	36.1%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	54%	**TGFβ1+SB431542 48 hours**	59.8%
TGFβ1+MK-2206 48 hours	308.4%		

3) β-catenin:

Overall, the expression of β-catenin in increased in all lysates from HaCaT cells that had been treated with the test conditions compared to SF-MEM at 48 hours (**Figure 130**). There were different levels of β-catenin expression among these test conditions. For instance, lysates from EGF-treated cells after 48 hours and TGFβ1 + PD98059 after 48 hours showed the highest expression of the protein compared to negative control and rest of the test conditions (**Figure 131**).

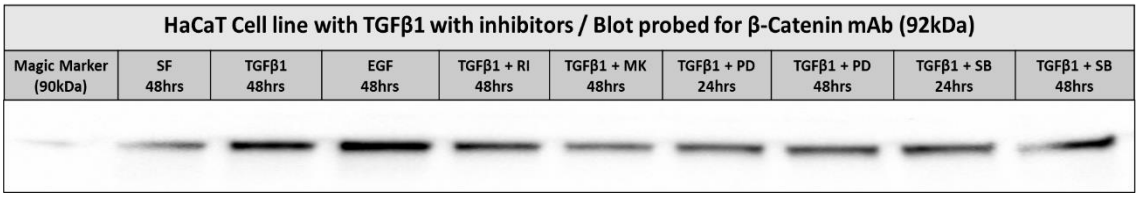


Figure 130: β-catenin expression in the HaCaT cell line using WB.

The HaCaT cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a β-catenin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from HaCaT cells treated with the different test conditions showed β-catenin expression. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 324).

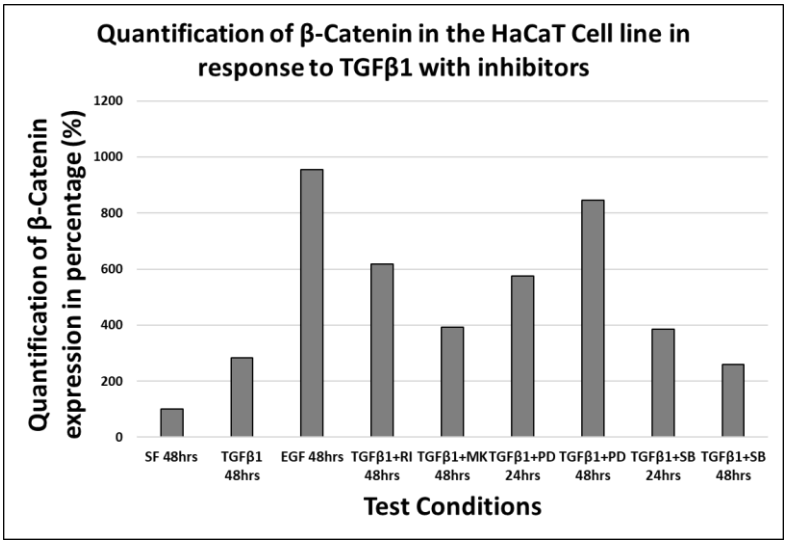


Figure 131: Quantification of β-Catenin expression in the HaCaT cell line.

The bar chart shows the quantification of β-catenin expression in the HaCaT cell line for phase I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. All lysates from HaCaT cells treated with the different test conditions showed an increase in β-catenin expression compared to the negative control, especially lysates from cells treated with EGF (10ng/ml) after 48 hours and TGFβ1 (10ng/ml)+PD98059 (5μM) after 48 hours compared to the baseline.

Table 76: Quantification of β-catenin expression in the HaCaT cell line with phase I assessment.

HaCaT Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	574.9%
10ng/ml TGFβ1 48 hours	284.1%	**TGFβ1+PD98059 48 hours**	845.2%
10ng/ml EGF 48 hrs	955.6%	**TGFβ1+SB431542 24 hours**	385.6%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	617.4%	**TGFβ1+SB431542 48 hours**	258.8%
TGFβ1+MK-2206 48 hours	391.6%		

4) Claudin-1:

Claudin-1 expression was found to be increased in lysates from HaCaT cells treated with TGFβ1 +SB431542 after 48 hours compared to SF-MEM at 48 hours and the other test conditions (**Figure 132**). Image lab software (BioRad) was used to quantify the protein expression and showed an increase in the level of Claudin-1 expression by more than tenfold, compared to the negative control (**Figure 133**).

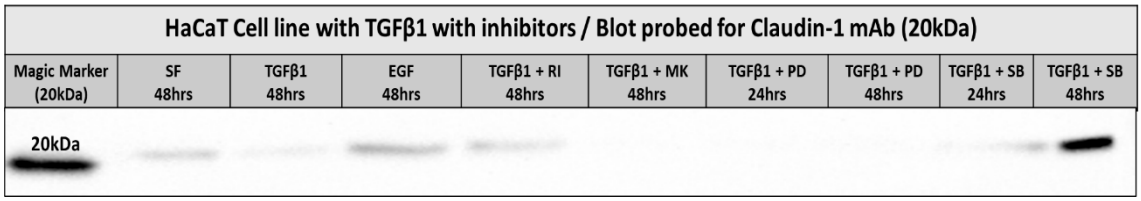


Figure 132: Claudin-1 expression in the HaCaT cell line using WB.

The HaCaT cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a Claudin-1 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Lysates from HaCaT cells treated with TGFβ1 (10ng/ml)+SB431542 (5μM) after 48 hours showed an increase in Claudin-1 expression compared to the negative control. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 324).

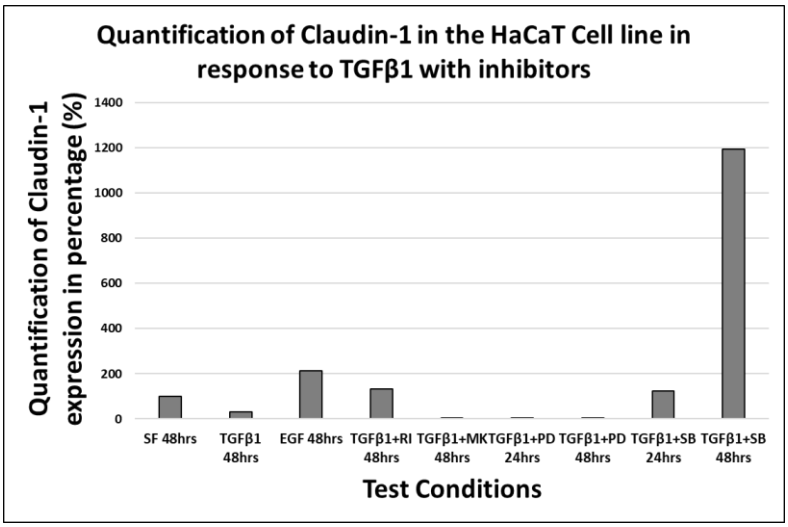


Figure 133: Quantification of Claudin-1 expression in the HaCaT cell line.

The bar chart shows the quantification of β-catenin expression in the HaCaT cell line for phase I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from HaCaT cells treated with TGFβ1 (10ng/ml)+SB431542 (5μM) after 48 hours showed an increase in Claudin-1 expression compared to the negative control.

Table 77: Quantification of Claudin-1 expression in the HaCaT cell line with phase I assessment.

HaCaT Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	3.9%
10ng/ml TGFβ1 48 hours	32.3%	**TGFβ1+PD98059 48 hours**	4.4%
10ng/ml EGF 48 hrs	213.5%	**TGFβ1+SB431542 24 hours**	123.1%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	133.4%	**TGFβ1+SB431542 48 hours**	1139.9%
TGFβ1+MK-2206 48 hours	2.4%		

5) WAVE-2:

The assessment of WAVE-2 expression in HaCaT cells using image lab software (BioRad) showed an increase compared to the negative control (**Figure 135**). WAVE-2 expression was varied in HaCaT cell line in response to different test conditions. For example, lysates from cells treated with TGFβ1 + MK-2206 (Akt inhibitor) showed the highest level of WAVE-2 expression compared to the other test conditions. In contrast, the lowest level of WAVE-2 expression, apart from SF-MEM at 48 hours, was observed in lysates from cells treated with TGFβ1 + SB431542 (Smad inhibitor) after 48 hours (**Figure 134**).

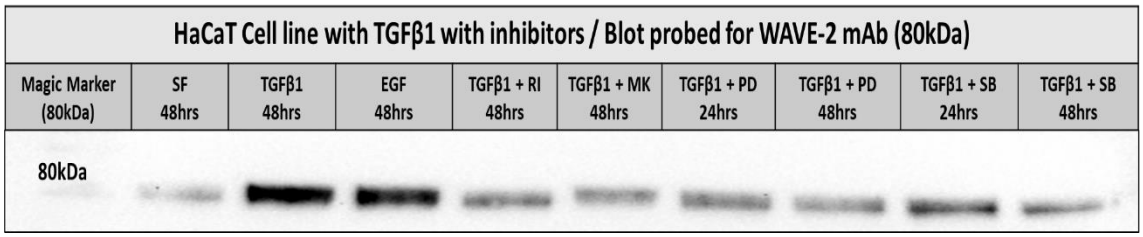


Figure 134: WAVE-2 expression in the HaCaT cell line using WB.

The HaCaT cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a WAVE-2 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from HaCaT cells treated with the different test conditions showed WAVE-2 expression. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 325).

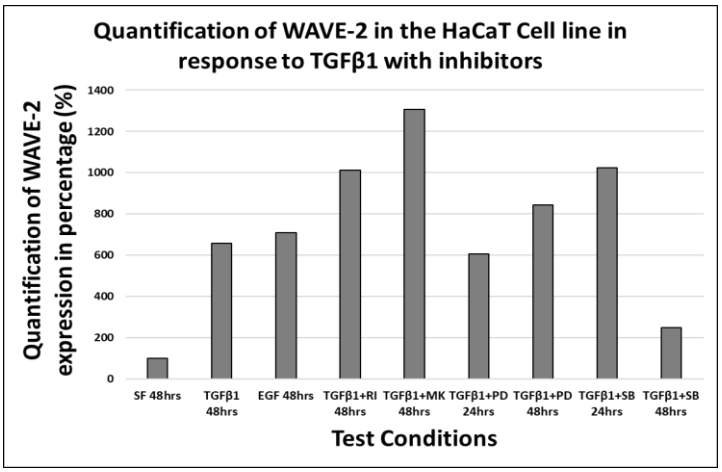


Figure 135: Quantification of WAVE-2 expression in the HaCaT cell line.

The bar chart shows the quantification of WAVE-2 expression in the HaCaT cell line for phase I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from HaCaT cells treated with TGFβ1 (10ng/ml)+MK2206 (1μM) after 48 hours showed the highest level of WAVE-2 expression compared to the negative control and the other test conditions.

Table 78: Quantification of WAVE-2 expression in the HaCaT cell line with phase I format.

HaCaT Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	605.9%
10ng/ml TGFβ1 48 hours	656.9%	**TGFβ1+PD98059 48 hours**	844.8%
10ng/ml EGF 48 hrs	709.1%	**TGFβ1+SB431542 24 hours**	1022.4%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	1013.4%	**TGFβ1+SB431542 48 hours**	248.4%
TGFβ1+MK-2206 48 hours	1307.2%		

6) Palladin:

The expression of Palladin was not detected in lysates from the HaCaT cell line treated with the different test conditions (**Figure 136**). This suggests HaCaT cells did not depend on this protein to change morphology or to migrate, especially in response TGFβ1.

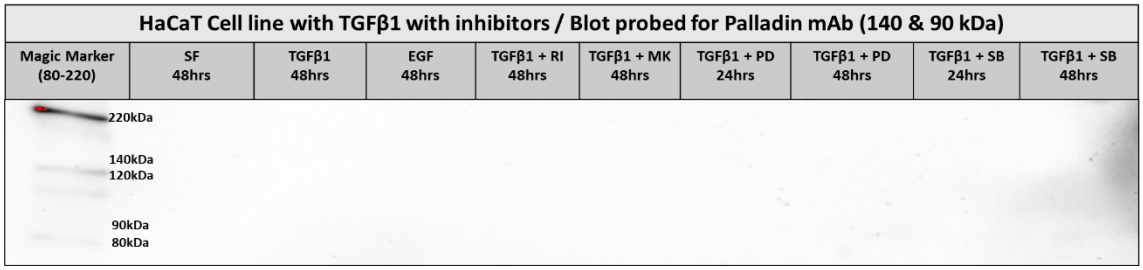


Figure 136: Palladin expression in the HaCaT cell line using WB.

The HaCaT cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a Palladin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from HaCaT cells treated with the different test conditions showed no expression of Palladin. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 325).

7) Gridin:

Similar to Palladin, there was no Gridin expression in lysates from the HaCaT cell line treated with the different test conditions (**Figure 137**). Therefore, the migratory cells, that were seen at the observational phase of the assessment, were not dependent on this protein.

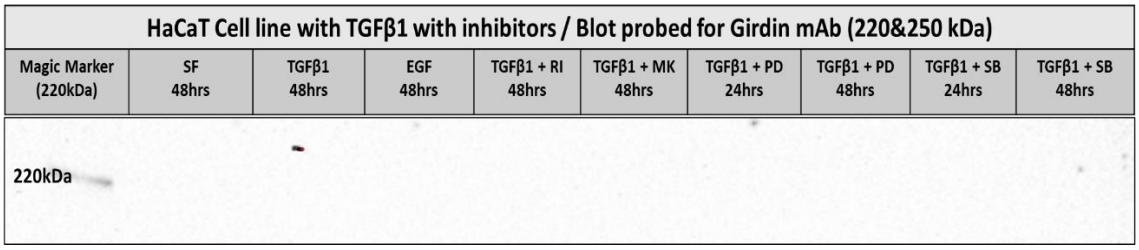


Figure 137: Gridin expression in the HaCaT cell line using WB.

The HaCaT cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a Gridin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from HaCaT cells treated with the different test conditions showed no expression of Gridin. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 325).

8) Vimentin:

Vimentin expression was not detected in lysates from the HaCaT cell line treated with the different test conditions including those, where migratory cells were observed (**Figure 138**). This indicates the role of TGFβ1 in HaCaT cell migration was not dependent on utilising vimentin.

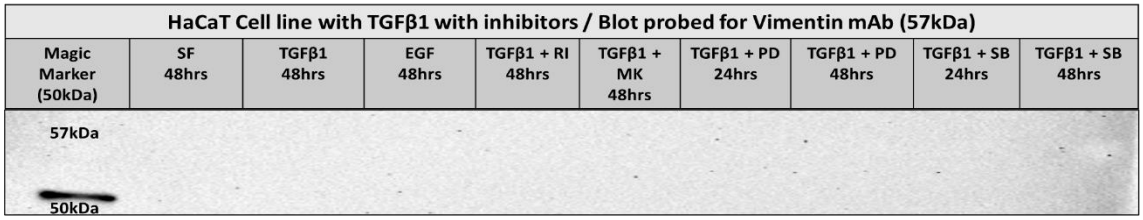


Figure 138: Vimentin expression in the HaCaT cells using WB.

The HaCaT cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a Vimentin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from HaCaT cells treated with the different test conditions showed no expression of vimentin. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 326).

(B) Phase II Assessment of the Migration Markers:

At this phase of assessment using ICC, four of the migration markers were evaluated based on their expression and location within the cell, plus establishing a link between the status of cells regarding migration and the markers. Those migration markers were E-cadherin, N-cadherin, β-catenin and Claudin-1. In addition, the protocol to calculate the correct total cell fluorescence (CTCF) was used to quantify the level of expression of each of the migration markers in cells treated with the different test conditions at 24 hours and 48 hours. The test conditions for this phase of assessment are described below:

Table 79: Design of phase II assessment for the HaCaT cell line.

Phase II assessment for migration marker in HaCaT cell line using ICC			
SF-MEM 24hrs	TGFβ1 24hrs	EGF 24hrs	TGFβ1 + TGF-β RI Kinase Inhibitor VII 24hrs
	TGFβ1 48hrs	EGF 48hrs	TGFβ1 + TGF-β RI Kinase Inhibitor VII 48hrs

SF-MEM 48hrs	TGFβ1 + MK-2206 24hrs	TGFβ1 + PD98059 24hrs	TGFβ1 + SB431542 24hrs
	TGFβ1 + MK-2206 48hrs	TGFβ1 + PD98059 48hrs	TGFβ1 + SB431542 48hrs

1) E-cadherin:

In cells treated with SF-MEM, the expression of E-cadherin was prominent especially at the cell membrane after 24 hours and 48 hours. Some variations in E-cadherin expression were observed in samples treated with TGFβ1 after 24 hours (**Figure 139-C**) and 48 hours (**Figure 139-D**); TGFβ1 + MK-2206 after 24 hours (**Figure 139-I**) and 48 hours (**Figure 139-L**); and TGFβ1 + PD98059 after 24 hours (**Figure 139-K**) and 48 hours (**Figure 139-L**). In the TGFβ1 treated cells, expression of E-cadherin was less compared to SF-MEM, in particular after 48 hours. In addition to the morphological change and alteration in colony appearance of the HaCaT cells, the expression of E-cadherin at the cell membrane became less than in cells grown in SF-MEM and cells treated with TGFβ1 after 24 hours. Changes in the E-cadherin expression, cell morphology, colony appearance and scattered cells were observed in cells treated with TGFβ1 + MK-2206 after 24 hours and 48 hours and TGFβ1 + PD98059 after 24 hours and 48 hours. The quantification of E-cadherin expression in the HaCaT cells treated with TGFβ1 + PD98059 for 48 hours, showed an increase in the level of the protein (**Figure 139-L**), accompanied by a change in cell morphology and colony appearance. In terms of E-cadherin expression, the rest of the samples with the other test conditions were similar to the control cells.

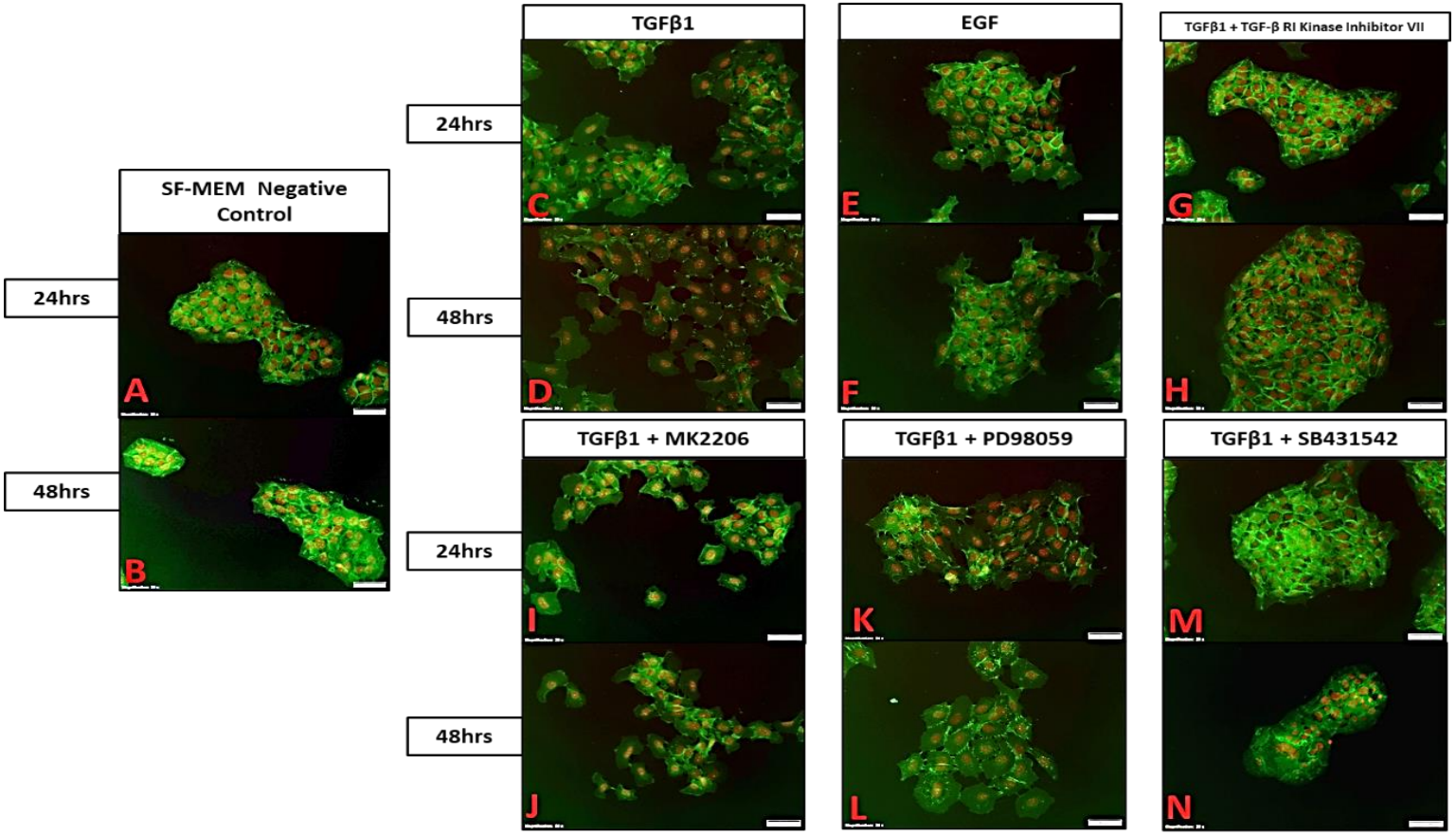


Figure 139: E-cadherin expression in the HaCaT cells using ICC.

Images of HaCaT cells that had been treated with seven test conditions for 24 hours and 48 hours including SF-MEM, TGFβ1 (10ng/ml), EGF (10ng/ml), TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM), TGFβ1 (10ng/ml)+MK-2206 (1μM), TGFβ1 (10ng/ml)+PD98059 (5μM), and TGFβ1 (10ng/ml)+SB431542 (5μM). The cells were fixed in situ and then prepared for the immunocytochemistry protocol The samples were then probed with an E-cadherin monoclonal antibody at 1:200 dilution, followed by secondary anti rabbit IgG (with fluorescent dye **green colour**) then DAPI (**red colour**). Immunofluorescence Images were taken at 200X magnification. 60mm culture dishes treated with TGFβ1 (10ng/ml) for 48 hours (**D**), TGFβ1 (10ng/ml)+MK-2206 (1μM) for 48 hours (**J**) and TGFβ1 (10ng/ml)+PD98059 (5μM) for 48 hours (**L**) showed localisation of E-cadherin in the cytoplasm compared to the negative control.

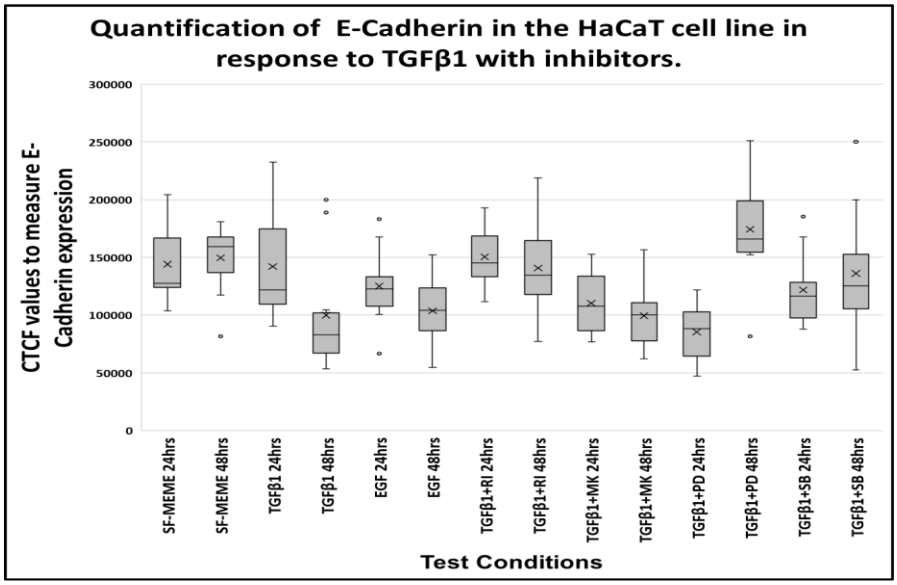


Figure 140: Quantification of E-cadherin expression in the HaCaT cell line by CTCF.

The graph shows the quantification of E-cadherin expression using ICC in the HaCaT cell line for the phase II assessment. The assessment of protein expression was carried out using ImageJ software and CTCF protocol (n=10). HaCaT cells treated with TGFβ1 (10ng/ml) after 48 hours showed a lower level of E-cadherin expression compared to the negative control. Raw data can be found in appendix 7 (page 333).

2) N-cadherin:

The assessment of N-cadherin expression and localisation within the HaCaT cell line was carried out using the ICC protocol. Cells incubated with SF-MEM medium showed no expression of the protein after 24 hours (**Figure 141-A**) and 48 hours (**Figure 141-B**). In contrast, the HaCaT cells treated with TGFβ1 for 24 and 48 hours (**Figure 141-C&D**), TGFβ1 + MK-2206 for 24 and 48 hours (**Figure 141-I&J**) and TGFβ1 + PD98059 for 24 and 48 hours (**Figure 141-K&L**) expressed N-cadherin. In addition, cells grown in these test conditions showed N-cadherin expression at the cell membrane, accompanied by changes in cell morphology and colony appearance. The difference in terms of cell morphology between these and cells treated with TGFβ1 for 48 hours and TGFβ1 + MK-2206 for 48 hours was that the latter contained long and spindle shaped cells with some completely separated from their colonies after 24 hours and 48 hours. Cells treated with TGFβ1 and TGFβ1 with PD98059 showed large round cells with less individual cells. The rest of the test conditions samples showed no N-cadherin expression by the cells (**Figure 142**).

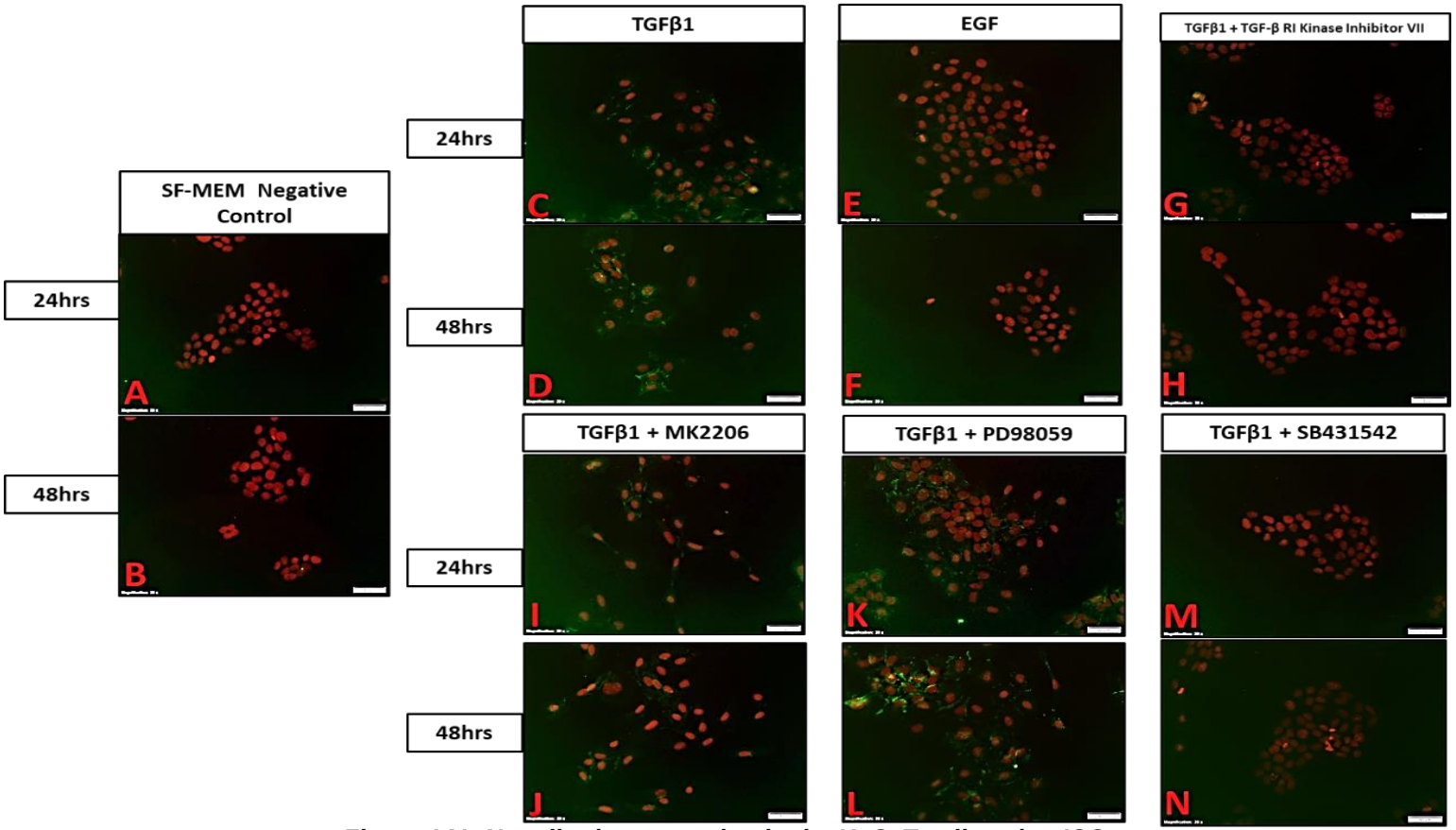


Figure 141: N-cadherin expression in the HaCaT cells using ICC.

Images of the HaCaT cells that had been treated with seven test conditions for 24 hours and 48 hours including SF-MEM, TGFβ1 (10ng/ml), EGF (10ng/ml), TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM), TGFβ1 (10ng/ml)+MK-2206 (1μM), TGFβ1 (10ng/ml)+PD98059 (5μM), and TGFβ1 (10ng/ml)+SB431542 (5μM). The cells were fixed in situ and then prepared for the immunocytochemistry protocol The samples were then probed with an N-cadherin monoclonal antibody at 1:200 dilution, followed by secondary anti rabbit IgG (with fluorescent dye **green colour**) then DAPI (**red colour**). Immunofluorescence Images were taken at 200X magnification. 60mm culture dishes treated with TGFβ1 (10ng/ml) for 24 hours and 48 hours (**C**, **D**), TGFβ1 (10ng/ml)+MK-2206 (1μM) for 24 hours and 48 hours (**I**, **J**) and TGFβ1 (10ng/ml)+PD98059 (5μM) for 24 hours and 48 hours (**K**, **L**) showed expression of N-cadherin on cell membrane compared to the negative control.

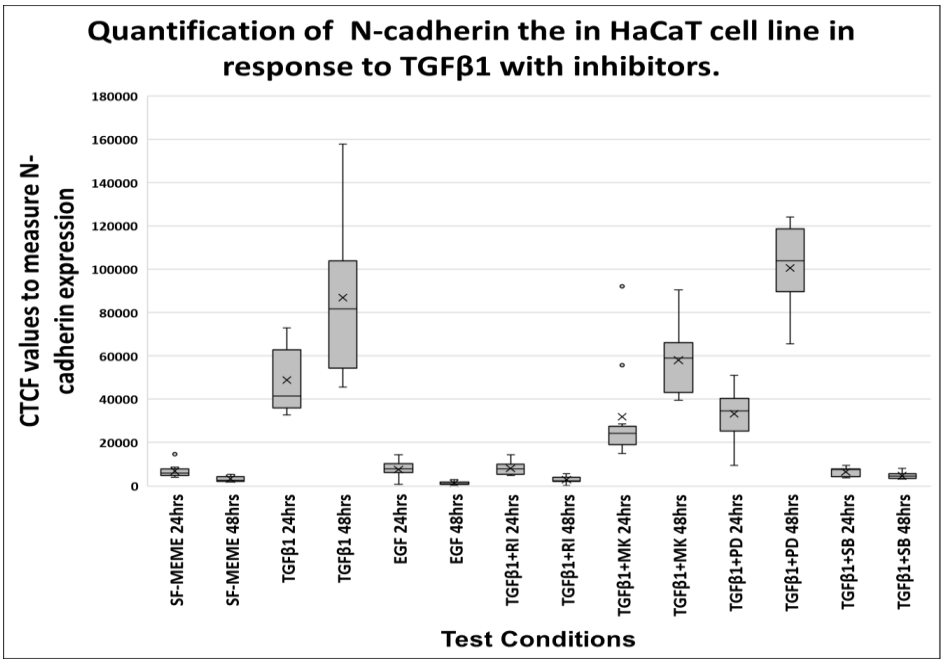


Figure 142: Quantification of N-cadherin expression in the HaCaT cell line by CTCF.

The graph shows the quantification of N-cadherin expression using ICC in the HaCaT cell line for phase II assessment. The assessment of the protein expression was carried out using ImageJ software and CTCF protocol (n=10). HaCaT cells treated with TGFβ1 (10ng/ml) after 24 hours and 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) and 24 hours and 48 hours and TGFβ1 (10ng/ml)+PD98059 (5μM) showed an increase in N-cadherin expression compared to the negative control. Raw data can be found in appendix 7 (page 337).

3) Claudin-1:

ICC was used to investigate the expression of Claudin-1 in the HaCaT cells as one of the epithelial markers. Claudin-1 expression in the HaCaT cell line was observed when cells were treated with SF-MEM for 48 hours (**Figure 143-B**), TGFβ1 + TGF-β RI Kinase Inhibitor VII for 48 hours (**Figure 143-H**), TGFβ1 + MK-2206 for 24 hours (**Figure 143-I**) and 48 hours (**Figure 143-J**), and TGFβ1 + SB431542 after 48 hours (**Figure 143-N**). The rest of the test conditions had no Claudin-1 expression after 24 hours or 48 hours (**Figure 144**). This indicates that the HaCaT cells needed 48 hours to produce Claudin-1, an essential component for tight junction adhesion, which might be hindered by the presence of some agents such as TGFβ1. Cells lacking Claudin-1 might help to establish cell morphological change and migration.

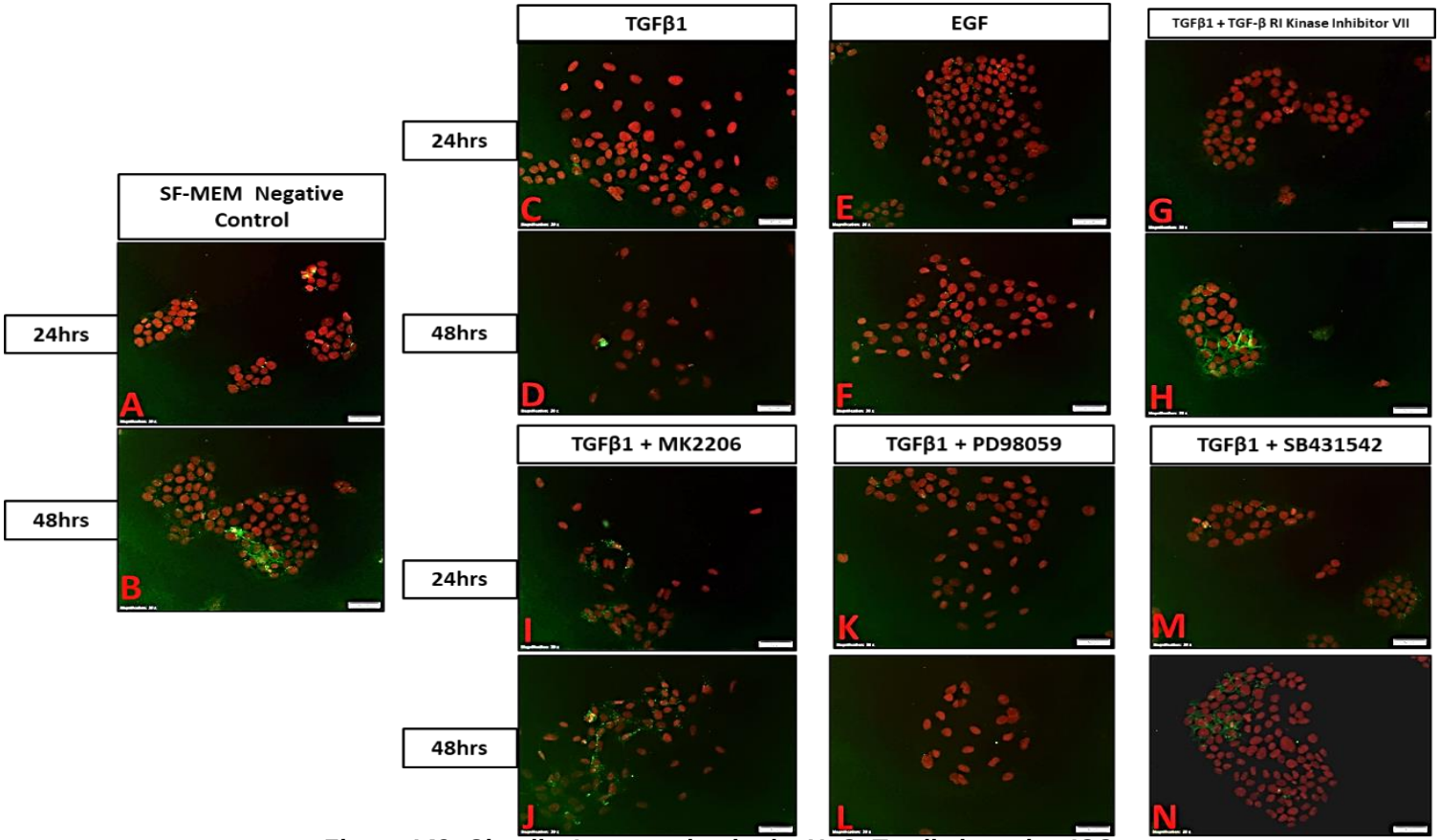


Figure 143: Claudin-1 expression in the HaCaT cells by using ICC.

Images of HaCaT cells after treatment with seven test conditions for 24 hours and 48 hours including SF-MEM, TGFβ1 (10ng/ml), EGF (10ng/ml), TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM), TGFβ1 (10ng/ml)+MK2206 (1μM), TGFβ1 (10ng/ml)+PD98059 (5μM), and TGFβ1 (10ng/ml)+SB431542 (5μM). The cells were fixed in situ and then prepared for the immunocytochemistry protocol The samples were then probed with a Claudin-1 monoclonal antibody at 1:200 dilution, followed by secondary anti rabbit IgG (with fluorescent dye **green colour**) then DAPI (**red colour**). Immunofluorescence Images were taken at 200X magnification. Claudin-1 expressed in culture dishes treated with SF-MEM after 48 hours (**B**), TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII after 48 hours (**H**) , TGFβ1 (10ng/ml)+MK-2206 (1μM)) after 24 hours and 48 hours (**I**, **J**) and TGFβ1 (10ng/ml)+SB431542 (5μM) after 48 hours (**N**).

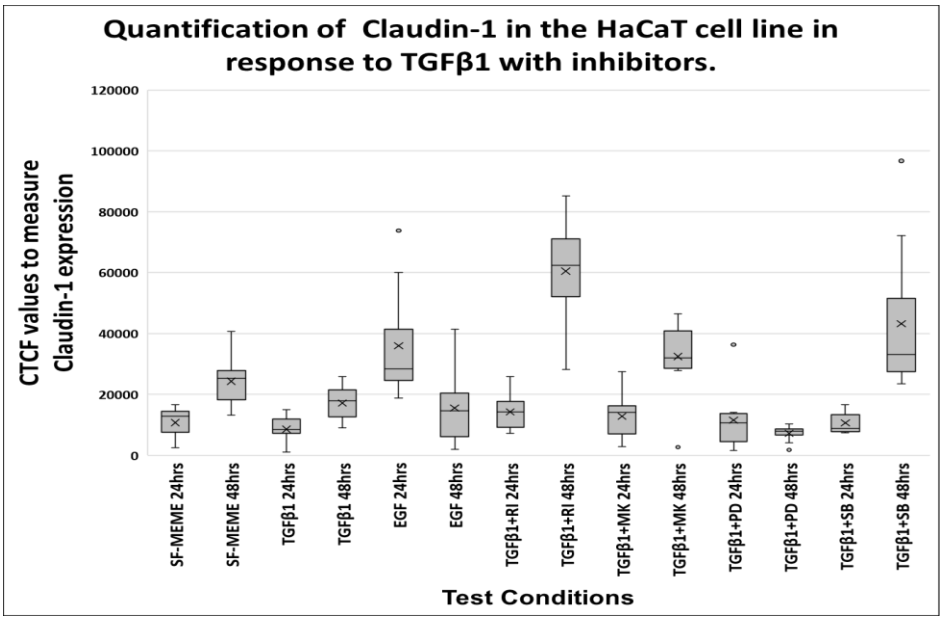


Figure 144: Quantification of Claudin-1 expression in the HaCaT cell line by CTCF.

The graph shows the quantification of Claudin-1 expression using ICC in the HaCaT cell line for phase II assessment. The assessment of the protein expression was carried out using ImageJ software and CTCF protocol (n=10). Claudin-1 expressed in HaCaT cells treated with TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) after 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours and TGFβ1 (10ng/ml)+SB431542 (5μM) after 48 hours. Raw data can be found in appendix 7 (page 340).

4) β-catenin:

In culture dishes treated with SF-MEM for 24 hours (**Figure 145-A**) and 48 hours (**Figure 145-B**), the HaCaT cells expressed β-catenin strongly and it was localised at the cell membrane in small round cells within closely compact colonies. A similar pattern of expression was observed in the phase II assessment of E-cadherin. These characteristics of β-catenin expression were observed in HaCaT cells treated with the test conditions phase II assessment i.e. three growth conditions (**Figure 146**). β-catenin expression in HaCaT cells after 48 hours in culture dishes treated with TGFβ1, EGF and TGFβ1 + PD98059 became higher than it was after 24 hours. The other test conditions showed similar level of β-catenin protein expression after 24 hours and 48 hours. The observational assessment of HaCaT cells, in terms of change in cell morphology and colony appearance, was observed in cells treated with TGFβ1 (**Figure 145-C&D**), TGFβ1 + MK-2206 (**Figure 145-I&J**) and TGFβ1 + PD98059 (**Figure 145-**

K&L). For these three test conditions, the expression of β-catenin at the cell membrane became less or absent compared to the negative control (SF-MEM). HaCaT cells treated with TGFβ1 and TGFβ1 + PD98059 showed large round cells with less compact colonies or even individual cells. In the case of cells with TGFβ1 + MK-2206, more individual cells were observed and were either small and round shape or an elongated shape in comparison to the other samples.

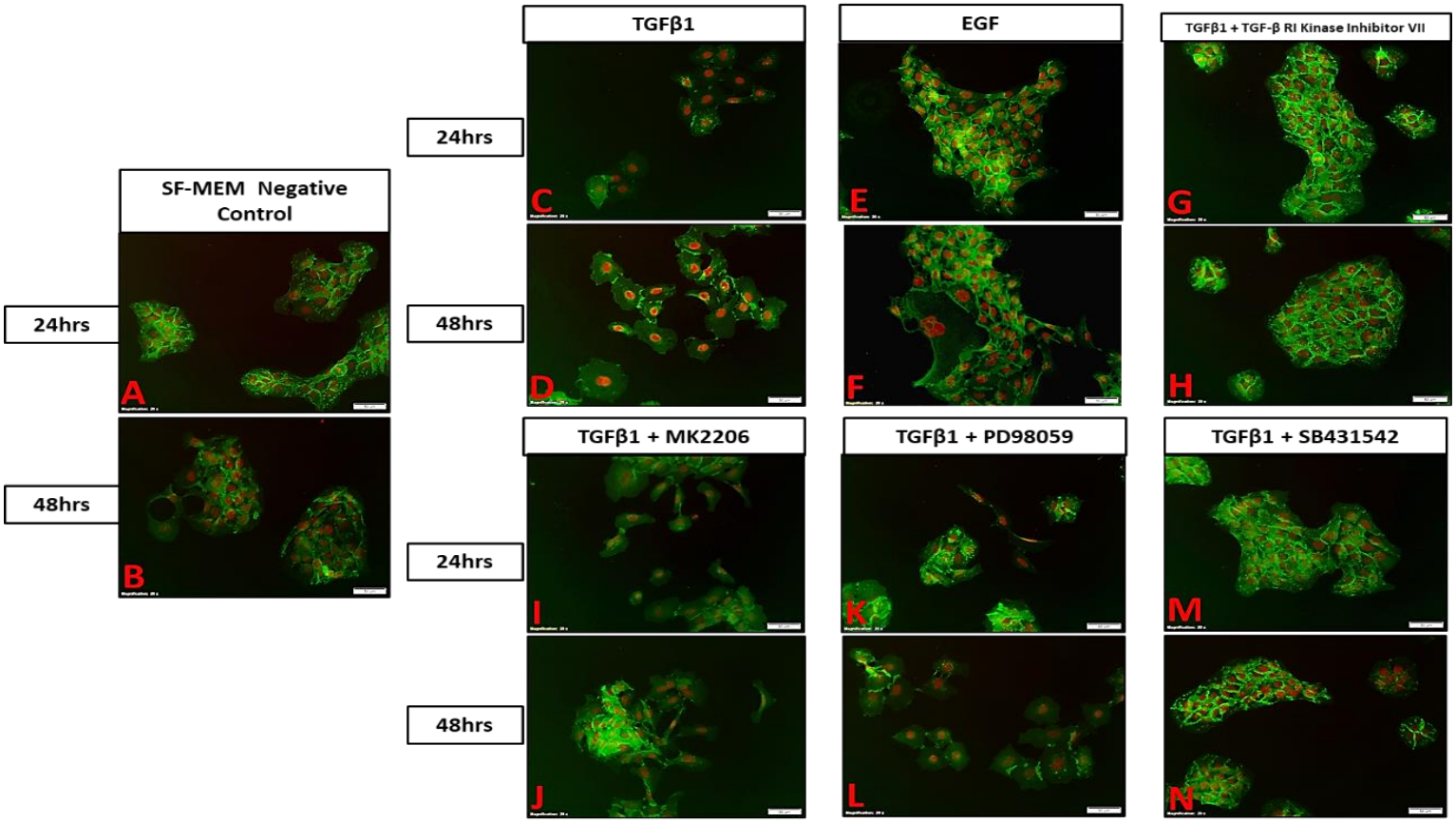


Figure 145: β-catenin expression in the HaCaT cells by ICC.

Images of HaCaT cells treated with seven test conditions for 24 hours and 48 hours including SF-MEM, TGFβ1 (10ng/ml), EGF (10ng/ml), TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM), TGFβ1 (10ng/ml)+MK-2206 (1μM), TGFβ1 (10ng/ml)+PD98059 (5μM), and TGFβ1 (10ng/ml)+SB431542 (5μM). These samples were then prepared for immunocytochemistry protocol starting with a treatment with Triton. The samples were then probed with a β-catenin monoclonal antibody at 1:50 dilution, followed by secondary anti rabbit IgG (with fluorescent dye **green colour**) then DAPI (**red colour**). Immunofluorescence Images were taken at 200X magnification. 60mm Culture dishes treated with TGFβ1 (10ng/ml) for 48 hours (**D**), TGFβ1 (10ng/ml)+MK-2206 (1μM) for 48 hours (**J**) and TGFβ1 (10ng/ml)+PD98059 (5μM) for 48 hours (**L**) showed localisation of β-catenin only in the cytoplasm compared to the negative control.

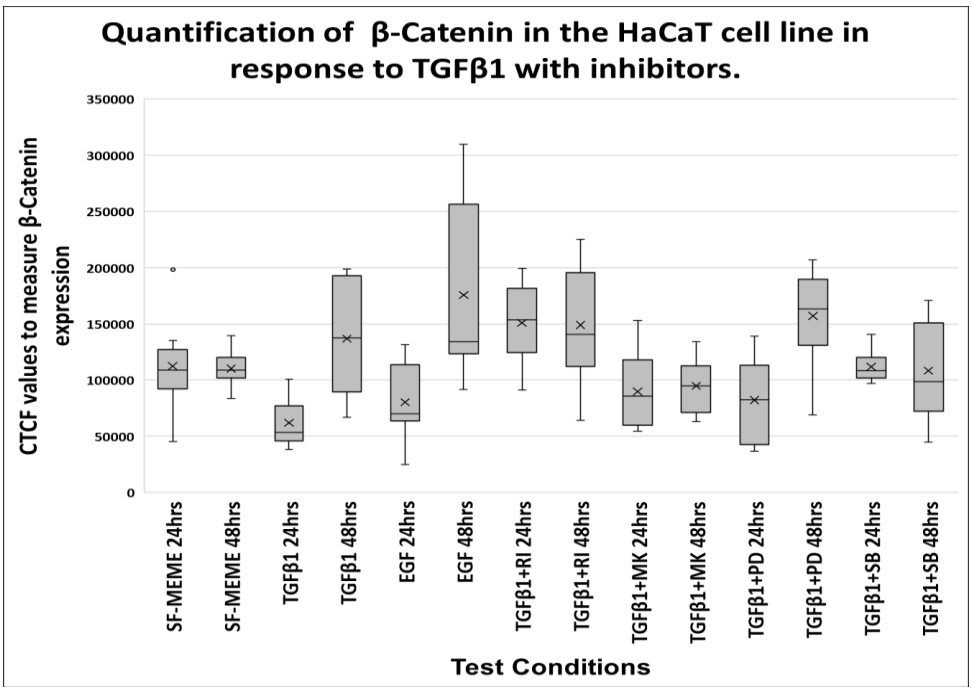


Figure 146: Quantification of β-catenin expression in the HaCaT cell line by CTCF.

The graph shows the quantification of β-catenin expression using ICC in the HaCaT cell line for the phase II assessment. The assessment of the protein expression was carried out using ImageJ software and CTCF protocol (n=10). β-catenin was expressed in the HaCaT cells treated with the different test conditions of phase II assessment. Raw data can be found in appendix 7 (page 344).

6.4.2 TYS Cell Line:

(A) Phase I Assessment of the Migration Markers:

The assessment of cell morphology and colony appearance of the TYS cell line was carried out after cells had been treated with seven test conditions after 24 hours and 48 hours. The test conditions for the phase I assessment included SF-MEM as the negative control group, TGFβ1, EGF, TGFβ1 + TGF-β RI Kinase Inhibitor VII, TGFβ1 + MK-2206, TGFβ1 + PD98059 and TGFβ1 + SB431542. The evaluation of the effect of the different test conditions was carried by taking photomicrographs of the TYS cells at 200x magnification, before the cell lysis protocol, which was followed by SDS-PAGE and Western blotting.

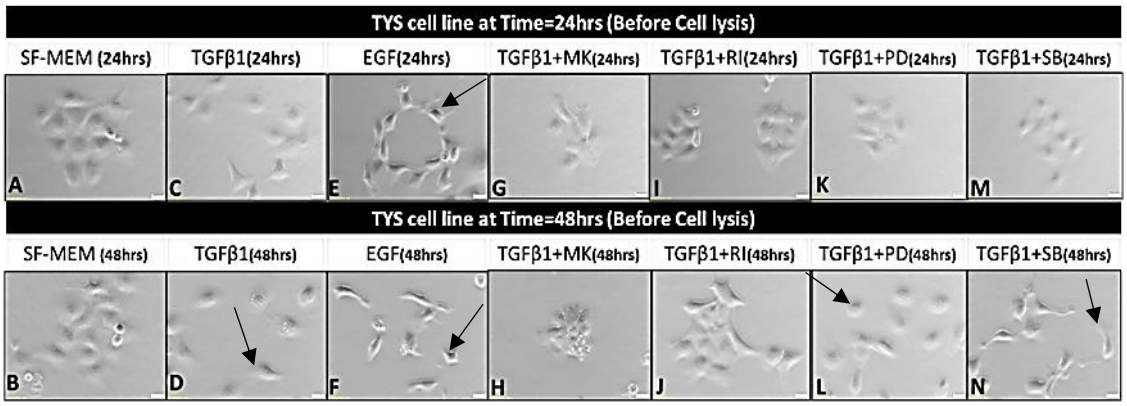


Figure 147: TYS cell line morphological assessment.

Images of the TYS cell line with SF-MEM at 48 hours, TGFβ1 at 48hours, EGF at 48 hours, TGFβ1+ TGF-β RI Kinase Inhibitor VII at 48 hours, TGFβ1+MK2206 at 48 hours, TGFβ1+PD98059 at 24 hours and 48 hours, and TGFβ1+SB431542 at 24 hours and 48 hours before cell lysis. All images were taken at 200x magnification to observe both cell morphology and colony appearance. (↑) scattered cells.

Using cell morphology and colony appearance as criteria for this assessment, the TYS cells showed morphological change when treated with four of the test conditions; TGFβ1(**Figure 147-C&D**), EGF (**Figure 147-E&F**), TGFβ1 + PD98059 (**Figure 147-L**) and TGFβ1 + SB431542 (**Figure 147-N**). For TYS treated with TGFβ1 for 48 hours and TGFβ1 +PD98059 for 48 hours, cells were scattered, large and rounded in shape, while those treated with EGF for 24 and 48 hours, and TGFβ1 + SB431542 for 48 hours showed cells with long and spindle-like shape. In contrast, cells treated with TGFβ1 + TGF-β RI Kinase Inhibitor VII after 24 hours and 48 hours (**Figure 147-I&J**), and TGFβ1 + MK-2206 after 24 hours and 48 hours (**Figure 147-G&H**) showed cell morphology and colony appearance similar to TYS cells incubated in SF-MEM.

1) E-cadherin:

The expression of E-cadherin was investigated in TYS cells treated with specific test conditions after 24 hours and 48 hours. The test conditions included SF-MEM at 48 hours, TGFβ1 at 48 hours, EGF at 48 hours, TGFβ1 + TGF-β RI Kinase Inhibitor VII at 48 hours, TGFβ1 + MK-2206 at 48 hours, TGFβ1 + PD98059 at 24 hours and 48 hours, and TGFβ1 + SB431542 at 24 hours and 48 hours. In comparison to lysates from cells treated with SF-MEM after 48 hours, the rest of test conditions showed an

increase in the expression of E-cadherin in TYS cells, except the one treated with TGFβ1 + SB431542 after 24 hours and 48 hours (**Figure 148**). According to protein quantification using image lab software (BioRad), lysates of cells treated with TGFβ1 + SB431542 showed similar or lower levels of E-cadherin expression in comparison to the negative control. Also, the highest expression of E-cadherin was detected in cells treated with TGFβ1 + PD98059 at 48 hours (**Figure 149**).

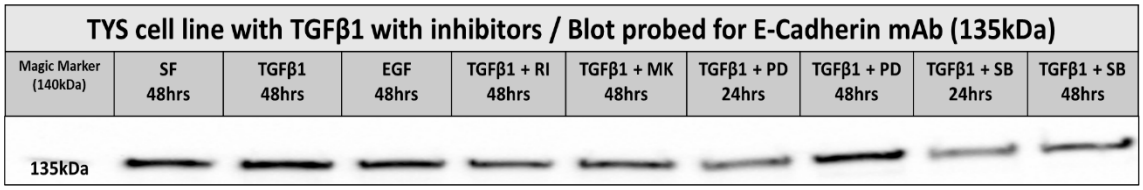


Figure 148: E-cadherin expression in the TYS cell line using WB.

The TYS cells were incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a E-cadherin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from TYS cells treated with the different test conditions showed E-cadherin expression. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 327).

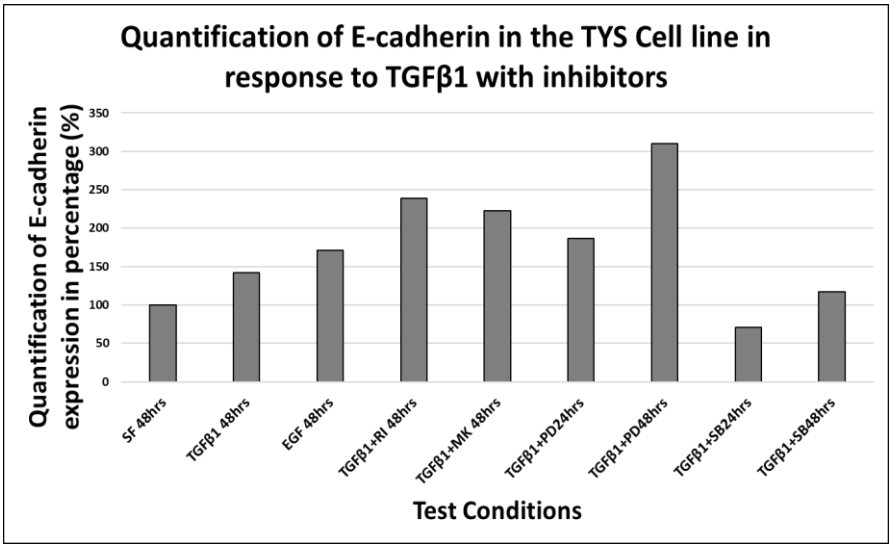


Figure 149: Quantification of E-cadherin expression in the TYS cell line.

The bar chart shows the quantification of E-cadherin expression in the TYS cell line for phase I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from TYS cells treated with TGFβ1 (10ng/ml)+PD98059 (5μM) after 48 hours showed an increase in E-cadherin expression compared to negative control. Also, lysates from cells treated with TGFβ1 (10ng/ml)+SB431542 (5μM) after 24 hours and 48 hours showed less expression of E-cadherin compared to the negative control.

Table 80: Quantification of E-cadherin expression in the TYS cell line with phase I assessment.

TYS Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	186.7%
10ng/ml TGFβ1 48 hours	142.3%	**TGFβ1+PD98059 48 hours**	309.7%
10ng/ml EGF 48 hrs	171.3%	**TGFβ1+SB431542 24 hours**	70.5%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	238.5%	**TGFβ1+SB431542 48 hours**	117.3%
TGFβ1+MK2206 48 hours	222.4%		

2) N-cadherin:

The test conditions of the phase I assessment showed N-cadherin expression in lysates from TYS cells treated with TGFβ1 for 48 hours, TGFβ1 + MK-2206 for 48 hours and TGFβ1 + PD98059 for 48 hours (**Figure 150**). The rest of the test conditions including SF-MEM did not induce the expression of N-cadherin in TYS cells, although some of these test conditions caused cells to migrate.

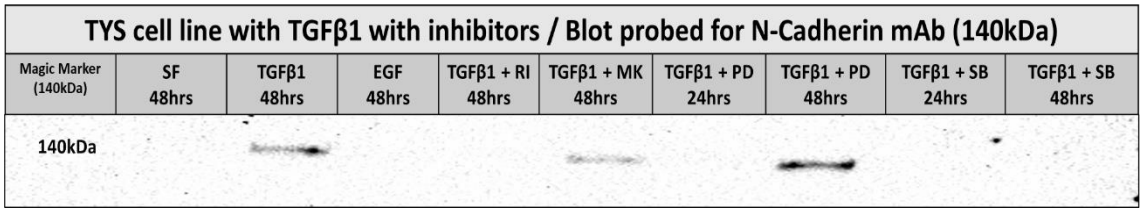


Figure 150: N-cadherin expression in the TYS cell line using WB.

The TYS cells were incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a N-cadherin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Lysates from TYS cells treated with TGFβ1 (10ng/ml) after 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours and TGFβ1 (10ng/ml)+PD98059 (5μM) after 48 hours showed an increase in N-cadherin expression to the negative control. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 327).

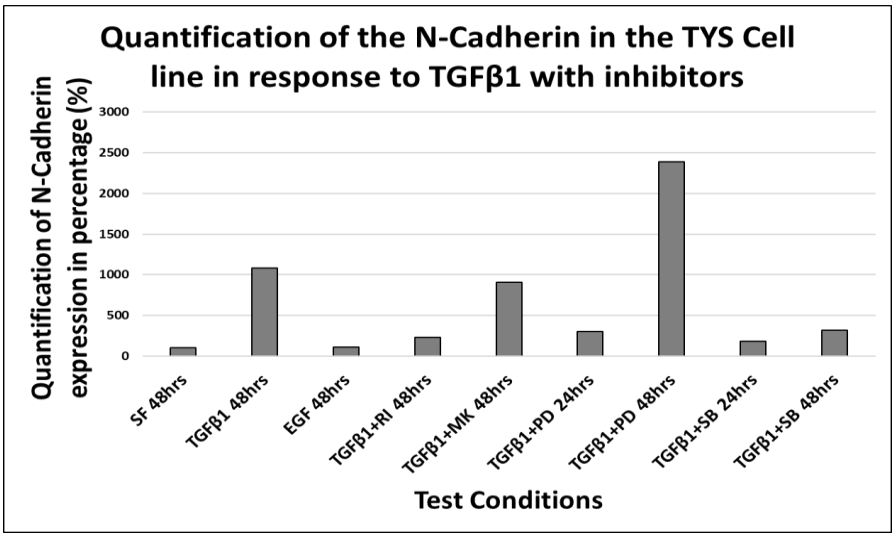


Figure 151: Quantification of N-cadherin expression in the TYS cell line.

The bar chart shows the quantification of N-cadherin expression in the TYS cell line for phase I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from TYS cells treated with TGFβ1 (10ng/ml) after 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours and TGFβ1 (10ng/ml)+PD98059 (5μM) after 48 hours showed an increase in N-cadherin expression compared to the negative control.

Table 81: Quantification of N-cadherin expression in the TYS cell line with phase I assessment.

TYS Cell line with test conditions and incubation periods			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	303.3%
10ng/ml TGFβ1 48 hours	1078.3%	**TGFβ1+PD98059 48 hours**	2389.6%
10ng/ml EGF 48 hrs	109.8%	**TGFβ1+SB431542 24 hours**	184.6%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	227%	**TGFβ1+SB431542 48 hours**	319.9%
TGFβ1+MK-2206 48 hours	902.3%		

3) β-catenin:

SDS-PAGE and Western blotting were used to assess β-catenin expression in lysates from TYS cells treated with the test conditions of phase I. The assessment using image lab software showed an increase and decrease in the levels of the protein in response to specific test conditions compared to SF-MEM after 48 hours (**Figure 152**). Lysates from TYS cells treated with EGF for 48 hours, TGFβ1 + TGF-β RI Kinase Inhibitor VII for 48 hours and TGFβ1 + MK-2206 for 48 hours showed an increase in β-catenin expression compared to the negative control. In

contrast, lysates from TYS cells treated with TGFβ1 after 48 hours, TGFβ1 + PD98059 after 24 hours and 48 hours, and TGFβ1 + SB431542 after 24 and 48 hours showed similar or lower levels of the protein compared to SF-MEM after 48 hours.

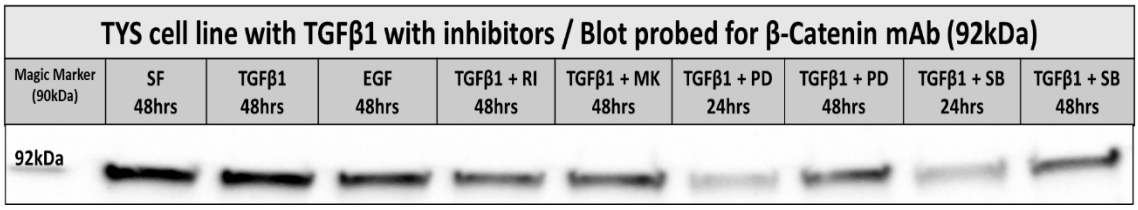


Figure 152: β-catenin expression in the TYS cell line using WB.

The TYS cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a β-catenin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from TYS cells treated with the different test conditions showed β-catenin expression. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 327).

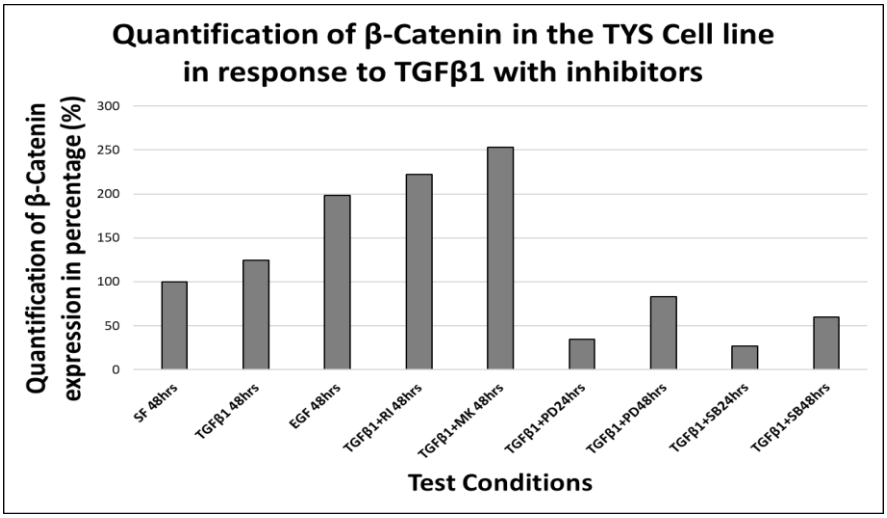


Figure 153: Quantification of β-catenin expression in the TYS cell line.

The bar chart shows the quantification of β-catenin expression in the TYS cell line for phase I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from TYS cells treated with EGF (10ng/ml) after 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) after 48 hours and TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours showed an increase in β-catenin expression compared to negative control. Lysates from TYS cells treated with TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) after 24 and 48 hours showed less β-catenin expression compared to the negative control.

Table 82: Quantification of β-catenin expression in the TYS cell line with phase I assessment.

TYS Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	34.7%
10ng/ml TGFβ1 48 hours	124.9%	**TGFβ1+PD98059 48 hours**	83%
10ng/ml EGF 48 hrs	198.6%	**TGFβ1+SB431542 24 hours**	27%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	222%	**TGFβ1+SB431542 48 hours**	60%
TGFβ1+MK-2206 48 hours	252.9%		

4) Claudin-1:

Lysates from TYS cells with the test conditions for phase I assessment showed no expression of Claudin-1. These test conditions included SF-MEM after 48 hours, TGFβ1 after 48 hours, EGF after 48 hours, TGFβ1 + TGF-β RI Kinase Inhibitor VII after 48 hours, TGFβ1 + MK-2206 after 48 hours, TGFβ1 + PD98059 after 24 hours and 48 hours, and TGFβ1 + SB431542 after 24 hours and 48 hours (**Figure 154**).

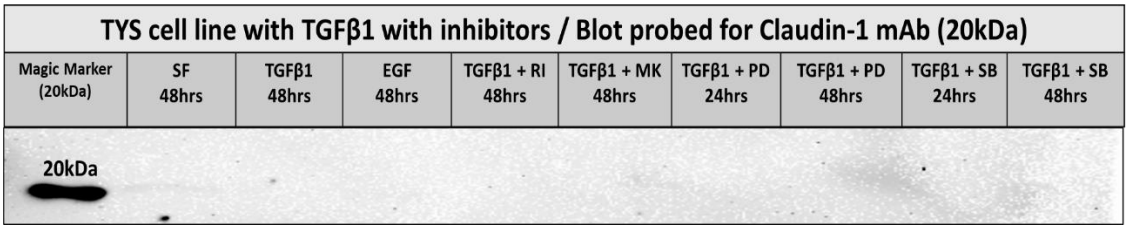


Figure 154: Claudin-1 expression in the TYS cell line using WB.

The TYS cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a Claudin-1 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Lysates from TYS cells treated with different test conditions showed no Claudin-1 expression. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 328).

5) WAVE-2:

The expression of WAVE-2 was reduced in lysates from TYS cells treated with phase I test conditions compared to those treated with SF-MEM for 48 hours. The low level of WAVE-2 expression was evident, using Image Lab software, in lysates from TYS cells treated with TGFβ1 + PD98059

for 24 and 48 hours, and TGFβ1 + SB431542 for 48 hours (**Figure 155** & **Figure 156**).

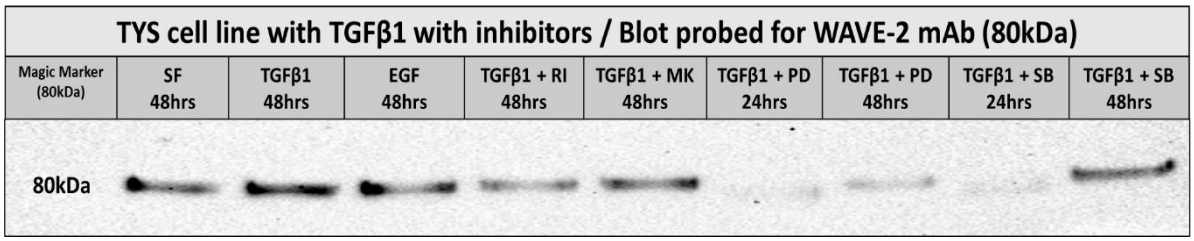


Figure 155: WAVE-2 expression in the TYS cell line using WB.

The TYS cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a WAVE-2 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from TYS cells treated with the different test conditions showed WAVE-2 expression, except those treated with TGFβ1 (10ng/ml)+PD98059 (5μM) after 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) after 24 hours. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 328).

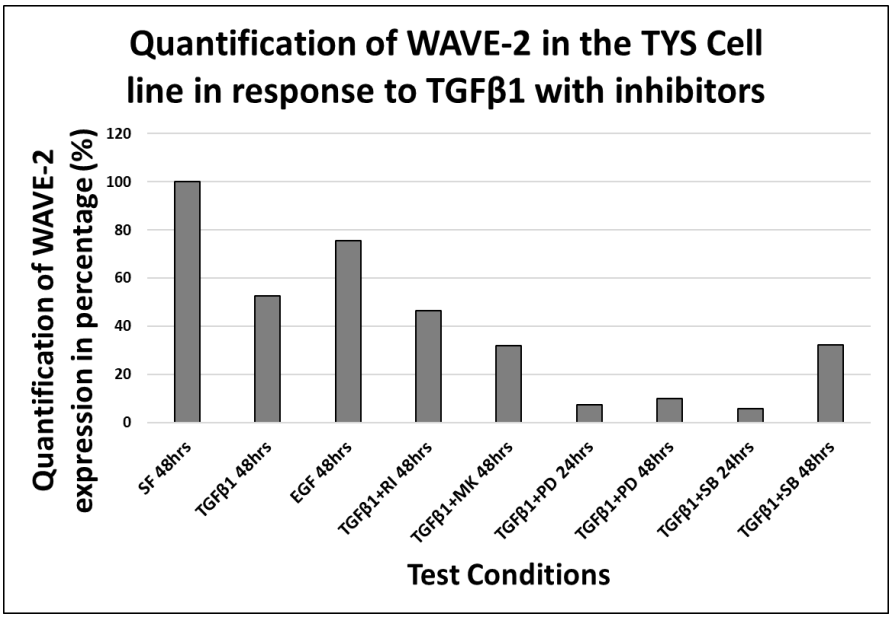


Figure 156: Quantification of WAVE-2 expression in the TYS cell line.

The bar chart shows the quantification of WAVE-2 expression in the TYS cell line for phase I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. All lysates from TYS cells treated with the test conditions of phase I assessment showed low WAVE-2 expression compared to the negative control, especially those with TGFβ1 (10ng/ml)+PD98059 (5μM) after 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) after 24 hours.

Table 83: Quantification of WAVE-2 expression in the TYS cell line with phase I assessment.

TYS Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	7.5%
10ng/ml TGFβ1 48 hours	52.6%	**TGFβ1+PD98059 48 hours**	9.9%
10ng/ml EGF 48 hrs	75.6%	**TGFβ1+SB431542 24 hours**	5.7%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	46.6%	**TGFβ1+SB431542 48 hours**	32.3%
TGFβ1+MK-2206 48 hours	31.8%		

6) Palladin:

All test conditions used for phase I assessment of previous migration markers, including SF-MEM for 48 hours, TGFβ1 for 48 hours, EGF for 48 hours, TGFβ1 + TGFβ RI Kinase Inhibitor VII for 48 hours, TGFβ1 with MK-2206 at 48 hours, TGFβ1 + PD98059 for 24 and 48 hours, and TGFβ1 + SB431542 for 24 and 48 hours, failed to induce Palladin expression in lysates from TYS cells (**Figure 157**).

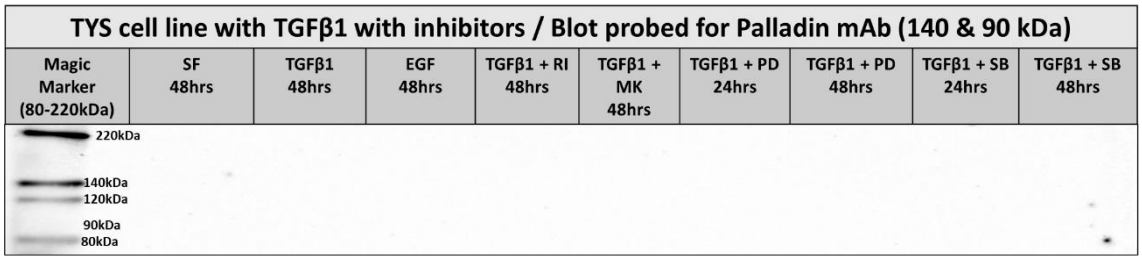


Figure 157: Palladin expression in the TYS cell line using WB.

The TYS cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a Palladin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from TYS cells treated with the different test conditions showed no expression of Palladin. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 328).

7) Gridin:

Using SDS-PAGE and Western blotting showed no expression of Gridin in lysates from TYS cells treated with the test conditions used in the phase I assessment, which included SF-MEM after 48 hours, TGFβ1 after 48

hours, EGF after 48 hours, TGFβ1 + TGFβ RI Kinase Inhibitor VII after 48 hours, TGFβ1 + MK-2206 after 48 hours, TGFβ1 + PD98059 after 24 and 48 hours, and TGFβ1 + SB 431542 after 24 and 48 hours (**Figure 158**).

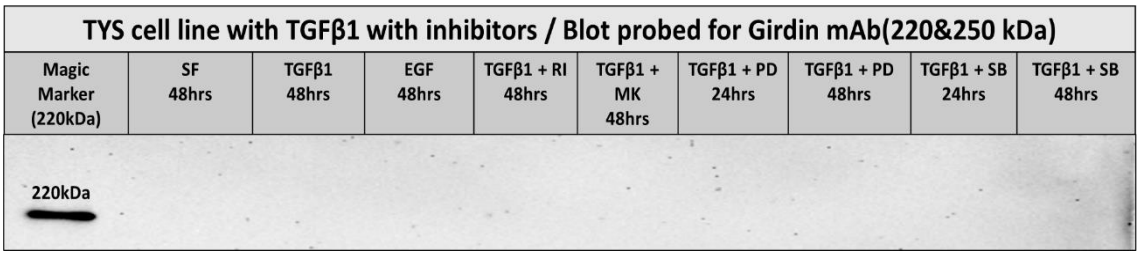


Figure 158: Gridin expression in the TYS cell line using WB.

The TYS cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a Gridin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from TYS cells treated with the different test conditions showed no expression of Gridin. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 329).

8) Vimentin:

Vimentin expression was undetectable in lysates from TYS cells treated with SF-MEM for 48 hours, TGFβ1 for 48 hours, EGF for 48 hours, TGFβ1 + TGFβ RI Kinase Inhibitor VII for 48 hours, TGFβ1 + MK-2206 for 48 hours, TGFβ1 + PD98059 for 24 hours, and TGFβ1 + SB431542 for 48 hours during phase I assessment (**Figure 159**).

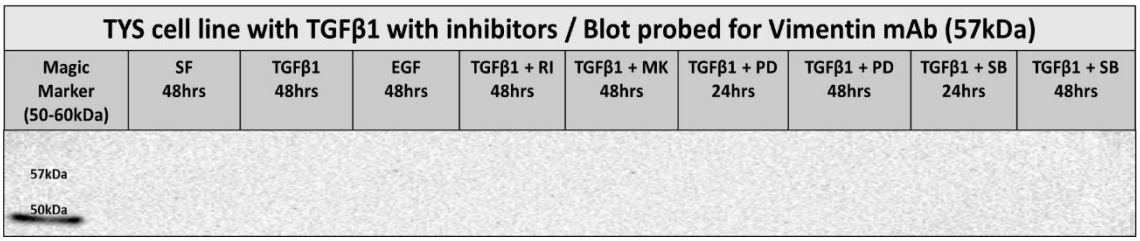


Figure 159: Vimentin expression in the TYS cell line using WB.

The TYS cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a Vimentin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from TYS cells treated with the different test conditions showed no expression of vimentin. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 329).

(B) Phase II Assessment of the Migration Markers:

At this phase of evaluation, ICC was used to investigate four migration markers. The assessment was based on protein expression and location within the cell. In addition, establishing a relationship between the migratory status of the cells and the markers was carried out. E-cadherin, N-cadherin, β-catenin, and Claudin-1 were used as migratory markers in this experiment. In addition, the protocol for calculating the correct total cell fluorescence (CTCF) was utilised to measure the level of expression of each migratory marker under the test conditions after 24 and 48 hours.

Table 84: Design of phase II assessment for the TYS cell line.

Phase II assessment for migration marker in TYS cell line using ICC			
SF-MEM 24hrs	TGFβ1 24hrs	EGF 24hrs	TGFβ1 + TGF-β RI Kinase Inhibitor VII 24hrs
	TGFβ1 48hrs	EGF 48hrs	TGFβ1 + TGF-β RI Kinase Inhibitor VII 48hrs
SF-MEM 48hrs	TGFβ1 + MK-2206 24hrs	TGFβ1 + PD98059 24hrs	TGFβ1 + SB431542 24hrs
	TGFβ1 + MK-2206 48hrs	TGFβ1 + PD98059 48hrs	TGFβ1 + SB431542 48hrs

(1) E-cadherin:

The ICC assay was used to assess the relationship between the expression of E-cadherin and cell morphology and colony appearance in the TYS cell line. This assessment was based on the expression of the protein and cell morphology in cells treated with SF-MEM after 24 and 48 hours as the control samples. The presence of E-cadherin in TYS cells treated with SF-MEM for 24 hours was mainly in the cell membrane, where cells maintained their small, rounded shape and close compact colonies (**Figure 160-A**). After 48 hours, TYS cells showed less compact colonies, large cells with less contact to or with their neighbour and E-cadherin expression was mainly in the cell cytoplasm (**Figure 160-B**).

With exogenous TGFβ1, the expression of E-cadherin in TYS cells slightly increased after 24 hours and was found to be localised in the

cytoplasm (**Figure 161**). In addition, TYS cells treated with this test condition showed a change or changes in cell morphology and colony appearance compared to the control samples at 24 hours (**Figure 160-C**). Similar findings were observed after 48 hours, there were changes in cell morphology and colony appearance with more individual cells. In terms of E-cadherin expression, TYS cells treated with TGFβ1 for 48 hours showed a similar level of expression of the protein to the negative control with a slight difference in the localisation, where E-cadherin was present in both the cytoplasm and nucleus in TGFβ1-treated cells (**Figure 160-F**).

Cells treated with EGF for 24 and 48 hours, showed a significant change in cell morphology and colony appearance, where cells became spindle-like in shape and lost adhesion to their neighbours, resulting in more single cells compared to the negative control (**Figure 160-E&F**). In addition, the presence of E-cadherin was mainly in the cytoplasm and not present at the cell membrane. Localised dense spots of E-cadherin were observed in the nucleus in cells treated with EGF for 48 hours.

Using TGFβ RI Kinase Inhibitor VII + TGFβ1 indicated that the expression of E-cadherin in TYS cells was higher than the negative control. It was observed at the cell membrane and cytoplasm of the TYS cells. The morphology and colony appearance of TYS cells treated with TGFβ1 + TGFβ RI Kinase Inhibitor VII remained similar to the negative control with more compact colonies after 48 hours (**Figure 160- G&H**).

The expression of E-cadherin in TYS cells treated with TGFβ1 + MK-2206 was at the same level as SF-MEM after 24 hours and 48 hours. However, there were some scattered cells and changes in colony appearance of TYS cells observed after 24 hours incubation with TGFβ1 + MK-2206 compared to the negative control (**Figure 160-I**). In contrast, these changes in cell morphology and colony appearance return to be similar to those cells treated with SF-MEM after cells be treated with TGFβ1 + MK-2206 for 48 hours (**Figure 160-J**).

In the TYS cells treated with TGFβ1 + PD98059 for 24 hours and 48 hours, the expression of E-cadherin was increased compared to the

negative control with similar protein localisation at the cell membrane. Also, changes in cell morphology and colony appearance of the TYS cells were observed, especially after 48 hours with TGFβ1 + PD98059, where cells appeared large with little or no contact with the neighbouring cells (**Figure 160-L**).

TYS cells incubated with SB432542 + TGFβ1 showed similar levels of E-cadherin expression to the negative control. However, regarding the localisation of E-cadherin, cell morphology and colony appearance, TYS cells treated with TGFβ1+ SB432542 showed two distinctive results at different incubation periods. After 24 hours, E-cadherin was localised in the cytoplasm and cell membrane with less compact colonies and large cells (**Figure 160-M**) while, after 48 hours, the presence of E-cadherin was detected only in the cytoplasm and colonies had dispersed, which resulted in small, rounded and spindled-shaped single cells (**Figure 160-N**).

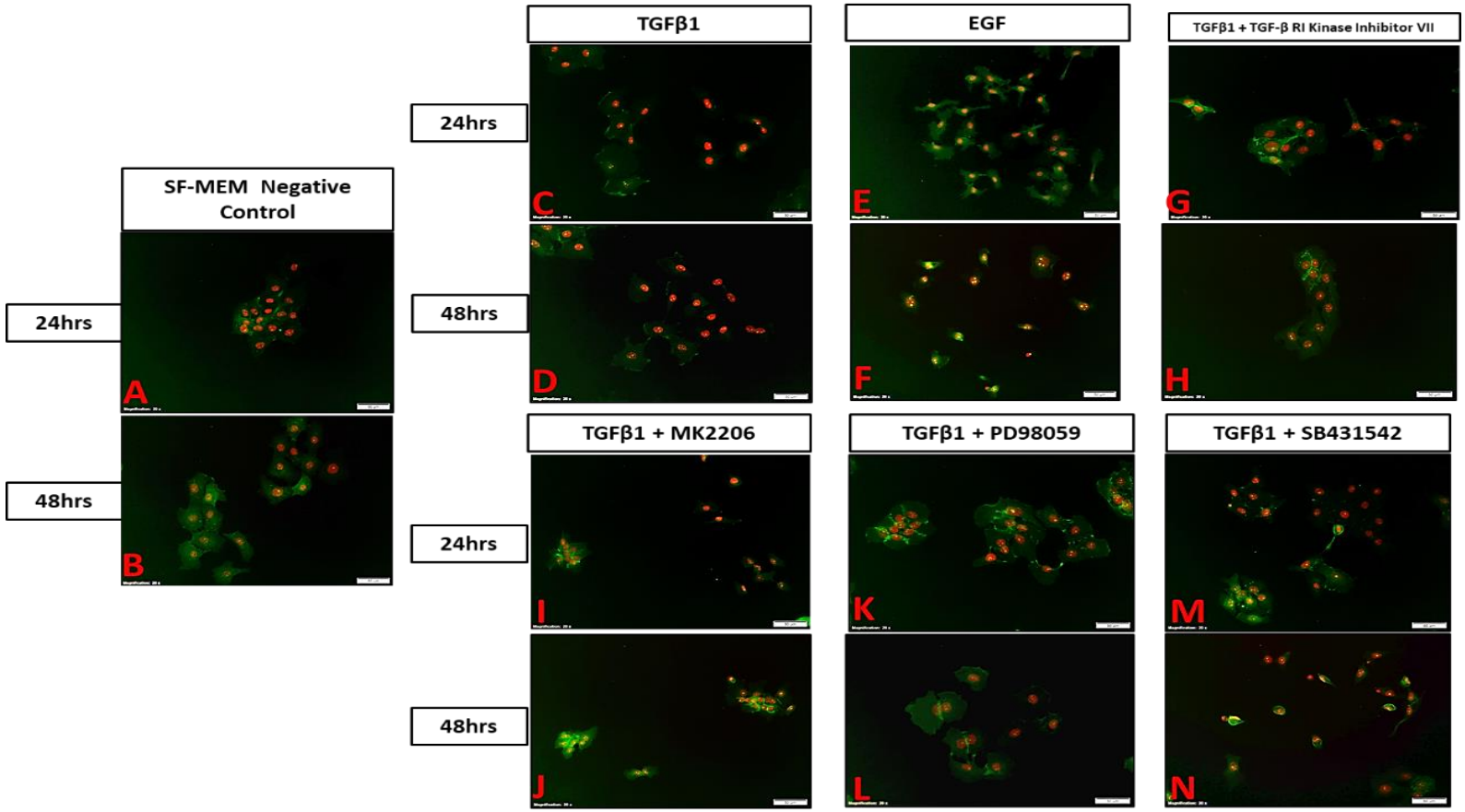


Figure 160: E-cadherin expression in the TYS cell line using ICC.

Images of TYS cells that had been cultured in seven test conditions for 24 hours and 48 hours (Table 59). These samples were then prepared for immunocytochemistry starting with a treatment with Triton. The samples were then probed with an E-cadherin monoclonal antibody at 1:200 dilution, followed by secondary anti rabbit IgG (with fluorescent dye **green colour**) then DAPI (**red colour**). Immunofluorescence Images were taken at 200X magnification. 60mm Culture dishes of TYS cells treated with TGFβ1 (10ng/ml) for 24 hours and 48 hours (**C**, **D**), EGF (10ng/ml) for 24 hours and 48 hours (**E**, **F**),TGFβ1 (10ng/ml)+MK-2206 (1μM) for 24 hours (**I**), TGFβ1 (10ng/ml)+PD98059 (5μM) for 48 hours (**L**) and TGFβ1 (10ng/ml)+SB431542 (5μM) for 48 hours (**N**) showed localisation of E-cadherin only in the cytoplasm compared to the negative control.

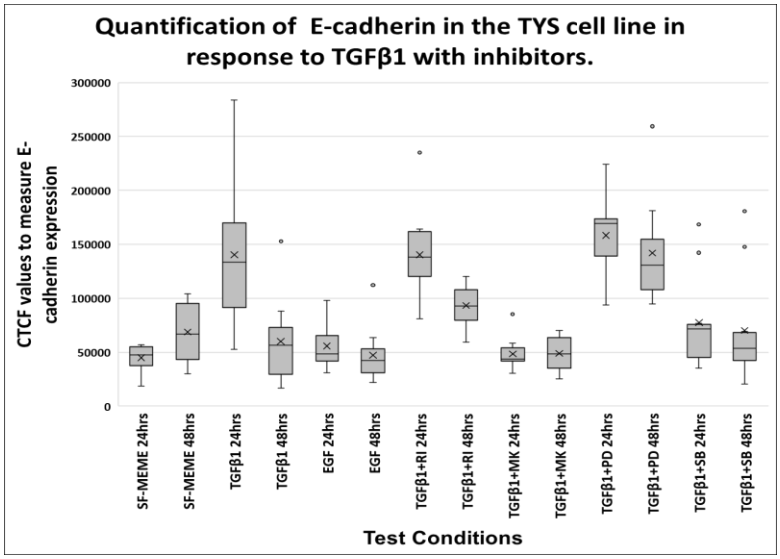


Figure 161: Quantification of E-cadherin expression in the TYS cell line by CTCF.

The graph shows the quantification of E-cadherin expression in the TYS cell line for the phase II assessment. The assessment of protein expression was carried out using ImageJ software and the CTCF protocol (n=10). TYS cells treated with TGFβ1 (10ng/ml) after 24 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) after 24 hours and 48 hours, and TGFβ1 (10ng/ml)+PD98059 (5μM) after 24 hours and 48 hours showed an increase in E-cadherin expression compared to the negative control. Raw data can be found in appendix 7 (page 347).

(2) N-cadherin:

N-cadherin expression in TYS cells, treated with the test conditions of the phase II assessment (**Table 84**), was difficult to visualise using only the immunofluorescence images (**Figure 162**). The protocol of corrected total cell fluorescence (CTCF) was used to evaluate the expression of the protein in the TYS cells in response to test conditions of phase II assessment. This protocol showed the expression of N-cadherin was slightly higher than the negative control in TYS cells treated with TGFβ1 for 24 hours and 48 hours, EGF for 48 hours, TGFβ1 + PD98059 for 24 hours and 48 hours, and TGFβ1 + SB431542 for 48 hours (**Figure 163**). The findings regarding cell morphology and colony appearance were similar to those for E-cadherin expression.

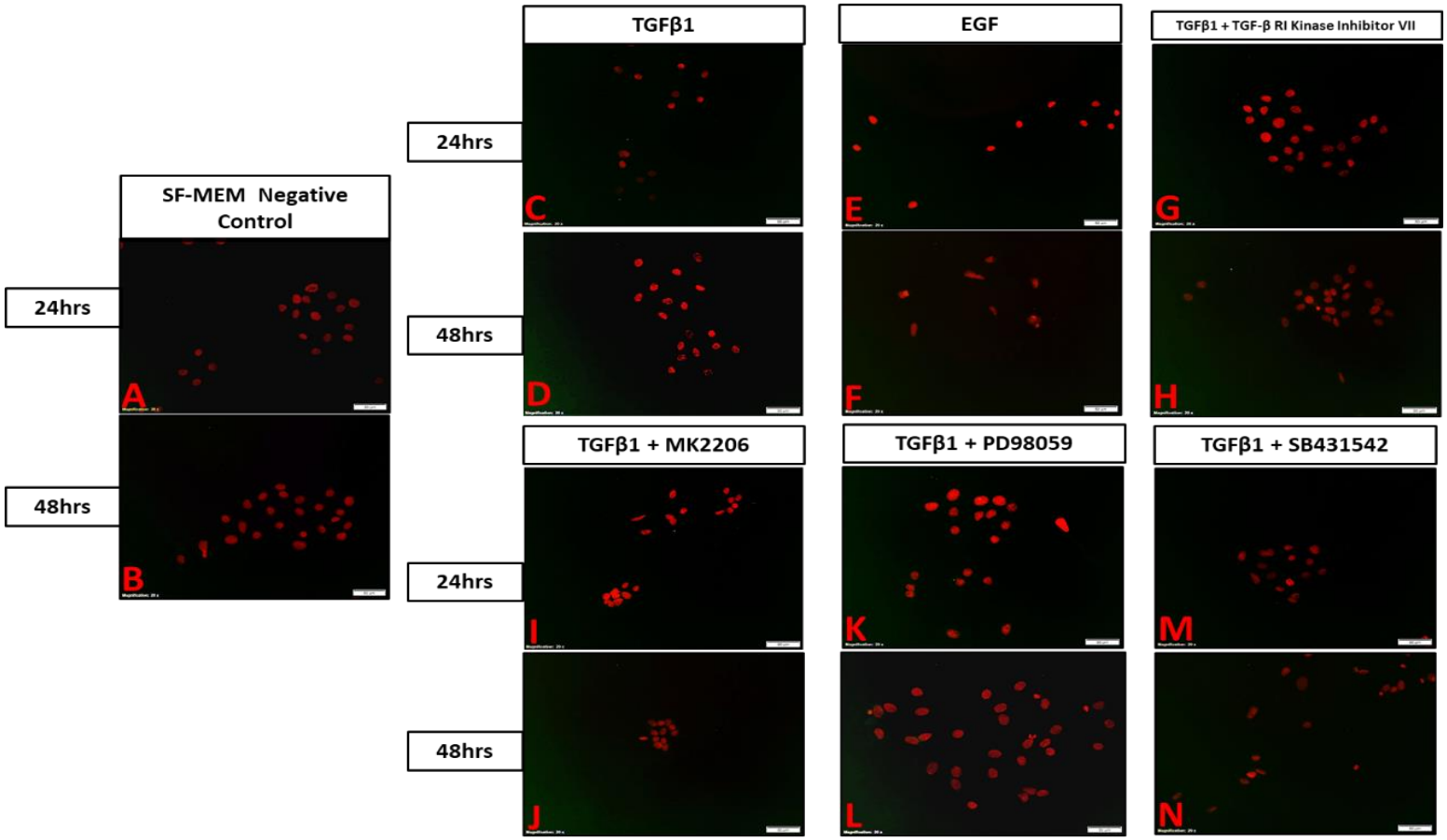


Figure 162: N-cadherin expression in the TYS cell line using ICC.

Images of TYS cells that had been treated with seven test conditions for 24 hours and 48 hours including SF-MEM, TGFβ1 (10ng/ml), EGF (10ng/ml), TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM), TGFβ1 (10ng/ml)+MK2206 (1μM), TGFβ1 (10ng/ml)+PD98059 (5μM), and TGFβ1 (10ng/ml)+SB431542 (5μM). The cells were fixed in situ and then prepared for the immunocytochemistry protocol The samples were then probed with an N-cadherin monoclonal antibody at 1:200 dilution, followed by secondary anti rabbit IgG (with fluorescent dye **green colour**) then DAPI (**red colour**). Immunofluorescence Images were taken at 200X magnification. No expression of N-cadherin was observed in any of the TYS cells treated with the test conditions of the phase II assessment.

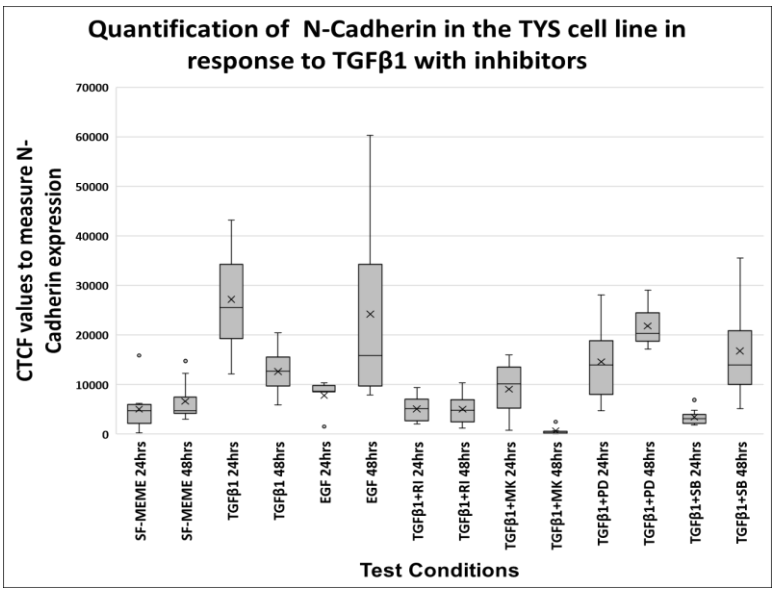


Figure 163: Quantification of N-cadherin expression in the TYS cell line by CTCF.

The graph shows the quantification of N-cadherin expression using ICC in the TYS cell line for phase II assessment. The assessment of the protein expression was carried out using ImageJ software and CTCF protocol (n=10). TYS cells treated with TGFβ1 (10ng/ml) after 24 hours, EGF (10ng/ml) after 24 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) after 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) after 48 hours showed an increase in N-cadherin expression compared to the negative control. Raw data can be found in appendix 7 (page 351).

(3) Claudin-1:

Immunofluorescence images of TYS cells treated with the test conditions of the phase II assessment (**Table 84**) showed no expression of Claudin-1 after 24 hours and 48 hours (**Figure 164**). Using the CTCF protocol for these photomicrographs showed a wide distribution of data for Claudin-1 expression in TYS cells treated with TGFβ1 for 24 hours, EGF for 24 hours, TGFβ1 + TGFβ RI Kinase Inhibitor VII for 24 hours and TGFβ1 + Mk-2206 for 24 hours compared to SF-MEM group after 24 hours (**Figure 165**). However, the distribution of the values of CTCF for Claudin-1 expression in TYS cells treated with TGFβ1 for 48 hours, EGF for 48 hours, TGFβ1 + TGFβ RI Kinase Inhibitor VII for 48 hours and TGFβ1 + MK-2206 for 48 hours were similar to the negative control (**Figure 165**).

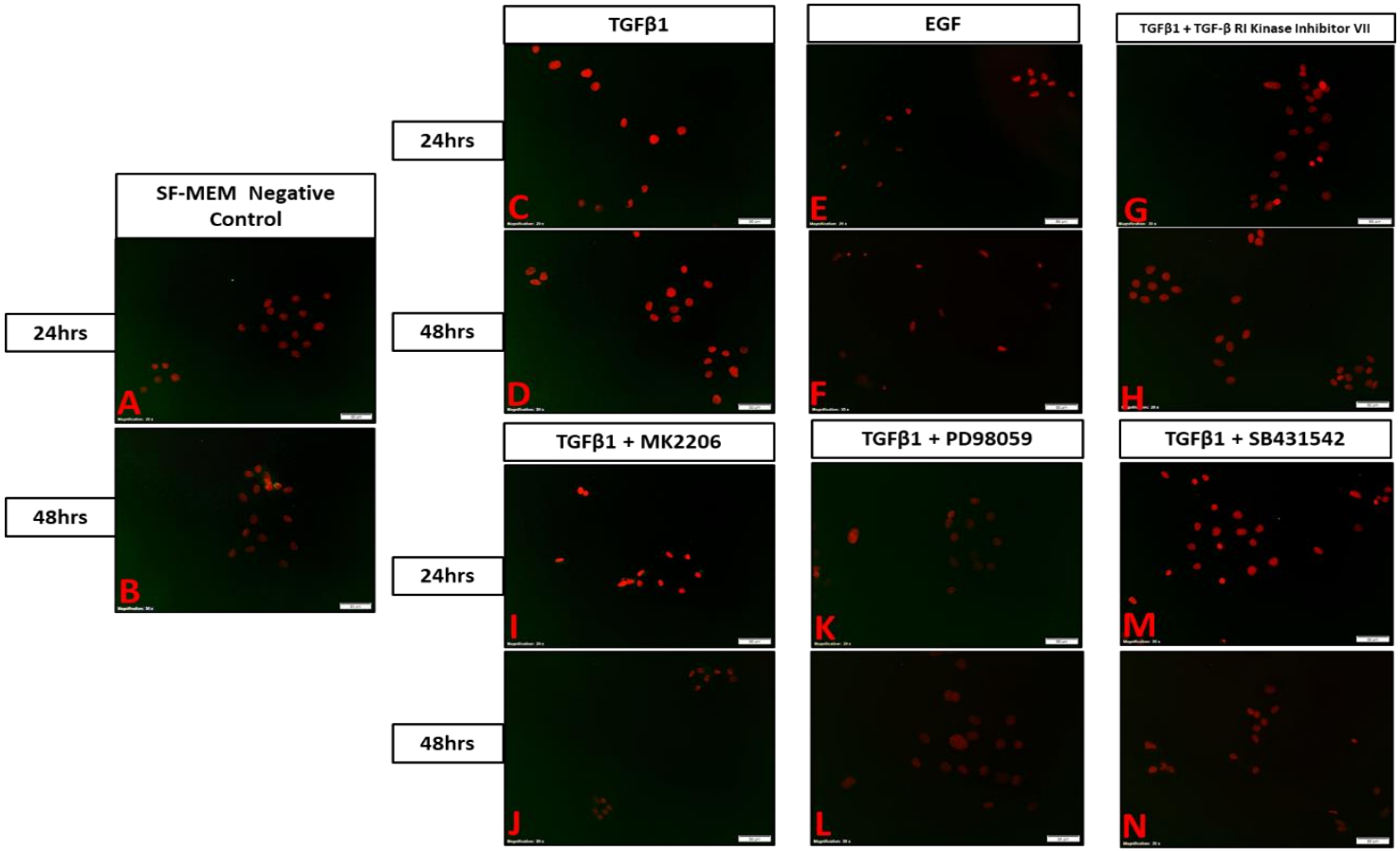


Figure 164: Claudin-1 expression in the TYS cells using ICC.

Images of TYS cells that had been cultured in seven test conditions for 24 hours and 48 hours including SF-MEM, TGFβ1 (10ng/ml), EGF (10ng/ml), TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM), TGFβ1 (10ng/ml)+MK-2206 (1μM), TGFβ1 (10ng/ml)+PD98059 (5μM), and TGFβ1 (10ng/ml)+SB431542 (5μM). The cells were fixed in situ and then prepared for the immunocytochemistry protocol The samples were then probed with a Claudin-1 monoclonal antibody at 1:200 dilution, followed by secondary anti rabbit IgG (with fluorescent dye **green colour**) then DAPI (**red colour**). Immunofluorescence Images were taken at 200X magnification. No expression of Claudin-1 was observed in any of the cells treated with the test conditions of phase II assessment.

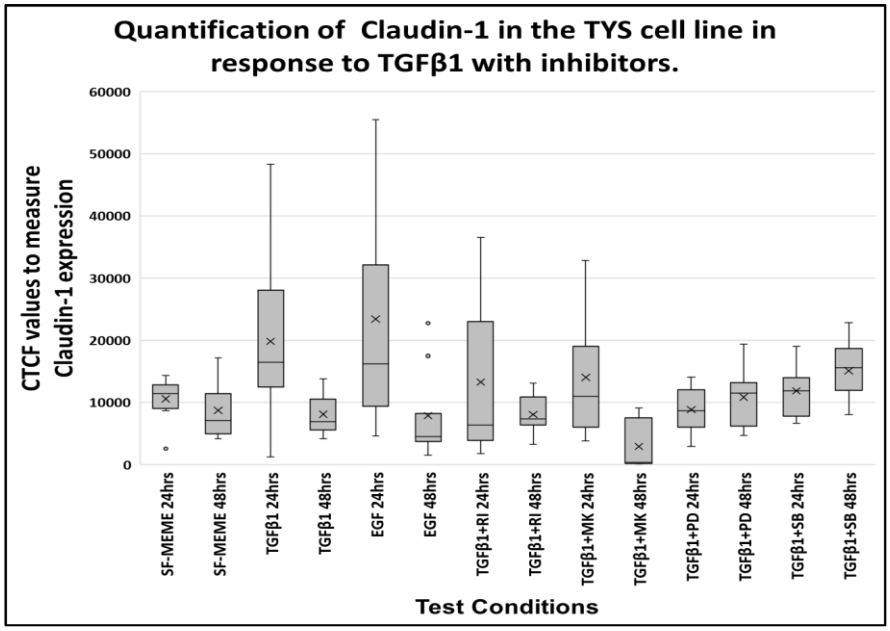


Figure 165: Quantification of Claudin-1 expression in the TYS cell line by CTCF.

The graph shows the quantification of Claudin-1 expression using ICC in the TYS cell line for phase II assessment. The assessment of the protein expression was carried out using ImageJ software and CTCF protocol (n=10). TYS cells treated with TGFβ1 for 24 hours, EGF for 24 hours, TGFβ1 + TGFβ RI Kinase Inhibitor VII for 24 hours and TGFβ1 + Mk-2206 for 24 hours showed a range of measurements of Claudin-1 expression compared to SF-MEM group after 24 hours. Raw data can be found in appendix 7 (page 354).

(4) β-catenin:

The expression and localisation of β-catenin was strongly associated with the cell morphology and colony appearance of the TYS cell line. TYS cells treated with SF-MEM for 24 hours showed a strong expression of β-catenin (**Figure 166-A**). The protein was localised at the cell membrane and the cytoplasm, with tight contact between cells, which helped them to maintain their colony appearance. The expression of β-catenin decreased and there was less at the cell membrane in the TYS cells after 48 hours in SF-MEM (**Figure 166-B**). The TYS cells treated with TGFβ1 for 24 hours showed similar levels of β-catenin expression to those incubated in SF-MEM after 24 hours accompanied by less compact colonies and large round cells (**Figure 166-C**). After 48 hours with the growth factor, scattered cells were observed and the expression of β-catenin was only detected in the cytoplasm of the TYS cells (**Figure 166-D**). EGF-treated TYS cells after 24 hours and 48 hours became elongated and spindle-like in shape

with less cellular adhesion (**Figure 166-E**). The expression of β-catenin was mainly in the cytoplasm, but not at the cell membrane.

The TYS cell line treated with TGFβ1 + TGFβ RI Kinase Inhibitor VII showed a resemblance to the negative control in terms of β-catenin expression, cell morphology and colony appearance after 24 hours and 48 hours (**Figure 166-G&H**). This indicates that the inhibitor completely blocked the effect, which was observed with cells treated with the growth factor alone.

Adding MK-2206 + TGFβ1 to TYS cells induced cell scattering and changes in colony appearance (**Figure 166-I&J**). Some of the single cells appeared large and round shape, while others appeared to have a spindle shape. The expression of β-catenin was observed in the cytoplasm and cell membrane.

TYS cells incubated with PD98059 + TGFβ1 for 48 hours and TGFβ1 + SB432542 for 24 hours and 48 hours showed less expression of β-catenin and less compact colonies compared to the negative control (**Figure 166-L, M, N**). For TYS cells treated with TGFβ1 + SB432542 for 24 hours, cells maintained their adhesion to the neighbouring cells compared to SF-MEM samples, but the localisation of β-Catenin became less at the cell membrane (**Figure 166-M**). After 48 hours, TYS cells had scattered and became spindle-like in shape with no β-catenin expression at the cell membrane (**Figure 166-N**).

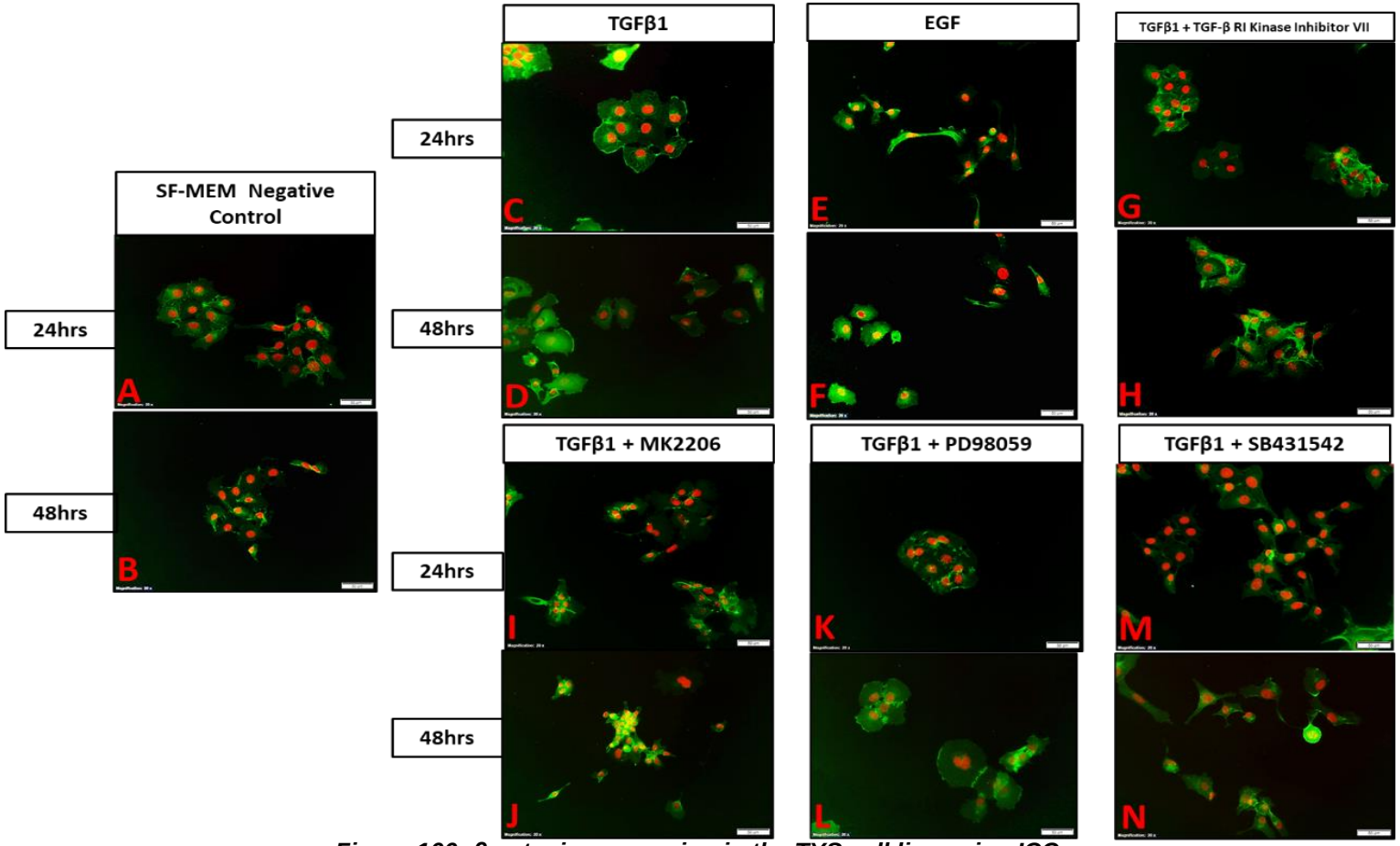


Figure 166: β-catenin expression in the TYS cell line using ICC.

Images TYS cells treated with seven test conditions for 24 hours and 48 hours (**Table 59**). These cells were then fixed in situ and prepared for the immunocytochemistry protocol. The fixed cells were then probed with a β-catenin monoclonal antibody at 1:50 dilution, followed by secondary anti rabbit IgG (with fluorescent dye **green colour**) then DAPI (**red colour**). Immunofluorescence Images were taken at 200X magnification. 60mm Culture dishes of TYS cells treated with TGFβ1 (10ng/ml) for 48 hours (**D**), EGF (10ng/ml) for 24 hours and 48 hours (**E**, **F**), and TGFβ1 10ng/ml)+SB431542 (5μM) for 48 hours (**N**) showed localisation of β-catenin only in the cytoplasm compared to the negative control.

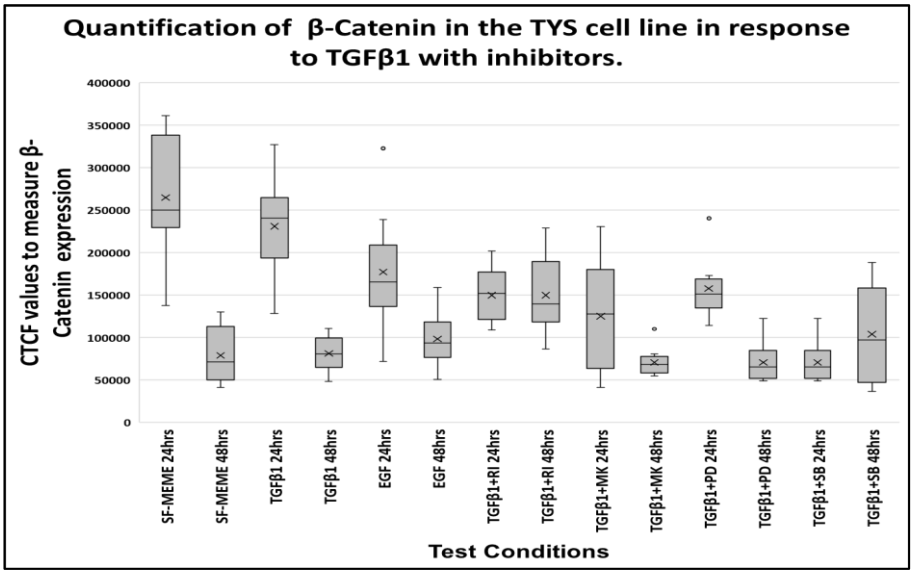


Figure 167: Quantification of β-catenin expression in the TYS cell line by CTCF.

The graph shows the quantification of β-catenin expression using ICC in the TYS cell line for phase II assessment. The assessment of protein expression was carried out using ImageJ software and CTCF protocol (n=10). β-catenin expression in TYS cells treated with the test conditions of phase II assessment showed similar levels to the negative control. Raw data can be found in appendix 7 (page 357).

6.4.3 SAS-H1 Cell Line:

(A) Phase I Assessment of the Migration Markers:

Prior to the cell lysis protocol, the SAS-H1 cells were observed after 24 hours and 48 hours of being treated with test conditions of phase I assessment for the migration markers. The test conditions for phase I assessment included SF-MEM as control group, TGFβ1, EGF, TGFβ1 + TGF-β RI Kinase Inhibitor VII, TGFβ1 + MK-2206, TGFβ1 + PD98059 and TGFβ1 + SB431542.

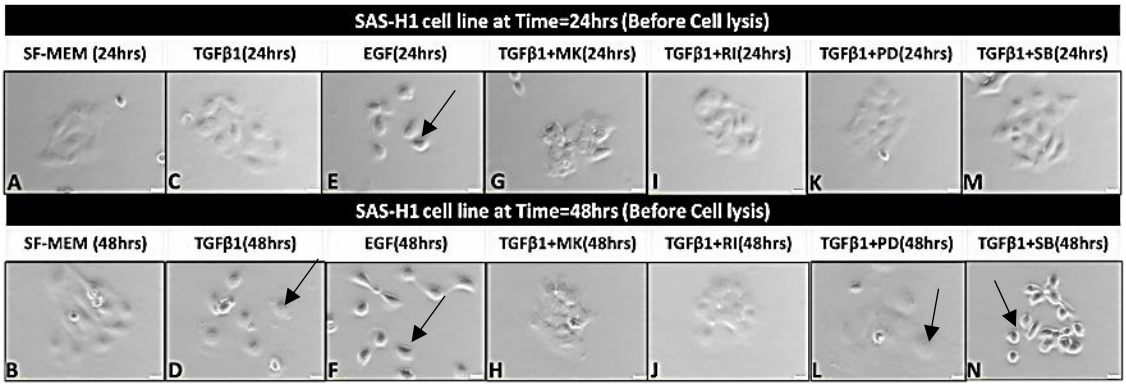


Figure 168: SAS-H1 cell line morphological assessment.

Images for the SAS-H1 cell line with SF-MEM at 48 hours, TGFβ1 at 48 hours, EGF at 48 hours, TGFβ1+ TGF-β RI Kinase Inhibitor VII at 48 hours, TGFβ1+MK2206 at 48 hours, TGFβ1+PD98059 at 24 hours and 48 hours, and TGFβ1+SB431542 at 24 hours and 48 hours before cell lysis. All images were taken at 200x magnification to observe both cell morphology and colony appearance. (↑) scattered cells.

The SAS-H1 cells treated with SF-MEM showed compact colonies with small round or cuboid cells with no indication of cell migration (**Figure 168-A&B**). However, cells migrating away from their colony were observed in culture dishes treated with TGFβ1 for 48 hours (**Figure 168-D**), EGF for 24 hours and 48 hours (**Figure 168-E&F**), TGFβ1 + PD98059 for 48 hours (**Figure 168-L**) and TGFβ1 + SB431542 for 48 hours (**Figure 168-N**). The SAS-H1 cells treated with TGFβ1 for 48 hours and TGFβ1 + PD98059 for 48 hours appeared to be large and round in shape. Also, those cells were separated from their colonies or in a less compact colonies. SAS-H1 cells treated with EGF for 24 hours and TGFβ1 + SB431542 after 48 hours appeared small, round and separated from their colonies.

(1) E-cadherin:

The investigation of E-cadherin expression in the SAS-H1 cells line in response to phase I assessment was carried out using SDS-PAGE and Western blotting. The aim of this assessment was to evaluate the level of E-cadherin expression in lysates from the SAS-H1 cells treated with the test conditions compared to the negative control, which was SF-MEM for 48 hours (**Figure 169**). All lysates from cells with those test conditions showed a similar level of E-cadherin to the control, except two groups. First, lysates from cells treated with PD98059 + TGFβ1 for 24 hours, which showed a slight increase in E-cadherin expression. Second, lysates from cells treated with TGFβ1 + MK-2206, which showed less expression of E-cadherin after 48 hours compared to the negative control (**Figure 170**).

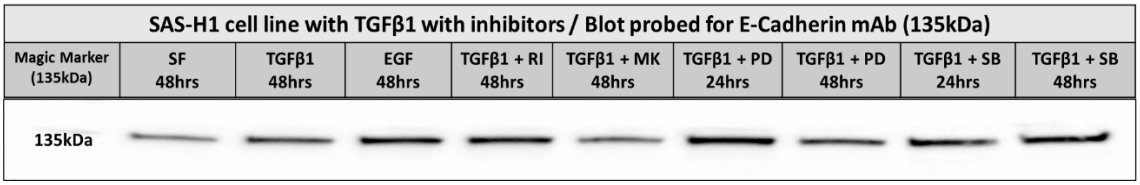


Figure 169: E-cadherin expression in the SAS-H1 cell line using WB.

The SAS-H1 cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a E-cadherin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from SAS-H1 cells treated with the different test conditions showed E-cadherin expression. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 330).

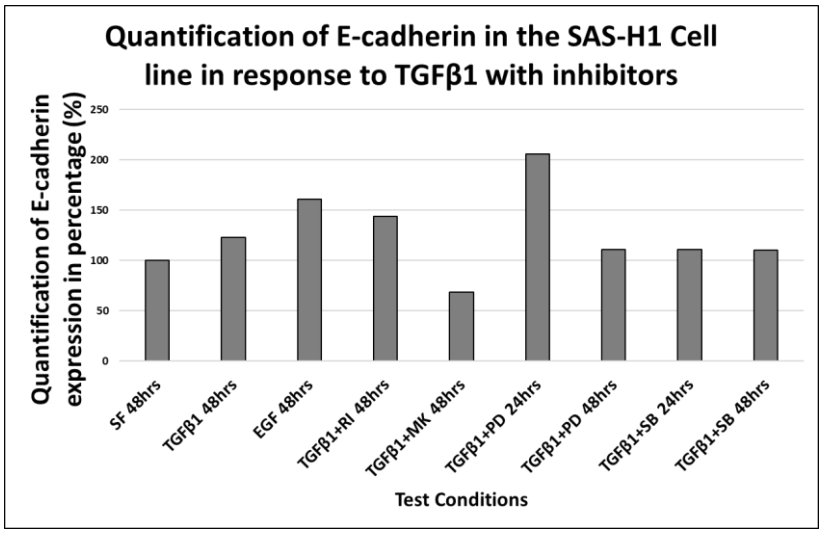


Figure 170: Quantification of E-Cadherin expression in the SAS-H1 cell line.

The bar chart shows the quantification of E-cadherin expression in the SAS-H1 cell line for phase I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from SAS-H1 cells treated with TGFβ1 (10ng/ml)+PD98059 (5μM) after 24 hours showed an increase in E-cadherin expression compared to the negative control. Also, lysates from cells treated with TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours showed less expression of E-cadherin compared to the negative control.

Table 85: Quantification of E-cadherin expression in the SAS-H1 cell line with phase I assessment.

SAS-H1 Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	205.6%
10ng/ml TGFβ1 48 hours	122.9%	**TGFβ1+PD98059 48 hours**	111.1%
10ng/ml EGF 48 hrs	160.9%	**TGFβ1+SB431542 24 hours**	110.8%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	143.6%	**TGFβ1+SB431542 48 hours**	110.2%
TGFβ1+MK-2206 48 hours	68.5%		

(2) N-cadherin:

N-cadherin expression was detected in lysates from SAS-H1 cells treated with TGFβ1 after 48 hours, TGFβ1 + MK-2206 after 48 hours and TGFβ1 + PD98059 after 24 hours and 48 hours (**Figure 171**). Using Image lab software (BioRad), lysates from cells treated with EGF after 48 hours and TGFβ1 + TGFβ RI Kinase Inhibitor VII after 48 hours showed a higher expression of N-cadherin compared to the negative control (**Figure 172**). Smad inhibitor (SB431542) blocked the effect of TGFβ1 on SAS-H1, which

resulted in similar level of N-cadherin expression to the control samples after 24 and 48 hours.

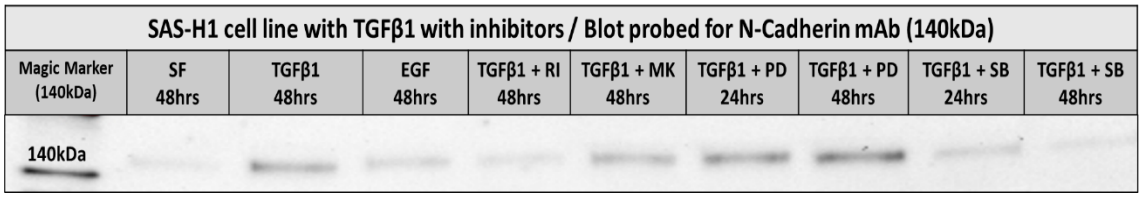


Figure 171: Phase I Assessment of N-cadherin expression in the SAS-H1 cell line.

The SAS-H1 cells were incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with an N-cadherin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Lysates from SAS-H1 cells treated with TGFβ1 (10ng/ml) after 48 hours, TGFβ1 (10ng/ml)+MK2206 (1μM) after 48 hours and TGFβ1 (10ng/ml)+PD98059 (5μM) after 24 hours and 48 hours showed an increase in N-cadherin expression in comparison to the negative control. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 330).

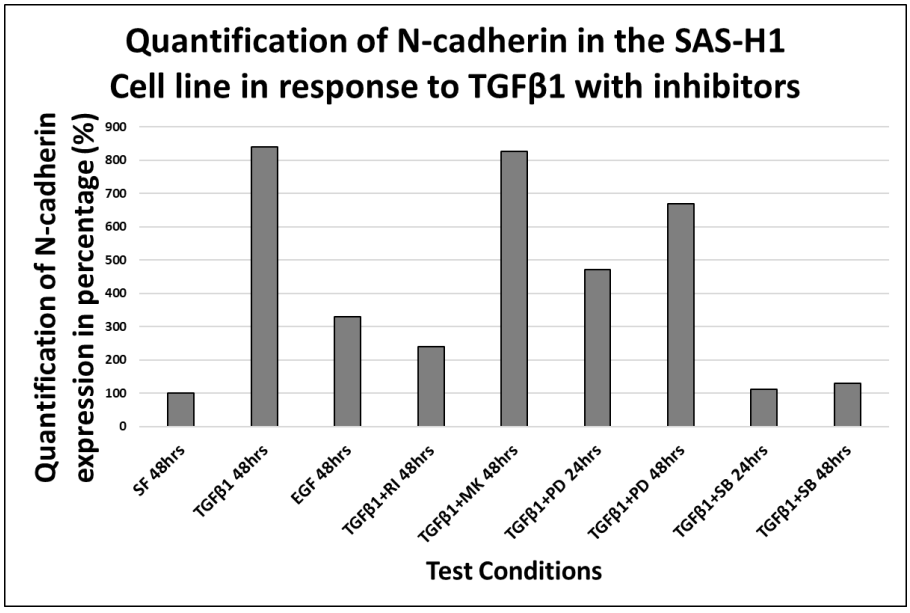


Figure 172: Quantification of N-cadherin expression in the SAS-H1 cell line.

The bar chart shows the quantification of N-cadherin expression in the SAS-H1 cell line for phase I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from SAS-H1 cells treated with TGFβ1 (10ng/ml) after 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours and TGFβ1 (10ng/ml)+PD98059 (5μM) after 24 hours and 48 hours showed an increase in N-cadherin expression compared to the negative control.

Table 86: Quantification of N-Cadherin expression in the SAS-H1 cell line with phase I assessment.

SAS-H1 Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	471.9%
10ng/ml TGFβ1 48 hours	840.8%	**TGFβ1+PD98059 48 hours**	670.1%
10ng/ml EGF 48 hrs	328.8%	**TGFβ1+SB431542 24 hours**	112.4%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	240.5%	**TGFβ1+SB431542 48 hours**	128.9%
TGFβ1+MK-2206 48 hours	826.3%		

(3) β-catenin:

All lysates from SAS-H1 treated with the test conditions of phase I assessment showed expression of β-catenin. The analysis of β-catenin expression using Image lab software (BioRad) showed an increase in the lysates from SAS-H1 cells in response to the test conditions compared to the negative control, except those from cells treated with TGFβ1 + SB431242 after 24 hours and 48 hours. These samples showed similar levels of β-catenin expression to the negative control (**Figure 174**).

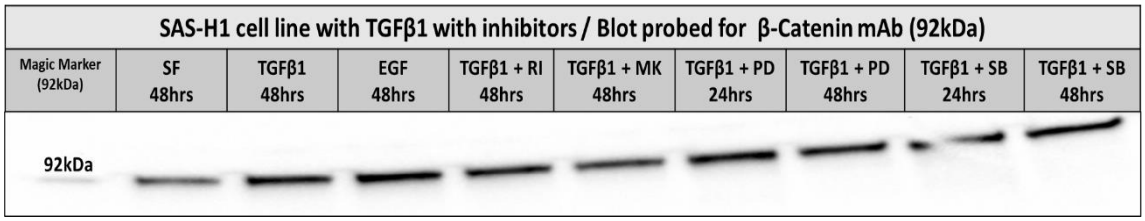


Figure 173: Phase I Assessment of β-catenin expression in the SAS-H1 cell line.

The SAS-H1 cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a β-catenin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from SAS-H1 cells treated with the different test conditions showed β-catenin expression. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 330).

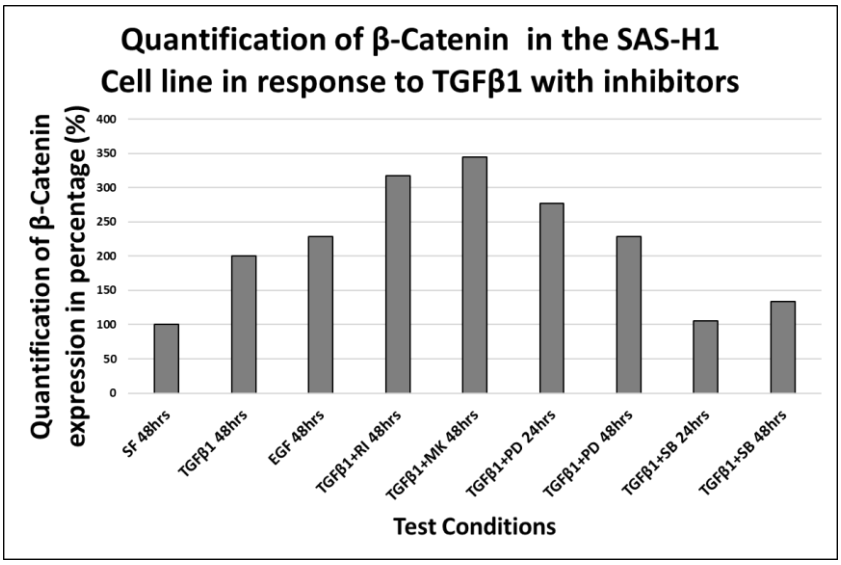


Figure 174: Quantification of β-catenin expression in the SAS-H1 cell line.

The bar chart shows the quantification of β-catenin expression in the SAS-H1 cell line for phase I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from TYS cells treated with TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) after 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) after 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) after 48 hours and TGFβ1 (10ng/ml)+PD98059 (5μM) after 24 hours and 48 hours showed an increase in β-catenin expression compared to negative control. Lysates from the SAS-H1 cells treated with TGFβ1 (10ng/ml)+SB431542 (5μM) after 24 hours and 48 hours showed less β-catenin expression compared to the negative control.

Table 87: Quantification of β-catenin expression in the SAS-H1 cell line with phase I assessment.

SAS-H1 Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	277.2%
10ng/ml TGFβ1 48 hours	200.7%	**TGFβ1+PD98059 48 hours**	228.1%
10ng/ml EGF 48 hrs	228.6%	**TGFβ1+SB431542 24 hours**	105.8%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	317.7%	**TGFβ1+SB431542 48 hours**	133.5%
TGFβ1+MK-2206 48 hours	344.5%		

(4) Claudin-1:

SDS-PAGE and Western showed no evidence of Claudin-1 expression in lysates from SAS-H1 cells treated with SF-MEM for 48 hours, TGFβ1 for 48 hours, EGF for 48 hours, TGF1 + TGFβ- RI Kinase Inhibitor VII for 48 hours, TGFβ1 + MK-2206 for 48 hours, TGFβ1 + PD98059 for 24 and 48 hours, and TGFβ1 + SB431542 (**Figure 175**).

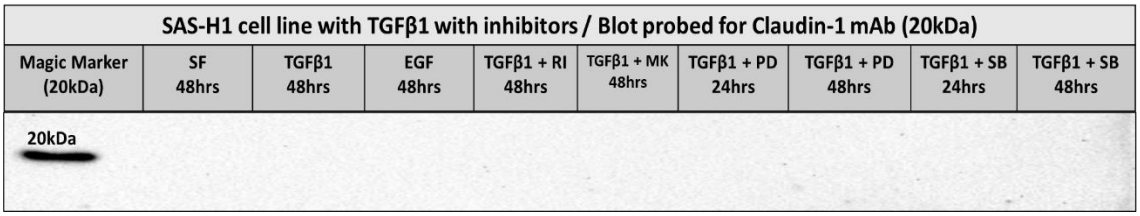


Figure 175: Claudin-1 expression in the SAS-H1 cell line using WB.

The SAS-H1 cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a Claudin-1 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Lysates from SAS-H1 cells treated with different test conditions showed no Claudin-1 expression. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 331).

(5) WAVE-2:

The expression of WAVE-2 was detected in lysates treated with TGFβ1 after 48 hours, EGF after 48 hours and TGFβ1 + PD98059 after 48 hours (**Figure 176**). Using Image lab software (BioRad) to analyse the Western blots, revealed that lysates from EGF-treated cells showed the highest level of WAVE-2 expression compared to the negative control and the other test conditions (**Figure 177**). TGFβ- RI Kinase Inhibitor VII and SB431542 reduced the levels of WAVE-2 in SAS-H1 cells to below the negative control level, suggesting an inhibition of TGFβ1 activity.

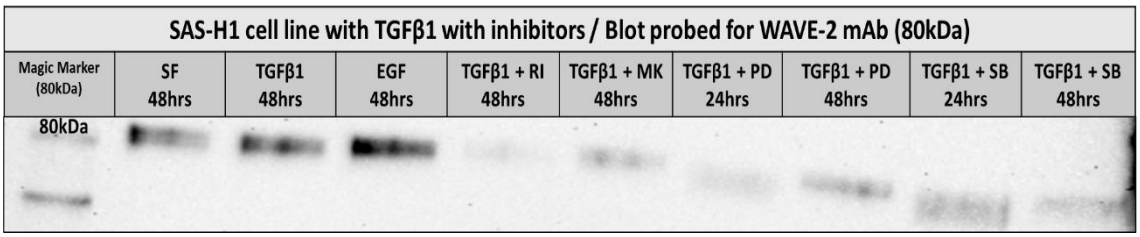


Figure 176: WAVE-2 expression in the SAS-H1 cell line using WB.

The SAS-H1 cells were incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a WAVE-2 monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. Lysates from SAS-H1 cells treated with TGFβ1 (10ng/ml) at 48 hours and EGF (10ng/ml) at 48 hours showed WAVE-2 expression. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 331).

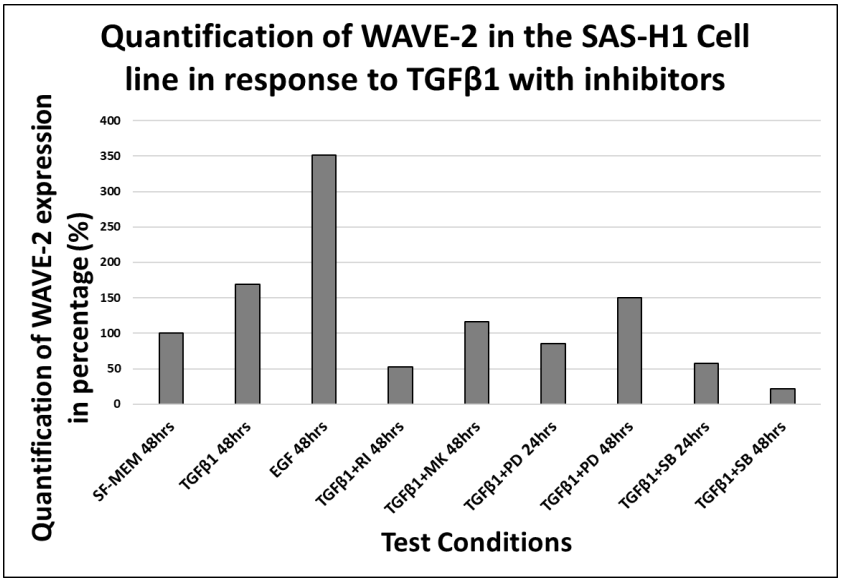


Figure 177: Quantification of WAVE-2 expression in the SAS-H1 cell line.

The bar chart shows the quantification of WAVE-2 expression in the SAS-H1 cell line for phase I assessment. The normalisation of the blot was carried out using Image Lab software (BioRad). The SF-MEM lane was considered as 100% for protein quantification. Lysates from SAS-H1 cells treated with TGFβ1 (10ng/ml) after 48 hours and EGF (10ng/ml) after 48 hours showed an increase in WAVE-2 expression compared to the negative control. Lysates from SAS-H1 treated with TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) after 48 hours and TGFβ1 (10ng/ml)+SB431542 (5μM) after 24 hours and 48 hours showed less WAVE-2 expression compared to the negative control.

Table 88: Quantification of WAVE-2 expression in the SAS-H1 cell line with phase I assessment.

SAS-H1 Cell line with test conditions and incubation periods.			
SF-MEM 48 hours	100%	**TGFβ1+PD98059 24 hours**	85.3%
10ng/ml TGFβ1 48 hours	169.5%	**TGFβ1+PD98059 48 hours**	150.1%
10ng/ml EGF 48 hrs	351.7%	**TGFβ1+SB431542 24 hours**	57.5%
TGFβ1+ TGF-β RI Kinase Inhibitor VII 48 hours	52.1%	**TGFβ1+SB431542 48 hours**	21.3%
TGFβ1+MK-2206 48 hours	116.2%		

(6) Palladin:

Palladin expression was not detected in lysates from SAS-H1 cells treated with SF-MEM for 48 hours, TGFβ1 for 48 hours, EGF for 48 hours, TGFβ1 + TGFβ- RI Kinase Inhibitor VII for 48 hours, TGFβ1 + MK-2206 for 48 hours, TGFβ1 + PD98059 for 24 and 48 hours, and TGFβ1 + SB431542 for 48 hours in phase I assessment (**Figure 178**).

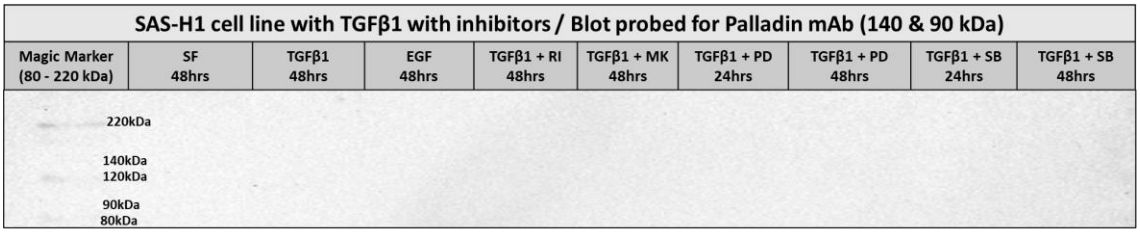


Figure 178: Palladin expression in the SAS-H1 cell line using WB.

The SAS-H1 cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a Palladin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from SAS-H1 cells treated with the different test conditions showed no expression of Palladin. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 331).

(7) Gridin:

In phase I assessment, Gridin expression was not observed in lysates from SAS-H1 cells treated with SF-MEM for 48 hours, TGFβ1 for 48 hours, EGF for 48 hours, TGFβ + TGFβ- RI Kinase Inhibitor VII for 48 hours, TGF β1 + MK-2206 for 48 hours, TGF β1 + PD98059 for 24 and 48 hours, and TGF β1 + SB431542 for 48 hours (**Figure 179**).

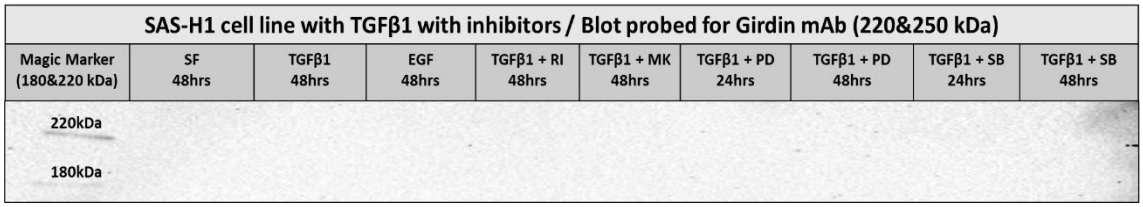


Figure 179: Gridin expression in the SAS-H1 cells using WB.

The SAS-H1 cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a Gridin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from SAS-H1 cells treated with the test conditions of phase I assessment showed no expression of Gridin. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 332).

(8) Vimentin:

Lysates from SAS-H1 cells did not contain vimentin after the cells were treated with SF-MEM for 48 hours, TGFβ1 for 48 hours, EGF for 48 hours, TGFβ1 + TGFβ-RI Kinase Inhibitor VII for 48 hours, TGFβ1 + MK-2206 for

48 hours, TGFβ1 + PD98059 for 24 and 48 hours, or TGFβ1 + SB431542 for 48 hours (**Figure 180**).

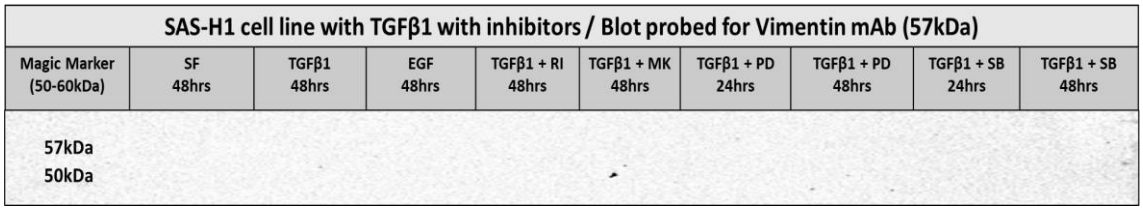


Figure 180: Vimentin expression in the SAS-H1 cell line using WB.

The SAS-H1 cell line was incubated with nine different test conditions: including SF-MEM at 48 hours, TGFβ1 (10ng/ml) at 48 hours, EGF (10ng/ml) at 48 hours, TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM) at 48 hours, TGFβ1 (10ng/ml)+MK-2206 (1μM) at 48 hours, TGFβ1 (10ng/ml)+PD98059 (5μM) at 24 and 48 hours, and TGFβ1 (10ng/ml)+SB431542 (5μM) at 24 and 48 hours. Lysates of the cells were then fractionated by SDS PAGE on 10% acrylamide gels, followed by transfer to PVDF by Western blotting. The blots were then probed with a Vimentin monoclonal antibody at a 1:1000 dilution, followed by goat anti-rabbit secondary. All lysates from SAS-H1 cells treated with the test conditions of phase I assessment showed no expression of vimentin. The figure shows the representative results from the majority of the experiments. Detailed methodology can be found in chapter two and raw data can be found in appendix 6 (page 332).

(B) Phase II Assessment of the Migration Markers:

At this phase of assessment, ICC was used to evaluate four migration markers in terms of their expression and location within the cells. This would establish a link between the state of cells regarding migration and the expression of markers. The proteins investigated were E-cadherin, N-cadherin, β-catenin, and Claudin-1. In addition, the procedure to compute the correct total cell fluorescence (CTCF) was utilised to measure the amount of expression of each of the migration markers with the test conditions described below:

Table 89: Design of Phase II assessment for the SAS-H1 cell line.

Phase II assessment for migration marker in SAS-H1 cell line using ICC			
SF-MEM 24hrs	TGFβ1 24hrs	EGF 24hrs	TGFβ1 + TGF-β RI Kinase Inhibitor VII 24hrs
	TGFβ1 48hrs	EGF 48hrs	TGFβ1 + TGF-β RI Kinase Inhibitor VII 48hrs
SF-MEM 48hrs	TGFβ1 + MK2206 24hrs	TGFβ1 + PD98059 24hrs	TGFβ1 + SB431542 24hrs
	TGFβ1 + MK-2206 48hrs	TGFβ1 + PD98059 48hrs	TGFβ1 + SB431542 48hrs

(1) E-cadherin:

Using SAS-H1 cells and the test conditions of phase II assessment (**Table 89**) helped to evaluate the expression of E-cadherin during cell migration. The assessment was based on comparing the effect of the test conditions on the expression of E-cadherin to the negative control after 24 and 48 hours. The two main criteria used in this assessment were protein expression and localisation within the cells. The SAS-H1 cells incubated with SF-MEM for 24 hours showed typical characteristics of epithelial cells, including a cuboid or round shape and compact colonies. Also, the expression of E-cadherin was detected at the cell membrane and in the cytoplasm (**Figure 181-A**). After 48 hours with SF-MEM, the SAS-H1 cell line showed less compact colonies with a few cells separated from their clusters. As a result the E-cadherin was localised at the cell membrane only, where contact between cells persisted (**Figure 181-B**).

After 48 hours incubation with exogenous TGFβ1, SAS-H1 cells dispersed, colony appearance changed and E-cadherin expression was lost from the cell membrane, accompanied by dense spots of the protein expression detected in the nucleus (**Figure 181-D**). With this test condition, the scattered cells appeared large and round in shape, plus a few cells were still attached to their colonies.

Changes in cell morphology, colony appearance and E-cadherin localisation for SAS-H1 treated with EGF for 24 hours and 48 hours were detected when compared to the negative control (**Figure 181-E&F**). There were few differences compared to TGFβ1-treated culture dishes. First, the cell morphology changed to a spindle shape or large rounded cells with extensions. Second, the expression of E-cadherin in the nucleus was less evident in comparison to cells treated with TGFβ1.

The effect of TGFβ1 on SAS-H1, was mentioned above, was inhibited by adding TGFβ- RI Kinase Inhibitor VII with the growth factor. The expression of E-cadherin was detected only at the cell membrane and cytoplasm, which was similar to the negative control. Cells appeared

as small, round and/or cuboid in tight compact colonies after 24 hours and 48 hours (**Figure 181-G&H**).

SAS-H1 cells incubated with TGFβ1 + MK-2206 showed lower expression of E-cadherin after 24 hours and 48 hours using CTCF protocol compared to the negative control (**Figure 182**). In the case of culture dishes incubated with TGFβ1 + PD98059 for 24 hours, the SAS-H1 cells expressed E-cadherin at the cell membrane and cytoplasm with no morphological changes in comparison to the SF-MEM negative control (**Figure 181-K**). However, after 48 hours incubation with the same test conditions, the SAS-H1 cells separated from their colonies and lost E-cadherin expression at the cell membrane (**Figure 181-L**).

Using a Smad inhibitor (SB431542) with TGFβ1 as a test condition for SAS-H1 cells, induced changes in cell morphology and the localisation of E-cadherin, which was similar to the findings from EGF-treated cells. For example, SAS-H1 cells treated with TGFβ1 + SB431542 after 24 hours, started to change their morphology to a spindle shape, with a few cells separated from their colonies and less E-cadherin expression at the cell membrane compared to the negative control (**Figure 181-M**). After 48 hours with TGFβ1 + SB431542, the SAS-H1 cells scattered, exhibiting a spindle shape and change in the location of E-cadherin expression, which included only the cytoplasm and nucleus (not the cell membrane) (**Figure 181-N**).

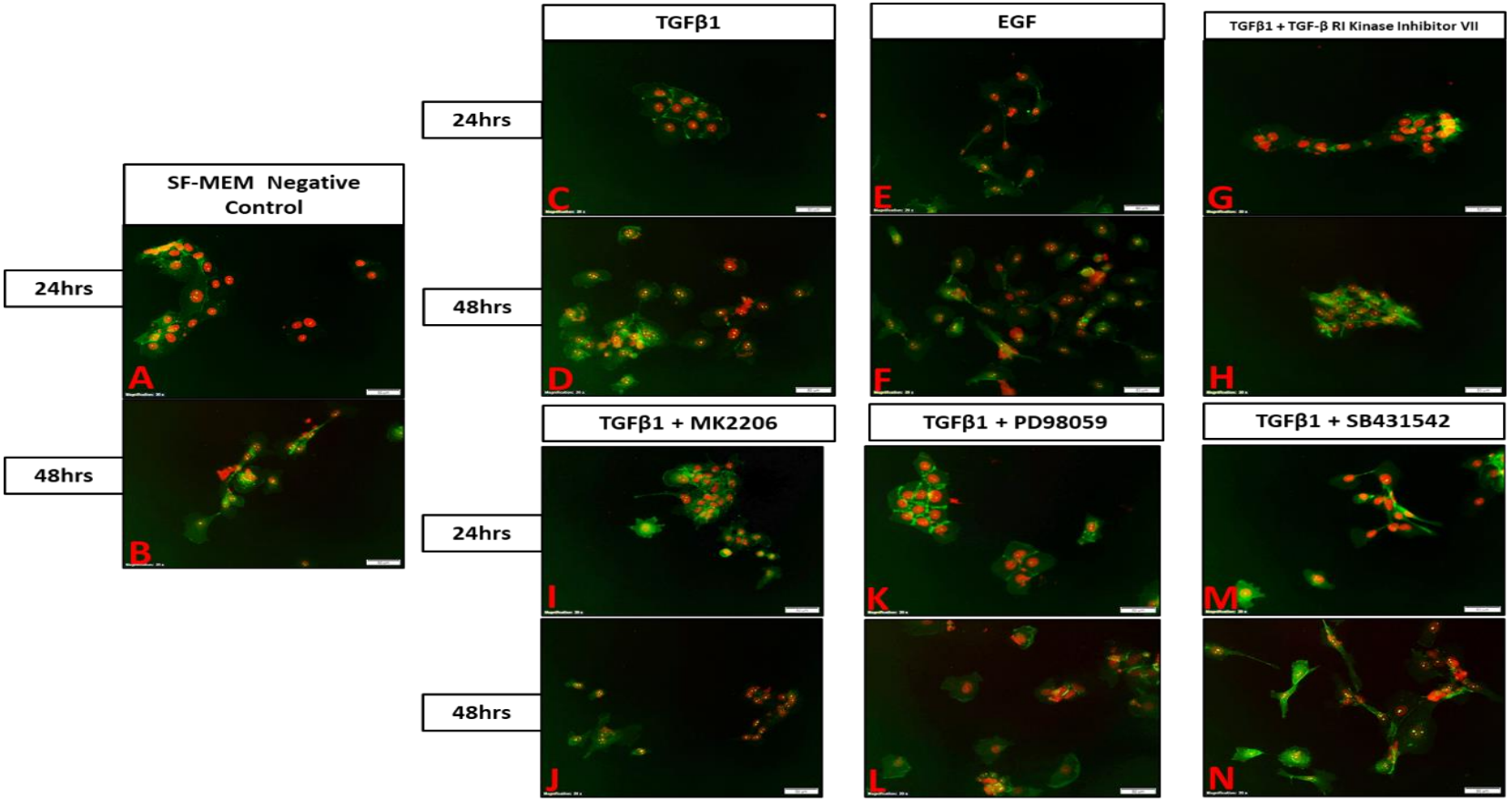


Figure 181: E-cadherin expression in the SAS-H1 cell line using ICC.

Images of SAS-H1 cells that had been treated with seven test conditions (**Table 64**) for 24 hours and 48 hours. The cells were fixed in situ and then prepared for the immunocytochemistry protocol The samples were then probed with an E-cadherin monoclonal antibody at 1:200 dilution, followed by secondary anti rabbit IgG (with fluorescent dye **green colour**) then DAPI (**red colour**). Immunofluorescence Images were taken at 200X magnification. 60mm Culture dishes of SAS-H1 treated with TGFβ1 (10ng/ml) for 48 hours (**D**), EGF (10ng/ml) for 24 hours and 48 hours (**E**, **F**), TGFβ1 (10ng/ml)+PD98059 (5μM) for 48 hours (**L**) and TGFβ1 (10ng/ml)+SB431542 (5μM) for 24 hours and 48 hours (**M**, **N**) showed localisation of E-cadherin in the cytoplasm only compared to the negative control, where it was localised in the cell membrane and cytoplasm.

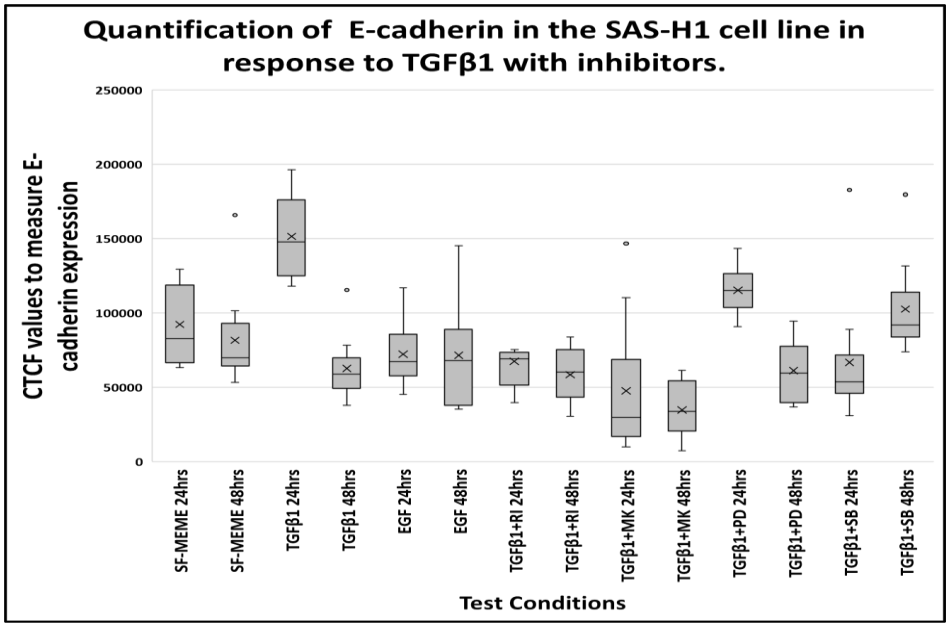


Figure 182: Quantification of E-cadherin expression in the SAS-H1 cell line by CTCF.

The graph shows the quantification of E-cadherin expression using ICC in the SAS-H1 cell line for the phase II assessment. The assessment of protein expression was carried out using ImageJ software and CTCF protocol (n=10). SAS-H1 cells treated with TGFβ1 (10ng/ml) after 24 hours and TGFβ1 (10ng/ml)+PD98059 (5μM) after 24 hours showed an increase in E-cadherin expression compared to the negative control. Raw data can be found in appendix 7 (page 361).

(2) N-cadherin:

SAS-H1 cells treated with TGFβ1 for 48 hours (**Figure 183-D**) and TGFβ1 + PD98059 for 48 hours (**Figure 183-L**) showed a low level of expression of N-cadherin in the immunofluorescence images compared to the negative control. In these test conditions, only a few SAS-H1 cells had N-cadherin expression at the cell membrane. In cells treated with the other test conditions of phase II assessment (**Table 89**), N-cadherin expression in SAS-H1 cells was difficult to visualise using only photomicrographs. The corrected total cell fluorescence (CTCF) methodology was used to measure the expression of the protein in SAS-H1 cells in response to the test conditions of phase II assessment. This analysis showed an increase in N-cadherin expression in the SAS-H1 cells treated with TGFβ1 for 48 hours, EGF for 48 hours and TGFβ1 + PD98059 after 48 hours compared to the negative control (**Figure 184**). Although EGF-treated cells had high levels of N-cadherin expression as revealed using ICC and CTCF protocol, it could not be visualised in the photomicrographs.

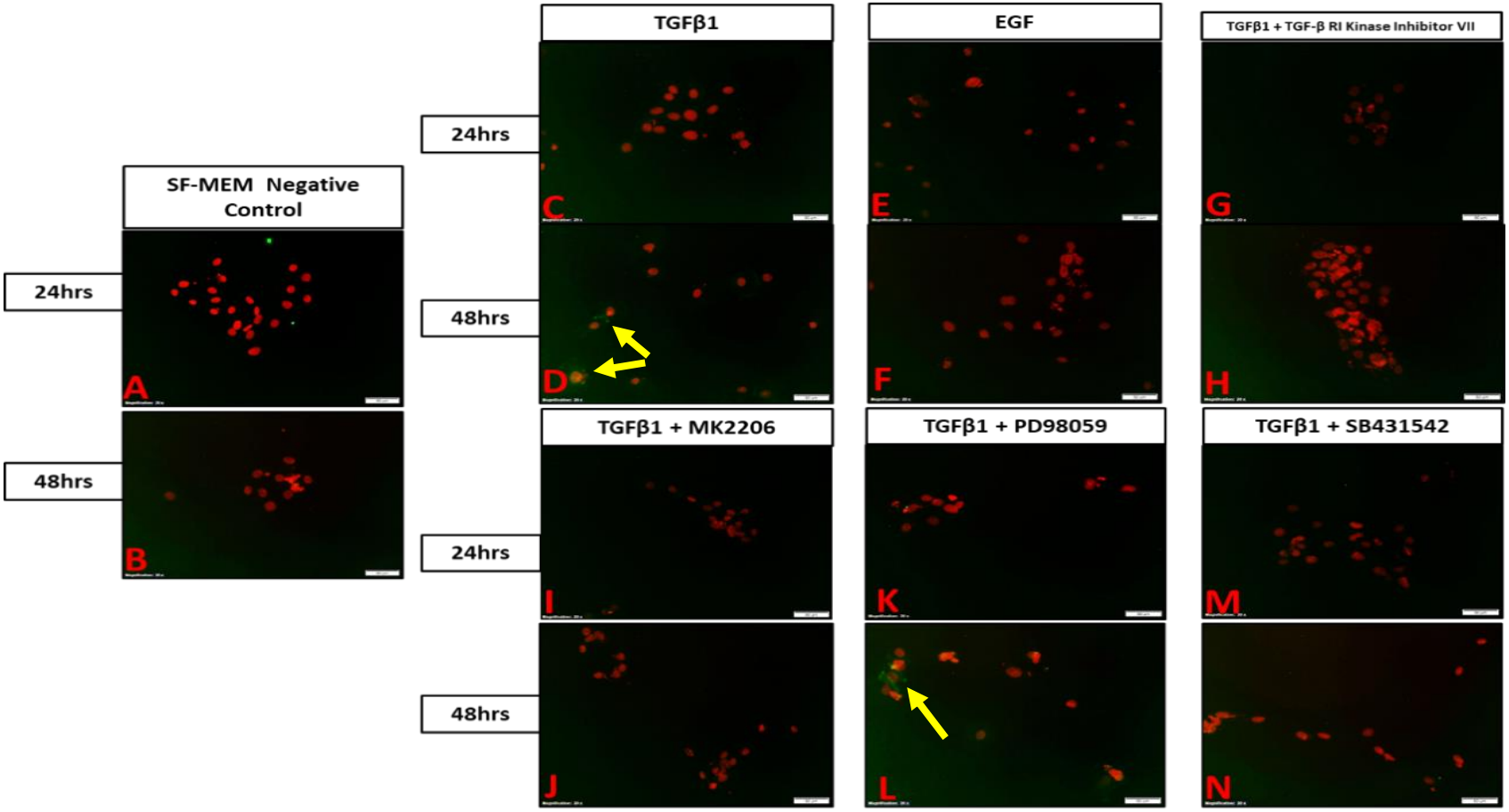


Figure 183: N-cadherin expression in the SAS-H1 cell line using ICC.

Images of SAS-H1 cells that had been treated with seven test conditions for 24 hours and 48 hours (**Table 64**). The cells were fixed in situ and then prepared for the immunocytochemistry protocol The samples were then probed with an N-cadherin monoclonal antibody at 1:200 dilution, followed by secondary anti rabbit IgG (with fluorescent dye **green colour**) then DAPI (**red colour**). Immunofluorescence Images were taken at 200X magnification. 60mm culture dishes of SAS-H1 cells treated with TGFβ1 (10ng/ml) for 48 hours (**D**) and TGFβ1 (10ng/ml)+PD98059 (5μM) for 48 hours (**L**) showed expression of N-cadherin o cell membrane compared to the negative control (yellow arrows).

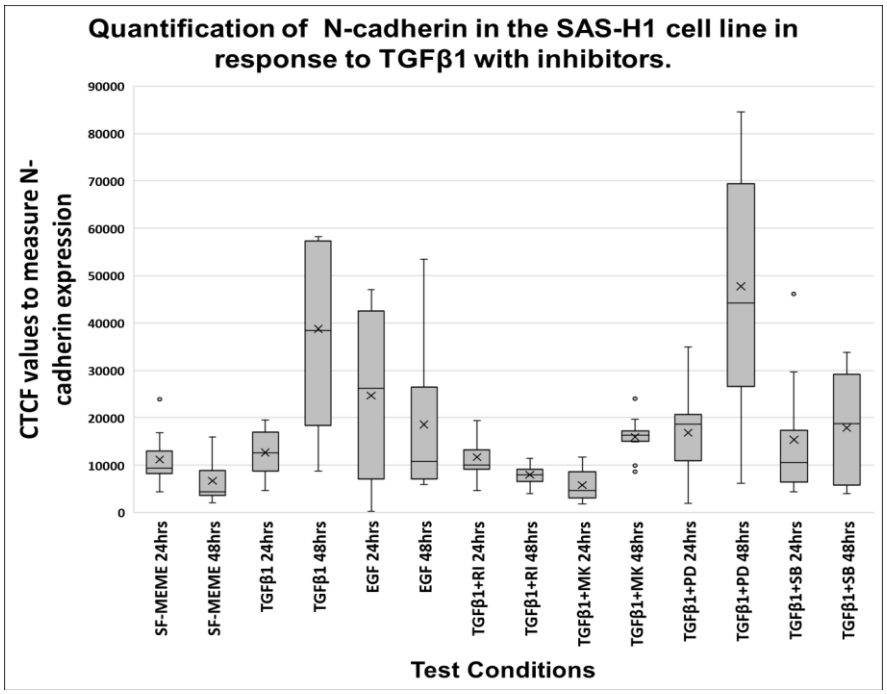


Figure 184: Quantification of N-cadherin expression in the SAS-H1 cell line by CTCF.

The graph shows the quantification of N-cadherin expression using ICC in the SAS-H1 cell line for phase II assessment. The assessment of the protein expression was carried out using ImageJ software and CTCF protocol (n=10). SAS-H1 cells treated with TGFβ1 (10ng/ml) after 48 hours and TGFβ1 (10ng/ml)+PD98059 (5μM) after 48 hours showed an increase in N-cadherin expression compared to the negative control. Raw data can be found in appendix 7 (page 364).

(3) Claudin-1:

Immunofluorescence images that were acquired for assessing SAS-H1 cells treated with the test conditions of phase II assessment () showed no expression of Claudin-1 (**Figure 185**). Using CTCF protocol, the measurements from SAS-H1 cells treated with TGFβ1 for 24 hours, TGFβ1 + Mk-2206 after 48 hours and TGFβ1 + PD98059 for 48 hours of expression of Claudin-1 was lower than the negative control. (**Figure 186**).

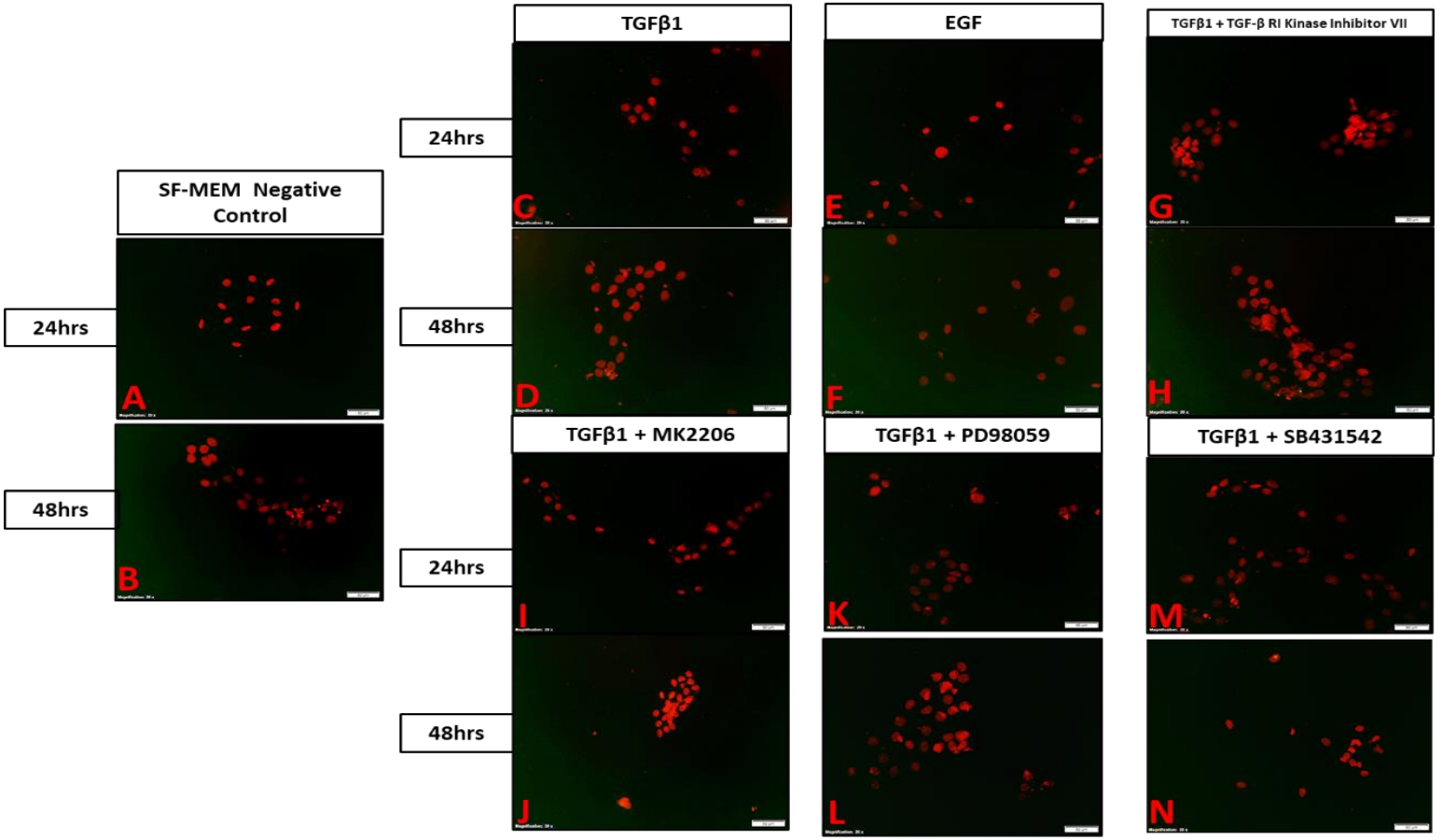


Figure 185: Claudin-1 expression in the SAS-H1 cell line using ICC.

Images of SAS-H1 cells that had been treated with seven test conditions for 24 hours and 48 hours including SF-MEM, TGFβ1 (10ng/ml), EGF (10ng/ml), TGFβ1 (10ng/ml)+TGF-β RI Kinase Inhibitor VII (5μM), TGFβ1 (10ng/ml)+MK2206 (1μM), TGFβ1 (10ng/ml)+PD98059 (5μM), and TGFβ1 (10ng/ml)+SB431542 (5μM). The cells were fixed in situ and then prepared for the immunocytochemistry protocol The samples were then probed with a Claudin-1 monoclonal antibody at 1:200 dilution, followed by secondary anti rabbit IgG (with fluorescent dye **green colour**) then DAPI (**red colour**). Immunofluorescence Images were taken at 200X magnification. No expression of Claudin-1 was observed in any of the SAS-H1 cells treated with the test conditions of the phase II assessment.

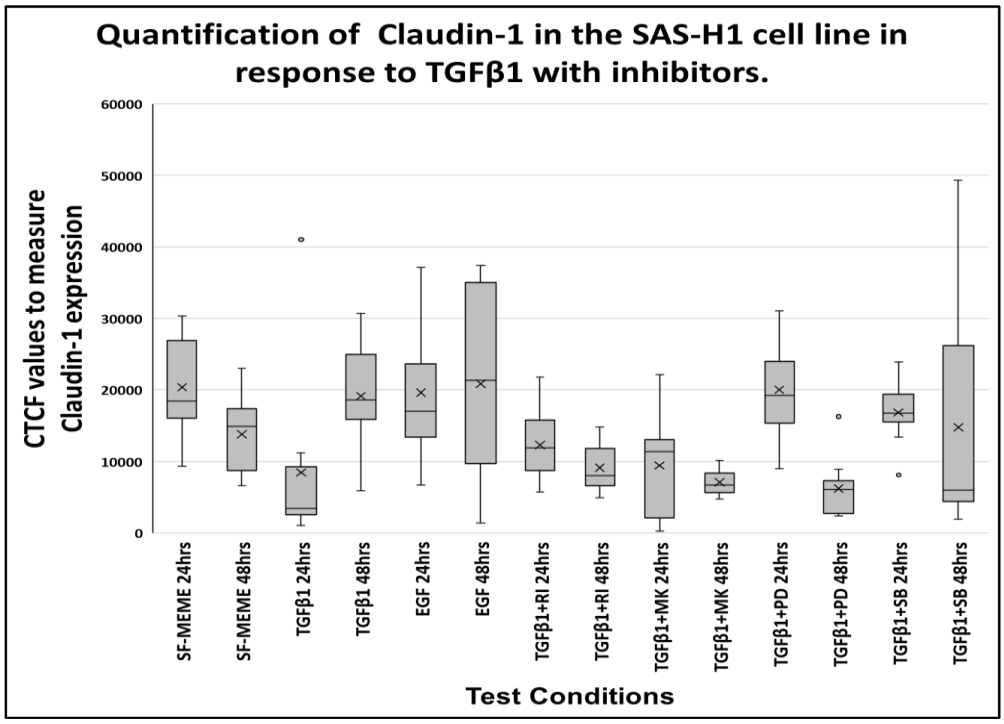


Figure 186: Quantification of Claudin-1 expression in the SAS-H1 cell line by CTCF.

The graph shows the quantification of Claudin-1 expression using ICC in the SAS-H1 cell line for phase II assessment. The assessment of the protein expression was carried out using ImageJ software and CTCF protocol (n=10). SAS-H1 cells treated with TGFβ1 for 24 hours, TGFβ1 + Mk-2206 for 48 and TGFβ1 (10ng/ml)+PD98059 (5μM) for 48 hours showed lower values for Claudin-1 expression. Raw data can be found in appendix 7 (page 368).

(4) β-catenin:

SAS-H1 cell morphology and colony appearance were correlated with β-catenin expression. The SAS-H1 cells treated with SF-MEM for 24 hours displayed a high level of β-Catenin expression. The protein was localised at the cell membrane and cytoplasm (**Figure 187-A**). After 48 hours, two distinct changes were detected in SAS-H1 cells treated with SF-MEM regarding β-catenin expression and cell behaviour. Firstly, β-catenin expression decreased at the cell membrane and secondly the presence of a few single cells separated from their colonies was observed (**Figure 187-B**).

Cultures dishes of SAS-H1 cells incubated with TGFβ1 for 24 hours exhibited comparable amounts of β-catenin expression to those treated with SF-MEM for 24 hours, although less compact colonies and larger round cells were observed (**Figure 187-C**). β-catenin was localised in the cytoplasm of SAS-H1 cells mainly after 48 hours with TGFβ1, and scattered cells were observed (**Figure 187-D**).

After 24 hours and 48 hours incubation with EGF, SAS-H1 cells becoming elongated and spindle-shaped with the loss of cellular contact (**Figure 187-E&F**). β -catenin expression was predominantly detected in the cytoplasm, but not at the cell membrane.

Culture dishes of SAS-H1 treated with TGFβ1 + TGFβ RI Kinase Inhibitor VII after 24 hours and 48 hours showed a resemblance to those incubated with SF-MEM for 24 hours in terms of β-catenin expression, cell shape and colony appearance (**Figure 187-G&H**). This suggests that the inhibitor entirely prevented the growth factor inducing cell migration.

The addition of MK-2206 + TGFβ1 to SAS-H1 cells for 24 hours and 48 hours showed comparable results to TGFβ1 + TGF RI Kinase Inhibitor VII treatment, with the exception of cells being more elongated and a change in colony morphology were observed (**Figure 187-I&J**).

After 48 hours, SAS-H1 cells treated with PD98059 + TGFβ1 showed a slight decrease in β-catenin expression and less compact colonies compared to cells treated with SF-MEM after 24 hours (**Figure 187-K&L**). SAS-H1 cells incubated with TGFβ1 + SB432542 expressed less β-catenin than the negative control. After 24 hours with this growth condition, cells maintained their adherence to neighbouring cells, however, the morphology of the colonies changed and less β-catenin was expressed at the cell membrane compared to the negative control (**Figure 187-M**). After 48 hours with TGFβ1 + SB432542, SAS-H1 cells dispersed, acquired a spindle shape and lacked β-catenin at the cell membrane (**Figure 187-N**)**.**

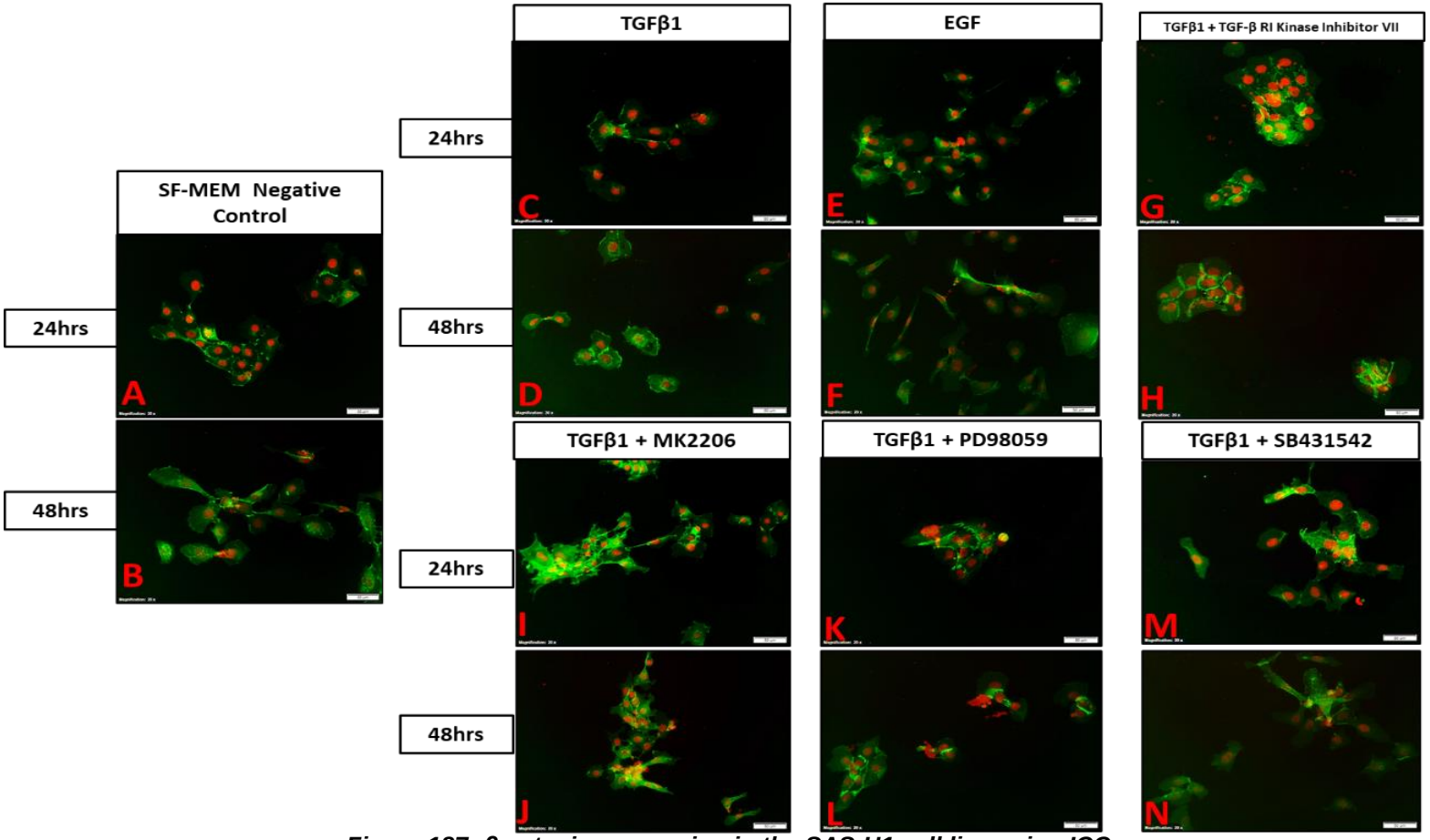


Figure 187: β-catenin expression in the SAS-H1 cell line using ICC.

Images of SAS-H1 cells that had been treated with seven test conditions for 24 hours and 48 hours (**Table 64**). The cells were fixed in situ and then prepared for the immunocytochemistry protocol The samples were then probed with a β-catenin monoclonal antibody at 1:50 dilution, followed by secondary anti rabbit IgG (with fluorescent dye **green colour**) then DAPI (**red colour**). Immunofluorescence Images were taken at 200X magnification. SAS-H1 treated with TGFβ1 (10ng/ml) for 48 hours (**D**), EGF (10ng/ml) for 24 hours and 48 hours (**E**, **F**), and TGFβ1 10ng/ml)+SB431542 (5μM) for 24 hours and 48 hours (**M**,**N**) showed the presence of β-catenin only in the cytoplasm compared to the negative control, which was found in the cell membrane and cytoplasm.

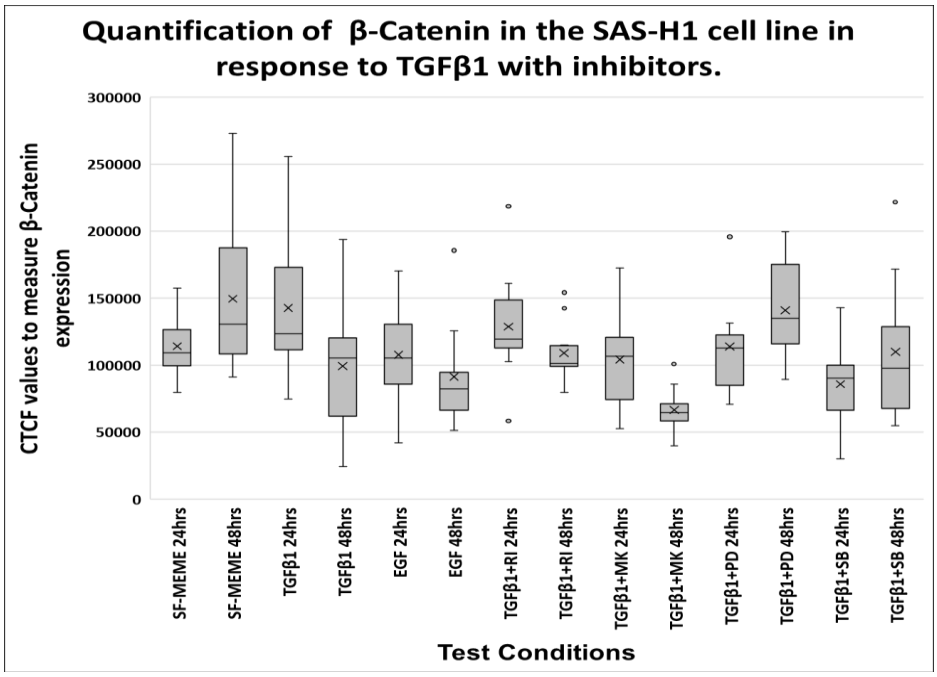


Figure 188: Quantification of β-catenin expression in the SAS-H1 cell line by CTCF.

The graph shows the quantification of β-catenin expression using ICC in the SAS-H1 cell line for phase II assessment. The assessment of the protein expression was carried out using ImageJ software and CTCF protocol (n=10). β-catenin expression in SAS-H1 cells treated with the test conditions of phase II assessment showed similar level to the negative control. Raw data can be found in appendix 7 (page 371).

6.5 Summary of Migration Markers Expression:

In terms of proteins expression, the HaCaT cell line showed no significant changes in the levels of the epithelial markers E-cadherin1 and β-catenin using SDS-PAGE and Western blotting, in response to the test conditions. The only exception was the increase in Claudin-1 expression in the HaCaT cell line after been treated with TGFβ1+ SB431542 for 48 hours, which was not observed with the oral cancer cell lines. This result suggests there is an establishment of the tight junctions between HaCaT cells after been treated with the Smad inhibitor. ICC evaluation of E-cadherin1 and β-catenin in the HaCaT cell line showed they were localised in the cytoplasm after the treatment with exogenous TGFβ1. The expression of N-cadherin was increased significantly in the HaCaT cell line in response to TGFβ1 after 48 hours, TGFβ1+ MK-2206 after 48 hours and TGFβ1+ PD98059 after 48 hours.

For oral cancer cell lines, only two epithelial markers were expressed: E-cadherin and β-catenin. However, both proteins were presence in the cytoplasm after cells were treated with TGFβ1 after 48 hours, EGF after 48 hours, TGFβ1+ PD98059 after 48 hours and TGFβ1+ SB431542 after 48 hours. The change of E-cadherin

and β-catenin localisation from the cell membrane to the cytoplasm only was not observed after the culture dishes been treated with TGFβ1+ TGF-β RI Kinase Inhibitor VII after 48 hours and TGFβ1+ MK-2206 for 48 hours. These findings suggested that the Akt pathway has a role in regulation of cell adhesion molecules. In terms of mesenchymal marker expression, N-cadherin was expressed in oral cancer cell lines after treatment with exogenous TGFβ1 after 48 hours, TGFβ1+ MK-2206 after 48 hours and TGFβ1+ PD98059 after 48 hours. N-cadherin expression in oral cancer cell lines (TYS and SAS-H1) indicates the acquisition of mesenchymal features by the tumour cells.

6.6 Discussion:

It was established from previous chapters that TGFβ1 induced cell migration in normal and oral cancer cell lines. The assessment included looking at the mechanisms that cells utilised to acquire motility by investigating the expression of proteins including epithelial markers, mesenchymal markers and proteins linked to the formation of lamellipodia and filopodia. Those migration markers were selected based on their link to epithelial to mesenchymal transition (EMT). It has been reported that EMT is identified by cells losing their epithelial components, especially at the cell junctions such as E-cadherin and acquiring mesenchymal cell characteristics including motility and invasiveness (Massagué, 2008; Ling, Cheng and Tao, 2021).

The expression of those migration markers was investigated in response to the same test conditions in “Chapter 6: Investigating the Expression of Signalling Pathways Molecules following TGFβ1 activation”. The assessment for the migration markers was carried out at two different stages. The first phase of assessment included using SDS-PAGE and Western blotting to evaluate the expression of a total of eight markers: E-cadherin, N-cadherin, β-catenin, Claudin-1, WAVE2, Palladin, Gridin and vimentin. The second phase of assessment included only four markers, E-cadherin, N-cadherin, β-catenin and Claudin-1, to evaluate their expression and localisation within the cells.

The assessment of E-cadherin expression in normal and oral cancer cell lines using SDS-PAGE, Western blotting and ICC showed no reduction in the protein levels in response to TGFβ1 and EGF. This was not the case when samples of breast cancer (Hazan *et al.*, 2004) and oral cancer showed a positive correlation between low E-cadherin expression and tumour progression (Zhong *et al.*, 2007; Attramadal *et al.*, 2015; López-Verdín *et al.*, 2019). Also, during EMT, loss of E-cadherin expression was associated with tumour development (Kourtidis *et al.*, 2017; Kushwaha *et al.*, 2019). Using the immunocytochemistry (ICC) assay to evaluate E-cadherin expression showed different localisation patterns of the protein compared to the negative control, where E-cadherin was mainly observed at the cell membrane with closely compact colonies. All three cell lines showed a change of E-cadherin from the cell membrane to the cell cytoplasm in response to TGFβ1 compared to the negative control. These findings agreed with results from metastatic cancers, where the presence of E-cadherin decreased at the cell

membrane and increased in the cytoplasm (Pannone *et al.*, 2014). According to a few studies, the presence of E-cadherin in the cytoplasm rather than cell membrane indicates a poor prognosis (Gupta *et al.*, 2018; Bendardaf *et al.*, 2019). Other *in vitro* studies on 2D cultures referred to the redistribution of E-cadherin as an outcome of only the scattering mode of cell migration rather than EMT (Grünert, Jechlinger and Beug, 2003). In addition, the ability of cancer cells to not lose the expression of their epithelial markers might indicate cells undergo partial EMT, which has been described to be an effective mechanism for tumour metastasis (Saitoh, 2018). Maintaining the cellular adhesion of cancer cells plays a role in their collective migration (Saitoh, 2018).

TGF-β RI Kinase Inhibitor VII blocked the migration of normal and oral cancer cell lines caused by TGFβ1. This inhibitory effect was manifested by the presence of E-cadherin at the cell membrane compared cells treated with the growth factor only. Other inhibitors also prevented TGFβ1 from causing cell migration and helped to maintain the cell membrane expression of E-cadherin. The effect of those inhibitors depended on the cell type, for example, SB431542 in the case of HaCaT cell line and MK-2206 in case of TYS and SAS-H1. The upregulation of E-cadherin is associated with preventing tumour cells from migrating (Kourtidis *et al.*, 2017).

In contrast to E-cadherin, N-cadherin was expressed in the normal and oral cancer cell lines in response to TGFβ1 as assessed with SDS-PAGE, Western blotting and ICC. The HaCaT, TYS and SAS-H1 cells showed high levels of N-cadherin after been incubated with TGFβ1 for 48 hours, which also resulted in cell migration compared to the negative control. These findings are in agreement with different studies which reported the positive expression of N-cadherin in lung cancer, prostate cancer and oral squamous cell carcinoma (Cao, Wang and Leng, 2019). Also, N-cadherin is considered as prognostic biomarker based on reports indicating that positive expression of the protein was 40% of invasive bladder cancer samples (Abufaraj *et al.*, 2017) and 51.9% of invasive ductal carcinoma samples (ElMoneim and Zaghloul, 2011). A meta-analysis for patients with different epithelial cancers showed the upregulation of N-cadherin is associated with poor prognosis and a more aggressive tumour phenotype (Luo *et al.*, 2018). For example, in patients with breast cancer (Baranwal and Alahari, 2009) and OSCC, overexpression of N-cadherin is associated with poor

prognosis (Chandolia *et al.*, 2017). A specific link was established between the expression of N-cadherin and the ability of cells to separate from their primary tumour mass (Islam *et al.*, 1996; Kuphal and Bosserhoff, 2006). This report was supported by different *in vitro* and *in vivo* experiments, which demonstrated a positive correlation between an increase in N-cadherin expression and the enhancement of the migratory capabilities of tumour cells (Cao, Wang and Leng, 2019). A few reports about the role of N-cadherin in tumour metastasis suggested the ability of the protein to increase matrix metalloproteinase-9 levels, to induce cell migration by activating the Fibroblast growth factor receptor (FGFR) pathway or to give the tumour cells the ability to adhere to the endothelial cells of blood vessel to avoid anoikis (Cao, Wang and Leng, 2019).

The expression of N-cadherin was low after adding TGF-β RI Kinase Inhibitor VII and SB431542, which indicates both inhibitors blocked the induction by TGFβ1 of N-cadherin expression. These results are in accord with reports from different studies indicating that TGFβ1 induces tumour progression via overexpression of N-cadherin (Cao, Wang and Leng, 2019). This process is mainly regulated by the Smad-dependent pathway (Yang *et al.*, 2015). MK-2206 (Akt inhibitor) and PD98059 (MAPK inhibitor) had no effect on N-cadherin expression in cells in response to TGFβ1, which suggests that there is no role of PI3K-Akt or Erk1/2-MAPK signalling pathways in N-cadherin upregulation. These outcomes are in conflict with the reports that support the activation of PI3K-Akt and MAPK leading to high N-cadherin expression in tumour cells (Cao, Wang and Leng, 2019). Using the ICC assay, the presence of N-cadherin was observed at the cell membrane. HaCaT and SAS-H1 cell lines showed a higher expression of N-cadherin compared to the TYS cell line after been treated with TGFβ1 and TGFβ1+ PD98059 for 48 hours.

For β-catenin expression in response to TGFβ1 and EGF, as quantified by SDS-PAGE and Western blotting showed an increase in the protein level in both normal and oral cancer cell lines. However, lysates from the TYS cell line incubated in TGFβ1+ PD98059 for 48 hours and TGFβ1 + SB431542 for 48 hours showed a decrease in β-catenin expression compared to the negative control. In the test conditions that induced cell migration of normal and oral cancer cell lines, such as TGFβ1 and EGF, β-catenin expression was localised in the cytoplasm only, especially around the nucleus compared to the negative control. The

relocation and accumulation of β-catenin in the nucleus was found in most types of cancer. The accumulation of β-catenin around the nucleus is linked to the induction of cancer stem cells traits by transactivation of the genes for cell adhesion molecules along with lymphoid enhancer factor/T-cell factor (Basu, Cheriyamundath and Ben-Ze'ev, 2018).

Claudin-1 expression was assessed using SDS-PAGE, Western blotting and ICC techniques. Claudin-1 was only expressed in the HaCaT cell line in response to the test conditions. The location of Claudin-1 within the cell was determined by using ICC. The expression of the protein was only seen in closely compact cell colonies. The expression of Claudin-1 was increased and associated with no cell migration, which was observed with the HaCaT cells treated with TGFβ1 + SB431542 for 48 hours. This is in agreement with studies that reported that Claudin-1 had a lower level of expression in different types of invasive cancers such as oesophageal and breast cancers (Bhat *et al.*, 2020). However, a report of Claudin-1 overexpression was found in oral squamous cell carcinoma (Oku *et al.*, 2006), while another study reported the absence of Claudin-1 from oral cancer samples linked to poorly differentiated tumours (Lourenço *et al.*, 2010).

The effect of TGFβ1 on WAVE-2 expression was investigated using normal and oral cancer cell lines, having been linked previously to the formation of filopodia via actin polymerisation (Bompard and Caron, 2004). The HaCaT and SAS-H1 cells showed an increase in WAVE-2 expression in response to TGFβ1 and EGF. In the case of SAS-H1, the inhibitors that were used kept the WAVE-2 expression at low level. The HaCaT cell line maintained high levels of WAVE-2 expression, even with the presence of the inhibitors. With the TYS cell line, WAVE-2 expression with TGFβ1 for 48 hours and EGF for 48 hours was less than the negative control. TYS cells treated with PD98059 + TGFβ1 showed a lower level of WAVE-2 compared to samples treated with TGFβ1 alone. All these data are in agreement with the report about the role of actin binding activity of WAVE2 in both actin polymerisation and branching (Suetsugu *et al.*, 2001).

In response to the different test conditions, Palladin was not found in any of the three cell lines. However, previous reports have stated that Palladin was found in pancreatic cancer and colorectal cancer (Ryu *et al.*, 2001). This suggests the cytoskeletal rearrangement related to invadopodia formation via Palladin was not

utilised by migratory cells, which might also indicate that different types of cancers achieve metastasis via different mechanisms.

Similar to Palladin, Gridin was not expressed in normal and oral cancer cell lines in response to the test conditions. However, studies have that reported that Gridin was expressed highly in colon cancer cells, which indicates a metastatic potential (Ghosh *et al.*, 2008). This expression of Gridin enhances PI3K-Akt signalling activities regarding actin remodelling during cancer cell migration (Ghosh, Garcia-Marcos and Farquhar, 2011).

Although vimentin is considered as a canonical marker of EMT (Thiery, 2002), it was not expressed in any of the three cell lines in response to the test conditions. A few studies have reported the association of high expression of vimentin with invasive forms of different cancers, such as breast cancer (Gilles *et al.*, 2003) and prostate cancer (Lang *et al.*, 2002). The upregulation of vimentin expression influences the induction of EMT by both Ras and Slug (Vuoriluoto *et al.*, 2011). Palladin, Gridin and vimentin were not detected using SDS-PAGE and Western bloting in normal and oral cancer cell lines. This suggests two possibilities either they were not present in the cells or they were presence in levels below the sensitivity of the assay. Therefore, a further investigation is needed to be carried out using a positive control sample for each of those proteins.

Chapter 7: General Discussion

7.1 Summary of Results:

To highlight the main findings from the different experiments of this project, a summary of the results from each experiment is given below:

- TGFβ1 promotes both normal and oral cancer cell lines to undergo single and collective cell migration.
- The induction of cell migration by TGFβ1 was regulated by a specific signalling pathway depending on the cell type. The migratory effect of TGFβ1 was controlled by different signalling pathways dependent on the cell line.
- The migration of the HaCaT cell line was regulated by the Smad signalling pathway.
- The TYS cell migration was controlled by the PI3K/Akt signalling pathway.
- The SAS-H1 migration was regulated by the Erk1/2/MAPK signalling pathway.
- TGF-β RI Kinase Inhibitor VII inhibits cell migration for both normal and oral cancer cell lines.
- TGFβ1 activated signalling pathways (Smad, PI3K/Akt, Erk1/2/MAPK and Rho-GTPase) in this project. TGFβ1 did not activate p-Rac1/Cdc42 in the HaCaT cell line and TYS cell line.
- TGFβ1 did not utilise the Smad signalling pathway to induce cell migration of the SAS-H1 cell line.
- The HaCaT cell line showed no change in the levels of the epithelial markers E-cadherin, Claudin-1 and β-catenin after been treated with TGFβ1, as quantified by SDS-PAGE and Western blotting. However, there was an increase of Claudin-1 in the HaCaT cell line after been treated with TGFβ1+ SB431542 for 48 hours. ICC evaluation of E-cadherin1 and β-catenin showed a change in the localisation of both proteins from cell membrane to cytoplasm after treatment with exogenous TGFβ1 and this was prevented by TGF-β RI Kinase Inhibitor VII and SB431542. The expression of N-cadherin in HaCaT cells was increased in response to TGFβ1.
- In the TYS and SAS-H1 cell lines, only two epithelial markers were expressed, E-cadherin and β-catenin. The localisation of these proteins changed from the cell membrane to cytoplasm after the cells were treated

with TGFβ1, but the redistribution of epithelial markers was not observed in the presence of TGF-β RI Kinase Inhibitor VII and MK-2206.

- N-cadherin was expressed in oral cancer cell lines after treatment with exogenous TGFβ1, which indicates the acquisition of mesenchymal features by the tumour cells.

7.2 General Discussion:

TGFβ signalling in a physiological context, regulates the development of both epithelial and neural tissues, the immune system and wound healing (Massagué, 2008). Also, TGFβ has a role in preventing the incipient tumour from progressing toward malignancy (Massagué, 2008; Jensen *et al.*, 2015). This involves the influence of TGFβ on cell proliferation, cell survival, cellular adhesion, cell differentiation and the cellular microenvironment. However, cancer cells could utilise TGFβ signalling to their own advantage by two main mechanisms. First, tumour cells could suppress the inhibitory arm of the TGFβ signalling pathway. The second mechanism is by using the pathway to induce cell migration, invasion, proliferation and evasion of immune surveillance (Massagué, 2008).

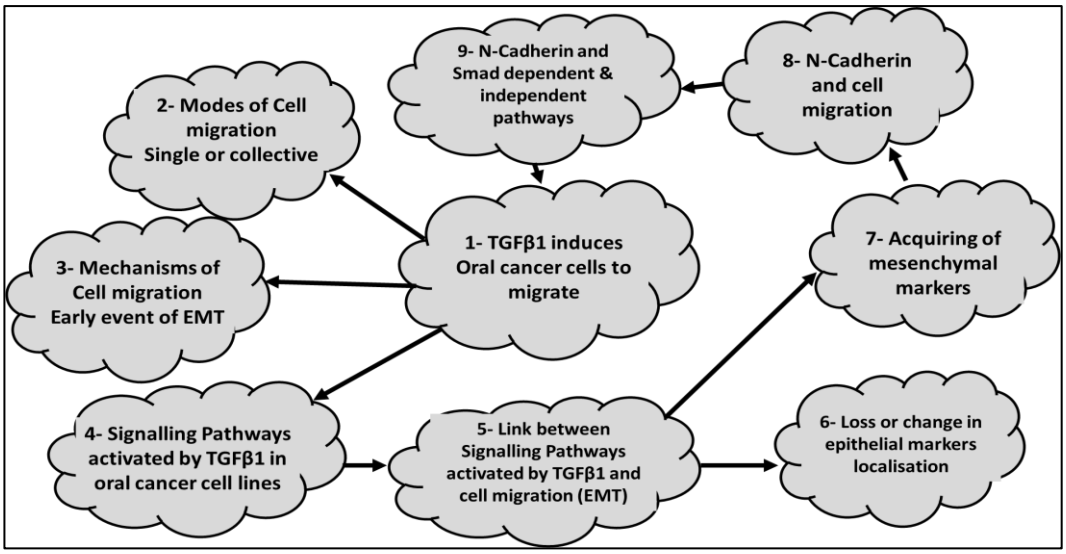


Figure 189: Illustration of the link between TGFβ and cell migration induction in oral cancer cells.

This figure shows the possible relation between the activation of TGFβ signalling pathways and oral cancer cell migration via EMT.

The role of TGFβ in cell migration via EMT in oral cancer cell lines was investigated. The assessment involved the effect of the growth factor in terms of cell migration, activation of the Smad-dependent and Smad-independent signalling pathways and also the effect of TGFβ on epithelial and mesenchymal

marker expression. In this project, EGF was used as positive control for cell migration via EMT based on previous work by Islam (Islam *et al.*, 2017) and others utilising OSCC cell lines (Xu *et al.*, 2017). The assessment of cell migration was based on the concept that cells have to go through cell polarisation, formation of leading protrusions, interaction with ECM and finally the contraction of the rear of the cells (Paňková *et al.*, 2010), which implies cells have to change their morphology to migrate.

Data from the migration assays (Scatter assay and Scratch assay) indicates that TGFβ1 induces cells motility by two modes of cell migration, single and collective cell migration. These finding are in agreement with reports about the ability of cancer cells to adapt different migration methods to invade the underlying tissue either individually or collectively (Saitoh, 2018; Janiszewska, Primi and Izard, 2020). Single cell migration of epithelial tumour cells is established by separation of the cells from the colony by losing cell-cell adhesion and changing cell morphology (Saitoh, 2018; Janiszewska, Primi and Izard, 2020). In contrast, the main feature of collective cancer cell migration is maintaining the cell-cell adhesion of group of cells while moving together (Saitoh, 2018; Janiszewska, Primi and Izard, 2020). Using TGF-RI Kinase Inhibitor VII with oral cancer cell lines blocked the migratory effect of TGFβ1 on these cells. For the collective cell migration assessment, it appeared that multiple signalling pathways have roles in the process, indicated by the ability of different inhibitors to prevent oral cancer cells from migration in the Scratch assay. The involvement of multiple signalling pathways might explain the hallmark of collective cell migration, where cells can maintain their physical and functional connection and the sustainability of cell-cell adhesion between the cells (Janiszewska, Primi and Izard, 2020).

Also, the migration assays revealed no features of a mesenchymal-type of cell migration of oral cancer cell lines, after being treated with exogenous TGFβ1, which includes elongated spindle-like shapes (Paňková *et al.*, 2010). This outcome would not support the occurrence of the transition from an epithelial to mesenchymal phenotype (EMT). The general definition of EMT describes the process as losing cell polarity, cell to cell adhesion and cell to matrix adhesion of the epithelial cells, in addition to acquiring mesenchymal cell features such as cell motility (Thiery, 2002). The earliest events during the EMT process were described to be loss of cell polarity and breakdown of tight junctions (Thiery,

2002). However, this order does not indicate a lineage switch (Thiery and Sleeman, 2006) which can be defined as an all or nothing change between epithelial and mesenchymal cell types (Basu, Cheriyamundath and Ben-Ze'ev, 2018). Reports from *in vivo* studies described the EMT program as a stepwise process during carcinogenesis (Basu, Cheriyamundath and Ben-Ze'ev, 2018), which indicates that full EMT is not required for tumour metastasis. All in all, changes in the morphology of cells, loss of their cell-to-cell adhesion and obtaining mesenchymal characteristics are essential for the transition (Thiery, 2002).

Based on the evidence from embryogenesis and tumour progression, it indicates that TGFβ induces cell migration via EMT (Saitoh, 2018). Also, reports have highlighted the overexpression of TGFβ in cancer tissues (Saitoh, 2018) such as breast cancer (Thiery, 2002) and oral cancer (Qiao, Johnson and Gao, 2010), where TGFβ was linked to regulation of EMT. This is supported by data from studies on human carcinoma samples, which showed the features of cells that had undergone EMT, in an environment rich with stromal TGFβ and other cytokines linked to EMT induction (Massagué, 2008). The activation of Smad-dependent and Smad-independent signalling pathways in oral cancer cells and their links to cell migration were also investigated.

The involvement of different TGFβ signalling pathways such as Smad, PI3K-Akt, Erk1/2-MAPK and Rho-GTPase signalling pathways in oral cancer cell migration was hypothesised. Although it was difficult to identify a specific role for each of the signalling pathways in oral cancer cell migration, their influence on the process was evident. An example of this was when the SAS-H1 cell line utilised TGFβ to activate the Smad-independent signalling pathway to induce cell migration. To explain why the Smad-dependent pathway was not involved in the process of cell migration, a further investigation will be needed. This investigation might include the assessment of early activation of the Smad pathway and the evaluation of the downstream activation of the Smad-dependent and independent pathways. Also, identifying the transcription factors linked to those signalling pathways and cell migration. This comes in agreement with studies suggesting the regulation of TGFβ during EMT is based on the combination of the Smad-dependent (Lv *et al.*, 2021) and Smad-independent pathways (Massagué, 2008). Those studies indicate that the Smad-dependent pathway is responsible for the

transcriptional event, while the Smad-independent pathways mediate the effect on cell junctions (Massagué, 2008). Also, reports indicated communication between TGFβ signalling pathways for example, the Ras signalling pathway enhances the effect of TGFβ inducing EMT via the Smad signalling pathway (Massagué, 2008). Other evidence for the role of TGFβ signalling is when patients with breast cancer that had metastasised to lung and liver showed positive biopsy staining for p-Smad2 (Massagué, 2008). These studies support the notion of TGFβ mediating cell migration via EMT by activating the Smad-dependent pathway (Lv *et al.*, 2021) and other Smad-independent pathways such as PI3K/Akt (Hong *et al.*, 2009; Wang and Chen, 2021) and/or the Erk/MAPK pathways (Thiery, 2002).

The final phase of this project was focusing on aberrant expression of epithelial, mesenchymal and migration markers that are linked with the EMT program. The expression of eight different markers was evaluated. This method of assessment was based on epithelial cells having to lose their epithelial markers and acquire mesenchymal markers to undergo EMT (Thiery, 2002). The best example of this concept is the cadherin switch which is considered to be one of the hallmarks of EMT, featuring a loss of E-cadherin (epithelial marker) and upregulation of N-cadherin (mesenchymal marker) (Janiszewska, Primi and Izard, 2020). The cadherin switch leads from cellular adhesion mediated by E-Cadherin, which includes cell to cell adhesion and cell to basement membrane, to N-Cadherin-dependent adhesion (Pyo *et al.*, 2007). This switch allows cells to adhere to collagen in the extracellular matrix instead of the basement membrane (Janiszewska, Primi and Izard, 2020). Regarding the expression of the epithelial markers, especially E-Cadherin in the oral cancer cell lines, downregulation was not observed. However, a consistent increase in N-cadherin levels in normal and oral cancer cell lines, in response to exogenous TGFβ1, was detected. The expression of N-Cadherin varied depending on the type of cell line, which might help with estimating the state of tumour progression (Walker, Frei and Lawson, 2014; Rai K and Ahmed, 2019). The upregulation of N-cadherin expression was inhibited by TGF-RI Kinase Inhibitor VII, which indicates the involvement of TGFβ1 signalling pathways in the increase of the mesenchymal markers in those epithelial cells. Various studies on different types of carcinomas associated cancer cell invasion with high N-cadherin expression (Abufaraj *et al.*, 2017). This supports the evidence regarding the involvement of N-cadherin in regulating the

Rho-family of GTPases and the Wnt signalling pathways (Janiszewska, Primi and Izard, 2020). Both of these pathways were linked to an aggressive cancer phenotype especially with the ability to migrate to secondary sites (Janiszewska, Primi and Izard, 2020).

In an attempt to correlate the findings from the migration assays and the expression of N-cadherin by the Immunocytochemistry assay, results from both experiments were combined. These results showed two main outcomes related to the normal and oral cancer cell lines that were used in this project. Cell migration and N-cadherin expression increased with TGFβ1 and were blocked by the TGF-RI Kinase Inhibitor VII. This correlation suggests that the migration of oral cancer cells via EMT was regulated by one of the TGFβ1 signalling pathways. However, it was difficult to identify a specific TGFβ signalling pathway responsible for both cell migration and N-cadherin expression in normal and oral cancer cell lines. This result highlights the involvement of more than one signalling pathway activated by TGFβ during EMT or that the activation of signalling pathways to induce cell migration is dependent on the cell type. The Smad-dependent pathway might have the major role in regulation cell migration of normal and oral cancer cell lines and the Smad-independent pathways, PI3K/Akt and Erk/MAPK, are involved in cell migration of oral cancer cell lines only. In addition, the cadherin switch from E-cadherin to N-cadherin seems to be regulated by the Smad signalling pathway and PI3K/Akt signalling pathways in normal and oral cancer cell lines.

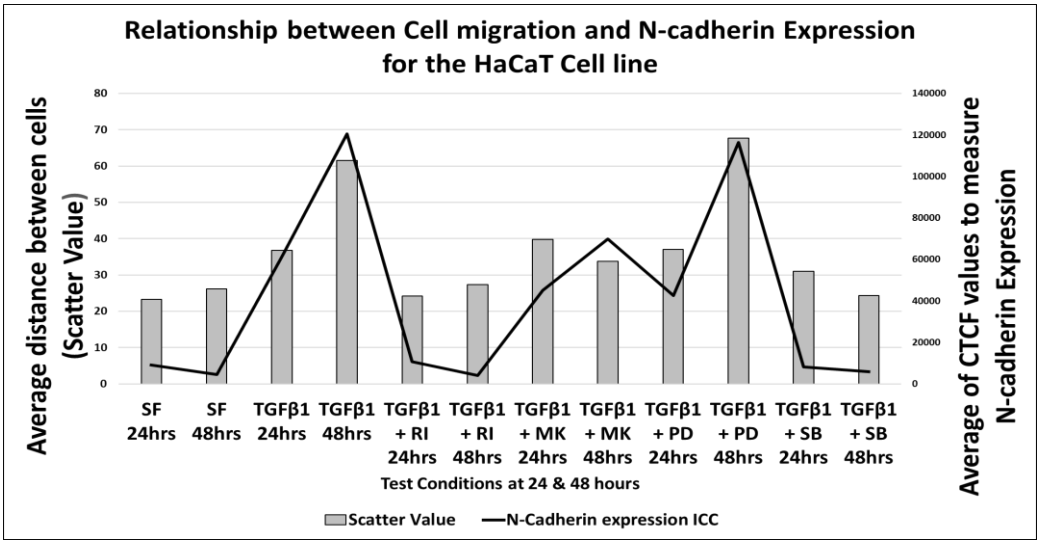


Figure 190: Correlation between the cell migration assessment and the expression of N-cadherin in the HaCaT cell line

In this figure the bar chart represents the average distance between the HaCaT cells and the line graph represents the expression of N-cadherin for the same test conditions.

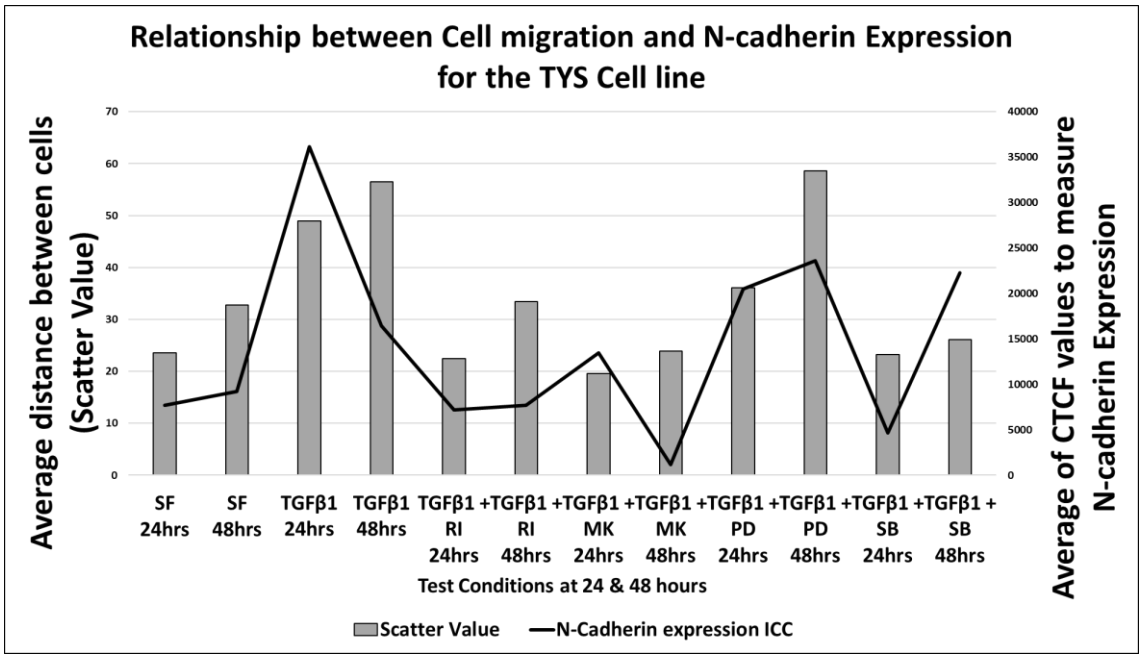


Figure 191: Correlation between the cell migration assessment and the expression of N-cadherin in the TYS cell line.

In this figure the bar chart represents the average distance between the TYS cells and the line graph represent the expression of N-cadherin for the same test conditions.

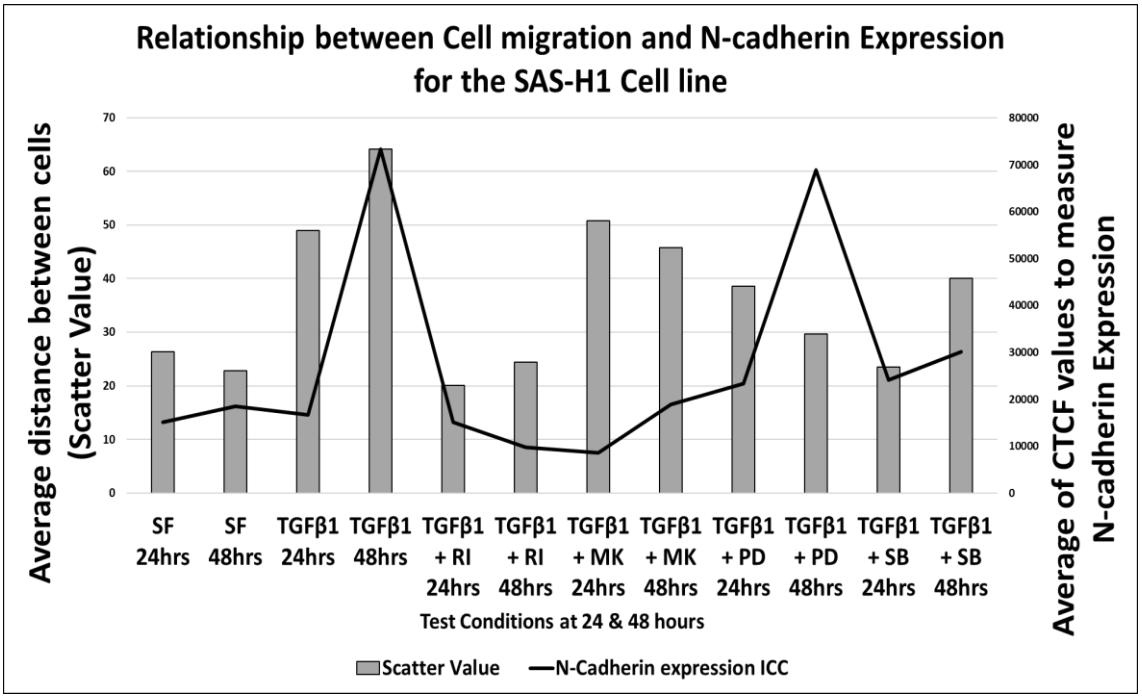


Figure 192: Correlation between the cell migration assessment and the expression of N-cadherin in the SAS-H1 cell line.

In this figure the bar chart represents the average distance between the SAS-H1 cells and the line graph represent the expression of N-cadherin for the same test conditions.

EMT is described as a sequential process and highly coordinated biological program. The features of EMT include the loss of epithelial tissue polarity, loss of

tight cell to cell junctions, remodelling of the extracellular matrix, gaining invasive abilities and resistance to apoptosis. All these features are essential for the transformation of epithelial cancer cells to a mesenchymal-type phenotype (Thiery, 2002; Grünert, Jechlinger and Beug, 2003). The completion of full EMT depends on a few factors to be established. Complete EMT needs four to six days of several repeated exposures to the same inducer in respect of the cell type (Grünert, Jechlinger and Beug, 2003). However, the description of the stages prior to full EMT varies in different studies, including scattering (Grünert, Jechlinger and Beug, 2003), partial EMT (Thiery, 2002; Saitoh, 2018), an intermediate stage of EMT (Huang, Guilford and Thiery, 2012; Pastushenko *et al.*, 2018) and early events of EMT. The features of early stage events in EMT include: morphological changes, loss of polarity in epithelial cells, redistribution or reduction in E-cadherin expression and a failure to acquire any mesenchymal markers (Grünert, Jechlinger and Beug, 2003). These changes occur as a result of short treatments (48 hours or less) with growth factors such as TGFβ and EGF, which resulted in activating PI3K/Akt, Erk/MAPK and Rho/GTPase signalling pathways (Grünert, Jechlinger and Beug, 2003; Marles and Biddle, 2022).

Overall data from this work highlighted only the behaviour of cells after 48 hours treatment with exogenous TGFβ1, therefore the requirements for complete EMT could not be achieved. However, the description of the transformation of the cancer cells in response to the growth factor as early events of the EMT program was established. The early events of EMT included loss of cell adhesion, cell migration and aberrant expression of E-cadherin and N-cadherin. Different reports have suggested a full EMT process is not required to achieve tumour metastasis (Basu, Cheriyamundath and Ben-Ze'ev, 2018). Moreover, the evidence indicated that effective tumour metastasis can be achieved by cells that have not undergone complete EMT (Saitoh, 2018). The ability of cancer cells to gain mesenchymal characteristics and maintain epithelial traits such as cell adhesion helps them to migrate collectively (Saitoh, 2018; Kisoda *et al.*, 2022).

Finally, using the TGF-RI Kinase Inhibitor VII to block TGFβ1 induced EMT showed promising results. These results suggested TGFβ signalling pathways as potential therapeutic targets to prevent oral cancer metastasis (**Figure 193**) (Takayama *et al.*, 2009; Hao, Baker and Ten Dijke, 2019). Studies on blocking TGFβ signalling in cancer patients indicate the possibility of preventing primary

tumour metastasis (Massagué, 2008; Ahmadi *et al.*, 2019). Concerns about the effect of blocking TGFβ signalling pathways might lead to autoimmune reactions or chronic inflammation (Massagué, 2008) or severe cardiotoxicities (Du and Shim, 2016). However, these concerns have not been observed in preclinical or clinical trials which used systemic TGFβ inhibitors (Massagué, 2008; Harsha *et al.*, 2020). There were also suggestions that blocking TGFβ signalling pathways could lead to progression of premalignant lesions (Massagué, 2008; MaruYama, Chen and Shibata, 2022). These suggestions were clarified with studies on animal models, where a systemic TGFβ blocker was administrated and did not lead to spontaneous tumour development (Massagué, 2008). Moreover, the different response of oral cancer cell lines to similar test conditions indicates that the possibility that each subtype of oral cancer has unique approaches to achieving metastasis. For example, in our study, the level of N-cadherin expression was variable depending on the cell line. The different responses of cell lines suggest that personalised therapeutic approaches might be effective in prevent oral cancer metastasis.

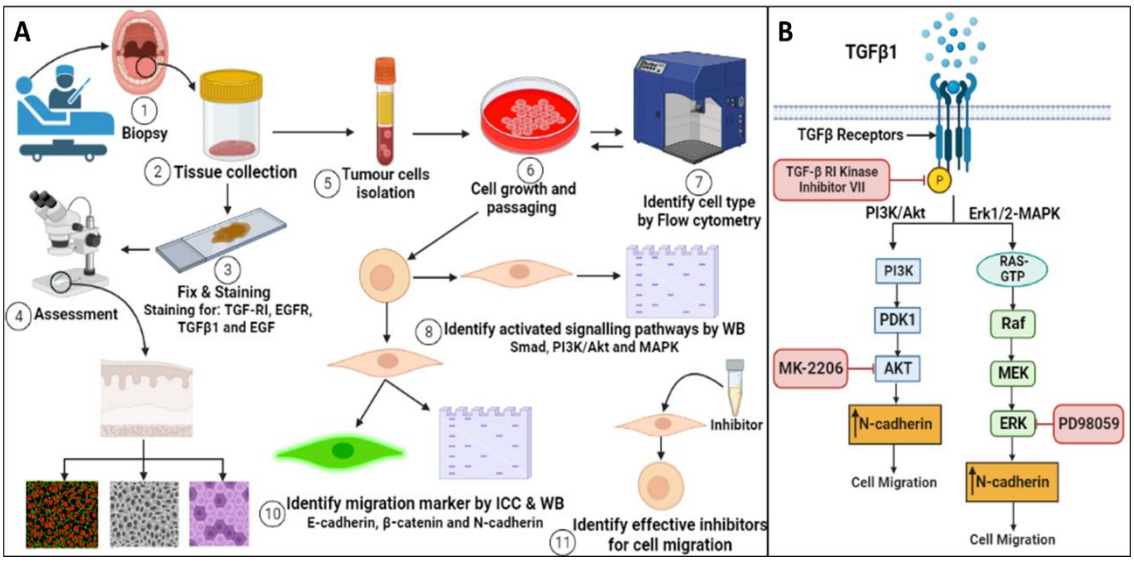


Figure 193: Personalised therapeutic approaches for oral cancer metastasis.

Figure (**A**) shows the protocol for samples collection and processing to identify the effective inhibitor for oral tumour metastasis. Figure (**B**) shows the potential therapeutic targets to prevent oral cancer metastasis.

Chapter 8: Conclusions and Future Work

8.1 Conclusion:

The main findings of this project regarding the role of TGFβ1 in oral cancer cell migration are summarised as follows:

1- TGFβ1 induces cell migration in both normal and oral cancer cell lines in comparison to EGF. However, each of the growth factors caused distinctive morphological changes of the cells.
2- The role of signalling pathways in cell migration induced by TGFβ1 was dependent on cell type.
3- TGFβ1 induces both modes of cell migration i.e. individual and collective. However, using specific inhibitors for any of signalling pathways activated by TGFβ1 (Smad, PI3K/Akt and Erk1/2 MAPK) inhibited the collective mode of cell migration of normal and oral cancer cell lines.
4- The activation of the Smad-dependent and Smad-independent pathways by TGFβ1 is cell type dependent.
5- TGFβ1 induces the movement of cell adhesion molecules from cell membrane to cytoplasm, which leads to breakdown of cell adhesion.
6- TGFβ1 upregulates N-cadherin, a mesenchymal marker, in both normal and oral cancer cell lines.
7- TGFβ1 induces migration of oral cancer cell lines via epithelial to mesenchymal transition (EMT).
8- TGFβ1-induced EMT is regulated by Smad, PI3K/Akt and Erk1/2 MAPK signalling pathways in normal and oral cancer cell lines.

Overall, the results from our work present sufficient evidence to support the claim that TGFβ1 induces cell migration via EMT of oral cancer cell lines via Smad-dependent and Smad-independent signalling pathways. Therefore, our conclusion rejected the null hypothesis.

8.2 Further Investigations:

We tried to build a model that was designed to evaluate the effect of the growth factors on oral cancer cell lines, in terms of cell migration. The model included three cell lines, two growth factors, three migration assay, four inhibitors and two assays for proteins expression evaluation. There are a few concepts that can be improved in the model to provide a better understanding:

A) Cell Lines:

1- Using a oral normal squamous cell line as the control group.
2- Using different cell lines, which represent each phase of cancer progression including a normal oral cell line, precancerous oral cell line, cell line of early OSCC and an aggressive OSCC cell line.
3- Using oral cancer cell lines from different sites in the oral cavity (different origins).

B) Growth Factors and Inhibitors:

1- Mixing the growth factors together as a test condition to compare the migratory effect on the cell lines to when they are applied separately.
2- Combining different inhibitors together to assess their effect on stopping cell migration.
3- Including inhibitors for proliferation in the study. These inhibitors will exclude the proliferative activities especially during the wound healing assays.

C) Signalling Pathways:

1- Investigating other signalling pathways which might be activated by the growth factors and evaluating if they have a role in cell migration.
2- Investigating downstream molecules that are associated with Smad dependent pathway and Smad-independent pathways.
3- Adding positive control samples to the SDS-PAGE and Western blotting to make sure the blot is working for migration markers such as Palladin, Gridin and vimentin.
4- Identifying the transcription factors that regulate cell migration via TGFβ-activated signalling pathways.
5- Investigating the cross-talk between TGFβ-activated signalling pathways.

D) Migration Assay:

1- Utilising 3D culture techniques to investigate cell migration of oral cancer cells to mimic an *in vivo* model to investigate the interactions between tumour cells and tumour microenvironment.

2- Distinguishing between complete and incomplete versions of epithelial to mesenchymal transition.

Chapter 9: References

References:

Abufaraj, M. *et al.* (2017) 'Prognostic role of N-cadherin expression in patients with non-muscle-invasive bladder cancer', *Urologic Oncology*, 35(5), pp. 264–271. Available at: https://doi.org/10.1016/j.urolonc.2017.01.012.

Ahmadi, A. *et al.* (2019) 'Transforming growth factor-β signaling: Tumorigenesis and targeting for cancer therapy', *Journal of Cellular Physiology*, 234(8), pp. 12173–12187. Available at: https://doi.org/10.1002/jcp.27955.

Ahmed, H. *et al.* (2020) *Oral Tumour Cell Migration and the Effect of the Local Soluble Factors from the Microenvironment on Signalling Pathways. Is It All about the Akt?* preprint. MEDICINE & PHARMACOLOGY. Available at: https://doi.org/10.20944/preprints202005.0445.v1.

Alessi, D.R. *et al.* (1996) 'Mechanism of activation of protein kinase B by insulin and IGF-1', *The EMBO journal*, 15(23), pp. 6541–6551.

Algayadh, I.G., Dronamraju, V. and Sylvester, P.W. (2016) 'Role of Rac1/WAVE2 Signaling in Mediating the Inhibitory Effects of γ-Tocotrienol on Mammary Cancer Cell Migration and Invasion', *Biological & Pharmaceutical Bulletin*, 39(12), pp. 1974–1982. Available at: https://doi.org/10.1248/bpb.b16-00461.

Ali, J. *et al.* (2017) 'Genetic etiology of oral cancer', *Oral Oncology*, 70, pp. 23–28. Available at: https://doi.org/10.1016/j.oraloncology.2017.05.004.

Alkhadar, H. *et al.* (2020) 'Nerve growth factor-induced migration in oral and salivary gland tumour cells utilises the PI3K/Akt signalling pathway: Is there a link to perineural invasion?', *Journal of Oral Pathology & Medicine*, 49(3), pp. 227–234. Available at: https://doi.org/10.1111/jop.12979.

Almangush, A. *et al.* (2020) 'Staging and grading of oral squamous cell carcinoma: An update', *Oral Oncology*, 107, p. 104799. Available at: https://doi.org/10.1016/j.oraloncology.2020.104799.

Alzawi, A. *et al.* (2022) 'Receptor, Signal, Nucleus, Action: Signals That Pass through Akt on the Road to Head and Neck Cancer Cell Migration', *Cancers*, 14(11), p. 2606. Available at: https://doi.org/10.3390/cancers14112606.

Amin, M.B. *et al.* (2017) *AJCC cancer staging manual*. Springer.

Angadi, P.V. *et al.* (2016) 'Immunoexpression of Epithelial Mesenchymal Transition Proteins E-Cadherin, β-Catenin, and N-Cadherin in Oral Squamous Cell Carcinoma', *International Journal of Surgical Pathology*, 24(8), pp. 696–703. Available at: https://doi.org/10.1177/1066896916654763.

Attramadal, C.G. *et al.* (2015) 'Tumor Budding, EMT and Cancer Stem Cells in T1-2/N0 Oral Squamous Cell Carcinomas', *Anticancer Research*, 35(11), pp. 6111–6120.

Bagan, J., Sarrion, G. and Jimenez, Y. (2010) 'Oral cancer: Clinical features', *Oral Oncology*, 46(6), pp. 414–417. Available at: https://doi.org/10.1016/j.oraloncology.2010.03.009.

Bagutti, C., Speight, P.M. and Watt, F.M. (1998) 'Comparison of integrin, cadherin, and catenin expression in squamous cell carcinomas of the oral cavity', *The Journal of Pathology*, 186(1), pp. 8–16. Available at: https://doi.org/10.1002/(SICI)1096-9896(199809)186:1<8::AID-PATH156>3.0.CO;2-H.

Bakin, A.V. *et al.* (2000) 'Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration', *The Journal of Biological Chemistry*, 275(47), pp. 36803–36810. Available at: https://doi.org/10.1074/jbc.M005912200.

Bánkfalvi, A. *et al.* (2002) 'Gains and losses of adhesion molecules (CD44, E-cadherin, and beta-catenin) during oral carcinogenesis and tumour progression', *The Journal of Pathology*, 198(3), pp. 343–351. Available at: https://doi.org/10.1002/path.1204.

Baranwal, S. and Alahari, S.K. (2009) 'Molecular mechanisms controlling E-cadherin expression in breast cancer', *Biochemical and Biophysical Research Communications*, 384(1), pp. 6–11. Available at: https://doi.org/10.1016/j.bbrc.2009.04.051.

Basu, S., Cheriyamundath, S. and Ben-Ze'ev, A. (2018) 'Cell-cell adhesion: linking Wnt/β-catenin signaling with partial EMT and stemness traits in tumorigenesis', *F1000Research*, 7, p. F1000 Faculty Rev-1488. Available at: https://doi.org/10.12688/f1000research.15782.1.

Batlle, E. *et al.* (2000) 'The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells', *Nature Cell Biology*, 2(2), pp. 84–89. Available at: https://doi.org/10.1038/35000034.

Bendardaf, R. *et al.* (2019) 'Cytoplasmic E-Cadherin Expression Is Associated With Higher Tumour Level of VEGFA, Lower Response Rate to Irinotecan-based Treatment and Poorer Prognosis in Patients With Metastatic Colorectal Cancer', *Anticancer Research*, 39(4), pp. 1953–1957. Available at: https://doi.org/10.21873/anticanres.13305.

Berridge, M.J. (2014) 'Module 1: Introduction', *Cell Signalling Biology*, 6, p. csb0001001. Available at: https://doi.org/10.1042/csb0001001.

Bhat, A.A. *et al.* (2020) 'Claudin-1, A Double-Edged Sword in Cancer', *International Journal of Molecular Sciences*, 21(2), p. E569. Available at: https://doi.org/10.3390/ijms21020569.

Bierie, B. and Moses, H.L. (2006) 'TGF-beta and cancer', *Cytokine & Growth Factor Reviews*, 17(1–2), pp. 29–40. Available at: https://doi.org/10.1016/j.cytogfr.2005.09.006.

Birchmeier, C., Birchmeier, W. and Brand-Saberi, B. (1996) 'Epithelial-mesenchymal transitions in cancer progression', *Acta Anatomica*, 156(3), pp. 217–226. Available at: https://doi.org/10.1159/000147848.

Bompard, G. and Caron, E. (2004) 'Regulation of WASP/WAVE proteins: making a long story short', *The Journal of Cell Biology*, 166(7), pp. 957–962. Available at: https://doi.org/10.1083/jcb.200403127.

Boukamp, P. *et al.* (1988) 'Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line', *The Journal of Cell Biology*, 106(3), pp. 761–771. Available at: https://doi.org/10.1083/jcb.106.3.761.

Bu, J.-Q. and Chen, F. (2017) 'TGF-β1 promotes cells invasion and migration by inducing epithelial mesenchymal transformation in oral squamous cell carcinoma', *European Review for Medical and Pharmacological Sciences*, 21(9), pp. 2137–2144.

Bugshan, A. and Farooq, I. (2020) 'Oral squamous cell carcinoma: metastasis, potentially associated malignant disorders, etiology and recent advancements in diagnosis', *F1000Research*, 9, p. 229. Available at: https://doi.org/10.12688/f1000research.22941.1.

Caldeira, P.C. *et al.* (2020) 'Tumor depth of invasion and prognosis of early-stage oral squamous cell carcinoma: A meta-analysis', *Oral Diseases*, 26(7), pp. 1357–1365. Available at: https://doi.org/10.1111/odi.13194.

Cannonier, S.A. *et al.* (2016) 'Hedgehog and TGFβ signaling converge on Gli2 to control bony invasion and bone destruction in oral squamous cell carcinoma', *Oncotarget*, 7(46), pp. 76062–76075. Available at: https://doi.org/10.18632/oncotarget.12584.

Cao, Z.-Q., Wang, Z. and Leng, P. (2019) 'Aberrant N-cadherin expression in cancer', *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, 118, p. 109320. Available at: https://doi.org/10.1016/j.biopha.2019.109320.

Cariati, P. *et al.* (2022) 'Impact of histological tumor grade on the behavior and prognosis of squamous cell carcinoma of the oral cavity', *Journal of Stomatology, Oral and Maxillofacial Surgery*, 123(6), pp. e808–e813. Available at: https://doi.org/10.1016/j.jormas.2022.07.005.

Chandolia, B. *et al.* (2017) 'Prognostic Potential of N-Cadherin in Oral Squamous Cell Carcinoma via Immunohistochemical Methods', *Journal of the College of Physicians and Surgeons--Pakistan: JCPSP*, 27(8), pp. 475–478.

Chang, H.W. *et al.* (2002) 'Loss of E-cadherin expression resulting from promoter hypermethylation in oral tongue carcinoma and its prognostic significance', *Cancer*, 94(2), pp. 386–392. Available at: https://doi.org/10.1002/cncr.10211.

Chang, J.W. *et al.* (2022) 'Claudin-1 mediates progression by regulating EMT through AMPK/TGF-β signaling in head and neck squamous cell carcinoma', *Translational Research*, 247, pp. 58–78. Available at: https://doi.org/10.1016/j.trsl.2022.04.003.

Chang, M.-C. *et al.* (2019) 'Stimulation of MMP-9 of oral epithelial cells by areca nut extract is related to TGF-β/Smad2-dependent and -independent pathways and prevented by betel leaf extract, hydroxychavicol and melatonin', *Aging*, 11(23), pp. 11624–11639. Available at: https://doi.org/10.18632/aging.102565.

Chaturvedi, P. *et al.* (2019) 'Tobacco related oral cancer', *BMJ (Clinical research ed.)*, 365, p. l2142. Available at: https://doi.org/10.1136/bmj.l2142.

Chaw, S.Y. *et al.* (2012) 'Epithelial to mesenchymal transition (EMT) biomarkers--E-cadherin, beta-catenin, APC and Vimentin--in oral squamous cell carcinogenesis and transformation', *Oral Oncology*, 48(10), pp. 997–1006. Available at: https://doi.org/10.1016/j.oraloncology.2012.05.011.

Chen, H. *et al.* (2021) 'Regulatory mechanism of oral mucosal rete peg formation', *Journal of Molecular Histology*, 52(5), pp. 859–868. Available at: https://doi.org/10.1007/s10735-021-10016-y.

Chen, I.-C. *et al.* (2014) 'Role of SIRT1 in regulation of epithelial-to-mesenchymal transition in oral squamous cell carcinoma metastasis', *Molecular Cancer*, 13, p. 254. Available at: https://doi.org/10.1186/1476-4598-13-254.

Chen, L. *et al.* (2020) 'MiR-132 inhibits migration and invasion and increases chemosensitivity of cisplatin-resistant oral squamous cell carcinoma cells via targeting TGF-β1', *Bioengineered*, 11(1), pp. 91–102. Available at: https://doi.org/10.1080/21655979.2019.1710925.

Chen, M.-F. *et al.* (2012) 'Significance of the TGF-β1/IL-6 axis in oral cancer', *Clinical Science (London, England: 1979)*, 122(10), pp. 459–472. Available at: https://doi.org/10.1042/CS20110434.

Chen, W. *et al.* (2018) 'Grainyhead-like 2 (GRHL2) knockout abolishes oral cancer development through reciprocal regulation of the MAP kinase and TGF-β signaling pathways', *Oncogenesis*, 7(5), p. 38. Available at: https://doi.org/10.1038/s41389-018-0047-5.

Chiba, T. *et al.* (2017) 'Transforming growth factor-β1 suppresses bone morphogenetic protein-2-induced mesenchymal-epithelial transition in HSC-4 human oral squamous cell carcinoma cells via Smad1/5/9 pathway suppression', *Oncology Reports*, 37(2), pp. 713–720. Available at: https://doi.org/10.3892/or.2016.5338.

Cho, K.-J. *et al.* (2017) 'Basaloid Squamous Cell Carcinoma of the Head and Neck: Subclassification into Basal, Ductal, and Mixed Subtypes Based on Comparison of Clinico-pathologic Features and Expression of p53, Cyclin D1, Epidermal Growth Factor Receptor, p16, and Human Papillomavirus', *Journal of Pathology and Translational Medicine*, 51(4), pp. 374–380. Available at: https://doi.org/10.4132/jptm.2017.03.03.

Choi, S. and Myers, J.N. (2008) 'Molecular pathogenesis of oral squamous cell carcinoma: implications for therapy', *Journal of Dental Research*, 87(1), pp. 14–32. Available at: https://doi.org/10.1177/154405910808700104.

Conway, D.I., Purkayastha, M. and Chestnutt, I.G. (2018) 'The changing epidemiology of oral cancer: definitions, trends, and risk factors', *British Dental Journal*, 225(9), pp. 867–873. Available at: https://doi.org/10.1038/sj.bdj.2018.922.

Dang, D. *et al.* (2004) 'Matrix metalloproteinases and TGFbeta1 modulate oral tumor cell matrix', *Biochemical and Biophysical Research Communications*, 316(3), pp. 937–942. Available at: https://doi.org/10.1016/j.bbrc.2004.02.143.

Debnath, P. *et al.* (2022) 'Epithelial-mesenchymal transition and its transcription factors', *Bioscience Reports*, 42(1), p. BSR20211754. Available at: https://doi.org/10.1042/BSR20211754.

Derycke, L.D.M. and Bracke, M.E. (2004) 'N-cadherin in the spotlight of cell-cell adhesion, differentiation, embryogenesis, invasion and signalling', *The International Journal of Developmental Biology*, 48(5–6), pp. 463–476. Available at: https://doi.org/10.1387/ijdb.041793ld.

Derynck, R. and Zhang, Y.E. (2003) 'Smad-dependent and Smad-independent pathways in TGF-beta family signalling', *Nature*, 425(6958), pp. 577–584. Available at: https://doi.org/10.1038/nature02006.

Detchokul, S. and Frauman, A.G. (2014) 'Editorial: cell movement', *British Journal of Pharmacology*, 171(24), pp. 5459–5461. Available at: https://doi.org/10.1111/bph.12849.

Dey, S. *et al.* (2022) 'Critical pathways of oral squamous cell carcinoma: molecular biomarker and therapeutic intervention', *Medical Oncology (Northwood, London, England)*, 39(3), p. 30. Available at: https://doi.org/10.1007/s12032-021-01633-4.

Diamond, M.E. *et al.* (2008) 'Differential growth factor regulation of N-cadherin expression and motility in normal and malignant oral epithelium', *Journal of Cell Science*, 121(Pt 13), pp. 2197–2207. Available at: https://doi.org/10.1242/jcs.021782.

Dik, E.A. *et al.* (2018) 'The value of histological grading of biopsy and resection specimens in early stage oral squamous cell carcinomas', *Journal of Cranio-Maxillofacial Surgery*, 46(6), pp. 1001–1006. Available at: https://doi.org/10.1016/j.jcms.2018.03.019.

Du, B. and Shim, J.S. (2016) 'Targeting Epithelial-Mesenchymal Transition (EMT) to Overcome Drug Resistance in Cancer', *Molecules (Basel, Switzerland)*, 21(7), p. 965. Available at: https://doi.org/10.3390/molecules21070965.

Duff, D. and Long, A. (2017) 'Roles for RACK1 in cancer cell migration and invasion', *Cellular Signalling*, 35, pp. 250–255. Available at: https://doi.org/10.1016/j.cellsig.2017.03.005.

Edlund, S. *et al.* (2002) 'Transforming growth factor-beta-induced mobilization of actin cytoskeleton requires signaling by small GTPases Cdc42 and RhoA', *Molecular Biology of the Cell*, 13(3), pp. 902–914. Available at: https://doi.org/10.1091/mbc.01-08-0398.

Ellis, I.R. (2021) 'The Migration and Invasion of Oral Squamous Carcinoma Cells: Matrix, Growth Factor and Signalling Involvement', *Cancers*, 13(11), p. 2633. Available at: https://doi.org/10.3390/cancers13112633.

ElMoneim, H.M.A. and Zaghloul, N.M. (2011) 'Expression of E-cadherin, N-cadherin and snail and their correlation with clinicopathological variants: an immunohistochemical study of 132 invasive ductal breast carcinomas in Egypt', *Clinics (Sao Paulo, Brazil)*, 66(10), pp. 1765–1771. Available at: https://doi.org/10.1590/s1807-59322011001000015.

El-Sharkawey, A.E. (2016) 'Calculate the Corrected Total Cell Fluorescence (CTCF).' Available at: https://doi.org/10.13140/RG.2.1.1307.8008.

Ernani, V. and Saba, N.F. (2015) 'Oral Cavity Cancer: Risk Factors, Pathology, and Management', *Oncology*, 89(4), pp. 187–195. Available at: https://doi.org/10.1159/000398801.

Etienne-Manneville, S. and Hall, A. (2002) 'Rho GTPases in cell biology', *Nature*, 420(6916), pp. 629–635. Available at: https://doi.org/10.1038/nature01148.

Friedl, P. and Gilmour, D. (2009) 'Collective cell migration in morphogenesis, regeneration and cancer', *Nature Reviews. Molecular Cell Biology*, 10(7), pp. 445–457. Available at: https://doi.org/10.1038/nrm2720.

Friedl, P. and Wolf, K. (2003) 'Tumour-cell invasion and migration: diversity and escape mechanisms', *Nature Reviews. Cancer*, 3(5), pp. 362–374. Available at: https://doi.org/10.1038/nrc1075.

Gaudet, M.M. *et al.* (2015) 'Anthropometry and head and neck cancer:a pooled analysis of cohort data', *International Journal of Epidemiology*, 44(2), pp. 673–681. Available at: https://doi.org/10.1093/ije/dyv059.

Ghosh, P. *et al.* (2008) 'Activation of Galphai3 triggers cell migration via regulation of GIV', *The Journal of Cell Biology*, 182(2), pp. 381–393. Available at: https://doi.org/10.1083/jcb.200712066.

Ghosh, P., Garcia-Marcos, M. and Farquhar, M.G. (2011) 'GIV/Girdin is a rheostat that fine-tunes growth factor signals during tumor progression', *Cell Adhesion & Migration*, 5(3), pp. 237–248. Available at: https://doi.org/10.4161/cam.5.3.15909.

Gilles, C. *et al.* (2003) 'Transactivation of vimentin by beta-catenin in human breast cancer cells', *Cancer Research*, 63(10), pp. 2658–2664.

Goicoechea, S.M., Arneman, D. and Otey, C.A. (2008) 'The role of palladin in actin organization and cell motility', *European Journal of Cell Biology*, 87(8–9), pp. 517–525. Available at: https://doi.org/10.1016/j.ejcb.2008.01.010.

Groeger, S. and Meyle, J. (2019) 'Oral Mucosal Epithelial Cells', *Frontiers in Immunology*, 10, p. 208. Available at: https://doi.org/10.3389/fimmu.2019.00208.

Groeger, S.E. and Meyle, J. (2015) 'Epithelial barrier and oral bacterial infection', *Periodontology 2000*, 69(1), pp. 46–67. Available at: https://doi.org/10.1111/prd.12094.

Grünert, S., Jechlinger, M. and Beug, H. (2003) 'Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis', *Nature Reviews. Molecular Cell Biology*, 4(8), pp. 657–665. Available at: https://doi.org/10.1038/nrm1175.

Gumbiner, B.M. (2005) 'Regulation of cadherin-mediated adhesion in morphogenesis', *Nature Reviews. Molecular Cell Biology*, 6(8), pp. 622–634. Available at: https://doi.org/10.1038/nrm1699.

Gupta, A. *et al.* (2018) 'Role of E-cadherin in Progression of Oral Squamous Cell Carcinoma: A Retrospective Immunohistochemical Study', *The Journal of Contemporary Dental Practice*, 19(9), pp. 1105–1110.

Gupta, B. *et al.* (2017) 'Associations between oral hygiene habits, diet, tobacco and alcohol and risk of oral cancer: A case-control study from India', *Cancer Epidemiology*, 51, pp. 7–14. Available at: https://doi.org/10.1016/j.canep.2017.09.003.

Hale, M.D., Hayden, J.D. and Grabsch, H.I. (2013) 'Tumour-microenvironment interactions: role of tumour stroma and proteins produced by cancer-associated fibroblasts in chemotherapy response', *Cellular Oncology (Dordrecht)*, 36(2), pp. 95–112. Available at: https://doi.org/10.1007/s13402-013-0127-7.

Hall, A. (2009) 'The cytoskeleton and cancer', *Cancer Metastasis Reviews*, 28(1–2), pp. 5–14. Available at: https://doi.org/10.1007/s10555-008-9166-3.

Hanein, D. and Horwitz, A.R. (2012) 'The structure of cell-matrix adhesions: the new frontier', *Current Opinion in Cell Biology*, 24(1), pp. 134–140. Available at: https://doi.org/10.1016/j.ceb.2011.12.001.

Hao, Y., Baker, D. and Ten Dijke, P. (2019) 'TGF-β-Mediated Epithelial-Mesenchymal Transition and Cancer Metastasis', *International Journal of Molecular Sciences*, 20(11), p. 2767. Available at: https://doi.org/10.3390/ijms20112767.

Harsha, C. *et al.* (2020) 'Targeting AKT/mTOR in Oral Cancer: Mechanisms and Advances in Clinical Trials', *International Journal of Molecular Sciences*, 21(9), p. 3285. Available at: https://doi.org/10.3390/ijms21093285.

Hasina, R. *et al.* (1999) 'Autocrine and paracrine motility factors and their involvement in invasiveness in a human oral carcinoma cell line', *British Journal of Cancer*, 80(11), pp. 1708–1717. Available at: https://doi.org/10.1038/sj.bjc.6690587.

Hazan, R.B. *et al.* (2004) 'Cadherin switch in tumor progression', *Annals of the New York Academy of Sciences*, 1014, pp. 155–163. Available at: https://doi.org/10.1196/annals.1294.016.

Hino, M. *et al.* (2016) 'Transforming growth factor-β1 induces invasion ability of HSC-4 human oral squamous cell carcinoma cells through the Slug/Wnt-5b/MMP-10 signalling axis', *Journal of Biochemistry*, 159(6), pp. 631–640. Available at: https://doi.org/10.1093/jb/mvw007.

Hirai, H. *et al.* (2010) 'MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo', *Molecular Cancer Therapeutics*, 9(7), pp. 1956–1967. Available at: https://doi.org/10.1158/1535-7163.MCT-09-1012.

Hirano, T. *et al.* (2020) 'TGF-β1 induces N-cadherin expression by upregulating Sox9 expression and promoting its nuclear translocation in human oral squamous cell carcinoma cells', *Oncology Letters*, 20(1), pp. 474–482. Available at: https://doi.org/10.3892/ol.2020.11582.

Hong, K.-O. *et al.* (2009) 'Inhibition of Akt activity induces the mesenchymal-to-epithelial reverting transition with restoring E-cadherin expression in KB and KOSCC-25B oral squamous cell carcinoma cells', *Journal of experimental & clinical cancer research: CR*, 28(1), p. 28. Available at: https://doi.org/10.1186/1756-9966-28-28.

Hsu, P.J. *et al.* (2020) 'Molecular biology of oral cavity squamous cell carcinoma', *Oral Oncology*, 102, p. 104552. Available at: https://doi.org/10.1016/j.oraloncology.2019.104552.

Hsu, P.-Y. *et al.* (2022) 'San-Zhong-Kui-Jian-Tang Exerts Antitumor Effects Associated With Decreased Cell Proliferation and Metastasis by Targeting ERK and the Epithelial-Mesenchymal Transition Pathway in Oral Cavity Squamous Cell Carcinoma', *Integrative Cancer Therapies*, 21, p. 15347354221134920. Available at: https://doi.org/10.1177/15347354221134921.

Huang, R.Y.-J., Guilford, P. and Thiery, J.P. (2012) 'Early events in cell adhesion and polarity during epithelial-mesenchymal transition', *Journal of Cell Science*, 125(Pt 19), pp. 4417–4422. Available at: https://doi.org/10.1242/jcs.099697.

Huang, S.H. and O'Sullivan, B. (2017) 'Overview of the 8th Edition TNM Classification for Head and Neck Cancer', *Current Treatment Options in Oncology*, 18(7), p. 40. Available at: https://doi.org/10.1007/s11864-017-0484-y.

ibidi, G. (2021) *Wound Healing Assay*. Protocol one, p. five.

Imamichi, Y. and Menke, A. (2007) 'Signaling pathways involved in collagen-induced disruption of the E-cadherin complex during epithelial-mesenchymal transition', *Cells, Tissues, Organs*, 185(1–3), pp. 180–190. Available at: https://doi.org/10.1159/000101319.

Islam, M. *et al.* (2017) 'The motogenic effect of EGF and TGF-α on the migration of tumor cells from the oral region: A role of epithelial-to-mesenchymal transition in cancer and a route for translation into the clinic', *Translational Research in Oral Oncology*, 2, p. 2057178X1769848. Available at: https://doi.org/10.1177/2057178X17698481.

Islam, M.R. *et al.* (2014) 'Is there a pAkt between VEGF and oral cancer cell migration?', *Cellular Signalling*, 26(6), pp. 1294–1302. Available at: https://doi.org/10.1016/j.cellsig.2014.02.004.

Islam, M.R. (2018) *Is it all just an Akt - you'd be SMAD to believe it! Role of TGFβ1 in oral cancer metastasis*. Science Repository Oü. Available at: https://doi.org/10.31487/j.DOBCR.2018.03.004.

Islam, S. *et al.* (1996) 'Expression of N-cadherin by human squamous carcinoma cells induces a scattered fibroblastic phenotype with disrupted cell-cell adhesion', *The Journal of Cell Biology*, 135(6 Pt 1), pp. 1643–1654. Available at: https://doi.org/10.1083/jcb.135.6.1643.

Janiszewska, M., Primi, M.C. and Izard, T. (2020) 'Cell adhesion in cancer: Beyond the migration of single cells', *The Journal of Biological Chemistry*, 295(8), pp. 2495–2505. Available at: https://doi.org/10.1074/jbc.REV119.007759.

Jardim, J.F. *et al.* (2015) 'Prognostic impact of perineural invasion and lymphovascular invasion in advanced stage oral squamous cell carcinoma', *International Journal of Oral and Maxillofacial Surgery*, 44(1), pp. 23–28. Available at: https://doi.org/10.1016/j.ijom.2014.10.006.

Javelaud, D. and Mauviel, A. (2005) 'Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-beta: implications for carcinogenesis', *Oncogene*, 24(37), pp. 5742–5750. Available at: https://doi.org/10.1038/sj.onc.1208928.

Jensen, D.H. *et al.* (2015) 'Molecular profiling of tumour budding implicates TGFβ-mediated epithelial-mesenchymal transition as a therapeutic target in oral squamous cell carcinoma', *The Journal of Pathology*, 236(4), pp. 505–516. Available at: https://doi.org/10.1002/path.4550.

Jin, X. *et al.* (2001) '[Expression of transforming growth factor beta(TGF-beta) subtypes in oral squamous cell carcinoma]', *Hua Xi Kou Qiang Yi Xue Za Zhi = Huaxi Kouqiang Yixue Zazhi = West China Journal of Stomatology*, 19(6), pp. 377–379.

Jones, H.B. *et al.* (2009) 'The impact of lymphovascular invasion on survival in oral carcinoma', *Oral Oncology*, 45(1), pp. 10–15. Available at: https://doi.org/10.1016/j.oraloncology.2008.03.009.

Joseph, M.J. *et al.* (2009) 'Slug is a downstream mediator of transforming growth factor-beta1-induced matrix metalloproteinase-9 expression and invasion of oral cancer cells', *Journal of Cellular Biochemistry*, 108(3), pp. 726–736. Available at: https://doi.org/10.1002/jcb.22309.

Kalluri, R. and Weinberg, R.A. (2009) 'The basics of epithelial-mesenchymal transition', *The Journal of Clinical Investigation*, 119(6), pp. 1420–1428. Available at: https://doi.org/10.1172/JCI39104.

Kedrin, D. *et al.* (2007) 'Cell motility and cytoskeletal regulation in invasion and metastasis', *Journal of Mammary Gland Biology and Neoplasia*, 12(2–3), pp. 143–152. Available at: https://doi.org/10.1007/s10911-007-9046-4.

Kielkopf, C.L., Bauer, W. and Urbatsch, I.L. (2021) 'Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of Proteins', *Cold Spring Harbor Protocols*, 2021(12). Available at: https://doi.org/10.1101/pdb.prot102228.

Kim, S.-A. *et al.* (2019) 'Receptor tyrosine kinase, RON, promotes tumor progression by regulating EMT and the MAPK signaling pathway in human oral squamous cell carcinoma', *International Journal of Oncology*, 55(2), pp. 513–526. Available at: https://doi.org/10.3892/ijo.2019.4836.

Kingsley, C. and Kourtidis, A. (2023) 'Critical roles of adherens junctions in diseases of the oral mucosa', *Tissue Barriers*, 11(2), p. 2084320. Available at: https://doi.org/10.1080/21688370.2022.2084320.

Kisoda, S. *et al.* (2022) 'The role of partial-EMT in the progression of head and neck squamous cell carcinoma', *Journal of Oral Biosciences*, 64(2), pp. 176–182. Available at: https://doi.org/10.1016/j.job.2022.02.004.

Kourtidis, A. *et al.* (2017) 'A central role for cadherin signaling in cancer', *Experimental Cell Research*, 358(1), pp. 78–85. Available at: https://doi.org/10.1016/j.yexcr.2017.04.006.

Kudo, Y. *et al.* (2004) 'Invasion and metastasis of oral cancer cells require methylation of E-cadherin and/or degradation of membranous beta-catenin', *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, 10(16), pp. 5455–5463. Available at: https://doi.org/10.1158/1078-0432.CCR-04-0372.

Kumar, M. *et al.* (2016) 'Oral cancer: Etiology and risk factors: A review', *Journal of Cancer Research and Therapeutics*, 12(2), pp. 458–463. Available at: https://doi.org/10.4103/0973-1482.186696.

Kuphal, S. and Bosserhoff, A.K. (2006) 'Influence of the cytoplasmic domain of E-cadherin on endogenous N-cadherin expression in malignant melanoma', *Oncogene*, 25(2), pp. 248–259. Available at: https://doi.org/10.1038/sj.onc.1209054.

Kurien, B.T. and Scofield, R.H. (2015) 'Western blotting: an introduction', *Methods in Molecular Biology (Clifton, N.J.)*, 1312, pp. 17–30. Available at: https://doi.org/10.1007/978-1-4939-2694-7_5.

Kushwaha, S.S. *et al.* (2019) 'Correlation of E-cadherin Immunohistochemical Expression with Histopathological Grading of Oral Squamous Cell Carcinoma', *Contemporary Clinical Dentistry*, 10(2), pp. 232–238. Available at: https://doi.org/10.4103/ccd.ccd_624_18.

Laine, F.J. and Smoker, W.R.K. (1995) 'Oral cavity: Anatomy and pathology', *Seminars in Ultrasound, CT and MRI*, 16(6), pp. 527–545. Available at: https://doi.org/10.1016/S0887-2171(06)80024-7.

Lämmermann, T. and Sixt, M. (2009) 'Mechanical modes of "amoeboid" cell migration', *Current Opinion in Cell Biology*, 21(5), pp. 636–644. Available at: https://doi.org/10.1016/j.ceb.2009.05.003.

Lamouille, S., Xu, J. and Derynck, R. (2014) 'Molecular mechanisms of epithelial-mesenchymal transition', *Nature Reviews. Molecular Cell Biology*, 15(3), pp. 178–196. Available at: https://doi.org/10.1038/nrm3758.

Lang, S.H. *et al.* (2002) 'Enhanced expression of vimentin in motile prostate cell lines and in poorly differentiated and metastatic prostate carcinoma', *The Prostate*, 52(4), pp. 253–263. Available at: https://doi.org/10.1002/pros.10088.

Laping, N.J. *et al.* (2002) 'Inhibition of transforming growth factor (TGF)-beta1-induced extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542', *Molecular Pharmacology*, 62(1), pp. 58–64. Available at: https://doi.org/10.1124/mol.62.1.58.

Lauffenburger, D.A. and Horwitz, A.F. (1996) 'Cell migration: a physically integrated molecular process', *Cell*, 84(3), pp. 359–369. Available at: https://doi.org/10.1016/s0092-8674(00)81280-5.

Lewis, M.A.O. (2018) 'Mouth cancer: presentation, detection and referral in primary dental care', *British Dental Journal*, 225(9), pp. 833–840. Available at: https://doi.org/10.1038/sj.bdj.2018.931.

Li, Y.-C. *et al.* (2017) 'SATB1 promotes tumor metastasis and invasiveness in oral squamous cell carcinoma', *Oral Diseases*, 23(2), pp. 247–254. Available at: https://doi.org/10.1111/odi.12602.

Lindenblatt, R. de C.R. *et al.* (2012) 'Oral squamous cell carcinoma grading systems--analysis of the best survival predictor', *Journal of Oral Pathology & Medicine: Official Publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology*, 41(1), pp. 34–39. Available at: https://doi.org/10.1111/j.1600-0714.2011.01068.x.

Ling, Z., Cheng, B. and Tao, X. (2021) 'Epithelial-to-mesenchymal transition in oral squamous cell carcinoma: Challenges and opportunities', *International Journal of Cancer*, 148(7), pp. 1548–1561. Available at: https://doi.org/10.1002/ijc.33352.

Liu, G. *et al.* (2019) 'KIF18A promotes head and neck squamous cell carcinoma invasion and migration via activation of Akt signaling pathway', *Translational Cancer Research*, 8(6), pp. 2252–2263. Available at: https://doi.org/10.21037/tcr.2019.09.38.

Liu, L.-K. *et al.* (2010) 'Upregulation of vimentin and aberrant expression of E-cadherin/beta-catenin complex in oral squamous cell carcinomas: correlation with the clinicopathological features and patient outcome', *Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc*, 23(2), pp. 213–224. Available at: https://doi.org/10.1038/modpathol.2009.160.

Liu, O. *et al.* (2012) 'Stereology study of oral verrucous carcinoma', *Journal of B.U.ON.: official journal of the Balkan Union of Oncology*, 17(2), pp. 343–349.

Lo Muzio, L. *et al.* (1999) 'Beta- and gamma-catenin expression in oral squamous cell carcinomas', *Anticancer Research*, 19(5B), pp. 3817–3826.

Lo Muzio, L. *et al.* (2007) 'Oral squamous cell carcinoma and biological markers: an update on the molecules mainly involved in oral carcinogenesis', *Minerva Stomatologica*, 56(6), pp. 341–347.

Logullo, A.F. *et al.* (2003) 'Transforming growth factor beta1 (TGFbeta1) expression in head and neck squamous cell carcinoma patients as related to prognosis', *Journal of Oral Pathology & Medicine: Official Publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology*, 32(3), pp. 139–145. Available at: https://doi.org/10.1034/j.1600-0714.2003.00012.x.

López-Verdín, S. *et al.* (2019) 'E-Cadherin gene expression in oral cancer: Clinical and prospective data', *Medicina Oral, Patologia Oral Y Cirugia Bucal*, 24(4), pp. e444–e451. Available at: https://doi.org/10.4317/medoral.23029.

Lourenço, S.V. *et al.* (2010) 'Oral squamous cell carcinoma: status of tight junction claudins in the different histopathological patterns and relationship with clinical parameters. A tissue-microarray-based study of 136 cases', *Journal of Clinical Pathology*, 63(7), pp. 609–614. Available at: https://doi.org/10.1136/jcp.2009.070409.

Luo, Y. *et al.* (2018) 'Upregulated N-cadherin expression is associated with poor prognosis in epithelial-derived solid tumours: A meta-analysis', *European Journal of Clinical Investigation*, 48(4). Available at: https://doi.org/10.1111/eci.12903.

Lv, S. *et al.* (2021) 'Naa10p and IKKα interaction regulates EMT in oral squamous cell carcinoma via TGF-β1/Smad pathway', *Journal of Cellular and Molecular Medicine*, 25(14), pp. 6760–6772. Available at: https://doi.org/10.1111/jcmm.16680.

Markopoulos, A.K. (2012) 'Current aspects on oral squamous cell carcinoma', *The Open Dentistry Journal*, 6, pp. 126–130. Available at: https://doi.org/10.2174/1874210601206010126.

Marles, H. and Biddle, A. (2022) 'Cancer stem cell plasticity and its implications in the development of new clinical approaches for oral squamous cell carcinoma', *Biochemical Pharmacology*, 204, p. 115212. Available at: https://doi.org/10.1016/j.bcp.2022.115212.

Martin, T.A. (2014) 'The role of tight junctions in cancer metastasis', *Seminars in Cell & Developmental Biology*, 36, pp. 224–231. Available at: https://doi.org/10.1016/j.semcdb.2014.09.008.

MaruYama, T., Chen, W. and Shibata, H. (2022) 'TGF-β and Cancer Immunotherapy', *Biological & Pharmaceutical Bulletin*, 45(2), pp. 155–161. Available at: https://doi.org/10.1248/bpb.b21-00966.

Massagué, J. (1998) 'TGF-beta signal transduction', *Annual Review of Biochemistry*, 67, pp. 753–791. Available at: https://doi.org/10.1146/annurev.biochem.67.1.753.

Massagué, J. (2008) 'TGFbeta in Cancer', *Cell*, 134(2), pp. 215–230. Available at: https://doi.org/10.1016/j.cell.2008.07.001.

Mattavelli, D. *et al.* (2020) 'The 8th TNM classification for oral squamous cell carcinoma: What is gained, what is lost, and what is missing', *Oral Oncology*, 111, p. 104937. Available at: https://doi.org/10.1016/j.oraloncology.2020.104937.

Miettinen, P.J. *et al.* (1994) 'TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors', *The Journal of Cell Biology*, 127(6 Pt 2), pp. 2021–2036. Available at: https://doi.org/10.1083/jcb.127.6.2021.

Miki, H., Suetsugu, S. and Takenawa, T. (1998) 'WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac', *The EMBO journal*, 17(23), pp. 6932–6941. Available at: https://doi.org/10.1093/emboj/17.23.6932.

Mincione, G. *et al.* (2008) 'Loss of expression of TGF-beta1, TbetaRI, and TbetaRII correlates with differentiation in human oral squamous cell carcinomas', *International Journal of Oncology*, 32(2), pp. 323–331.

Mishra, A. *et al.* (2022) 'Worst pattern of invasion in oral squamous cell carcinoma is an independent prognostic factor', *Journal of Oral Biology and Craniofacial Research*, 12(6), pp. 771–776. Available at: https://doi.org/10.1016/j.jobcr.2022.08.027.

Mishra, Y.G. and Manavathi, B. (2021) 'Focal adhesion dynamics in cellular function and disease', *Cellular Signalling*, 85, p. 110046. Available at: https://doi.org/10.1016/j.cellsig.2021.110046.

Morikawa, M., Derynck, R. and Miyazono, K. (2016) 'TGF-β and the TGF-β Family: Context-Dependent Roles in Cell and Tissue Physiology', *Cold Spring Harbor Perspectives in Biology*, 8(5), p. a021873. Available at: https://doi.org/10.1101/cshperspect.a021873.

Najm, P. and El-Sibai, M. (2014) 'Palladin regulation of the actin structures needed for cancer invasion', *Cell Adhesion & Migration*, 8(1), pp. 29–35. Available at: https://doi.org/10.4161/cam.28024.

Nanci, A. (2017) *Ten Cate's Oral Histology-e-book: development, structure, and function*. Elsevier Health Sciences.

Niaz, K. *et al.* (2017) 'Smokeless tobacco (paan and gutkha) consumption, prevalence, and contribution to oral cancer', *Epidemiology and Health*, 39, p. e2017009. Available at: https://doi.org/10.4178/epih.e2017009.

Nielsen, M.S. *et al.* (2012) 'Gap junctions', *Comprehensive Physiology*, 2(3), pp. 1981–2035. Available at: https://doi.org/10.1002/cphy.c110051.

Odell, E.W. *et al.* (1994) 'The prognostic value of individual histologic grading parameters in small lingual squamous cell carcinomas. The importance of the pattern of invasion', *Cancer*, 74(3), pp. 789–794. Available at: https://doi.org/10.1002/1097-0142(19940801)74:3<789::AID-CNCR2820740302>3.0.CO;2-A.

Oku, N. *et al.* (2006) 'Tight junction protein claudin-1 enhances the invasive activity of oral squamous cell carcinoma cells by promoting cleavage of laminin-5 gamma2 chain via matrix metalloproteinase (MMP)-2 and membrane-type MMP-1', *Cancer Research*, 66(10), pp. 5251–5257. Available at: https://doi.org/10.1158/0008-5472.CAN-05-4478.

Okumura, K. *et al.* (1996) 'Establishment of high- and low-invasion clones derived for a human tongue squamous-cell carcinoma cell line SAS', *Journal of Cancer Research and Clinical Oncology*, 122(4), pp. 243–248. Available at: https://doi.org/10.1007/BF01209653.

Pang, X., Tang, Y.-L. and Liang, X.-H. (2018) 'Transforming growth factor-β signaling in head and neck squamous cell carcinoma: Insights into cellular responses', *Oncology Letters*, 16(4), pp. 4799–4806. Available at: https://doi.org/10.3892/ol.2018.9319.

Paňková, K. *et al.* (2010) 'The molecular mechanisms of transition between mesenchymal and amoeboid invasiveness in tumor cells', *Cellular and Molecular Life Sciences*, 67(1), pp. 63–71. Available at: https://doi.org/10.1007/s00018-009-0132-1.

Pannone, G. *et al.* (2014) 'The role of E-cadherin down-regulation in oral cancer: CDH1 gene expression and epigenetic blockage', *Current Cancer Drug Targets*, 14(2), pp. 115–127. Available at: https://doi.org/10.2174/1568009613666131126115012.

Park, J. *et al.* (2017) 'Loss of RUNX3 expression inhibits bone invasion of oral squamous cell carcinoma', *Oncotarget*, 8(6), pp. 9079–9092. Available at: https://doi.org/10.18632/oncotarget.14071.

Pastushenko, I. *et al.* (2018) 'Identification of the tumour transition states occurring during EMT', *Nature*, 556(7702), pp. 463–468. Available at: https://doi.org/10.1038/s41586-018-0040-3.

Pereira, M.C. *et al.* (2007) 'Histologic subtypes of oral squamous cell carcinoma: prognostic relevance', *Journal (Canadian Dental Association)*, 73(4), pp. 339–344.

Pramanik, K.K. and Mishra, R. (2022) 'ERK-mediated upregulation of matrix metalloproteinase-2 promotes the invasiveness in human oral squamous cell carcinoma (OSCC)', *Experimental Cell Research*, 411(1), p. 112984. Available at: https://doi.org/10.1016/j.yexcr.2021.112984.

Pyo, S.W. *et al.* (2007) 'Expression of E-cadherin, P-cadherin and N-cadherin in oral squamous cell carcinoma: correlation with the clinicopathologic features and patient outcome', *Journal of Cranio-Maxillo-Facial Surgery: Official Publication of the European Association for Cranio-Maxillo-Facial Surgery*, 35(1), pp. 1–9. Available at: https://doi.org/10.1016/j.jcms.2006.11.004.

Qiao, B., Johnson, N.W. and Gao, J. (2010) 'Epithelial-mesenchymal transition in oral squamous cell carcinoma triggered by transforming growth factor-beta1 is Snail family-dependent and correlates with matrix metalloproteinase-2 and -9 expressions', *International Journal of Oncology*, 37(3), pp. 663–668. Available at: https://doi.org/10.3892/ijo_00000715.

Rai K, H. and Ahmed, J. (2019) 'A Correlative Study of N-Cadherin Expression with Different Grades of Oral Squamous Cell Carcinoma Projecting as a Marker of Epithelial to Mesenchymal Transition in Tumor Progression', *Asian Pacific journal of cancer prevention: APJCP*, 20(8), pp. 2327–2332. Available at: https://doi.org/10.31557/APJCP.2019.20.8.2327.

Ramos-García, P. and González-Moles, M.Á. (2022) 'Prognostic and Clinicopathological Significance of the Aberrant Expression of β-Catenin in Oral Squamous Cell Carcinoma: A Systematic Review and Meta-Analysis', *Cancers*, 14(3), p. 479. Available at: https://doi.org/10.3390/cancers14030479.

Rivera, C. (2015) 'Essentials of oral cancer', *International Journal of Clinical and Experimental Pathology*, 8(9), pp. 11884–11894.

Rivera, C. *et al.* (2017) 'Prognostic biomarkers in oral squamous cell carcinoma: A systematic review', *Oral Oncology*, 72, pp. 38–47. Available at: https://doi.org/10.1016/j.oraloncology.2017.07.003.

Roberts, P.J. and Der, C.J. (2007) 'Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer', *Oncogene*, 26(22), pp. 3291–3310. Available at: https://doi.org/10.1038/sj.onc.1210422.

Rodríguez-Molinero, J. *et al.* (2021) 'Association between Oral Cancer and Diet: An Update', *Nutrients*, 13(4), p. 1299. Available at: https://doi.org/10.3390/nu13041299.

Rørth, P. (2009) 'Collective cell migration', *Annual Review of Cell and Developmental Biology*, 25, pp. 407–429. Available at: https://doi.org/10.1146/annurev.cellbio.042308.113231.

Rosen, L.B. *et al.* (1994) 'Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras', *Neuron*, 12(6), pp. 1207–1221. Available at: https://doi.org/10.1016/0896-6273(94)90438-3.

Rosenthal, E. *et al.* (2004) 'Elevated expression of TGF-beta1 in head and neck cancer-associated fibroblasts', *Molecular Carcinogenesis*, 40(2), pp. 116–121. Available at: https://doi.org/10.1002/mc.20024.

van Roy, F. (2014) 'Beyond E-cadherin: roles of other cadherin superfamily members in cancer', *Nature Reviews. Cancer*, 14(2), pp. 121–134. Available at: https://doi.org/10.1038/nrc3647.

Ryu, B. *et al.* (2001) 'Invasion-specific genes in malignancy: serial analysis of gene expression comparisons of primary and passaged cancers', *Cancer Research*, 61(5), pp. 1833–1838.

Saitoh, M. (2018) 'Involvement of partial EMT in cancer progression', *Journal of Biochemistry*, 164(4), pp. 257–264. Available at: https://doi.org/10.1093/jb/mvy047.

Salehiniya, H. and Raei, M. (2020) 'Oral cavity and lip cancer in the world: An epidemiological review', *Biomedical Research and Therapy*, 7(8), pp. 3898–3905. Available at: https://doi.org/10.15419/bmrat.v7i8.619.

Santibáñez, J.F. *et al.* (2002) 'Transforming growth factor-beta1 modulates matrix metalloproteinase-9 production through the Ras/MAPK signaling pathway in transformed keratinocytes', *Biochemical and Biophysical Research Communications*, 296(2), pp. 267–273. Available at: https://doi.org/10.1016/s0006-291x(02)00864-1.

Sarode, G. *et al.* (2020) 'Epidemiologic aspects of oral cancer', *Disease-a-month: DM*, 66(12), p. 100988. Available at: https://doi.org/10.1016/j.disamonth.2020.100988.

Satelli, A. and Li, S. (2011) 'Vimentin in cancer and its potential as a molecular target for cancer therapy', *Cellular and molecular life sciences: CMLS*, 68(18), pp. 3033–3046. Available at: https://doi.org/10.1007/s00018-011-0735-1.

Schmitz, A.A. *et al.* (2000) 'Rho GTPases: signaling, migration, and invasion', *Experimental Cell Research*, 261(1), pp. 1–12. Available at: https://doi.org/10.1006/excr.2000.5049.

Shi, Y. and Massagué, J. (2003) 'Mechanisms of TGF-beta signaling from cell membrane to the nucleus', *Cell*, 113(6), pp. 685–700. Available at: https://doi.org/10.1016/s0092-8674(03)00432-x.

Shimizu, T. *et al.* (2008) 'Synthesis and evaluation of [2-(4-quinolyloxy)phenyl]methanone derivatives: novel selective inhibitors of transforming growth factor-beta kinase', *Journal of Medicinal Chemistry*, 51(11), pp. 3326–3329. Available at: https://doi.org/10.1021/jm701626z.

Speight, P.M. and Farthing, P.M. (2018) 'The pathology of oral cancer', *British Dental Journal*, 225(9), pp. 841–847. Available at: https://doi.org/10.1038/sj.bdj.2018.926.

Sreekumar, V.N. (2019) 'Global Scenario of Research in Oral Cancer', *Journal of Maxillofacial and Oral Surgery*, 18(3), pp. 354–359. Available at: https://doi.org/10.1007/s12663-018-1166-4.

Suetsugu, S. *et al.* (2001) 'Enhancement of branching efficiency by the actin filament-binding activity of N-WASP/WAVE2', *Journal of Cell Science*, 114(Pt 24), pp. 4533–4542. Available at: https://doi.org/10.1242/jcs.114.24.4533.

Sulzmaier, F.J. and Ramos, J.W. (2013) 'RSK isoforms in cancer cell invasion and metastasis', *Cancer Research*, 73(20), pp. 6099–6105. Available at: https://doi.org/10.1158/0008-5472.CAN-13-1087.

Sun, L. *et al.* (2008) 'Transforming growth factor-beta 1 promotes matrix metalloproteinase-9-mediated oral cancer invasion through snail expression', *Molecular cancer research: MCR*, 6(1), pp. 10–20. Available at: https://doi.org/10.1158/1541-7786.MCR-07-0208.

Sun, Y. *et al.* (2015) 'Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis', *Journal of Receptor and Signal Transduction Research*, 35(6), pp. 600–604. Available at: https://doi.org/10.3109/10799893.2015.1030412.

Sung, H. *et al.* (2021) 'Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries', *CA: a cancer journal for clinicians*, 71(3), pp. 209–249. Available at: https://doi.org/10.3322/caac.21660.

Suzuki, S. *et al.* (2019) 'CD147 mediates transforming growth factor-β1-induced epithelial-mesenchymal transition and cell invasion in squamous cell carcinoma of the tongue', *Experimental and Therapeutic Medicine*, 17(4), pp. 2855–2860. Available at: https://doi.org/10.3892/etm.2019.7230.

Takayama, S. *et al.* (2009) 'Inhibition of TGF-beta1 suppresses motility and invasiveness of oral squamous cell carcinoma cell lines via modulation of integrins and down-regulation of matrix-metalloproteinases', *Oncology Reports*, 21(1), pp. 205–210.

Tang, W. *et al.* (2004) '[In situ expression of transforming growth factor beta 1 in the process of induction chemotherapy for oral squamous cell carcinoma]', *Sichuan Da Xue Xue Bao. Yi Xue Ban = Journal of Sichuan University. Medical Science Edition*, 35(1), pp. 42–43, 90.

Theveneau, E. and Mayor, R. (2011) 'Can mesenchymal cells undergo collective cell migration? The case of the neural crest', *Cell Adhesion & Migration*, 5(6), pp. 490–498. Available at: https://doi.org/10.4161/cam.5.6.18623.

Thiery, J.P. (2002) 'Epithelial-mesenchymal transitions in tumour progression', *Nature Reviews. Cancer*, 2(6), pp. 442–454. Available at: https://doi.org/10.1038/nrc822.

Thiery, J.P. and Sleeman, J.P. (2006) 'Complex networks orchestrate epithelial-mesenchymal transitions', *Nature Reviews. Molecular Cell Biology*, 7(2), pp. 131–142. Available at: https://doi.org/10.1038/nrm1835.

Thijssen – van Loosdregt, I. *et al.* (2021) *Collective cell migration assays An extensive overview of currently available assays and tips on how to select the most suitable assay for your research*. CytoSMART Technologies B.V. Emmasingel 33 5611 AZ Eindhoven Netherlands: CytoSMART Technologies or its affiliates. Available at: https://cytosmart.com/resources/e-books/e-book-collective-cell-migration-assays.

Tirelli, G. *et al.* (2018) 'Prognosis of oral cancer: a comparison of the staging systems given in the 7th and 8th editions of the American Joint Committee on Cancer Staging Manual', *British Journal of Oral and Maxillofacial Surgery*, 56(1), pp. 8–13. Available at: https://doi.org/10.1016/j.bjoms.2017.11.009.

Trepat, X., Chen, Z. and Jacobson, K. (2012) 'Cell migration', *Comprehensive Physiology*, 2(4), pp. 2369–2392. Available at: https://doi.org/10.1002/cphy.c110012.

Ulrich, F. and Heisenberg, C.-P. (2009) 'Trafficking and cell migration', *Traffic (Copenhagen, Denmark)*, 10(7), pp. 811–818. Available at: https://doi.org/10.1111/j.1600-0854.2009.00929.x.

Vallina, C. *et al.* (2021) 'Genes involved in the epithelial-mesenchymal transition in oral cancer: A systematic review', *Oral Oncology*, 117, p. 105310. Available at: https://doi.org/10.1016/j.oraloncology.2021.105310.

Vega, F.M. and Ridley, A.J. (2008) 'Rho GTPases in cancer cell biology', *FEBS letters*, 582(14), pp. 2093–2101. Available at: https://doi.org/10.1016/j.febslet.2008.04.039.

Vuoriluoto, K. *et al.* (2011) 'Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer', *Oncogene*, 30(12), pp. 1436–1448. Available at: https://doi.org/10.1038/onc.2010.509.

Walker, A., Frei, R. and Lawson, K.R. (2014) 'The cytoplasmic domain of N-cadherin modulates MMP-9 induction in oral squamous carcinoma cells', *International Journal of Oncology*, 45(4), pp. 1699–1706. Available at: https://doi.org/10.3892/ijo.2014.2549.

Wang, J. *et al.* (2020) 'The circEPSTI1/mir-942-5p/LTBP2 axis regulates the progression of OSCC in the background of OSF via EMT and the PI3K/Akt/mTOR pathway', *Cell Death & Disease*, 11(8), p. 682. Available at: https://doi.org/10.1038/s41419-020-02851-w.

Wang, S.-S. *et al.* (2019) 'The maintenance of an oral epithelial barrier', *Life Sciences*, 227, pp. 129–136. Available at: https://doi.org/10.1016/j.lfs.2019.04.029.

Wang, X. and Chen, Q. (2021) 'FERMT1 knockdown inhibits oral squamous cell carcinoma cell epithelial-mesenchymal transition by inactivating the PI3K/AKT signaling pathway', *BMC oral health*, 21(1), p. 598. Available at: https://doi.org/10.1186/s12903-021-01955-9.

Webb, D.J., Zhang, H. and Horwitz, A.F. (2005) 'Cell migration: an overview', *Methods in Molecular Biology (Clifton, N.J.)*, 294, pp. 3–11.

Wennerberg, K. and Der, C.J. (2004) 'Rho-family GTPases: it's not only Rac and Rho (and I like it)', *Journal of Cell Science*, 117(Pt 8), pp. 1301–1312. Available at: https://doi.org/10.1242/jcs.01118.

White, R.A., Malkoski, S.P. and Wang, X.-J. (2010) 'TGFβ signaling in head and neck squamous cell carcinoma', *Oncogene*, 29(40), pp. 5437–5446. Available at: https://doi.org/10.1038/onc.2010.306.

Woolgar, J.A. (2006) 'Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma', *Oral Oncology*, 42(3), pp. 229–239. Available at: https://doi.org/10.1016/j.oraloncology.2005.05.008.

Xie, F. *et al.* (2018) 'TGF-β signaling in cancer metastasis', *Acta Biochimica Et Biophysica Sinica*, 50(1), pp. 121–132. Available at: https://doi.org/10.1093/abbs/gmx123.

Xie, W. *et al.* (2003) 'Frequent alterations of Smad signaling in human head and neck squamous cell carcinomas: a tissue microarray analysis', *Oncology Research*, 14(2), pp. 61–73. Available at: https://doi.org/10.3727/000000003108748612.

Xu, J., Lamouille, S. and Derynck, R. (2009) 'TGF-β-induced epithelial to mesenchymal transition', *Cell Research*, 19(2), pp. 156–172. Available at: https://doi.org/10.1038/cr.2009.5.

Xu, Q. *et al.* (2017) 'EGF induces epithelial-mesenchymal transition and cancer stem-like cell properties in human oral cancer cells via promoting Warburg effect', *Oncotarget*, 8(6), pp. 9557–9571. Available at: https://doi.org/10.18632/oncotarget.13771.

Xuan, Y.-Z., Jin, C.-R. and Yang, K.-J. (2020) 'TGF-β downregulation overcomes gemcitabine resistance in oral squamous cell carcinoma', *Cancer Biomarkers: Section A of Disease Markers*, 29(2), pp. 179–187. Available at: https://doi.org/10.3233/CBM-201456.

Yamaguchi, H., Wyckoff, J. and Condeelis, J. (2005) 'Cell migration in tumors', *Current Opinion in Cell Biology*, 17(5), pp. 559–564. Available at: https://doi.org/10.1016/j.ceb.2005.08.002.

Yamamura, Y. *et al.* (2015) 'Akt-Girdin signaling in cancer-associated fibroblasts contributes to tumor progression', *Cancer Research*, 75(5), pp. 813–823. Available at: https://doi.org/10.1158/0008-5472.CAN-14-1317.

Yanagawa, T. *et al.* (1986) 'An adenoid squamous carcinoma-forming cell line established from an oral keratinizing squamous cell carcinoma expressing carcinoembryonic antigen', *The American Journal of Pathology*, 124(3), pp. 496–509.

Yang, H. *et al.* (2015) 'TGF-β-activated SMAD3/4 complex transcriptionally upregulates N-cadherin expression in non-small cell lung cancer', *Lung Cancer (Amsterdam, Netherlands)*, 87(3), pp. 249–257. Available at: https://doi.org/10.1016/j.lungcan.2014.12.015.

Yates, C.B. and Phillips, C.D. (2001) 'Oral cavity and oropharynx', *Current Problems in Diagnostic Radiology*, 30(2), pp. 38–59. Available at: https://doi.org/10.1067/mdr.2001.113657.

Yilmaz, M. and Christofori, G. (2010) 'Mechanisms of motility in metastasizing cells', *Molecular cancer research: MCR*, 8(5), pp. 629–642. Available at: https://doi.org/10.1158/1541-7786.MCR-10-0139.

Yu, C. *et al.* (2011) 'TGF-β1 mediates epithelial to mesenchymal transition via the TGF-β/Smad pathway in squamous cell carcinoma of the head and neck', *Oncology Reports*, 25(6), pp. 1581–1587. Available at: https://doi.org/10.3892/or.2011.1251.

Zargaran, M. (2020) 'Alternation of β-catenin and CD44s Immunoexpression in Different Histopathological Grades of Oral Squamous Cell Carcinoma', *Asian Pacific journal of cancer prevention: APJCP*, 21(5), pp. 1181–1185. Available at: https://doi.org/10.31557/APJCP.2020.21.5.1181.

Zavadil, J. *et al.* (2004) 'Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition', *The EMBO journal*, 23(5), pp. 1155–1165. Available at: https://doi.org/10.1038/sj.emboj.7600069.

Zavadil, J. and Böttinger, E.P. (2005) 'TGF-beta and epithelial-to-mesenchymal transitions', *Oncogene*, 24(37), pp. 5764–5774. Available at: https://doi.org/10.1038/sj.onc.1208927.

Zhang, Y.E. (2009) 'Non-Smad pathways in TGF-beta signaling', *Cell Research*, 19(1), pp. 128–139. Available at: https://doi.org/10.1038/cr.2008.328.

Zhao, X.-W. *et al.* (2019) 'The role of MAPK signaling pathway in formation of EMT in oral squamous carcinoma cells induced by TNF-α', *Molecular Biology Reports*, 46(3), pp. 3149–3156. Available at: https://doi.org/10.1007/s11033-019-04772-0.

Zhong, L.-P. *et al.* (2007) 'Expression of E-cadherin in cervical lymph nodes from primary oral squamous cell carcinoma patients', *Archives of Oral Biology*, 52(8), pp. 740–747. Available at: https://doi.org/10.1016/j.archoralbio.2007.01.013.

Zhou, J. *et al.* (2015) 'Expression of E-cadherin and vimentin in oral squamous cell carcinoma', *International Journal of Clinical and Experimental Pathology*, 8(3), pp. 3150–3154.

Zhu, H.J. and Burgess, A.W. (2001) 'Regulation of transforming growth factor-beta signaling', *Molecular cell biology research communications: MCBRC*, 4(6), pp. 321–330. Available at: https://doi.org/10.1006/mcbr.2001.0301.

Chapter 10: Appendices

Appendix 1: Data of Scatter Assay

Scatter assay assessment by measuring the distance between the cells:

Statistical Analysis:

To evaluate if the method to measure the distance between cells is reproducible using R software. R software was used to find intra and Interclass Correlation agreement for 20% of subjects.

1) Intraclass correlation: (Same observer assessing at two different periods of time)

Average Score Intraclass Correlation
Model: One-way
Type: agreement
Subjects = 20
Raters = 2 ICC(2) = **0.986**
95%-Confidence Interval for ICC Population Values: 0.965 < ICC < 0.994

N.B: Two months gap between first observation and second observation

2) Interclass correlation: (Two different observers)

Average Score Intraclass Correlation
Model: Two-way
Type: agreement
Subjects = 20

Raters = 2 ICC(A,2) = **0.94**
95%-Confidence Interval for ICC Population Values: 0.85 < ICC < 0.976

HaCaT Cell line/Scatter Assay/Date 07-09-2021/ ImageJ software.

Observer1/ First assessment 17-3-2022, Second assessment 12-5-2022 (Two months gap).

Test Conditions	Observer1 #1/ Incubation periods			Observer1 #2/ Incubation periods		
Serum Free MEM	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	27.6	19.6	35.1	17.1	20.7	26.3
	28	29.2	28.7	19.3	21.4	16.9
	12.5	19.1	17.8	22.2	19.5	25.2
	23.7	24.7	21.7	21.4	26.8	29.8
	19.7	23.2	27.4	24.3	19.9	27.4
10ng/ml of TGFβ1	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	25.2	32.9	53.1	26.1	46.7	70.7
	31.5	28.4	60	25.9	28.4	63.8
	34.6	45.3	57.3	32.4	33.6	55.3
	43.5	41.6	66.5	33.5	33.8	43.4
	26.6	35.1	70.4	26.7	42.2	54.4
10ng/ml of EGF	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	17.7	31.7	37.1	18.1	20.5	27.5
	34.4	34.5	45.5	33.4	16.9	22.5
	34.1	40	34.8	33.5	26.9	20.5
	36.9	38.3	25.5	38.4	26.4	21.6
	14.8	38.4	29.8	17.9	21.5	24.1
10ng/ml of TGFβ1 + 5µM of TGF-β RI Kinase Inhibitor VII	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	26.3	21.3	25.4	33.8	20.7	17.1
	33.8	28.8	36.6	28.4	20.7	22.6
	22.6	20.7	26.7	32.3	30.2	25.7
	32.7	19.3	26.4	20.8	23.9	18.9
	43.8	30.2	21.2	42.8	20.1	19.7
10ng/ml of EGF + 5µM of TGF-β RI Kinase Inhibitor VII	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	17.6	24.1	24.3	19.1	21.5	21.9
	14.6	29	14.8	19.2	22.9	17.8
	26.8	30.8	18.7	15.5	29.9	22.9
	32.8	22.6	25.7	22.3	30.9	25.5
	19	21.8	26.7	19.4	23.5	25.1
10ng/ml of TGFβ1 + 1µM of MK-2206	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	24.5	23.7	37.2	19.2	47.3	25.9
	23.1	44.5	42.3	22.3	30.3	27.1
	21.8	37.6	37.7	21.7	28	36.9
	18.4	47.7	28.4	20.5	28.1	37.2
	27.6	44.8	23.1	25.2	27.2	41.2

10ng/ml of EGF + 1µM of MK-2206	Day0 in µm	Day1 in µm	Day2 in µm	Day0 in µm	Day1 in µm	Day2 in µm
	21.5	22.6	48.1	20.3	20.2	38.5
	20.7	19.5	44.6	20.7	20.2	32.1
	21.1	20.6	44	19.2	28.1	31.1
	18.4	27.1	42.6	20.3	26.3	33.7
	18.1	25.5	62.1	19.3	27.6	40.8
10ng/ml of TGFβ1 + 5µM of PD98059	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	17.7	28.5	82.4	29.4	27.6	72.6
	36.5	29.8	59.6	31.4	24.8	61
	33.8	41.8	67.4	38.8	38.8	67.5
	29.2	46.8	66.1	37.2	45.4	78.7
	25.7	37.9	62.6	28.8	38.6	59.2
10ng/ml of EGF + 5µM of PD98059	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	24.2	14.5	26.4	22.1	24.9	26.4
	36.9	29	32.5	38.9	29.7	22.9
	34.4	36.8	26.4	36.6	22.8	21.1
	42.9	20.7	23.5	42.6	30.9	28.5
	40.7	33.1	22.3	38.3	31.3	36.2
10ng/ml of TGFβ1 + 5µM of SB431542	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	26.5	28.6	24.4	26.9	14.8	19
	33.4	30.7	22.2	21.7	21.3	19.9
	30.8	24.4	21.1	26.2	23.8	16.3
	36	34.3	25.8	25.9	21.7	18
	44.7	36.5	28	35.8	20.5	23.4
10ng/ml of EGF + 5µM of SB431542	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	39	20.2	15.8	21.9	22.2	16.1
	37	23.5	15.3	25.8	18.2	14.6
	37.6	27.2	15.7	18.3	24.4	18.2
	25	20	19.8	31.8	21.5	20
	33.2	28	19.4		23.2	16.9

HaCaT Cell line/Scatter Assay/Date 07-09-2021/ ImageJ software.

Observer2/ Assessment Date 12-5-2022

Test Conditions	Incubation periods		
Serum Free MEM	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	21.4	25.5	30.8
	29.3	24.6	20.6
	14.9	36.8	32.8
	20.2	32.5	29.4
	19.5	26.5	25.4
10ng/ml of TGFβ1	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	33.4	30.2	41.3
	27.2	48.1	68.5
	38.8	45.4	67.7
	33.2	33.3	56.3
	27.7	34.7	52.1
10ng/ml of EGF	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	18.2	24.6	23.8
	20.5	14.8	36.7
	20.6	23.2	30.3
	37.3	33.1	26.5
	37.1	20.2	31
10ng/ml of TGFβ1 + 5µM of TGF-β RI Kinase Inhibitor VII	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	19.1	26.5	27.7
	44	35.7	23.1
	45.7	31.8	29.3
	32.4	33.2	32.7
	26.4	27	26.9
10ng/ml of EGF + 5µM of TGF-β RI Kinase Inhibitor VII	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	17.5	24.2	13.3
	22.6	27	26.2
	14.7	30.1	18.8
	22.5	30.3	19.6
	19.4	23.7	26
10ng/ml of TGFβ1 + 1µM of MK-2206	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	26.9	38.6	34.4
	21.9	19.2	28.3
	23.6	22.7	26.1
	21.5	41.6	51.9
	22.9	47.5	25.8
10ng/ml of EGF + 1µM of MK-2206	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	21.3	21.5	19.7
	21.2	25.1	24.2
	20.7	22.2	34
	17.2	23.8	37.7
	20.8	33	32.5
	Day0 in µm	**Day1 in µm**	**Day2 in µm**

10ng/ml of TGFβ1 + 5μM of PD98059	28.5	25.7	69.4
	44	31.4	63.6
	26.3	26	60.6
	23	62.1	
	36.9	33.1	
10ng/ml of EGF + 5μM of PD98059	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	25.2	25.7	18.7
	36.6	24.3	30.8
	32.9	24.1	36.2
	41	34.6	28.4
	41.1	37.9	30.5
10ng/ml of TGFβ1 + 5μM of SB431542	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	33.2	22.3	17.4
	28.8	23.5	18.8
	28.6	22	24.6
	34.3	32.3	20.5
	23	30.5	28.1
10ng/ml of EGF + 5μM of SB431542	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	17.5	26.4	14.7
	30.7	24.8	22.8
	21.2	23.7	22.7
	31.6	30.8	20.3
	30	32	30.5

TYS Cell line/Scatter Assay/Date 07-09-2021/ ImageJ software.

Observer1/ Assessment Date 21-03-2022

Test Conditions	**Incubation periods**		
Serum Free MEM	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	26.1	18.6	27.1
	29.7	23.1	31.3
	37.1	18.7	36.5
	25.3	26.6	35.8
	35.7	30.7	32.7
10ng/ml of TGFβ1	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	20.4	45.5	49.9
	31.4	43.2	62.5
	33.2	56	48.1
	32	52.9	68.3
	35.4	46.9	53.4
10ng/ml of EGF	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	21.2	49.5	50.2
	33.8	58.4	66.7
	23.5	52.3	49.8
	19.6	56.8	44.8

	38.9	51.7	80.6
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	24.7	20.9	38.9
	26.8	14.2	30.4
	32.1	28.7	33.3
	28.7	21.4	24.9
	37.2	26.6	39.5
10ng/ml of EGF + 5μM of TGF-β RI Kinase Inhibitor VII	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	19.3	31.3	56.1
	22.2	44.7	48.1
	24.9	54.7	49.9
	29.3	46.6	38.7
	27.5	47.6	69.3
10ng/ml of TGFβ1 + 1μM of MK-2206	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	29.7	18.7	25.1
	38.1	21.1	23.2
	34.4	15.7	18.7
		20.8	21.2
		21.5	30.9
10ng/ml of EGF + 1μM of MK-2206	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	31.6	48.3	47.5
	34.7	33.4	38.8
	29.2	58.2	56.8
	32.4	42	70.3
	39.3	38.4	
10ng/ml of TGFβ1 + 5μM of PD98059	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	30.3	31.8	48.6
	35.3	27.3	54
	26.3	37.1	45.8
	35.6	42	69.6
	41.7	42	74.7
10ng/ml of EGF + 5μM of PD98059	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	26.3	67.1	56.2
	32	60	50
	24.2	59.7	55
	25.5	56.8	54.3
	24.7	51.5	35.4
10ng/ml of TGFβ1 + 5μM of SB431542	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	23.2	23.4	21.2
	29.6	23	21.2
	23	20.8	31.2
	23.7	22.2	31.4
	32.6	26.3	25.6
10ng/ml of EGF + 5μM of SB431542	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	32.7	45.3	47.4
	19.3	47.4	71.5

	30.9	39.9	57.5
	23.4	55.6	44.4
	26	53.5	32.9

SAS-H1 Cell line/Scatter Assay/Date 07-09-2021/ ImageJ software.

Observer1/ Assessment Date 21-03-2022

Test Conditions	**Incubation periods**		
Serum Free MEM	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	31.4	20.7	18.5
	36.5	21.6	23.1
	22.9	28.7	34.2
	31.5	25.6	20.4
	31	35	17.8
10ng/ml of TGFβ1	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	32.6	45.5	72.1
	34	43.2	56.7
	23.1	56	48
	33.6	52.9	79.6
	24.2	46.9	
10ng/ml of EGF	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	36.8	54.3	37.9
	37.4	36.3	57.5
	21.6	41.3	51.6
	22.5	46.1	64.9
	31.2	42.1	51.2
10ng/ml of TGFβ1 + 5µM of TGF-β RI Kinase Inhibitor VII	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	21.5	20.9	16.2
	17.9	20.7	21.4
	32.6	17.5	29.4
	30	22.2	23.2
		18.5	31.7
10ng/ml of EGF + 5µM of TGF-β RI Kinase Inhibitor VII	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	31.7	56.9	74.5
	25.4	49.2	50.3
	33.1	41.7	53.9
	28.4		59.3
	32		62.6
10ng/ml of TGFβ1 + 1µM of MK-2206	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	30.6	46.3	42.9
	35.9	55.1	37.5
	33	45.7	38.6
	34.2	49.8	59.1
	42.2	57	50.7
10ng/ml of EGF + 1µM of MK-2206	**Day0 in µm**	**Day1 in µm**	**Day2 in µm**
	20.2	48.4	30.9

	21.8	53.1	25.7
	24.5	33.3	34.2
	25.1	32.6	26.5
	21.9	29	28.8
10ng/ml of TGFβ1 + 5μM of PD98059	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	23	36.7	24.6
	21.2	45.1	31.1
	34.1	36.2	24
	19.8	31.8	35.9
	35.7	42.6	32.7
10ng/ml of EGF + 5μM of PD98059	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	26.1	51.2	77
	22.2	69.9	54
	20.4	45.4	57.2
	18.7		61.4
	27.8		52.9
10ng/ml of TGFβ1 + 5μM of SB431542	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	21.2	19.3	42
	27.8	21.3	37
	19.9	27.4	40.7
	19.1	26	40.4
	34	22.9	
10ng/ml of EGF + 5μM of SB431542	**Day0 in μm**	**Day1 in μm**	**Day2 in μm**
	22.1	17	23.7
	31.7	24.1	28.7
	38.9	27.3	42.3
	35.8	19.1	28
	44	21.6	24.9

Appendix 2: Data of Gap Closure Assay

HaCaT Cell line/Gap Closure Assay/Date 03-03-2021/ ImageJ software.

SF-MEM	Inserts no.	Area/pixel T= 0min	Area/pixel T= 1hr	Area/pixel T= 2hrs	Area/pixel T= 6hrs	Area/pixel T= 24hrs
	2	1905674	1919144	1900663	1917871	1706017
		1904387	1850761	1883978	1877687	1484728
		1911817	1883595	1908364	1889434	1761365
	4	1666725	1643884	1656883	1673479	1527451
		1658601	1680728	1652499	1612908	1156009
		1567267	1545553	1548603	1573441	1036182
	5	1694195	1674219	1685021	1706455	1569482
		1623174	1711937	1653989	1669617	1454817
		1643792	1613953	1632626	1634371	1356835
10ng/ml of TGFβ1	**Inserts no.**	**Area/pixel T= 0min**	**Area/pixel T= 1hr**	**Area/pixel T= 2hrs**	**Area/pixel T= 6hrs**	**Area/pixel T= 24hrs**
	1	1821935	1668183	1626197	1667096	669662
		1690276	1484173	1636962	1570649	281599
		1525426	1494883	1502808	1506924	511335
	2	1627529	1635753	1627154	1603568	153583
		1595860	1778744	1580027	1574959	679147
		1510807	1503217	1534151	1527796	584717
	4	1715945	1700080	1693508	1739365	258341
		1614905	1603180	1600756	1565094	0
		1606032	1585608	1590324	1630349	0
10ng/ml of EGF	**Inserts no.**	**Area/pixel T= 0min**	**Area/pixel T= 1hr**	**Area/pixel T= 2hrs**	**Area/pixel T= 6hrs**	**Area/pixel T= 24hrs**
	2	1649687	1801240	1879086	2124587	826450
		1663992	1806080	1927698	2046593	0
		1660008	1781757	1811874	2015134	0
	3	1795588	1874680	2034968	2239174	565147
		1798096	1907152	1969810	2201106	1545118
		1772747	1871550	1975563	2259629	953322
	4	1495762	1644576	1641802	1710669	519832
		1568817	1619717	1598248	1632392	0
		1549678	1663596	1700397	1676411	482110

TYS Cell line/Gap Closure Assay/Date 13-01-2020/ ImageJ software.

SF-MEM	Inserts no.	Area/pixel T= 0min	Area/pixel T= 1hr	Area/pixel T= 2hrs	Area/pixel T= 6hrs	Area/pixel T= 24hrs
	1	1781374	1573990	1422507	1059499	0
		1921335	1593760	1572978	487455	0
	3	1690312	1353265	1253135	624537	88604.03
		1634605	1425359	1354764	157440	0
	5	1690392	1353326	1093698	302184	0
		1615560	1319779	1175952	283232	0
10ng/ml of TGFβ1	**Inserts no.**	**Area/pixel T= 0min**	**Area/pixel T= 1hr**	**Area/pixel T= 2hrs**	**Area/pixel T= 6hrs**	**Area/pixel T= 24hrs**
	2	1688315	1505886	1403695	627922	0
		1738670	1471016	1409040	961870	0
	4	1596656	1422827	1262673	876303	0
		1610168	1366364	1255574	785360	0
	5	1641003	1408769	1240532	565885	0
		1625096	1361146	1254758	599456	0
10ng/ml of EGF	**Inserts no.**	**Area/pixel T= 0min**	**Area/pixel T= 1hr**	**Area/pixel T= 2hrs**	**Area/pixel T= 6hrs**	**Area/pixel T= 24hrs**
	3	1768760	1649650	1611187	1206116	0
		1719077	1679620	1656612	1093796	0
	4	1505894	1452595	1471103	456458	0
		1515240	1498317	1471937	714242	0
	5	1608640	1462086	1479290	1061952	0
		1559307	1521137	1418641	701727	0

SAS-H1 Cell line/Gap Closure Assay/Date 15-04-2021/ ImageJ software.

SF-MEM	Inserts no.	Area/pixel T= 0min	Area/pixel T= 1hr	Area/pixel T= 2hrs	Area/pixel T= 6hrs	Area/pixel T= 24hrs
	2	2080393	1990627	1899676	1878498	0
		2022591	1903910	1785909	1942847	2064970
		1919834	1883597	1862221	1761649	1246306
	3	2125443	2060184	2024864	2281692	2362511
		2235857	2122788	2169252	2161170	1579312
		2027758	1951482	1918875	1714189	0
	4	1772672	1687911	1633489	1697982	659737
		1948709	1909793	1892995	2038143	1458317
		1873162	1839073	1826809	2043800	944862
10ng/ml of TGFβ1	**Inserts no.**	**Area/pixel T= 0min**	**Area/pixel T= 1hr**	**Area/pixel T= 2hrs**	**Area/pixel T= 6hrs**	**Area/pixel T= 24hrs**
	1	2069893	2019327	1926082	1797771	240831
		2142458	2044805	2017920	1742149	76197
		2091361	2020218	1856753	1894163	132421
	2	1859095	1744357	1675411	1336319	0
		1910402	1865924	1812689	1679509	49331
		1887420	1801080	1719698	1546108	124502
	3	2138958	2104946	2071245	1986850	315862
		2125736	2209409	2122189	2073955	563551
		2100859	2022386	1935325	1684845	417261
10ng/ml of EGF	**Inserts no.**	**Area/pixel T= 0min**	**Area/pixel T= 1hr**	**Area/pixel T= 2hrs**	**Area/pixel T= 6hrs**	**Area/pixel T= 24hrs**
	1	2199566	2190373	2180872	2103325	301359
		1928853	1912026	1935972	1754805	0
		2017127	1985636	1962392	2105967	546367
	2	1784272	1666865	1658552	1594875	142285
		1797143	1699396	1585943	1253754	128550
		1798181	1722111	1556674	1606504	0
	3	2003105	1974823	1969303	2300486	0
		1974592	1957072	1935059	2767740	0
		2066527	2031899	2034412	2148162	0

Appendix 3: Data Scratch Assay

HaCaT Cell line/Scratch Assay/Date 02-11-2021/ ImageJ software.

	Sample No.	Area/pixel T=0	Area/pixel T=24hrs				
SF-MEM	**1**	4665092	3774738				
	2	4649542	3993266				
	3	3862586	3787755				
10ng/ml of TGFβ1	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**	**10ng/ml of EGF**	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**
	1	4447751	3412547		**1**	4306676	2286995
	2	4607021	4040217		**2**	4403005	2330824
	3	4691594	4123911		**3**	4276675	2524575
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**	**10ng/ml of EGF + 5μM of TGF-β RI Kinase Inhibitor VII**	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**
	1	4652848	4770506		**1**	4619573	4649476
	2	4702436	4781452		**2**	4271759	4506954
	3	4714445	4769819		**3**	4449592	4696722
10ng/ml of TGFβ1 + 1μM of MK-2206	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**	**10ng/ml of EGF +1μM of MK-2206**	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**
	1	4794030	4783970		**1**	4812632	3801844
	2	4611577	4710586		**2**	4807824	4438917
	3	4713304	4774679		**3**	4797223	3673735
10ng/ml of TGFβ1 + 5μM of PD98059	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**	**10ng/ml of EGF + 5μM of PD98059**	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**
	1	4778871	4706219		**1**	4532857	4797681
	2	4663661	4794353		**2**	4788217	4764023
	3	4092066	4589968		**3**	4682246	4717996
10ng/ml of TGFβ1 + 5μM of SB431542	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**	**10ng/ml of EGF + 5μM of SB431542**	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**
	1	4727243	4699596		**1**	4764337	4576120
	2	4538901	4731770		**2**	4742657	4425553
	3	4713607	4689482		**3**	4813839	4650158

TYS Cell line/Scratch Assay/Date 10-10-2021/ ImageJ software.

	Sample No.	Area/pixel T=0	Area/pixel T=24hrs				
SF-MEM	1	4128067	2585415				
	2	4131267	2327345				
	3	4178523	1904729				
10ng/ml of TGFβ1	Sample No.	Area/pixel T=0	Area/pixel T=24hrs	10ng/ml of EGF	Sample No.	Area/pixel T=0	Area/pixel T=24hrs
	1	4725854	1378782		1	4662972	611402
	2	4686698	887149		2	4624144	952256
	3	4593122	893693		3	4683884	1376182
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII	Sample No.	Area/pixel T=0	Area/pixel T=24hrs	10ng/ml of EGF + 5μM of TGF-β RI Kinase Inhibitor VII	Sample No.	Area/pixel T=0	Area/pixel T=24hrs
	1	4737238	4425386		1	4426281	3533760
	2	4755302	4406818		2	4564492	4205403
	3	4767085	4101960		3	4420367	3645213
10ng/ml of TGFβ1 + 1μM of MK-2206	Sample No.	Area/pixel T=0	Area/pixel T=24hrs	10ng/ml of EGF +1μM of MK-2206	Sample No.	Area/pixel T=0	Area/pixel T=24hrs
	1	4794603	4679368		1	4773917	4191176
	2	4831646	4713331		2	4706366	4051128
	3	4785036	4539438		3	4793006	4401611
10ng/ml of TGFβ1 + 5μM of PD98059	Sample No.	Area/pixel T=0	Area/pixel T=24hrs	10ng/ml of EGF + 5μM of PD98059	Sample No.	Area/pixel T=0	Area/pixel T=24hrs
	1	4568976	3576340		1	4589653	4481254
	2	4598249	3715133		2	4690652	4240285
	3	4669649	3359654		3	4098731	4268541
10ng/ml of TGFβ1 + 5μM of SB431542	Sample No.	Area/pixel T=0	Area/pixel T=24hrs	10ng/ml of EGF + 5μM of SB431542	Sample No.	Area/pixel T=0	Area/pixel T=24hrs
	1	4647165	4059711		1	4439182	3276164
	2	4700144	4100232		2	4244285	3198999
	3	4555787	4308430		3	4473319	3884712

SAS-H1 Cell line/Scratch Assay/Date 21-11-2021/ ImageJ software.

	Sample No.	Area/pixel T=0	Area/pixel T=24hrs				
SF-MEM	**1**	4721936	2688686				
	2	4679634	2868906				
	3	4737941	4436227				
10ng/ml of TGFβ1	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**	**10ng/ml of EGF**	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**
	1	4771940	3132625		**1**	4741466	2690887
	2	4458439	2818425		**2**	4769651	2436573
	3	4757575	3747608		**3**	4753520	2160007
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**	**10ng/ml of EGF + 5μM of TGF-β RI Kinase Inhibitor VII**	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**
	1	4803553	4331682		**1**	4660301	4733415
	2	4794539	2543971		**2**	4461803	4461803
	3	4685204	3594293		**3**	4775414	4706626
10ng/ml of TGFβ1 + 1μM of MK-2206	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**	**10ng/ml of EGF +1μM of MK-2206**	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**
	1	4570649	3438058		**1**	4291965	3141010
	2	4705784	4453660		**2**	4707780	2892100
	3	4530720	3404758		**3**	4773937	2627661
10ng/ml of TGFβ1 + 5μM of PD98059	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**	**10ng/ml of EGF + 5μM of PD98059**	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**
	1	4412701	3642190		**1**	4494127	3936511
	2	4129546	3156257		**2**	4688600	3971903
	3	3861291	3292793		**3**	4644597	3745795
10ng/ml of TGFβ1 + 5μM of SB431542	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**	**10ng/ml of EGF + 5μM of SB431542**	**Sample No.**	**Area/pixel T=0**	**Area/pixel T=24hrs**
	1	4759695	4424141		**1**	4732660	4473334
	2	4713031	4376042		**2**	4662088	3792283
	3	4781448	3828793		**3**	4807101	3717023

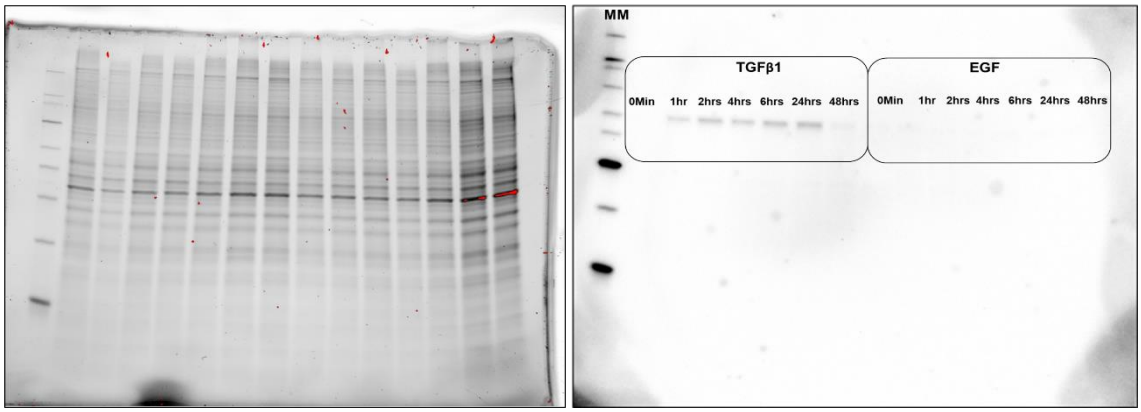
Appendix 4: Data SDS-PAGE & Western Blot (1)

Report of WB experiment regarding signalling pathways activation in responses to growth factors at different incubation periods.

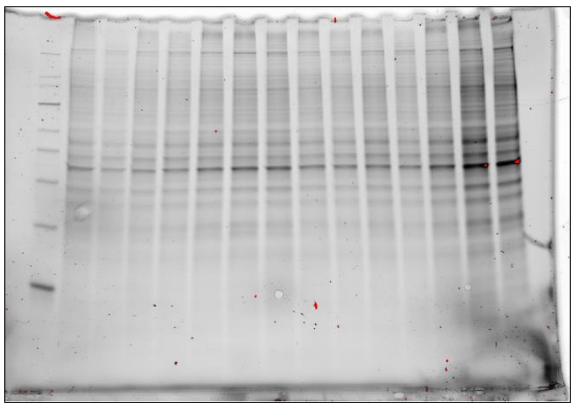
Gel & Blot Design:

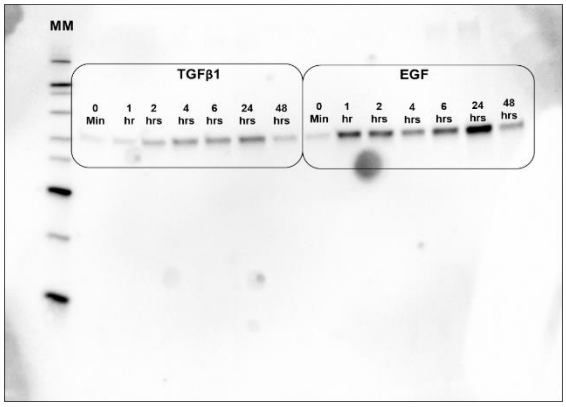
WB experiment repot	Date: 03/08/2021
Cell line: HaCaT	
Gel and Blot 1 design (Different incubation periods with growth factors)	
Lane 1	Magic Marker
Lane 2	10ng/ml of TGFβ1 (T = 0 Min)
Lane 3	10ng/ml of TGFβ1 (T = 1 hour)
Lane 4	10ng/ml of TGFβ1 (T = 2 hours)
Lane 5	10ng/ml of TGFβ1 (T = 4 hours)
Lane 6	10ng/ml of TGFβ1 (T = 6 hours)
Lane 7	10ng/ml of TGFβ1 (T = 24 hours)
Lane 8	10ng/ml of TGFβ1 (T = 48 hours)
Lane 9	10ng/ml of EGF (T = 0 Min)
Lane 10	10ng/ml of EGF (T = 1 hour)
Lane 11	10ng/ml of EGF (T = 2 hours)
Lane 12	10ng/ml of EGF (T = 4 hours)
Lane 13	10ng/ml of EGF (T = 6 hours)
Lane 14	10ng/ml of EGF (T = 24 hours)
Lane 15	10ng/ml of EGF (T = 48 hours)

Gel & Blot No.1 (p-Smad2)

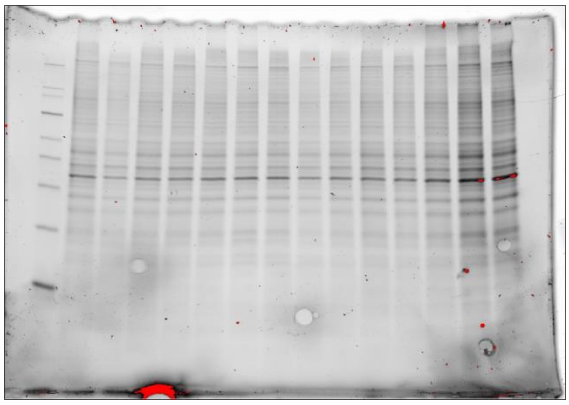


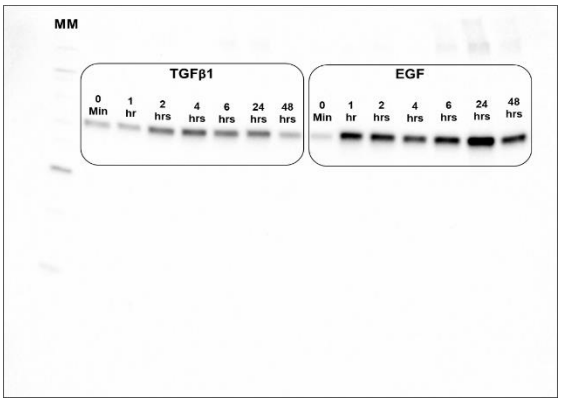
Gel & Blot No.2 (p-Akt T308)



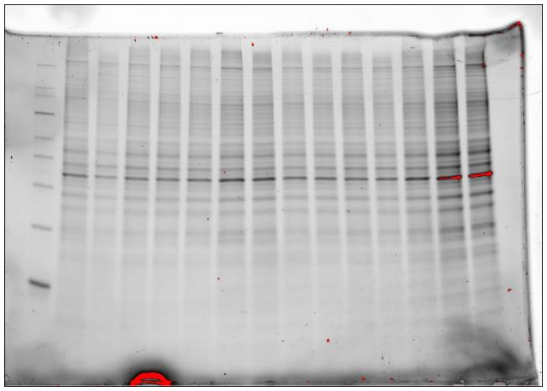


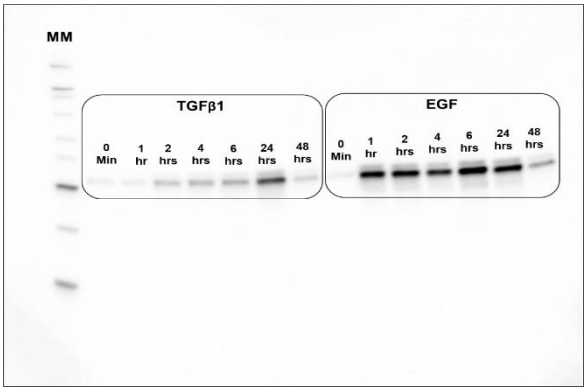
Gel & Blot No.3 (p-Akt S473)



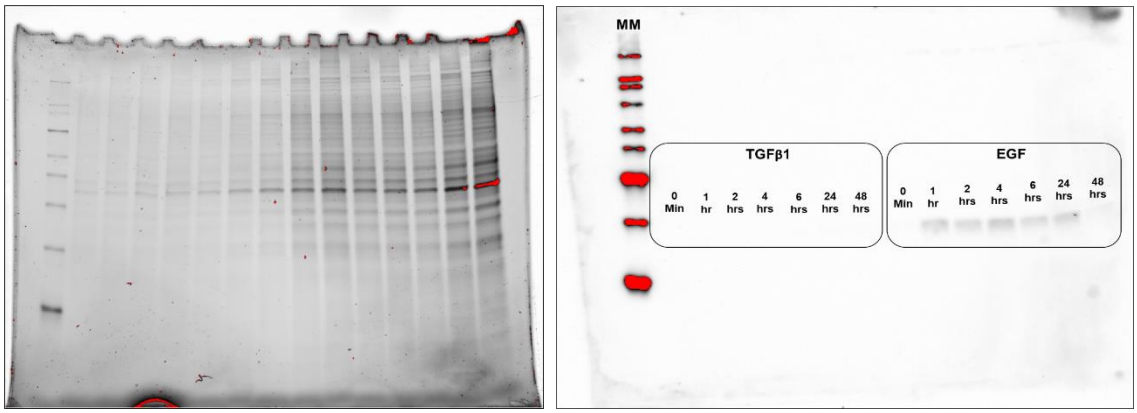


Gel & Blot No.4 (p-p44/p42)





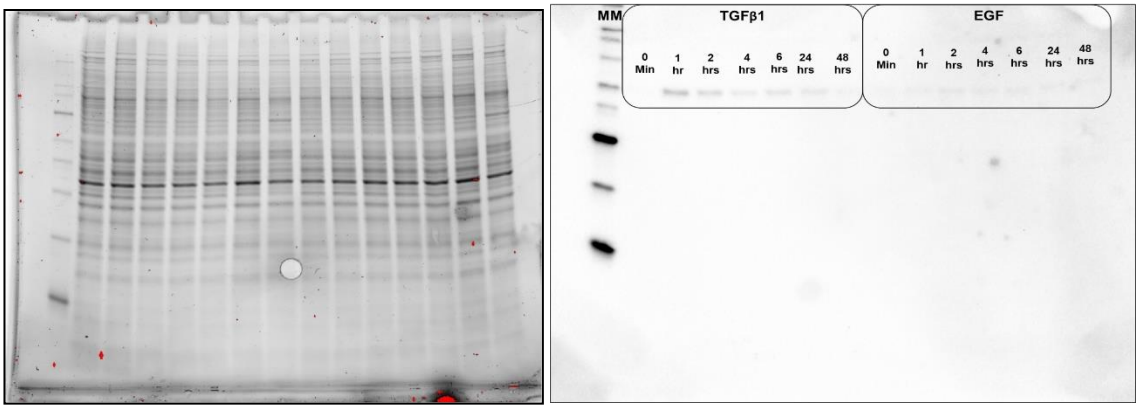
Gel & Blot No.5 (p-Rac1/Cdc42)



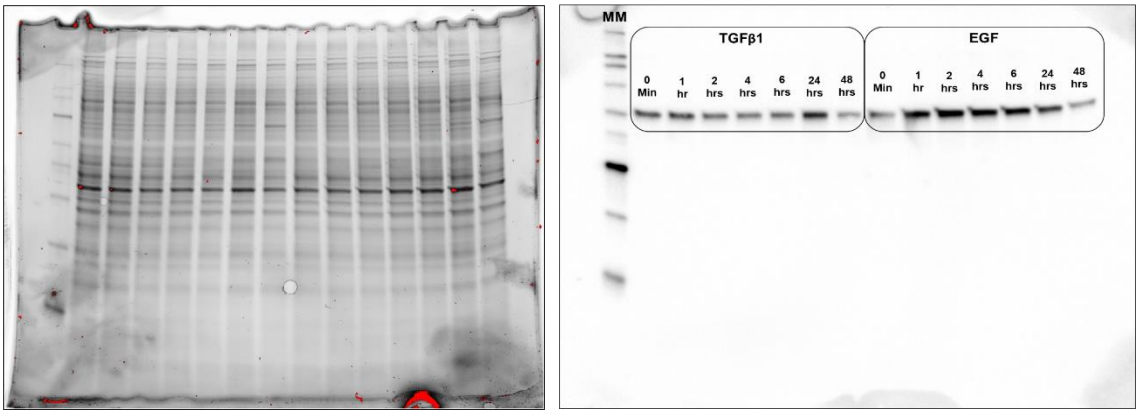
Gel & Blot Design:

WB experiment repot	Date: 16/11/2020
Cell line: TYS	
Gel and Blot 6 design (Different incubation periods with growth factors)	
Lane 1	Magic Marker
Lane 2	10ng/ml of TGFβ1 (T = 0 Min)
Lane 3	10ng/ml of TGFβ1 (T = 1 hour)
Lane 4	10ng/ml of TGFβ1 (T = 2 hours)
Lane 5	10ng/ml of TGFβ1 (T = 4 hours)
Lane 6	10ng/ml of TGFβ1 (T = 6 hours)
Lane 7	10ng/ml of TGFβ1 (T = 24 hours)
Lane 8	10ng/ml of TGFβ1 (T = 48 hours)
Lane 9	10ng/ml of EGF (T = 0 Min)
Lane 10	10ng/ml of EGF (T = 1 hour)
Lane 11	10ng/ml of EGF (T = 2 hours)
Lane 12	10ng/ml of EGF (T = 4 hours)
Lane 13	10ng/ml of EGF (T = 6 hours)
Lane 14	10ng/ml of EGF (T = 24 hours)
Lane 15	10ng/ml of EGF (T = 48 hours)

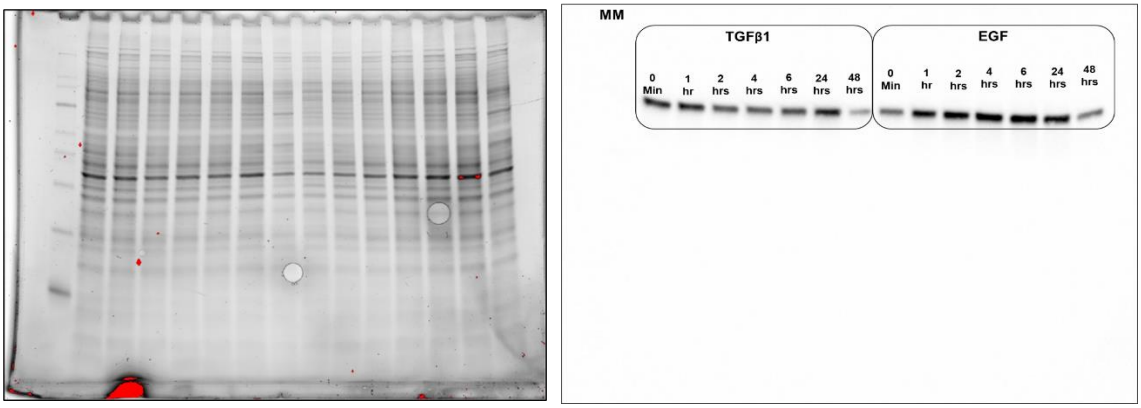
Gel & Blot No.6 (p-Smad2)



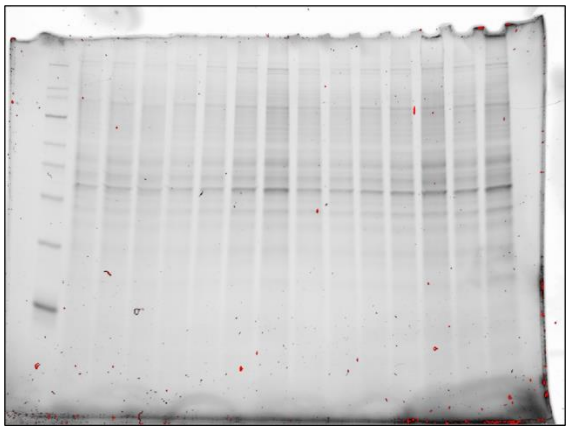
Gel & Blot No.7 (p-Akt T308)

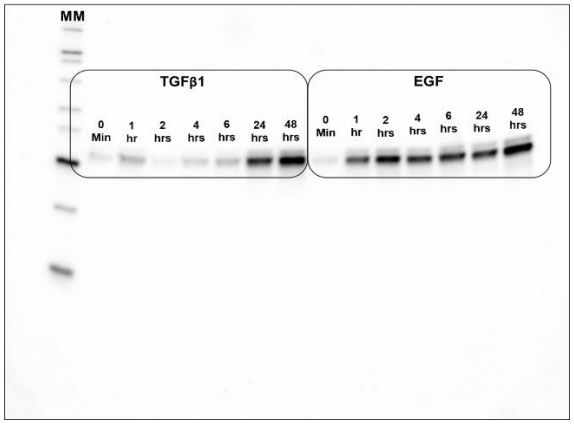


Gel & Blot No.8 (p-Akt S473)

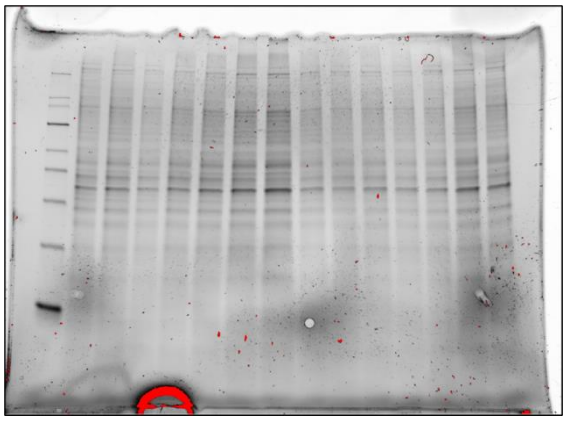


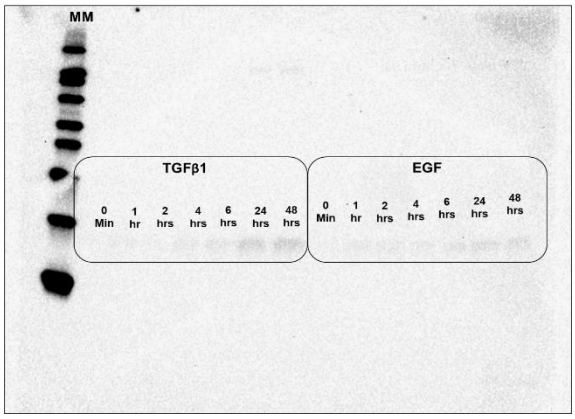
Gel & Blot No.9 (p-p44/p42)





Gel & Blot No.10 (p-Rac1/Cdc42)



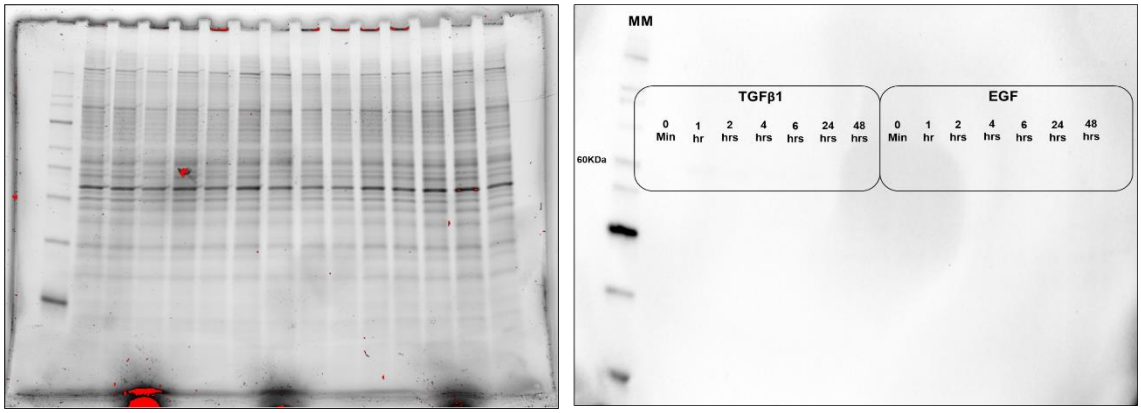


Gel & Blot Design:

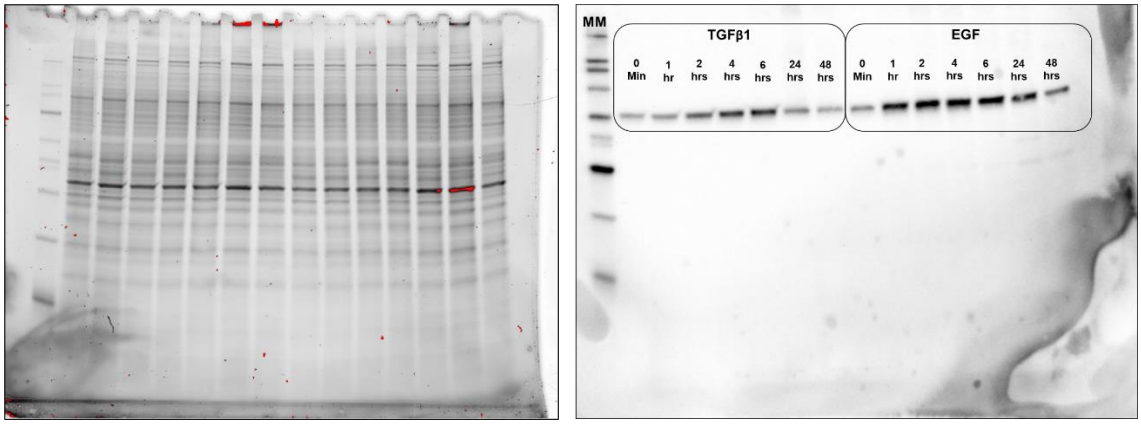
WB experiment repot	Date: 12/04/2021
Cell line: SAS-H1	
Gel and Blot 11 design (Different incubation periods with growth factors)	
Lane 1	Magic Marker
Lane 2	10ng/ml of TGFβ1 (T = 0 Min)
Lane 3	10ng/ml of TGFβ1 (T = 1 hour)
Lane 4	10ng/ml of TGFβ1 (T = 2 hours)
Lane 5	10ng/ml of TGFβ1 (T = 4 hours)
Lane 6	10ng/ml of TGFβ1 (T = 6 hours)
Lane 7	10ng/ml of TGFβ1 (T = 24 hours)
Lane 8	10ng/ml of TGFβ1 (T = 48 hours)
Lane 9	10ng/ml of EGF (T = 0 Min)
Lane 10	10ng/ml of EGF (T = 1 hour)
Lane 11	10ng/ml of EGF (T = 2 hours)

Lane 12	**10ng/ml of EGF (T = 4 hours)**
Lane 13	**10ng/ml of EGF (T = 6 hours)**
Lane 14	**10ng/ml of EGF (T = 24 hours)**
Lane 15	**10ng/ml of EGF (T = 48 hours)**

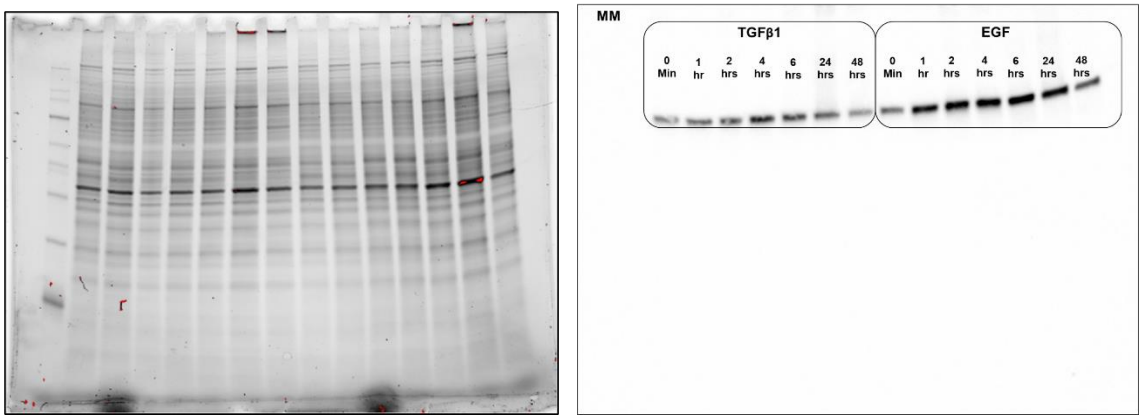
Blot & Gel No.11 (p-Smad2)



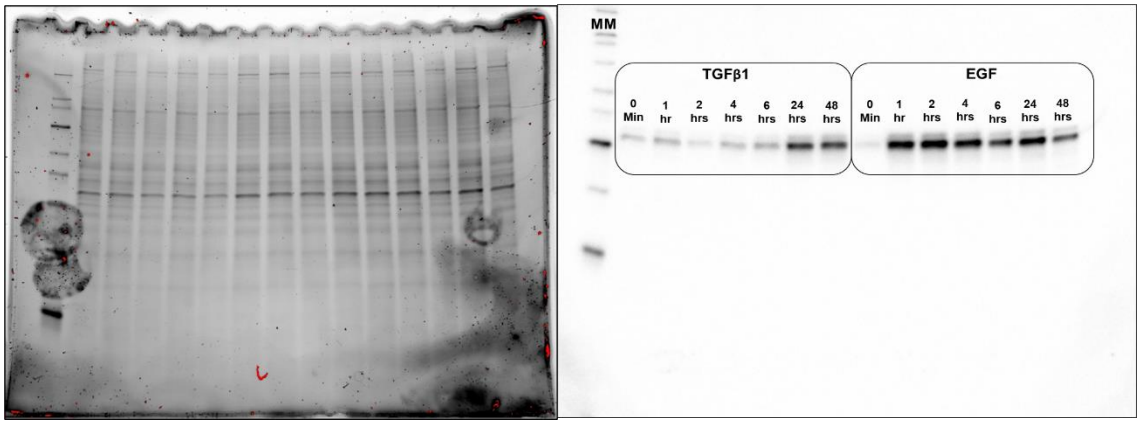
Blot & Gel No.12 (p-Akt T308)



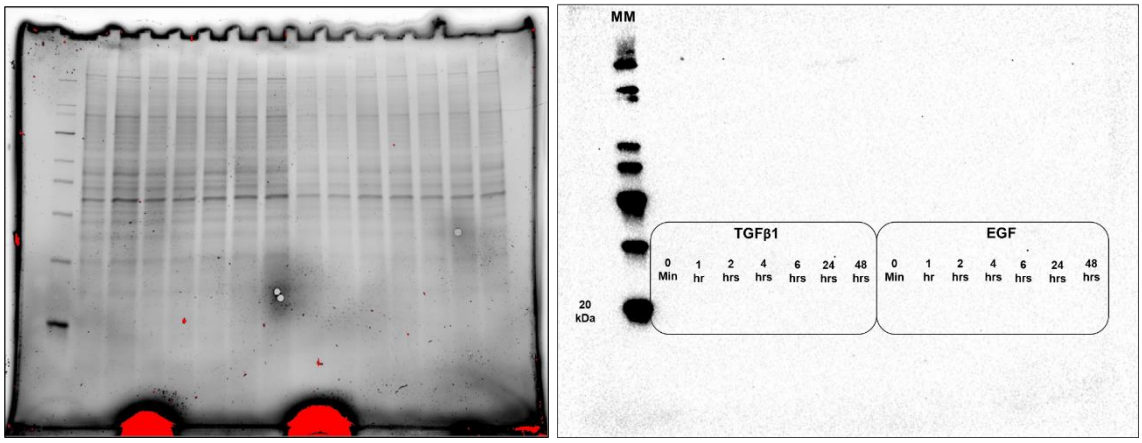
Blot & Gel No.13 (p-Akt S473)



Gel & Blot No.14 (p-p44/p42)



Gel & Blot No. 15 (p-Rac1/Cdc42)



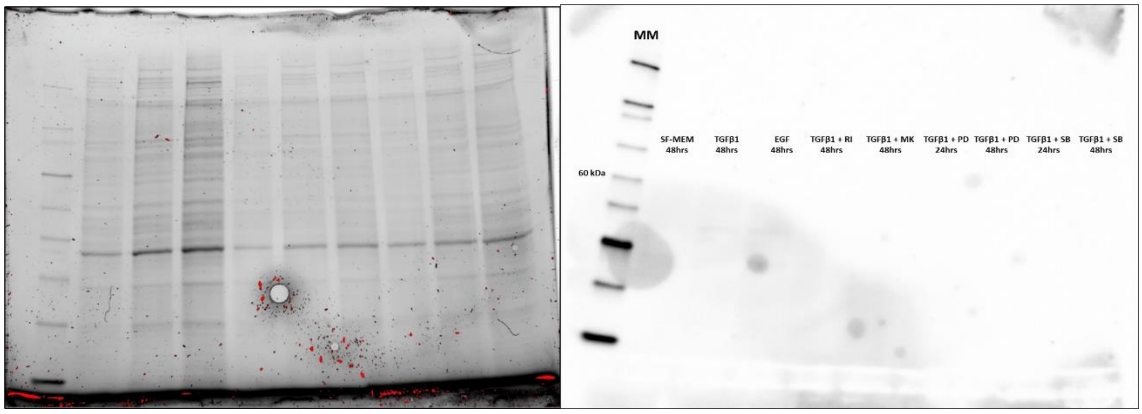
Appendix 5: Data SDS-PAGE & Western Blot (2)

Report of WB Experiment Regarding Signalling Pathways Activation in Responses to Different Test Conditions at 24 hours and 48 hours.

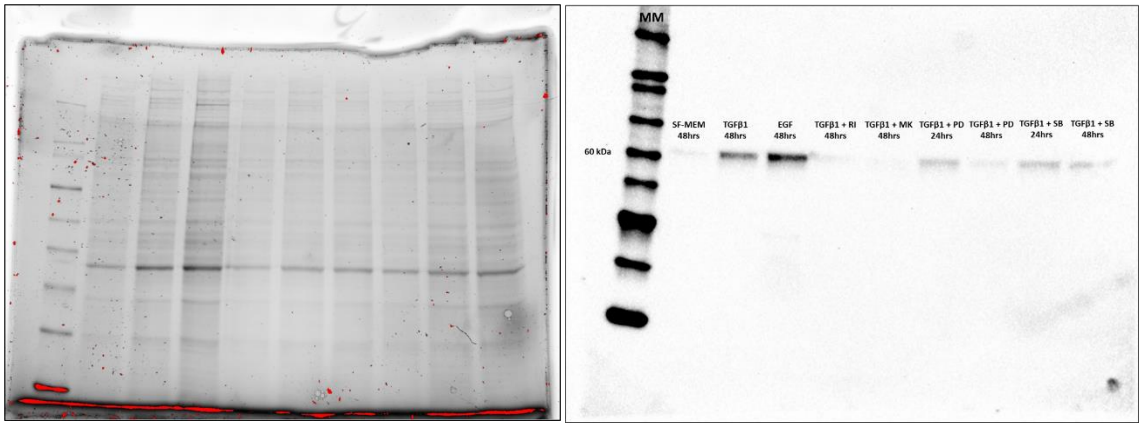
Gel & Blot Design

WB experiment repot	Date: 10/1/2022
Cell line: HaCaT	
Gel and Blot 1 design (Different test conditions)	
Lane 1	Magic Marker
Lane 2	SF-MEM (T = 48 hours)
Lane 3	10ng/ml of TGFβ1 (T = 48 hours)
Lane 4	10ng/ml of EGF (T = 48 hours)
Lane 5	10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII (T = 48 hours)
Lane 6	10ng/ml of TGFβ1 + 1μM of MK-2206 (T = 48 hours)
Lane 7	10ng/ml of TGFβ1 + 5μM of PD98059 (T = 24 hours)
Lane 8	10ng/ml of TGFβ1 + 5μM of PD98059 (T = 48 hours)
Lane 9	10ng/ml of TGFβ1 + 5μM of SB431542 (T = 24 hours)
Lane 10	10ng/ml of TGFβ1 + 5μM of SB431542 (T = 48 hours)

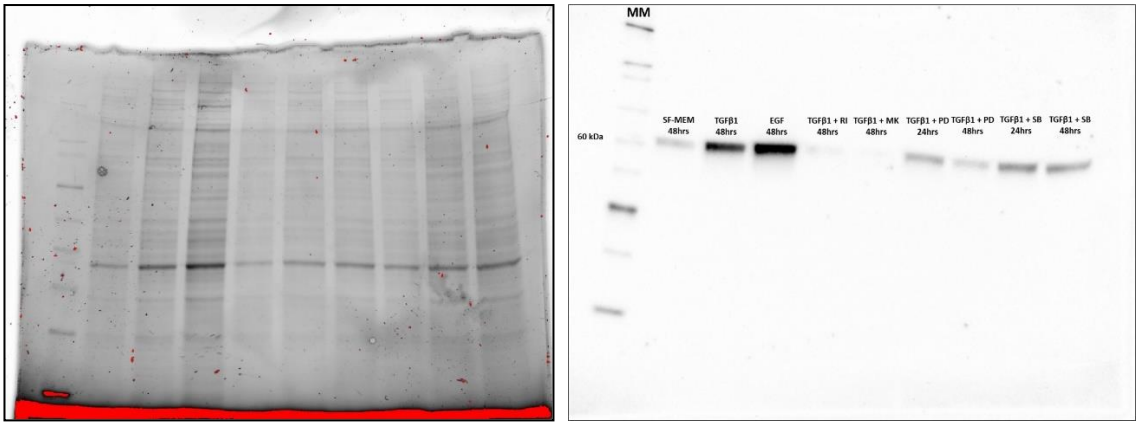
Gel & Blot No.1 (p-Smad2)



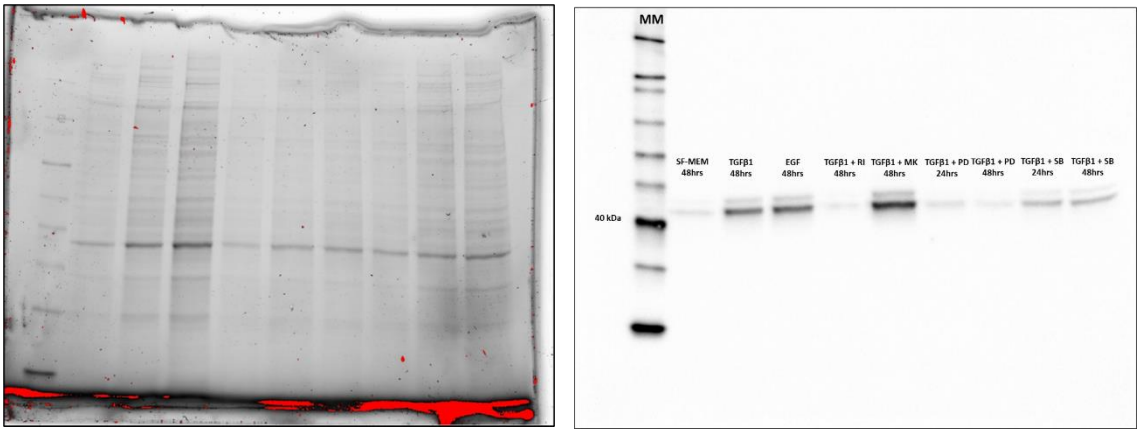
Gel & Blot No.2 (p-Akt T308)



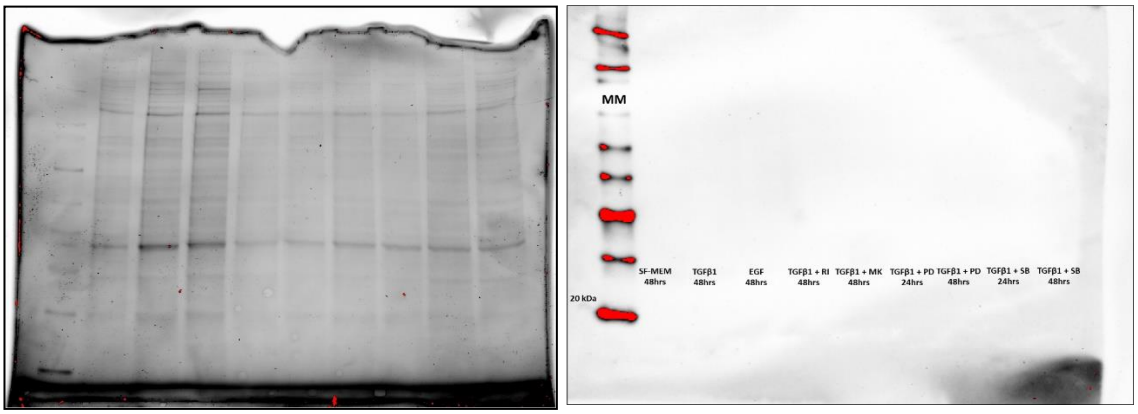
Gel & Blot No.3 (p-Akt S473)



Gel & Blot No.4 (p-p44/42)



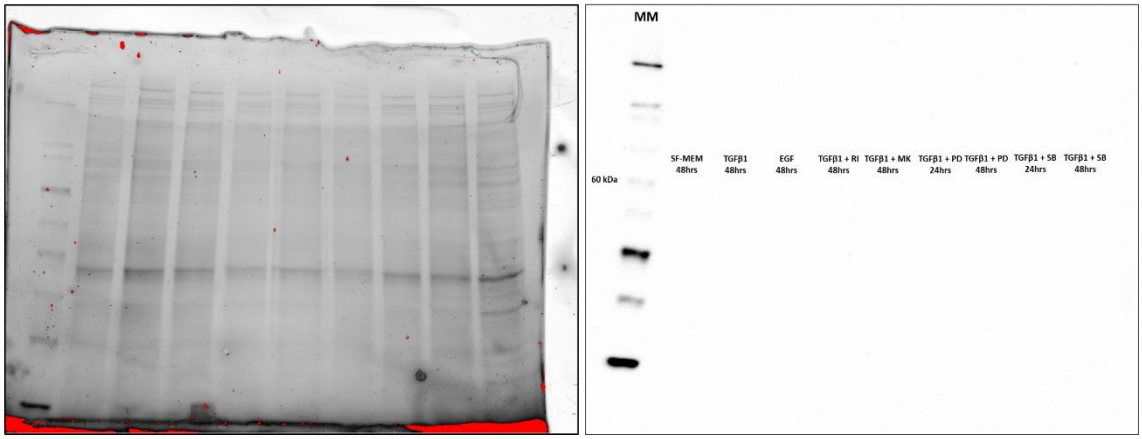
Gel & Blot No.5 (p-Rac1/Cdc42)



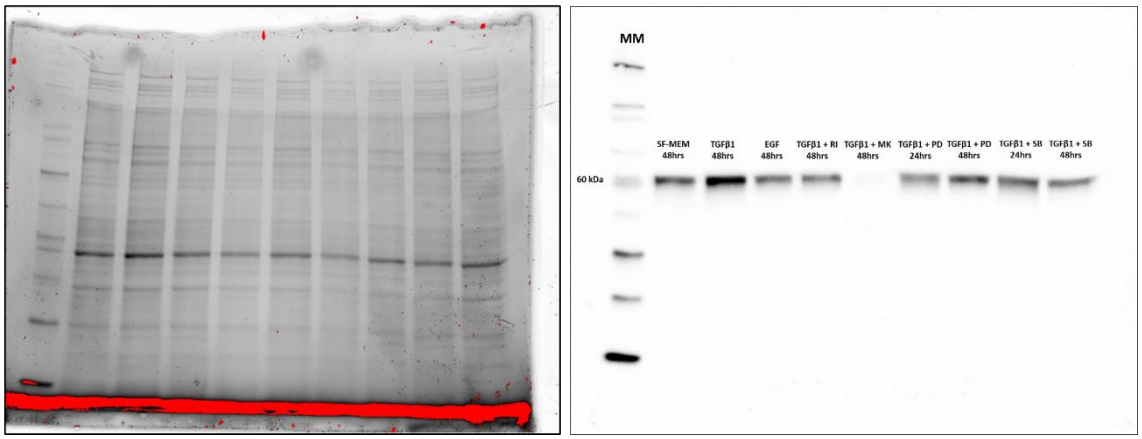
Gel & Blot Design:

WB experiment repot	Date: 6/4/2022
Cell line: TYS	
Gel and Blot 6 design (Different test conditions)	
Lane 1	Magic Marker
Lane 2	SF-MEM (T = 48 hours)
Lane 3	10ng/ml of TGFβ1 (T = 48 hours)
Lane 4	10ng/ml of EGF (T = 48 hours)
Lane 5	10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII (T = 48 hours)
Lane 6	10ng/ml of TGFβ1 + 1μM of MK-2206 (T = 48 hours)
Lane 7	10ng/ml of TGFβ1 + 5μM of PD98059 (T = 24 hours)
Lane 8	10ng/ml of TGFβ1 + 5μM of PD98059 (T = 48 hours)
Lane 9	10ng/ml of TGFβ1 + 5μM of SB431542 (T = 24 hours)
Lane 10	10ng/ml of TGFβ1 + 5μM of SB431542 (T = 48 hours)

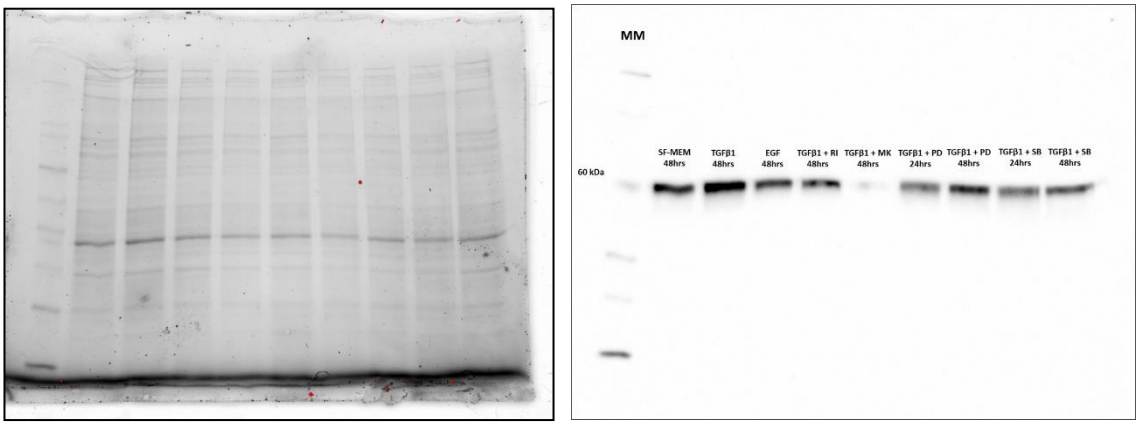
Gel & Blot No.6 (p-Smad2)



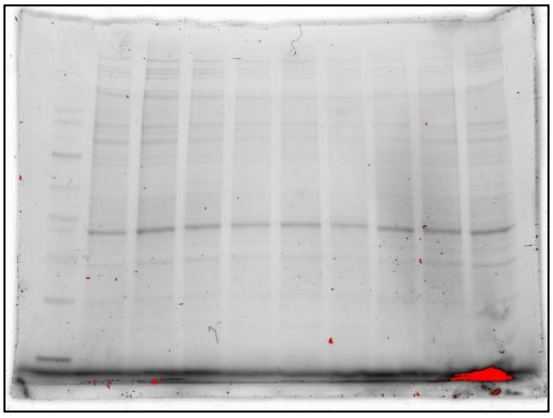
Gel & Blot No.7 (p-Akt T308)

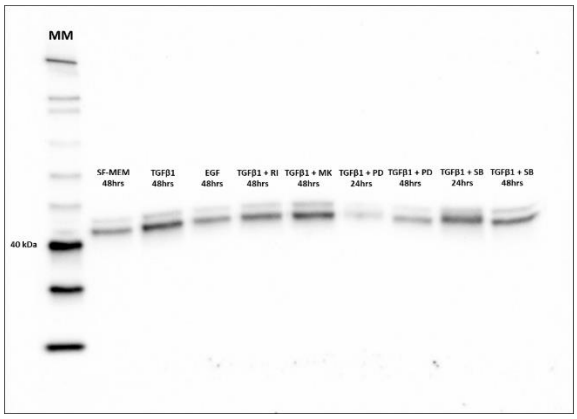


Gel & Blot No.8 (p-Akt S473)

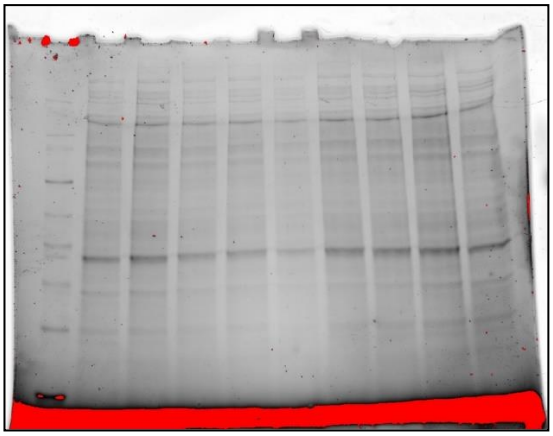


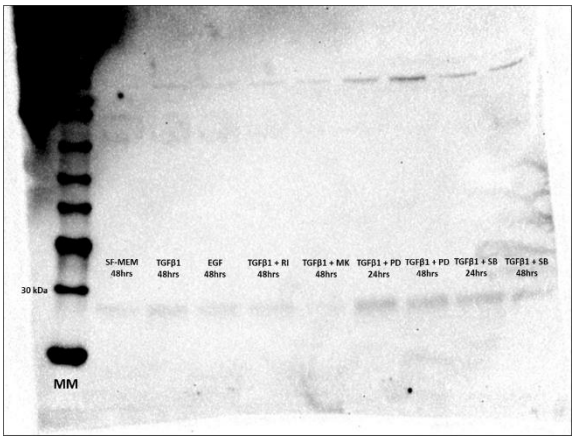
Gel & Blot No.9 (p-p44/42)





Gel & Blot No.10 (p-Rac1/Cdc42)

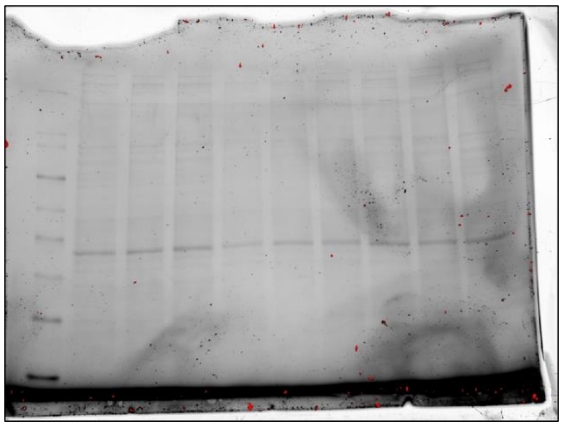


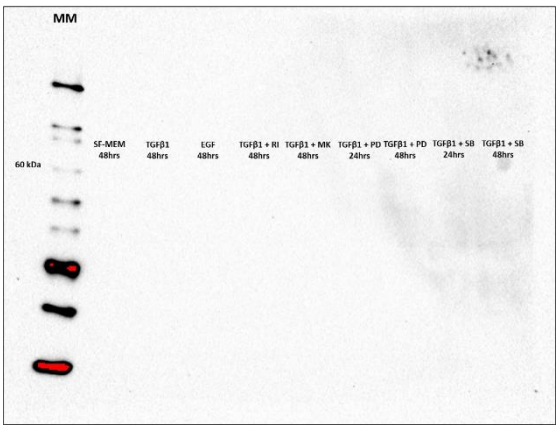


Gel & Blot Design

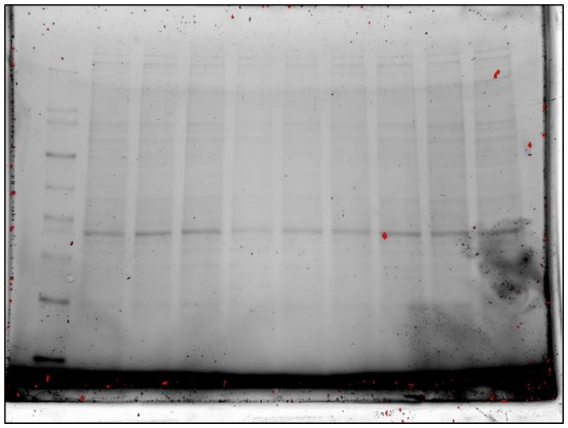
WB experiment repot	Date: 10/4/2022
Cell line: SAS-H1	
Gel and Blot 11 design (Different test conditions)	
Lane 1	Magic Marker
Lane 2	SF-MEM (T = 48 hours)
Lane 3	10ng/ml of TGFβ1 (T = 48 hours)
Lane 4	10ng/ml of EGF (T = 48 hours)
Lane 5	10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII (T = 48 hours)
Lane 6	10ng/ml of TGFβ1 + 1μM of MK-2206 (T = 48 hours)
Lane 7	10ng/ml of TGFβ1 + 5μM of PD98059 (T = 24 hours)
Lane 8	10ng/ml of TGFβ1 + 5μM of PD98059 (T = 48 hours)
Lane 9	10ng/ml of TGFβ1 + 5μM of SB431542 (T = 24 hours)
Lane 10	10ng/ml of TGFβ1 + 5μM of SB431542 (T = 48 hours)

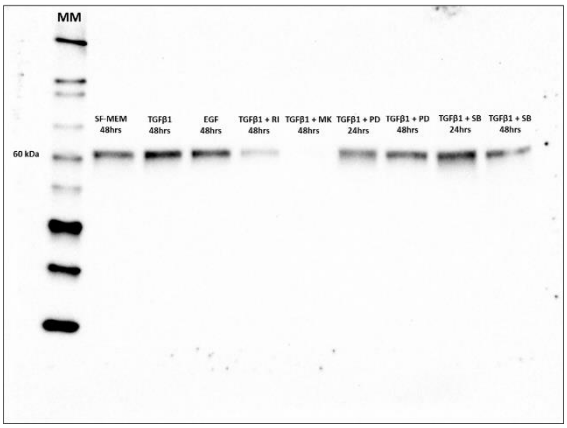
Gel & Blot No.11 (p-Smad)



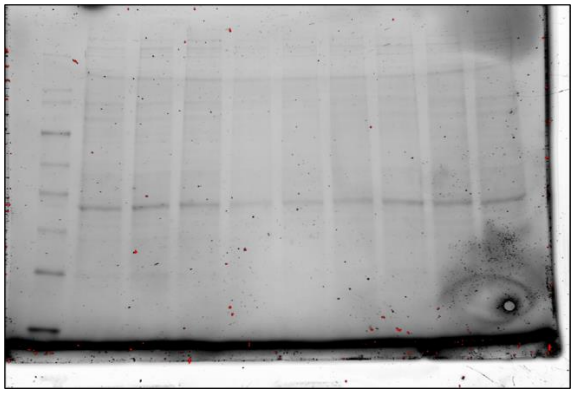


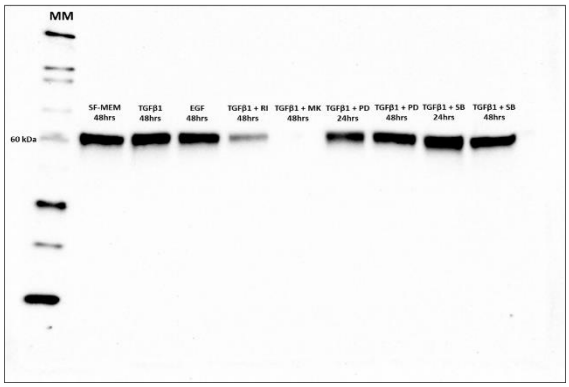
Gel & Blot No.12 (p-Akt T308)



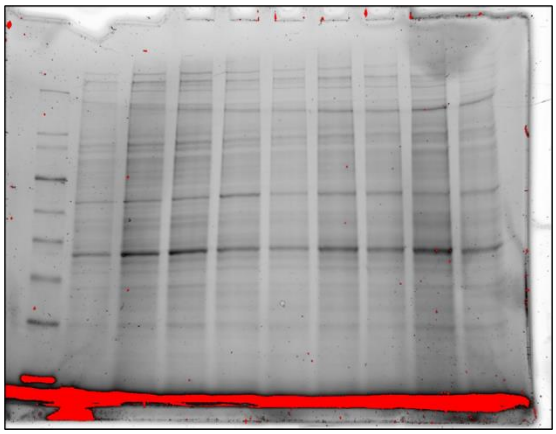


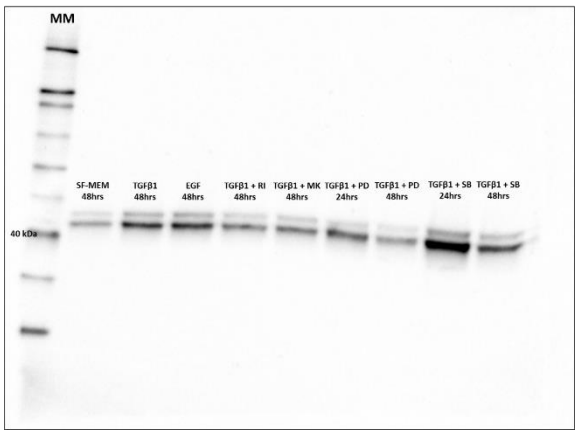
Gel & Blot No.13 (p-Akt S473)



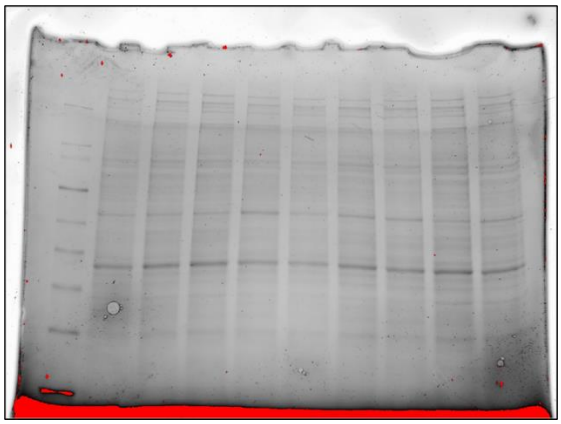


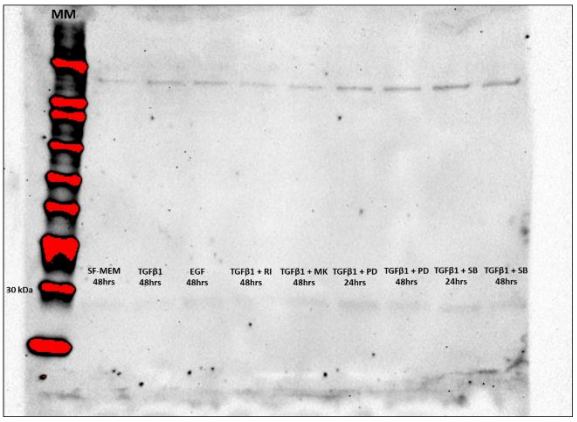
Gel & Blot No.14 (p-p44/42)





Gel & Blot No.15 (p-Rac1/Cdc42)





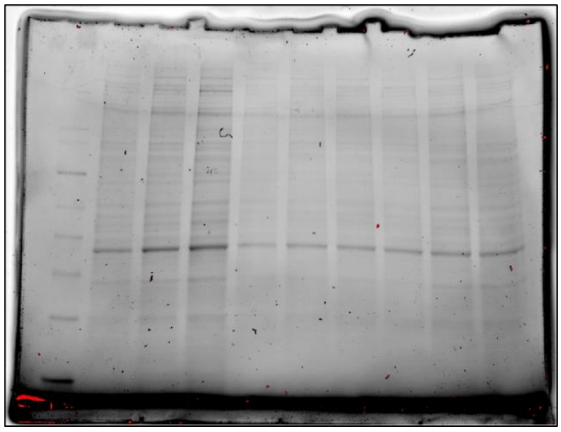
Appendix 6: Data SDS-PAGE & Western Blot (3)

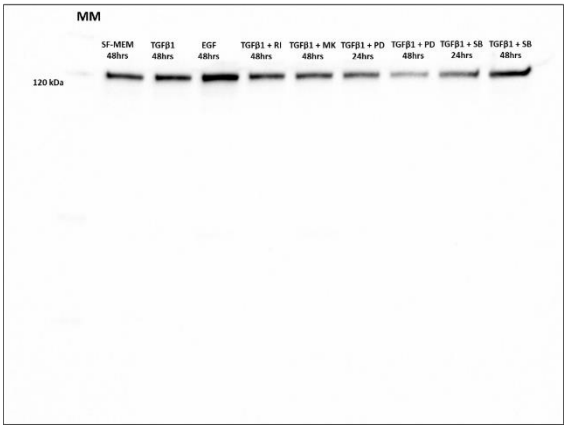
Report of WB Experiment Regarding Expression of Migration Marker(s) in Responses to Different Test Conditions at 24 hours and 48 hours.

Gel & Blot Design

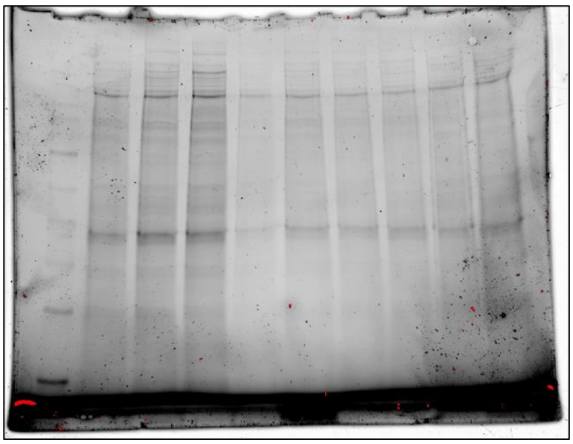
WB experiment repot	Date: 13/12/2021
Cell line: HaCaT	
Gel and Blot 1 design (Different test conditions)	
Lane 1	Magic Marker
Lane 2	SF-MEM (T = 48 hours)
Lane 3	10ng/ml of TGFβ1 (T = 48 hours)
Lane 4	10ng/ml of EGF (T = 48 hours)
Lane 5	10ng/ml of TGFβ1 + 5uM of TGF-β RI Kinase Inhibitor VII (T = 48 hours)
Lane 6	10ng/ml of TGFβ1 + 1μM of MK-2206 (T = 48 hours)
Lane 7	10ng/ml of TGFβ1 + 5μM of PD98059 (T = 24 hours)
Lane 8	10ng/ml of TGFβ1 + 5μM of PD98059 (T = 48 hours)
Lane 9	10ng/ml of TGFβ1 + 5μM of SB431542 (T = 24 hours)
Lane 10	10ng/ml of TGFβ1 + 5μM of SB431542 (T = 48 hours)

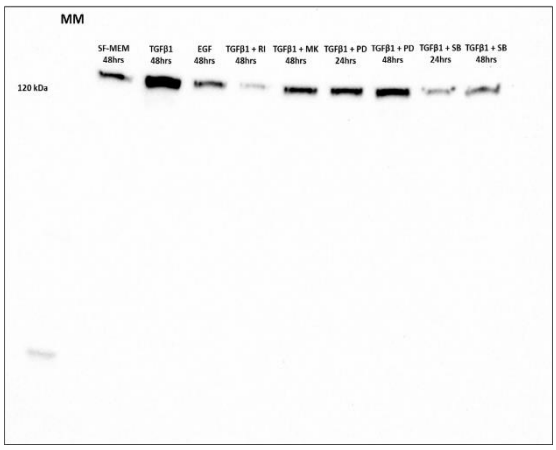
Gel & Blot no.1 (E-cadherin)



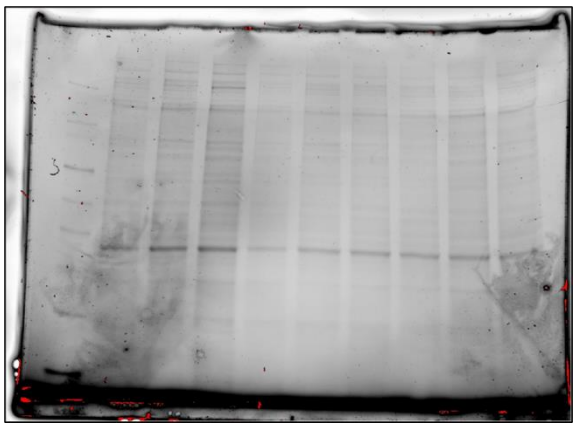


Gel & Blot No.2 (N-cadherin)

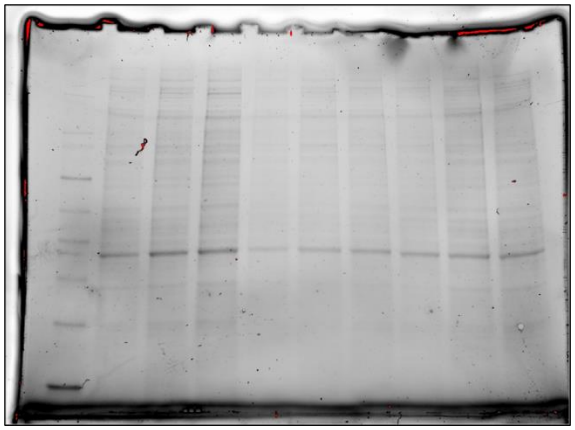


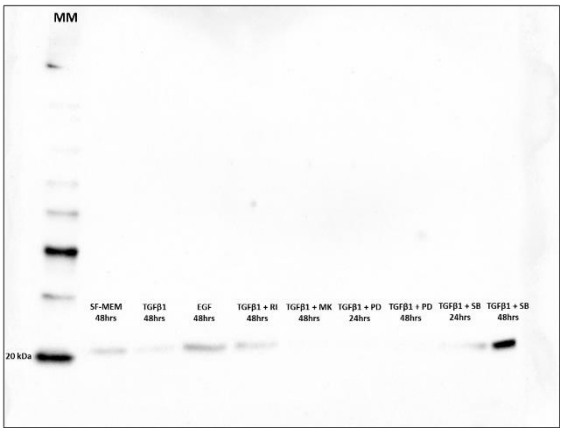


Gel & Blot No.3 (β-Catenin)

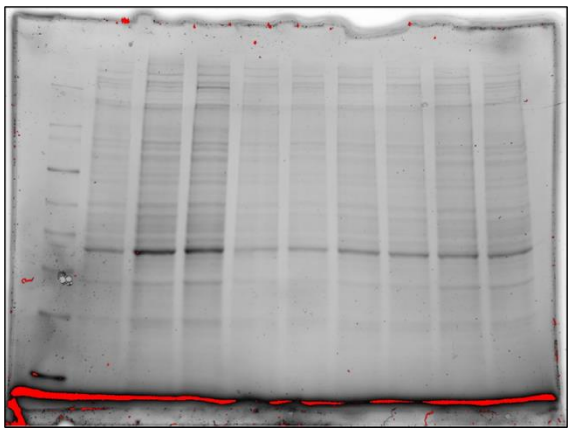


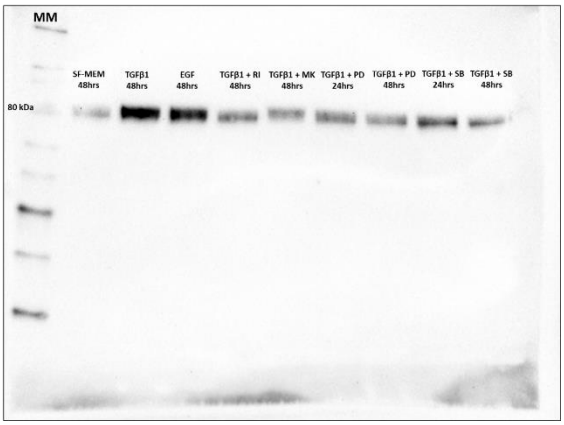
Gel & Blot No.4 (Claudin-1)



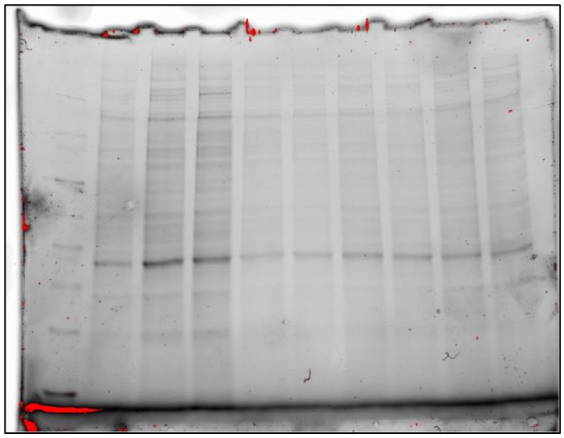


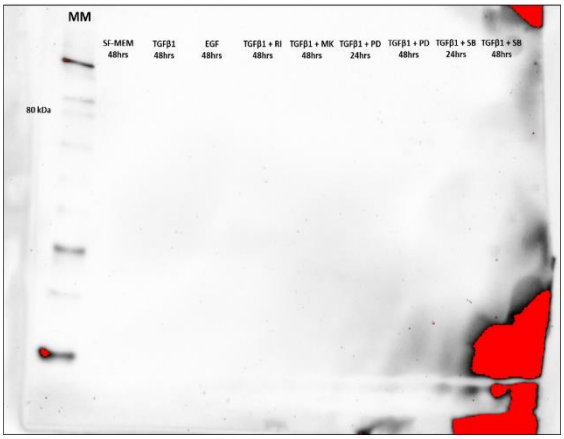
Gel & Blot No.5 (WAVE-2)



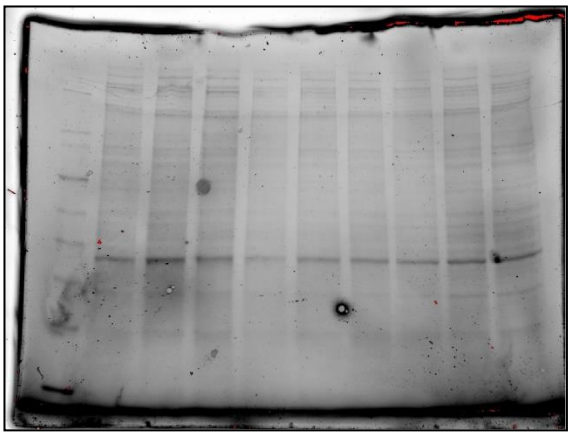


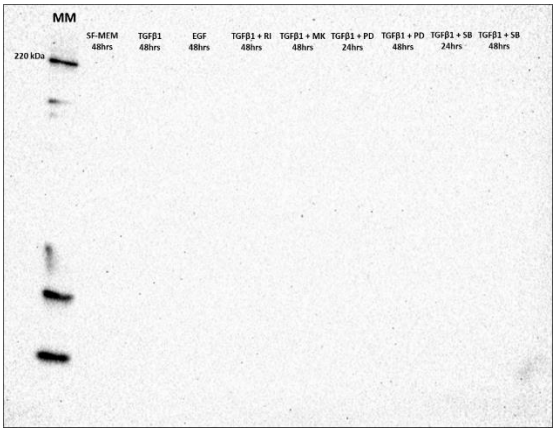
Gel & Blot No.6 (Palladin)



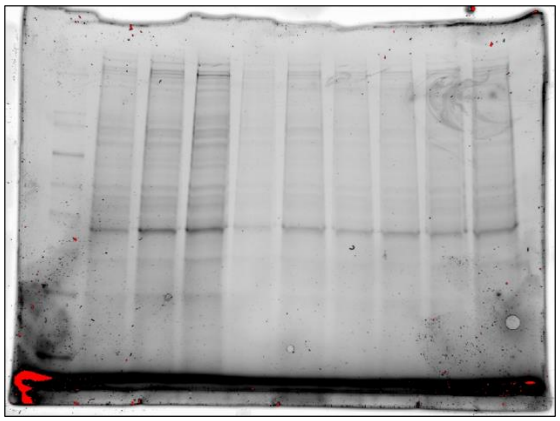


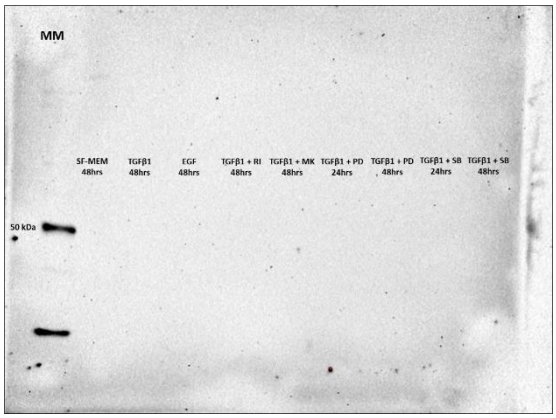
Gel & Blot No.7 (Gridin)





Gel & Blot No.8 (Vimentin)

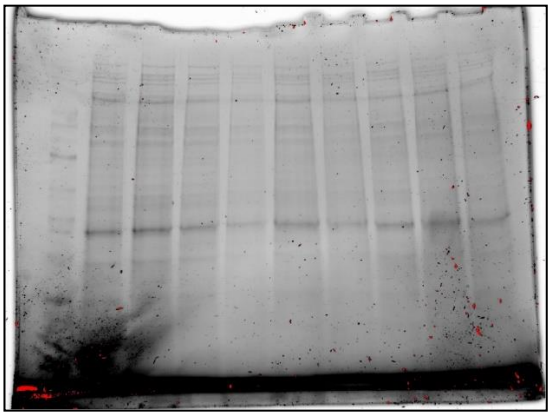


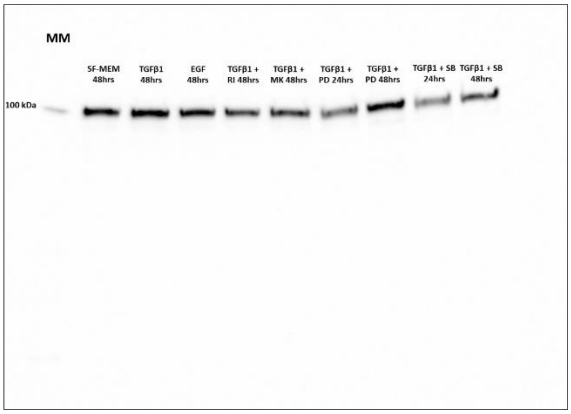


Gel & Blot Design

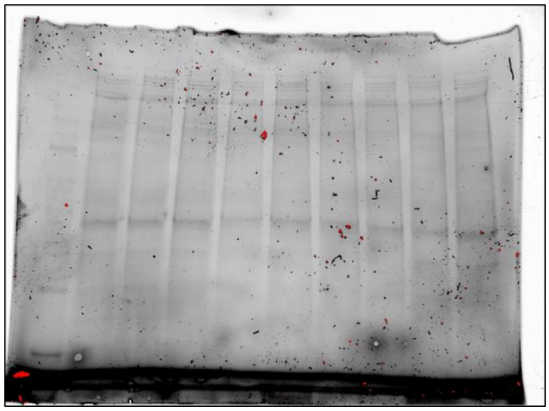
WB experiment repot	Date: 22/6/2022
Cell line: TYS	
Gel and Blot 9 design (Different test conditions)	
Lane 1	Magic Marker
Lane 2	SF-MEM (T = 48 hours)
Lane 3	10ng/ml of TGFβ1 (T = 48 hours)
Lane 4	10ng/ml of EGF (T = 48 hours)
Lane 5	10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII (T = 48 hours)
Lane 6	10ng/ml of TGFβ1 + 1μM of MK-2206 (T = 48 hours)
Lane 7	10ng/ml of TGFβ1 + 5μM of PD98059 (T = 24 hours)
Lane 8	10ng/ml of TGFβ1 + 5μM of PD98059 (T = 48 hours)
Lane 9	10ng/ml of TGFβ1 + 5μM of SB431542 (T = 24 hours)
Lane 10	10ng/ml of TGFβ1 + 5μM of SB431542 (T = 48 hours)

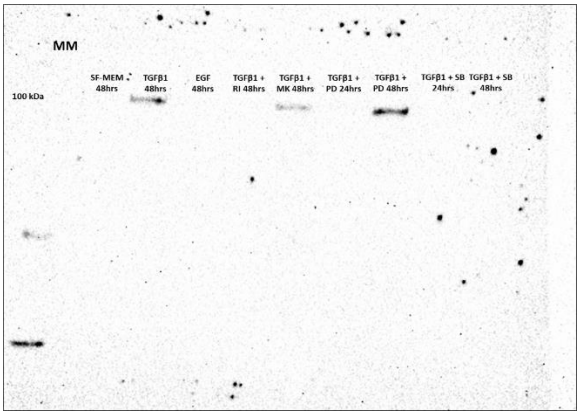
Gel & Blot No. 9 (E-cadherin)



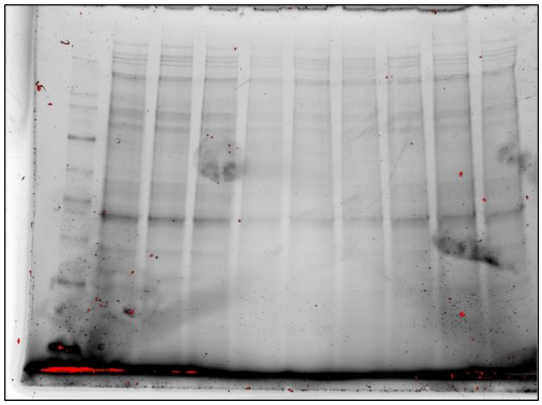


Gel & Blot No.10 (N-cadherin)

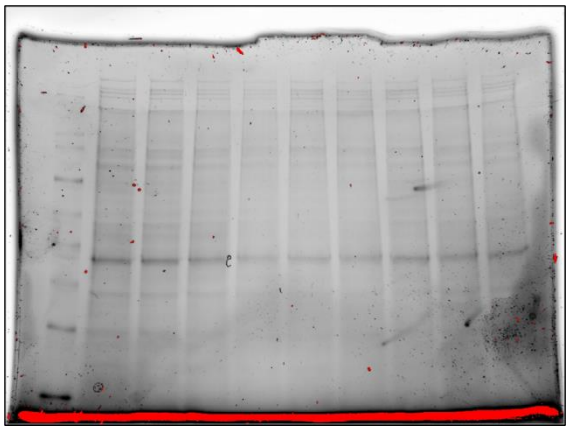


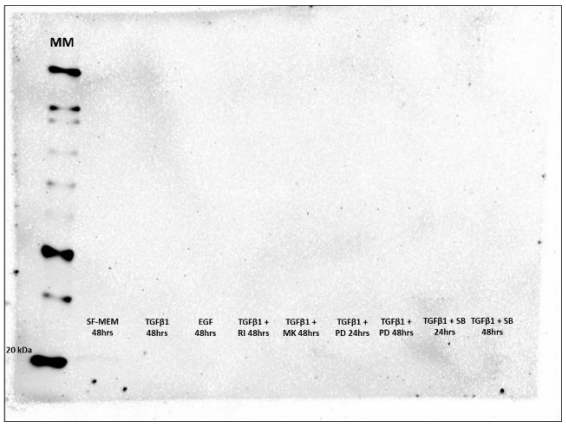


Gel & Blot No.11 (β-Catenin)

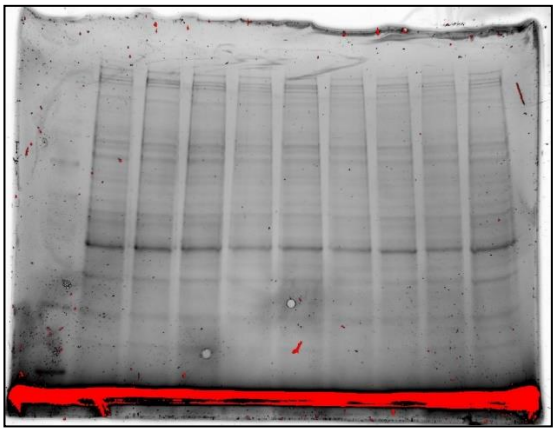


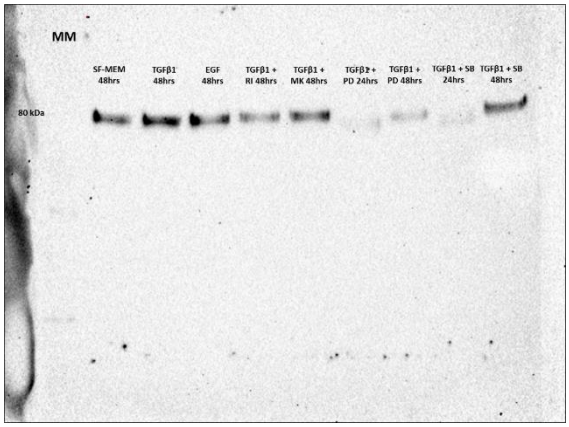
Gel & Blot No.12 (Claudin-1)



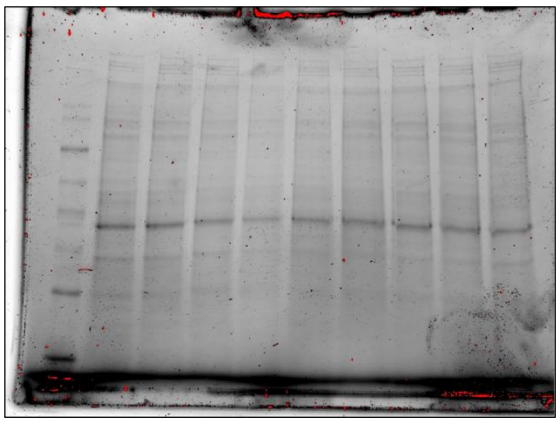


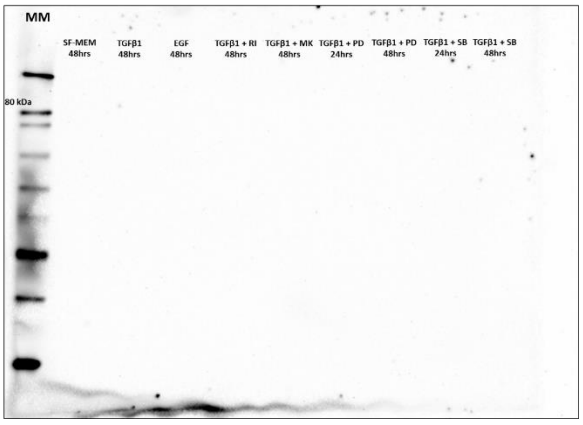
Gel & Blot No.13 (WAVE-2)



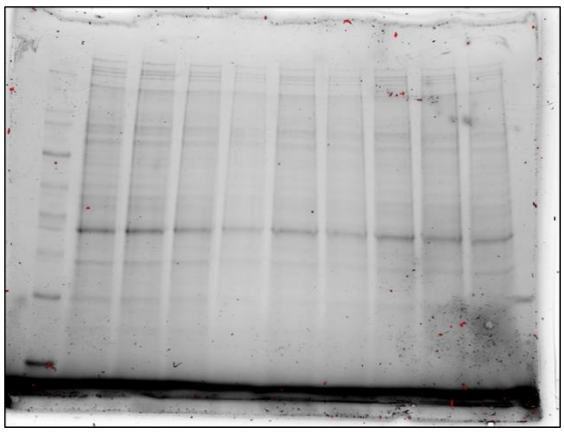


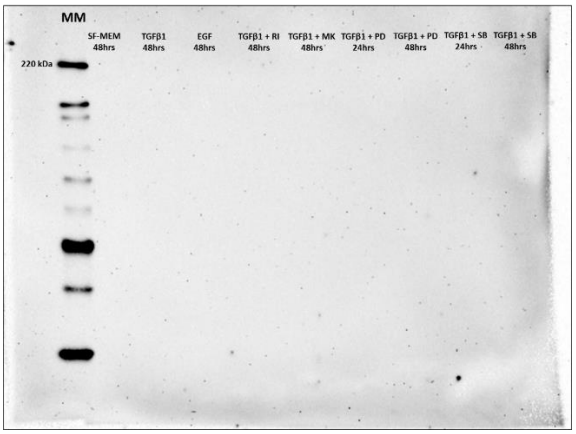
Gel & Blot No.14 (Palladin)



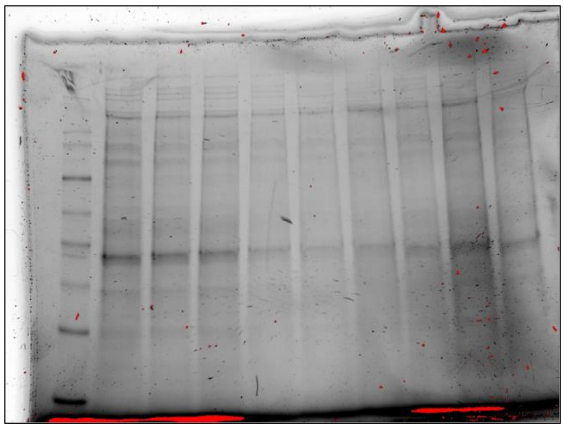


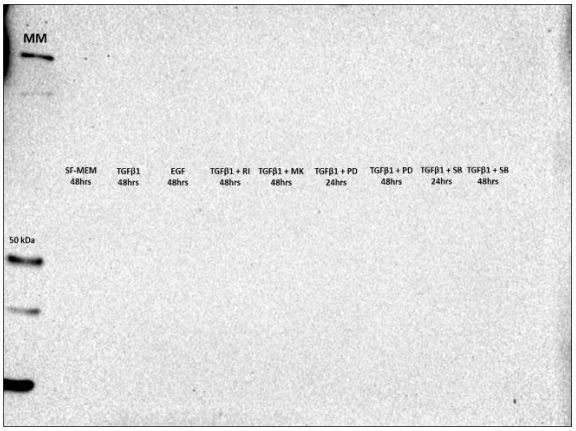
Gel & Blot No.15 (Gridin)





Gel & Blot No.16 (Vimentin)

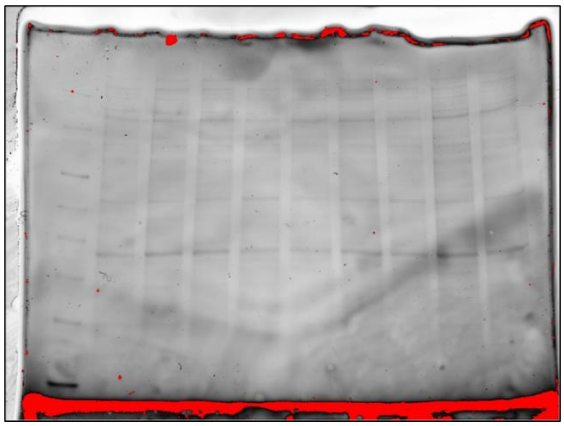


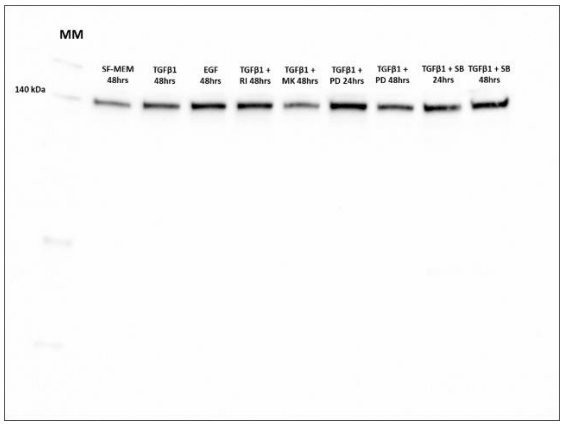


Gel & Blot No. 17

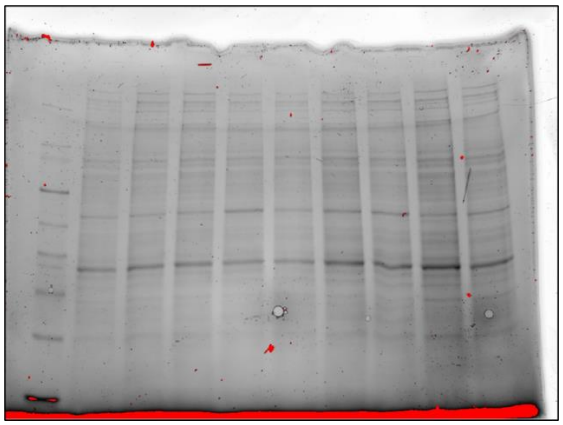
WB experiment repot	Date: 6/12/2022
Cell line: SAS-H1	
Gel and Blot 17 design (Different test conditions)	
Lane 1	Magic Marker
Lane 2	SF-MEM (T = 48 hours)
Lane 3	10ng/ml of TGFβ1 (T = 48 hours)
Lane 4	10ng/ml of EGF (T = 48 hours)
Lane 5	10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII (T = 48 hours)
Lane 6	10ng/ml of TGFβ1 + 1μM of MK-2206 (T = 48 hours)
Lane 7	10ng/ml of TGFβ1 + 5μM of PD98059 (T = 24 hours)
Lane 8	10ng/ml of TGFβ1 + 5μM of PD98059 (T = 48 hours)
Lane 9	10ng/ml of TGFβ1 + 5μM of SB431542 (T = 24 hours)
Lane 10	10ng/ml of TGFβ1 + 5μM of SB431542 (T = 48 hours)

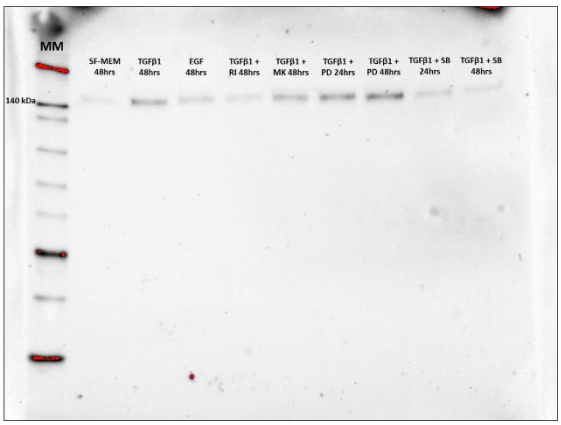
Gel & Blot No.17 (E-cadherin)



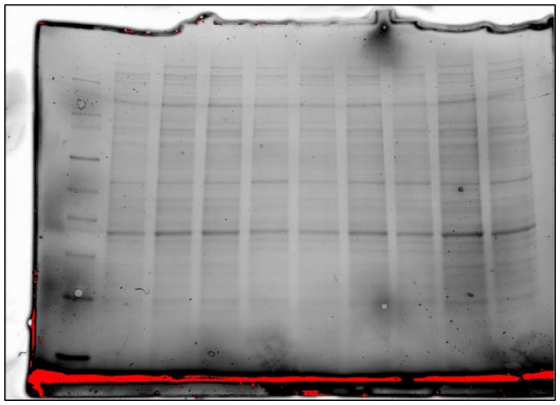


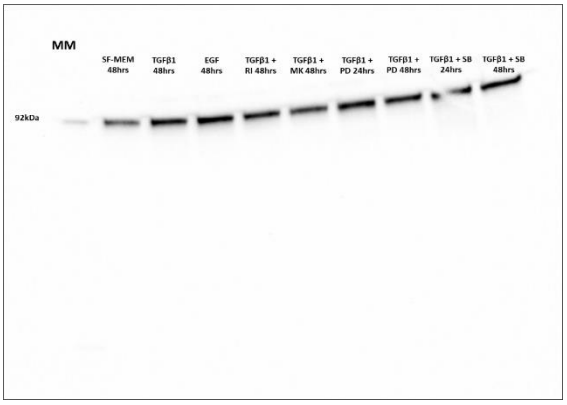
Gel & Blot No.18 (N-cadherin)



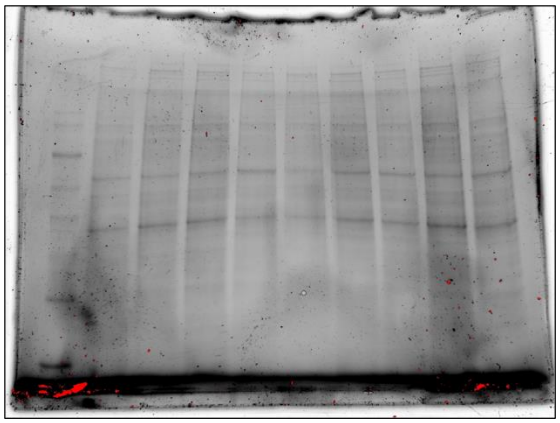


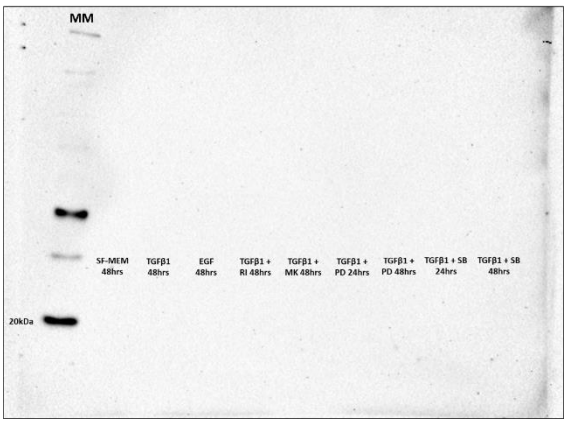
Gel & Blot No.19 (β-catenin)



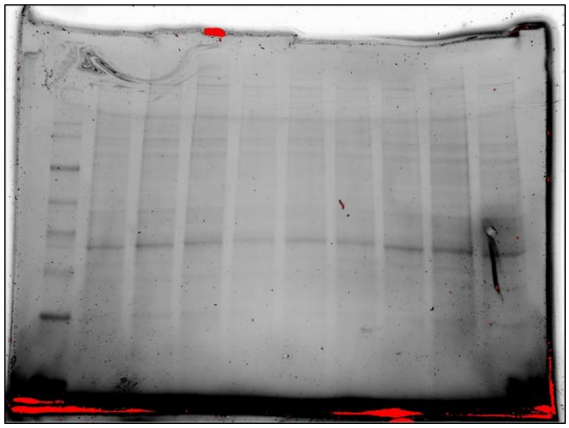


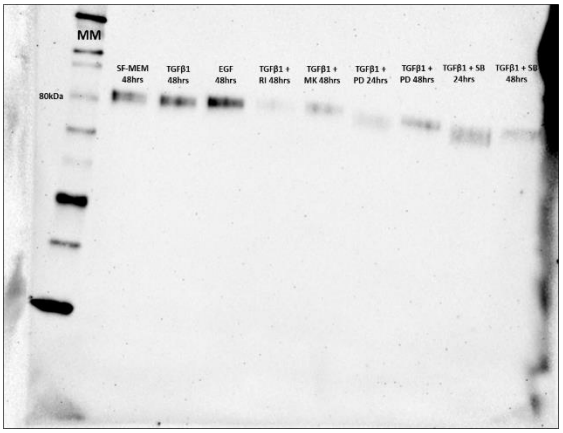
Gel & Blot No.20 (Claudin-1)



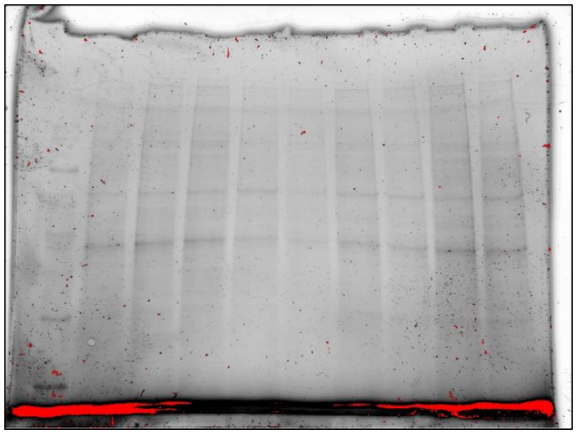


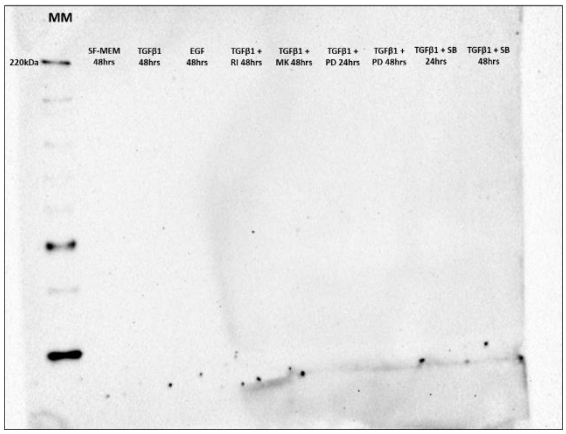
Gel & Blot No.21 (WAVE-2)



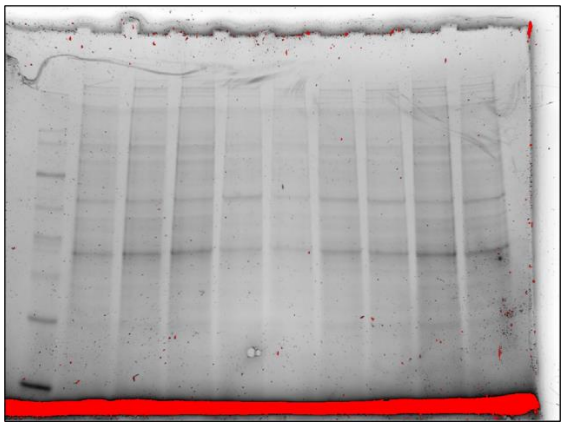


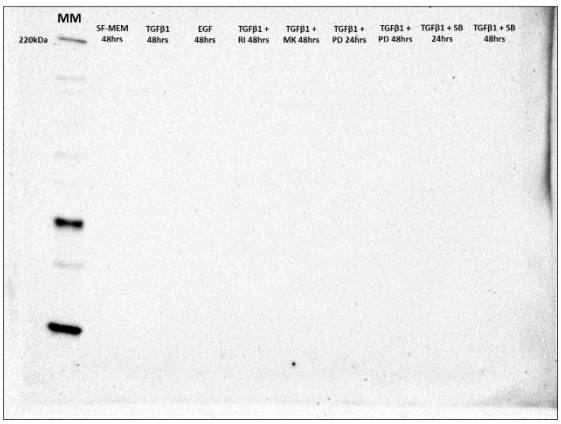
Gel & Blot No.22 (Palladin)



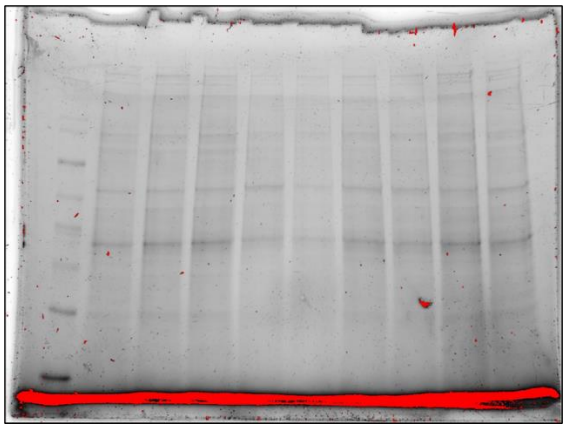


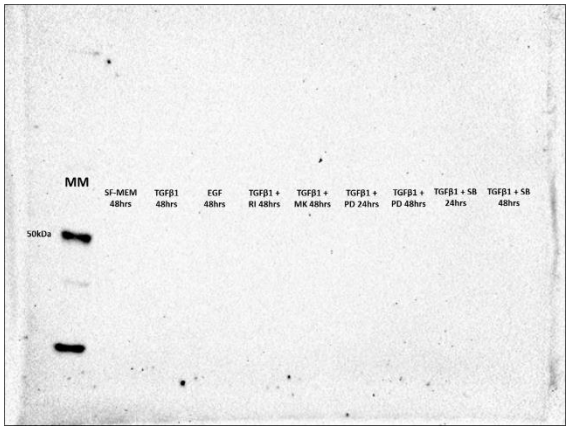
Gel & Blot No.23 (Gridin)





Gel & Blot No.24 (Vimentin)





Appendix 7: Data Immunocytochemistry Assessment.

Report of ICC Experiment Regarding Expression of Migration Marker(s) in Responses to Different Test Conditions at 24 hours and 48 hours.

The formula to calculate the corrected total cell fluorescence (CTCF) was to measure cell fluorescence using ImageJ.

The of CTCF was contributed by Luke Hammond at University of Queensland, Australia.

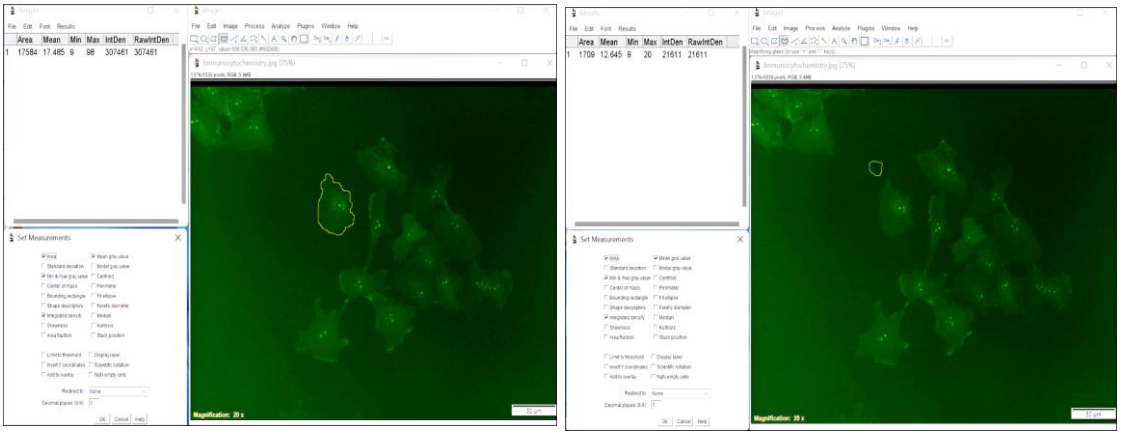
Open ImageJ and select the cell of interest using of the drawing tool (Freeform).

From the Analyze list, Set Measurements and select Area integrated intensity and Mean Grey Value were selected.

Press Measure from Analyze list, which led to a box contain values for the first cell.

Next select the area next to cell where no fluorescence, which will be the background value.

The previous steps were repeated for all cells will be selected in the assessment.



HaCaT Cell Line		ICC for Migration Markers			Date:12/10/2021	
Calculate Correlated total cell fluorescence					Antibody: E-Cadherin	
SF-MEM/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4296	30.131	129444	0.703	126423.9
	2	4114	31.638	130158	0.703	127265.9
	3	7204	23.193	167082	0.703	162017.6
	4	9157	20.466	187407	0.703	180969.6
	5	4133	25.751	106429	0.703	103523.5
	6	4852	27.033	131162	0.703	127751
	7	6021	34.621	208455	0.703	204222.2
	8	4066	29.437	119690	0.703	116831.6
	9	4852	26.033	126310	0.703	122899
	10	8272	21.049	174116	0.703	168300.8
SF-MEM/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	7576	16.058	121655	0.539889	117564.8
	2	7313	23.113	169026	0.539889	165077.8

	3	9271	19.474	180548	0.539889	175542.7
	4	4046	20.72	83834	0.539889	81649.61
	5	7215	22.415	161721	0.539889	157825.7
	6	6673	27.657	184557	0.539889	180954.3
	7	6476	26.515	171709	0.539889	168212.7
	8	8394	19.294	161953	0.539889	157421.2
	9	5469	24.302	132909	0.539889	129956.3
	10	8635	19.162	165463	0.539889	160801.1
10ng/ml of TGFβ1 /24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	15235	8.403	128022	2.4752	90312.33
	2	20424	9.405	192088	2.4752	141534.5
	3	16924	9.506	160873	2.4752	118982.7
	4	15994	9.264	148162	2.4752	108573.7
	5	20020	8.102	162211	2.4752	112657.5
	6	24774	11.857	293733	2.4752	232412.4
	7	18989	12.253	232664	2.4752	185662.4
	8	18707	13.88	259646	2.4752	213342.4
	9	10139	14.775	149803	2.4752	124706.9
	10	18505	7.48	138417	2.4752	92613.42
10ng/ml of TGFβ1 /48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	32500	8.616	280022	2.4664	199864
	2	25049	6.635	166211	2.4664	104430.1
	3	26961	9.472	255376	2.4664	188879.4
	4	19900	6.039	120184	2.4664	71102.64
	5	18004	6.661	119923	2.4664	75517.93
	6	20657	5.059	104496	2.4664	53547.58
	7	23489	6.511	152934	2.4664	95000.73
	8	18349	6.041	110854	2.4664	65598.03
	9	20879	6.778	141528	2.4664	90032.03
	10	25871	4.638	119987	2.4664	56178.77
10ng/ml of EGF/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	10160	16.886	171558	0.3761	167736.8
	2	10156	18.41	186977	0.3761	183157.3
	3	6223	19.54	121596	0.3761	119255.5
	4	9604	14.059	135025	0.3761	131412.9
	5	8473	14.138	119793	0.3761	116606.3
	6	14065	9.884	139018	0.3761	133728.2
	7	13116	9.988	131008	0.3761	126075.1
	8	10735	10.151	108973	0.3761	104935.6
	9	6420	16.058	103094	0.3761	100679.4
	10	6403	10.799	69146	0.3761	66737.83
10ng/ml of EGF/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	20222	9.84	198979	2.3142	152181.2
	2	7570	15.969	120886	2.3142	103367.5
	3	7498	15.483	116095	2.3142	98743.13
	4	7899	20.697	163486	2.3142	145206.1

	5	4498	20.595	92637	2.3142	82227.73
	6	5938	21.345	126744	2.3142	113002.3
	7	10409	12.388	128942	2.3142	104853.5
	8	15515	5.866	91010	2.3142	55105.19
	9	13247	11.902	157671	2.3142	127014.8
	10	11263	7.177	80840	2.3142	54775.17
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	8287	19.291	159866	0	159866
	2	7543	17.354	130902	0	130902
	3	6783	19.568	132727	0	132727
	4	6555	17.058	111812	0	111812
	5	6721	26.705	179485	0	179485
	6	8937	16.433	146865	0	146865
	7	12566	15.345	192830	0	192830
	8	5387	26.647	143546	0	143546
	9	8333	20.57	171409	0	171409
	10	4365	31.014	135378	0	135378
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	8994	13.933	125312	0.8563	117610.4
	2	9793	17.012	166594	0.8563	158208.3
	3	6823	12.154	82926	0.8563	77083.47
	4	8541	13.765	117569	0.8563	110255.3
	5	11715	15.092	176801	0.8563	166769.4
	6	8203	21.689	177912	0.8563	170887.8
	7	6341	21.231	134624	0.8563	129194.2
	8	6182	19.989	123573	0.8563	118279.4
	9	12506	18.366	229689	0.8563	218980.1
	10	7602	19.253	146361	0.8563	139851.4
10ng/ml of TGFβ1 + 1μM of MK-2206/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	10753	9.704	104345	0.2973	101148.1
	2	9411	8.45	79519	0.2973	76721.11
	3	11278	12.85	144926	0.2973	141573.1
	4	8452	14.161	119687	0.2973	117174.2
	5	10616	11.063	117450	0.2973	114293.9
	6	8867	9.791	86813	0.2973	84176.84
	7	7484	12.882	96407	0.2973	94182.01
	8	7940	10.639	84470	0.2973	82109.44
	9	9367	15.163	142029	0.2973	139244.2
	10	15047	10.433	156983	0.2973	152509.5
10ng/ml of TGFβ1 + 1μM of MK-2206/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	15745	10.792	169925	0.8454	156614.2
	2	10779	10.124	109127	0.8454	100014.4
	3	7371	9.302	68562	0.8454	62330.56
	4	7371	11.019	81218	0.8454	74986.56
	5	12013	8.032	96490	0.8454	86334.21
	6	16694	8.458	141205	0.8454	127091.9

	7	13275	9.224	122443	0.8454	111220.3
	8	8368	12.82	107279	0.8454	100204.7
	9	8620	8.478	73080	0.8454	65792.65
	10	9218	12.701	117076	0.8454	109283.1
10ng/ml of TGFβ1 + + 5μM of PD98059/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	11854	8.711	103260	0.9469	92035.45
	2	11714	9.879	115722	0.9469	104630
	3	10363	6.92	71708	0.9469	61895.28
	4	8514	9.358	79677	0.9469	71615.09
	5	16474	8.345	137481	0.9469	121881.8
	6	12751	7.599	96899	0.9469	84825.08
	7	21079	6.351	133877	0.9469	113917.3
	8	11278	5.124	57787	0.9469	47107.86
	9	13493	5.228	70547	0.9469	57770.48
	10	16384	6.919	113358	0.9469	97843.99
10ng/ml of TGFβ1 + + 5μM of PD98059/ 48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	24605	10.139	249471	3.3929	165988.7
	2	25439	11.732	298439	3.3929	212127
	3	43378	14.683	636928	3.3929	489750.8
	4	15340	13.45	206323	3.3929	154275.9
	5	14666	13.765	201873	3.3929	152112.7
	6	12884	9.732	125387	3.3929	81672.88
	7	26333	12.922	340275	3.3929	250929.8
	8	18589	14.087	261872	3.3929	198801.4
	9	10087	19.479	196485	3.3929	162260.8
	10	13736	17.279	237350	3.3929	190745.1
10ng/ml of TGFβ1 + 5μM of SB431542/ 24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	8162	19.789	161518	3.8069	130446.1
	2	11083	20.512	227333	3.8069	185141.1
	3	8485	18.158	154067	3.8069	121765.5
	4	7463	15.59	116345	3.8069	87934.11
	5	7106	16.727	118860	3.8069	91808.17
	6	6266	23.084	144644	3.8069	120790
	7	5930	19.724	116963	3.8069	94388.08
	8	7539	17.983	135575	3.8069	106874.8
	9	5374	24.626	132342	3.8069	111883.7
	10	5303	35.384	187639	3.8069	167451
10ng/ml of TGFβ1 + 5μM of SB431542/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	17149	14.439	247615	2.7914	199745.3
	2	9618	18.988	182629	2.7914	155781.3
	3	8868	18.015	159760	2.7914	135005.9
	4	9296	18.236	169523	2.7914	143574.1
	5	10928	25.684	280678	2.7914	250173.6
	6	9121	14.99	136722	2.7914	111261.6
	7	6483	20.61	133612	2.7914	115515.4
	8	5443	20.019	108961	2.7914	93767.41

	9	7834	16.028	125566	2.7914	103698.2
	10	3687	17.021	62758	2.7914	52466.11

HaCaT Cell Line		**ICC for Migration Markers**			**Date:12/10/2021**	
Calculate Correlated total cell fluorescence					**Antibody: N-Cadherin**	
SF-MEM/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4560	1.915	8733	0.8716	4758.504
	2	4265	2.076	8853	0.8716	5135.626
	3	3667	1.975	7242	0.8716	4045.843
	4	4064	2.708	11007	0.8716	7464.818
	5	5203	2.555	13295	0.8716	8760.065
	6	3475	2.841	9871	0.8716	6842.19
	7	3929	1.936	7608	0.8716	4183.484
	8	4001	2.849	11397	0.8716	7909.728
	9	3113	2.472	7696	0.8716	4982.709
	10	5349	3.623	19379	0.8716	14716.81
SF-MEM/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4946	1.277	6315	0.4559	4060.119
	2	3768	0.969	3651	0.4559	1933.169
	3	4766	1.603	7640	0.4559	5467.181
	4	3900	1.6	6239	0.4559	4460.99
	5	5117	1.525	7804	0.4559	5471.16
	6	4627	0.888	4109	0.4559	1999.551
	7	3907	0.956	3735	0.4559	1953.799
	8	4559	1.175	5356	0.4559	3277.552
	9	4927	0.81	3989	0.4559	1742.781
	10	3740	1.014	3791	0.4559	2085.934
10ng/ml of TGFβ1 /24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	13599	4.937	67133	1.9367	40795.82
	2	16477	5.344	88053	1.9367	56141.99
	3	14044	6.557	92082	1.9367	64882.99
	4	13135	4.519	59354	1.9367	33915.45
	5	19701	5.645	111206	1.9367	73051.07
	6	7922	6.356	50355	1.9367	35012.46
	7	13026	7.312	95242	1.9367	70014.55
	8	8539	6.886	58800	1.9367	42262.52
	9	13186	4.41	58152	1.9367	32614.67
	10	12490	5.097	63659	1.9367	39469.62
10ng/ml of TGFβ1 /48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	38423	10.183	391276	6.075	157856.3
	2	10256	11.874	121782	6.075	59476.8
	3	7819	12.015	93947	6.075	46446.58
	4	7501	12.159	91202	6.075	45633.43
	5	9474	12.707	120384	6.075	62829.45

	6	11335	10.711	121414	6.075	52553.88
	7	26002	10.106	262782	6.075	104819.9
	8	22352	10.582	236521	6.075	100732.6
	9	21375	10.812	231102	6.075	101248.9
	10	32855	10.281	337797	6.075	138202.9
10ng/ml of EGF/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	3646	5.697	20773	1.7247	14484.74
	2	2813	4.586	12900	1.7247	8048.419
	3	2865	3.905	11187	1.7247	6245.735
	4	3249	3.558	11561	1.7247	5957.45
	5	3476	4.902	17039	1.7247	11043.94
	6	3995	3.709	14817	1.7247	7926.824
	7	3826	1.928	7378	1.7247	779.2978
	8	4041	2.115	8545	1.7247	1575.487
	9	5223	3.178	16601	1.7247	7592.892
	10	4901	4.154	20361	1.7247	11908.25
10ng/ml of EGF/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	3186	3.526	11235	3.4745	165.243
	2	3214	3.702	11899	3.4745	731.957
	3	2556	3.731	9537	3.4745	656.178
	4	3025	4.221	12769	3.4745	2258.638
	5	2744	4.07	11168	3.4745	1633.972
	6	3757	3.68	13826	3.4745	772.3035
	7	3808	4.209	16029	3.4745	2798.104
	8	2519	3.826	9637	3.4745	884.7345
	9	2378	3.416	8124	3.4745	-138.361
	10	2676	2.531	6774	3.4745	-2523.76
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	3576	3.714	13281	0.9095	10028.63
	2	2884	4.333	12496	0.9095	9873.002
	3	3312	3.929	13012	0.9095	9999.736
	4	3446	3.502	12069	0.9095	8934.863
	5	4048	2.596	10510	0.9095	6828.344
	6	4121	2.38	9807	0.9095	6058.951
	7	4341	2.033	8826	0.9095	4877.861
	8	5393	1.858	10021	0.9095	5116.067
	9	5457	1.77	9659	0.9095	4695.859
	10	3494	5.035	17591	0.9095	14413.21
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4969	3.538	17579	2.4138	5584.828
	2	3338	3.281	10952	2.4138	2894.736
	3	4345	3.475	15100	2.4138	4612.039
	4	3874	3.007	11648	2.4138	2296.939
	5	2893	3.082	8916	2.4138	1932.877
	6	3630	3.085	11198	2.4138	2435.906
	7	4721	2.417	11410	2.4138	14.4502

	8	4380	2.815	12331	2.4138	1758.556
	9	3930	3.019	11864	2.4138	2377.766
	10	3317	3.685	12223	2.4138	4216.425
10ng/ml of TGFβ1 + 1μM of MK-2206/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	7400	3.827	28319	1.0219	20756.94
	2	10429	3.328	34711	1.0219	24053.6
	3	8639	2.743	23701	1.0219	14872.81
	4	8708	3.133	27278	1.0219	18379.29
	5	3001	6.243	18734	1.0219	15667.28
	6	5269	5.623	29628	1.0219	24243.61
	7	12218	3.364	41102	1.0219	28616.43
	8	14293	4.924	70380	1.0219	55773.98
	9	17966	6.149	110473	1.0219	92113.54
	10	15952	2.546	40612	1.0219	24310.65
10ng/ml of TGFβ1 + 1μM of MK-2206/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	14759	5.045	74465	1.0843	58461.82
	2	7302	6.513	47556	1.0843	39638.44
	3	16668	6.507	108467	1.0843	90393.89
	4	10106	7.431	75102	1.0843	64144.06
	5	10075	7.014	70662	1.0843	59737.68
	6	9418	5.349	50373	1.0843	40161.06
	7	12638	6.503	82183	1.0843	68479.62
	8	11452	6.912	79161	1.0843	66743.6
	9	11478	5.648	64830	1.0843	52384.4
	10	7343	6.491	47663	1.0843	39700.99
10ng/ml of TGFβ1 + + 5μM of PD98059/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	6974	4.351	30345	0.8263	24582.38
	2	14953	3.541	52950	0.8263	40594.34
	3	9307	3.657	34032	0.8263	26341.63
	4	14671	2.519	36962	0.8263	24839.35
	5	12329	3.556	43841	0.8263	33653.55
	6	8036	5.75	46205	0.8263	39564.85
	7	11397	5.316	60585	0.8263	51167.66
	8	7519	5.58	41953	0.8263	35740.05
	9	10478	5.22	54697	0.8263	46039.03
	10	7079	2.178	15415	0.8263	9565.622
10ng/ml of TGFβ1 + + 5μM of PD98059/ 48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	26333	10.173	267891	5.4599	124115.5
	2	22526	10.013	225546	5.4599	102556.3
	3	21581	11.046	238387	5.4599	120556.9
	4	23518	10.355	243525	5.4599	115119.1
	5	14047	10.12	142162	5.4599	65466.78
	6	13766	10.558	145342	5.4599	70181.02
	7	13056	12.209	159396	5.4599	88111.55
	8	18789	11.84	222469	5.4599	119882.9
	9	11036	14.047	155018	5.4599	94762.54

	10	17941	11.32	203100	5.4599	105143.9
10ng/ml of TGFβ1 + 5μM of SB431542/ 24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	3951	3.879	15325	1.924	7723.276
	2	2875	3.203	9209	1.924	3677.5
	3	4534	2.985	13536	1.924	4812.584
	4	3516	3.095	10881	1.924	4116.216
	5	5006	3.511	17575	1.924	7943.456
	6	3440	3.084	10609	1.924	3990.44
	7	4219	3.608	15223	1.924	7105.644
	8	4843	3.89	18837	1.924	9519.068
	9	4005	3.871	15502	1.924	7796.38
	10	4032	3.986	16073	1.924	8315.432
10ng/ml of TGFβ1 + 5μM of SB431542/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	2562	12.676	32477	11.4491	3144.406
	2	3234	12.865	41605	11.4491	4578.611
	3	2768	13.225	36606	11.4491	4914.891
	4	2945	13.391	39437	11.4491	5719.401
	5	3039	14.083	42798	11.4491	8004.185
	6	2407	13.91	33482	11.4491	5924.016
	7	3464	12.803	44348	11.4491	4688.318
	8	2872	12.536	36003	11.4491	3121.185
	9	2549	12.914	32917	11.4491	3733.244
	10	3547	12.31	43665	11.4491	3055.042

HaCaT Cell Line		**ICC for Migration Markers**			**Date:12/10/2021**	
Calculate Correlated total cell fluorescence					**Antibody: Claudin-1**	
SF-MEM/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	3041	5.235	15920	1.2695	12059.45
	2	3768	5.134	19344	1.2695	14560.52
	3	3422	5.157	17648	1.2695	13303.77
	4	3485	5.37	18713	1.2695	14288.79
	5	3965	5.47	21688	1.2695	16654.43
	6	3501	0.861	3015	1.2695	-1429.52
	7	3925	0.86	3375	1.2695	-1607.79
	8	3994	3.5	13980	1.2695	8909.617
	9	3342	2.007	6706	1.2695	2463.331
	10	3958	2.087	8259	1.2695	3234.319
SF-MEM/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5028	7.716	38797	1.7677	29909
	2	5587	9.041	50511	1.7677	40634.86
	3	3637	8.747	31812	1.7677	25382.88
	4	4644	7.271	33767	1.7677	25557.8
	5	4612	7.214	33271	1.7677	25118.37
	6	4630	7.925	36694	1.7677	28509.55

	7	5543	5.899	32699	1.7677	22900.64
	8	5133	4.749	24379	1.7677	15305.4
	9	3032	6.078	18429	1.7677	13069.33
	10	4708	5.278	24848	1.7677	16525.67
10ng/ml of TGFβ1 /24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	3621	5.012	18147	0.887	14935.17
	2	2906	4.926	14314	0.887	11736.38
	3	3071	5.006	15373	0.887	12649.02
	4	2393	4.649	11124	0.887	9001.409
	5	3319	4.493	14911	0.887	11967.05
	6	3991	2.63	10495	0.887	6954.983
	7	4056	2.84	11520	0.887	7922.328
	8	3920	2.785	10919	0.887	7441.96
	9	5155	1.2	6186	0.887	1613.515
	10	6079	1.04	6324	0.887	931.927
10ng/ml of TGFβ1 /48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	9428	4.811	45357	2.4697	22072.67
	2	9621	4.713	45343	2.4697	21582.02
	3	7046	3.733	26303	2.4697	8901.494
	4	9030	4.174	37687	2.4697	15385.61
	5	10727	4.867	52207	2.4697	25714.53
	6	8400	4.514	37920	2.4697	17174.52
	7	8091	5.06	40941	2.4697	20958.66
	8	9212	4.459	41078	2.4697	18327.12
	9	7883	3.702	29186	2.4697	9717.355
	10	6021	4.406	26529	2.4697	11658.94
10ng/ml of EGF/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4616	10.993	50744	5.881	23597.3
	2	4632	9.941	46048	5.881	18807.21
	3	3803	11.83	44989	5.881	22623.56
	4	5258	11.143	58590	5.881	27667.7
	5	4827	11.571	55851	5.881	27463.41
	6	5812	11.293	65637	5.881	31456.63
	7	7263	14.15	102772	5.881	60058.3
	8	4647	12.082	56144	5.881	28814.99
	9	13257	11.446	151740	5.881	73775.58
	10	9519	10.572	100633	5.881	44651.76
10ng/ml of EGF/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	14315	3.866	55348	0.9738	41408.05
	2	8160	4.104	33489	0.9738	25542.79
	3	4465	3.84	17144	0.9738	12795.98
	4	5164	4.624	23876	0.9738	18847.3
	5	4033	2.857	11521	0.9738	7593.665
	6	3291	1.994	6562	0.9738	3357.224
	7	5768	4.613	26605	0.9738	20988.12
	8	3974	5.1	20268	0.9738	16398.12

	9	4762	2.123	10112	0.9738	5474.764
	10	2722	1.692	4606	0.9738	1955.316
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	6108	4.964	30318	0.7276	25873.82
	2	4433	4.865	21565	0.7276	18339.55
	3	3633	4.52	16420	0.7276	13776.63
	4	4105	4.445	18247	0.7276	15260.2
	5	2574	4.525	11648	0.7276	9775.158
	6	4150	2.887	11980	0.7276	8960.46
	7	4749	3.797	18034	0.7276	14578.63
	8	3544	2.747	9734	0.7276	7155.386
	9	3301	3.31	10927	0.7276	8525.192
	10	6903	3.572	24656	0.7276	19633.38
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	6630	8.228	54553	1.8412	42345.84
	2	5708	10.764	61439	1.8412	50929.43
	3	7029	13.949	98049	1.8412	85107.21
	4	6071	12.063	73237	1.8412	62059.07
	5	6128	10.937	67020	1.8412	55737.13
	6	8354	9.694	80984	1.8412	65602.62
	7	9500	9.512	90360	1.8412	72868.6
	8	4472	8.121	36316	1.8412	28082.15
	9	6722	11.148	74934	1.8412	62557.45
	10	10420	9.443	98393	1.8412	79207.7
10ng/ml of TGFβ1 + 1μM of MK-2206/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5027	4.173	20976	0.3214	19360.32
	2	12061	1.618	19514	0.3214	15637.59
	3	4024	4.383	17636	0.3214	16342.69
	4	7119	4.179	29752	0.3214	27463.95
	5	4512	1.923	8675	0.3214	7224.843
	6	4295	3.718	15970	0.3214	14589.59
	7	5765	2.639	15214	0.3214	13361.13
	8	4605	1.824	8399	0.3214	6918.953
	9	5099	1.157	5897	0.3214	4258.181
	10	5436	0.839	4563	0.3214	2815.87
10ng/ml of TGFβ1 + 1μM of MK-2206/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	7110	5.321	37829	4.9433	2682.137
	2	10127	7.943	80443	4.9433	30382.2
	3	11456	7.368	84403	4.9433	27772.56
	4	6127	9.465	57992	4.9433	27704.4
	5	7635	10.306	78683	4.9433	40940.9
	6	7477	9.147	68391	4.9433	31429.95
	7	5928	12.313	72994	4.9433	43690.12
	8	7262	9.438	68541	4.9433	32642.76
	9	11640	8.401	97782	4.9433	40241.99
	10	7995	10.761	86035	4.9433	46513.32

10ng/ml of TGFβ1 + + 5μM of PD98059/24 hours	Cell	Area/ pixel	Mean	Integrated intensity	Background mean	CTCF
	1	8114	8.245	66900	3.7699	36311.03
	2	4723	6.573	31045	3.7699	13239.76
	3	4396	6.145	27014	3.7699	10441.52
	4	3657	6.723	24586	3.7699	10799.48
	5	4989	5.391	26898	3.7699	8089.969
	6	3965	4.607	18266	3.7699	3318.347
	7	8839	5.364	47416	3.7699	14093.85
	8	6502	4.02	26138	3.7699	1626.11
	9	10203	5.126	52302	3.7699	13837.71
	10	8671	4.137	35873	3.7699	3184.197
10ng/ml of TGFβ1 + + 5μM of PD98059/ 48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4982	5.522	27509	3.912	8019.416
	2	6105	5.307	32398	3.912	8515.24
	3	4750	5.403	25664	3.912	7082
	4	5817	5.402	31421	3.912	8664.896
	5	6185	5.569	34442	3.912	10246.28
	6	6724	5.289	35561	3.912	9256.712
	7	5386	5.091	27420	3.912	6349.968
	8	6561	4.175	27392	3.912	1725.368
	9	6225	4.554	28350	3.912	3997.8
	10	6944	5.016	34830	3.912	7665.072
10ng/ml of TGFβ1 + 5μM of SB431542/ 24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	2605	4.701	12245	1.3217	8801.972
	2	3237	4.013	12990	1.3217	8711.657
	3	3486	6.044	21068	1.3217	16460.55
	4	2303	4.761	10965	1.3217	7921.125
	5	2386	4.554	10865	1.3217	7711.424
	6	4362	4.571	19940	1.3217	14174.74
	7	2516	4.308	10838	1.3217	7512.603
	8	3789	4.197	15901	1.3217	10893.08
	9	3124	3.652	11410	1.3217	7281.009
	10	4526	4.973	22508	1.3217	16525.99
10ng/ml of TGFβ1 + 5μM of SB431542/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	3698	15.696	58045	8.3611	27125.65
	2	6295	13.872	87322	8.3611	34688.88
	3	3853	16.474	63475	8.3611	31259.68
	4	3343	16.097	53811	8.3611	25859.84
	5	14649	14.96	219146	8.3611	96664.25
	6	5061	15.342	77644	8.3611	35328.47
	7	10603	15.164	160784	8.3611	72131.26
	8	9647	14.269	137649	8.3611	56989.47
	9	4666	14.446	67406	8.3611	28393.11
	10	3864	14.439	55794	8.3611	23486.71

HaCaT Cell Line		ICC for Migration Markers			Date:12/10/2021	
Calculate Correlated total cell fluorescence					Antibody: β-Catenin	
SF-MEM/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5181	21.881	113367	2.0623	102682.2
	2	7018	17.887	125528	2.0623	111054.8
	3	2881	17.774	51207	2.0623	45265.51
	4	7353	29.031	213464	2.0623	198299.9
	5	6737	17.871	120397	2.0623	106503.3
	6	3946	31.715	125146	2.0623	117008.2
	7	7011	15.098	105851	2.0623	91392.21
	8	6289	16.749	105336	2.0623	92366.2
	9	5487	26.738	146714	2.0623	135398.2
	10	11212	13.186	147838	2.0623	124715.5
SF-MEM/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	9797	13.957	136736	1.6545	120526.9
	2	6736	19.08	128524	1.6545	117379.3
	3	7054	13.473	95041	1.6545	83370.16
	4	10356	13.215	136854	1.6545	119720
	5	9607	16.183	155467	1.6545	139572.2
	6	7663	16.064	123102	1.6545	110423.6
	7	9863	11.37	112144	1.6545	95825.67
	8	8883	13.77	122315	1.6545	107618.1
	9	10896	11.173	121739	1.6545	103711.6
	10	10896	11.173	121739	1.6545	103711.6
10ng/ml of TGFβ1 /24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	12543	8.4	105357	4.1481	53327.38
	2	8202	10.423	85486	4.1481	51463.28
	3	7453	9.281	69168	4.1481	38252.21
	4	14655	9.864	144564	4.1481	83773.59
	5	15817	8.876	140397	4.1481	74786.5
	6	18855	6.535	123213	4.1481	45000.57
	7	9725	9.63	93649	4.1481	53308.73
	8	9004	9.276	83521	4.1481	46171.51
	9	21245	8.884	188730	4.1481	100603.6
	10	12079	10.203	123248	4.1481	73143.1
10ng/ml of TGFβ1 /48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	48353	9.451	456977	2.0965	355604.9
	2	7313	11.265	82378	2.0965	67046.3
	3	27327	9.072	247923	2.0965	190631.9
	4	17061	7.375	125818	2.0965	90049.61
	5	23416	10.415	243868	2.0965	194776.4
	6	24102	10.347	249395	2.0965	198865.2
	7	12318	11.733	144529	2.0965	118704.3
	8	18355	9.568	175628	2.0965	137146.7
	9	14332	8.321	119254	2.0965	89206.96
	10	25187	7.837	197391	2.0965	144586.5

10ng/ml of EGF/24 hours	Cell	Area/ pixel	Mean	Integrated intensity	Background mean	CTCF
	1	6258	12.566	78636	0.8752	73159
	2	2437	11.124	27109	0.8752	24976.14
	3	4246	15.545	66002	0.8752	62285.9
	4	7559	9.395	71014	0.8752	64398.36
	5	8090	14.273	115468	0.8752	108387.6
	6	8905	15.487	137913	0.8752	130119.3
	7	6026	12.964	78120	0.8752	72846.04
	8	10627	13.243	140736	0.8752	131435.2
	9	8647	8.691	75152	0.8752	67584.15
	10	6823	10.741	73287	0.8752	67315.51
10ng/ml of EGF/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	7680	18.661	143318	6.7062	91814.38
	2	18372	14.003	257267	6.7062	134060.7
	3	18692	13.762	257234	6.7062	131881.7
	4	12187	16.836	205179	6.7062	123450.5
	5	15420	16.634	256494	6.7062	153084.4
	6	18692	18.046	337310	6.7062	211957.7
	7	22948	20.193	463382	6.7062	309488.1
	8	161432	16.217	2617899	6.7062	1535304
	9	24582	18.945	465703	6.7062	300851.2
	10	9781	19.33	189067	6.7062	123473.7
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	12390	17.019	210860	0.9257	199390.6
	2	7538	18.301	137950	0.9257	130972.1
	3	6380	20.467	130579	0.9257	124673
	4	5578	27.96	155960	0.9257	150796.4
	5	8430	19.912	167854	0.9257	160050.3
	6	6169	15.719	96972	0.9257	91261.36
	7	8684	18.897	164099	0.9257	156060.2
	8	7532	24.352	183422	0.9257	176449.6
	9	6945	18.658	129583	0.9257	123154
	10	12281	16.896	207497	0.9257	196128.5
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	9483	13.812	130983	1.7378	114503.4
	2	10268	13.78	141492	1.7378	123648.3
	3	9453	20.172	190689	1.7378	174261.6
	4	5336	22.591	120547	1.7378	111274.1
	5	3512	19.946	70049	1.7378	63945.85
	6	10330	17.035	175969	1.7378	158017.5
	7	5681	21.578	122587	1.7378	112714.6
	8	9193	26.245	241269	1.7378	225293.4
	9	9943	20.559	204419	1.7378	187140.1
	10	8865	26.559	235447	1.7378	220041.4
	Cell	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**

10ng/ml of TGFβ1 + 1μM of MK-2206/24 hours	**1**	14420	11.5	165835	0.9033	152809.4
	2	13357	9.37	125159	0.9033	113093.6
	3	5965	10.061	60014	0.9033	54625.82
	4	9997	6.805	68030	0.9033	58999.71
	5	10601	10.12	107282	0.9033	97706.12
	6	11451	6.188	70859	0.9033	60515.31
	7	7379	9.386	69260	0.9033	62594.55
	8	10762	8.864	95390	0.9033	85668.69
	9	39638	10.047	398253	0.9033	362448
	10	21202	6.684	141722	0.9033	122570.2
10ng/ml of TGFβ1 + 1μM of MK-2206/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	6864	13.617	93464	4.4078	63208.86
	2	14687	12.144	178362	4.4078	113624.6
	3	8929	14.67	130986	4.4078	91628.75
	4	9773	13.192	128926	4.4078	85848.57
	5	16757	11.095	185921	4.4078	112059.5
	6	16900	12.333	208425	4.4078	133933.2
	7	10030	15.178	152231	4.4078	108020.8
	8	15735	10.619	167088	4.4078	97731.27
	9	13578	9.577	130041	4.4078	70191.89
	10	13156	9.871	129863	4.4078	71873.98
10ng/ml of TGFβ1 + + 5μM of PD98059/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	9563	11.274	107812	1.2197	96148.01
	2	5086	14.673	74625	1.2197	68421.61
	3	10954	11.452	125441	1.2197	112080.4
	4	8807	17.023	149921	1.2197	139179.1
	5	11218	11.051	123975	1.2197	110292.4
	6	9107	5.868	53438	1.2197	42330.19
	7	12714	5.761	73245	1.2197	57737.73
	8	8804	5.387	47426	1.2197	36687.76
	9	6497	7.758	50404	1.2197	42479.61
	10	10712	12.1	129612	1.2197	116546.6
10ng/ml of TGFβ1 + + 5μM of PD98059/ 48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	18771	9.403	176509	3.2558	115394.4
	2	14765	7.938	117199	3.2558	69127.11
	3	16606	13.09	217380	3.2558	163314.2
	4	21151	10.193	215593	3.2558	146729.6
	5	25905	10.405	269540	3.2558	185198.5
	6	42849	11.612	497552	3.2558	358044.2
	7	22726	11.771	267512	3.2558	193520.7
	8	23549	12.028	283249	3.2558	206578.2
	9	19280	12.367	238438	3.2558	175666.2
	10	19739	11.265	222362	3.2558	158095.8
10ng/ml of TGFβ1 + 5μM of SB431542/ 24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	9921	11.455	113648	0.9197	104523.7
	2	10120	10.495	106206	0.9197	96898.64

	3	11092	10.654	118177	0.9197	107975.7
	4	15555	9.002	140021	0.9197	125715.1
	5	10821	13.902	150431	0.9197	140478.9
	6	8274	13.36	110537	0.9197	102927.4
	7	9190	13.417	123304	0.9197	114852
	8	8352	15.062	125802	0.9197	118120.7
	9	6915	15.237	105366	0.9197	99006.27
	10	8489	13.711	116395	0.9197	108587.7
10ng/ml of TGFβ1 + 5μM of SB431542/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	9121	21.966	200356	3.2735	170498.4
	2	7179	22.397	160786	3.2735	137285.5
	3	7726	25.258	195141	3.2735	169849.9
	4	7800	21.805	170081	3.2735	144547.7
	5	11518	12.207	140598	3.2735	102893.8
	6	8009	15.004	120171	3.2735	93953.54
	7	4194	13.909	58336	3.2735	44606.94
	8	3049	27.887	85026	3.2735	75045.1
	9	2557	28.371	72545	3.2735	64174.66
	10	3807	24.326	92609	3.2735	80146.79

TYS Cell Line		**ICC for Migration Markers**			**Date:12/10/2021**	
Calculate Correlated total cell fluorescence					**Antibody: E-cadherin**	
SF-MEM/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4111	11.168	45912	0.6349	43301.93
	2	4588	12.913	59247	0.6349	56334.08
	3	5905	9.582	56579	0.6349	52829.92
	4	7672	8.044	61711	0.6349	56840.05
	5	4392	11.617	51020	0.6349	48231.52
	6	3249	11.608	37714	0.6349	35651.21
	7	6218	8.152	50689	0.6349	46741.19
	8	10169	6.098	62015	0.6349	55558.7
	9	9654	4.187	40424	0.6349	34294.68
	10	4134	5.145	21268	0.6349	18643.32
SF-MEM/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	20406	5.422	110650	0.8031	94261.94
	2	8741	8.7	76046	0.8031	69026.1
	3	13808	5.507	76035	0.8031	64945.8
	4	9250	6.757	62505	0.8031	55076.33
	5	10935	3.824	41812	0.8031	33030.1
	6	10274	3.722	38241	0.8031	29989.95
	7	10134	4.69	47525	0.8031	39386.38

	8	12383	8.938	110677	0.8031	100732.2
	9	19417	5.733	111314	0.8031	95720.21
	10	14538	7.954	115636	0.8031	103960.5
10ng/ml of TGFβ1 /24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	9145	8.627	78894	1.0073	69682.24
	2	7158	8.339	59688	1.0073	52477.75
	3	8229	12.968	106714	1.0073	98424.93
	4	10228	9.955	101822	1.0073	91519.34
	5	20086	15.144	304187	1.0073	283954.4
	6	34201	12.52	428190	1.0073	393739.3
	7	13297	11.057	147023	1.0073	133628.9
	8	16544	12.753	210992	1.0073	194327.2
	9	17520	10.676	187048	1.0073	169400.1
	10	22915	8.419	192920	1.0073	169837.7
10ng/ml of TGFβ1 /48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	29514	8.969	264699	3.795	152693.4
	2	16144	8.393	135498	3.795	74231.52
	3	18638	8.52	158790	3.795	88058.79
	4	9411	6.763	63648	3.795	27933.26
	5	7634	6.828	52128	3.795	23156.97
	6	6106	6.552	40008	3.795	16835.73
	7	10694	8.084	86448	3.795	45864.27
	8	9383	7.398	69414	3.795	33805.52
	9	15403	8.341	128472	3.795	70017.62
	10	18686	7.364	137599	3.795	66685.63
10ng/ml of EGF/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5264	11.848	62367	1.5308	54308.87
	2	5881	9.432	55469	1.5308	46466.37
	3	10172	9.579	97435	1.5308	81863.7
	4	11672	9.919	115771	1.5308	97903.5
	5	4938	11.478	56678	1.5308	49118.91
	6	7165	7.026	50339	1.5308	39370.82
	7	6724	6.112	41100	1.5308	30806.9
	8	5945	8.344	49607	1.5308	40506.39
	9	4919	11.263	55403	1.5308	47872.99
	10	10426	8.163	85110	1.5308	69149.88
10ng/ml of EGF/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	23697	6.098	144497	1.3587	112299.9
	2	6676	4.619	30839	1.3587	21768.32
	3	9439	6.022	56843	1.3587	44018.23
	4	4371	7.549	32997	1.3587	27058.12
	5	3997	12.309	49200	1.3587	43769.28
	6	5915	10.861	64244	1.3587	56207.29
	7	7865	9.431	74173	1.3587	63486.82
	8	4603	7.954	36611	1.3587	30356.9
	9	6022	8.091	48721	1.3587	40538.91

	10	4750	8.191	38908	1.3587	32454.18
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	6074	26.743	162439	1.1229	155618.5
	2	7145	23.75	169696	1.1229	161672.9
	3	9570	18.284	174974	1.1229	164227.8
	4	18150	21.913	397713	1.1229	377332.4
	5	18210	14.028	255444	1.1229	234996
	6	9866	13.332	131536	1.1229	120457.5
	7	14543	10.616	154387	1.1229	138056.7
	8	16461	8.725	143623	1.1229	125138.9
	9	6860	13.032	89401	1.1229	81697.91
	10	11823	7.963	94149	1.1229	80872.95
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	12866	12.276	157939	2.921	120357.4
	2	12591	10.976	138196	2.921	101417.7
	3	7457	13.583	101288	2.921	79506.1
	4	13999	9.545	133616	2.921	92724.92
	5	9777	8.971	87707	2.921	59148.38
	6	12815	8.947	114657	2.921	77224.39
	7	16977	9.271	157401	2.921	107811.2
	8	13231	9.925	131314	2.921	92666.25
	9	17627	9.134	161009	2.921	109520.5
	10	12866	12.276	157939	2.921	120357.4
10ng/ml of TGFβ1 + 1μM of MK-2206/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4173	14.677	61248	0.6965	58341.51
	2	6493	7.571	49157	0.6965	44634.63
	3	5826	8.033	46798	0.6965	42740.19
	4	4834	10.195	49285	0.6965	45918.12
	5	4248	10.475	44497	0.6965	41538.27
	6	2410	13.372	32227	0.6965	30548.44
	7	6487	9.45	61299	0.6965	56780.8
	8	4407	10.292	45355	0.6965	42285.52
	9	3788	10.352	39213	0.6965	36574.66
	10	4940	17.941	88630	0.6965	85189.29
10ng/ml of TGFβ1 + 1μM of MK-2206/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4156	9.757	40551	1.8705	32777.2
	2	4944	10.445	51639	1.8705	42391.25
	3	6074	12.767	77546	1.8705	66184.58
	4	3079	12.331	37966	1.8705	32206.73
	5	4540	14.124	64122	1.8705	55629.93
	6	4546	17.306	78671	1.8705	70167.71
	7	3371	16.851	56804	1.8705	50498.54
	8	3384	21.965	74329	1.8705	67999.23
	9	6106	5.99	36573	1.8705	25151.73
	10	5022	11.055	55519	1.8705	46125.35

10ng/ml of TGFβ1 + + 5μM of PD98059/24 hours	Cell	Area/ pixel	Mean	Integrated intensity	Background mean	CTCF
	1	14974	11.539	172784	1.5233	149974.1
	2	12297	10.26	126164	1.5233	107432
	3	16775	9.829	164874	1.5233	139320.6
	4	17061	11.441	195191	1.5233	169202
	5	24477	13.656	334267	1.5233	296981.2
	6	16193	15.373	248938	1.5233	224271.2
	7	19566	10.223	200027	1.5233	170222.1
	8	17432	12.804	223205	1.5233	196650.8
	9	10454	10.476	109519	1.5233	93594.42
	10	14633	13.404	196140	1.5233	173849.6
10ng/ml of TGFβ1 + + 5μM of PD98059/ 48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	17561	8.425	147957	1.3016	125099.6
	2	22580	9.33	210662	1.3016	181271.9
	3	32995	9.162	302307	1.3016	259360.7
	4	19403	8.309	161222	1.3016	135967.1
	5	18643	7.186	133976	1.3016	109710.3
	6	23803	7.909	188265	1.3016	157283
	7	25184	7.15	180075	1.3016	147295.5
	8	27131	5.268	142918	1.3016	107604.3
	9	21516	5.695	122540	1.3016	94534.77
	10	25039	5.32	133214	1.3016	100623.2
10ng/ml of TGFβ1 + 5μM of SB431542/ 24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	7176	5.375	38572	0.4705	35195.69
	2	8292	5.866	48641	0.4705	44739.61
	3	11850	6.588	78067	0.4705	72491.58
	4	4578	10.229	46830	0.4705	44676.05
	5	7959	6.324	50334	0.4705	46589.29
	6	6939	10.988	76248	0.4705	72983.2
	7	14591	10.219	149100	0.4705	142234.9
	8	7133	10.365	73935	0.4705	70578.92
	9	20449	8.704	177986	0.4705	168364.7
	10	11555	7.129	82376	0.4705	76939.37
10ng/ml of TGFβ1 + 5μM of SB431542/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	20287	9.851	199856	0.9519	180544.8
	2	5573	27.466	153067	0.9519	147762.1
	3	6248	9.6	59981	0.9519	54033.53
	4	5297	14.033	74331	0.9519	69288.79
	5	4052	5.967	24177	0.9519	20319.9
	6	4986	9.227	46004	0.9519	41257.83
	7	4453	12.853	57235	0.9519	52996.19
	8	5601	9.088	50900	0.9519	45568.41
	9	5350	5.523	29547	0.9519	24454.34
	10	3943	17.556	69225	0.9519	65471.66

TYS Cell Line		ICC for Migration Markers			Date:12/10/2021	
Calculate Correlated total cell fluorescence					Antibody: N-cadherin	
SF-MEM/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4798	4.43	21257	3.2381	5720.596
	2	5726	3.089	17688	3.2381	-853.361
	3	4933	3.779	18644	3.2381	2670.453
	4	4833	3.63	17546	3.2381	1896.263
	5	5016	4.428	22210	3.2381	5967.69
	6	4722	4.232	19983	3.2381	4692.692
	7	5556	3.289	18273	3.2381	282.1164
	8	7638	4.042	30869	3.2381	6136.392
	9	7001	5.498	38490	3.2381	15820.06
	10	7056	3.537	24956	3.2381	2107.966
SF-MEM/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	6327	0.869	5500	0.0604	5117.849
	2	5717	0.773	4420	0.0604	4074.693
	3	5080	0.83	4215	0.0604	3908.168
	4	5774	0.803	4635	0.0604	4286.25
	5	5471	0.859	4701	0.0604	4370.552
	6	5382	1.214	6535	0.0604	6209.927
	7	8273	1.841	15234	0.0604	14734.31
	8	7200	1.761	12680	0.0604	12245.12
	9	9445	0.901	8508	0.0604	7937.522
	10	3818	0.856	3269	0.0604	3038.393
10ng/ml of TGFβ1 /24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	8296	8.083	67060	2.9656	42457.38
	2	7650	8.616	65909	2.9656	43222.16
	3	6214	7.351	45682	2.9656	27253.76
	4	5744	8.981	51586	2.9656	34551.59
	5	4208	8.621	36277	2.9656	23797.76
	6	5879	8.618	50667	2.9656	33232.24
	7	7233	5.488	39696	2.9656	18245.82
	8	9098	5.405	49172	2.9656	22190.97
	9	8760	4.663	40847	2.9656	14868.34
	10	4849	5.461	26478	2.9656	12097.81
10ng/ml of TGFβ1 /48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	13581	3.495	47466	2.3737	15228.78
	2	11258	3.885	43739	2.3737	17015.89
	3	7258	3.184	23106	2.3737	5877.685
	4	9854	3.966	39077	2.3737	15686.56
	5	10178	3.728	37939	2.3737	13779.48
	6	8963	4.649	41669	2.3737	20393.53
	7	5956	3.944	23493	2.3737	9355.243
	8	6595	3.975	26218	2.3737	10563.45
	9	4496	3.829	17213	2.3737	6540.845
	10	9067	3.638	32984	2.3737	11461.66

10ng/ml of EGF/24 hours	Cell	Area/ pixel	Mean	Integrated intensity	Background mean	CTCF
	1	4572	4.136	18910	1.984571	9836.539
	2	3254	5.171	16825	1.984571	10367.2
	3	8673	2.978	25831	1.984571	8618.812
	4	8249	3.018	24896	1.984571	8525.27
	5	5061	1.812	9173	1.984571	-870.916
	6	9124	1.8	16421	1.984571	-1686.23
	7	6054	2.227	13482	1.984571	1467.405
10ng/ml of EGF/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	14723	1.861	27405	1.3245	7904.387
	2	34278	1.964	67315	1.3245	21913.79
	3	7859	2.566	20164	1.3245	9754.755
	4	14670	1.961	28769	1.3245	9338.585
	5	6997	2.721	19039	1.3245	9771.474
	6	23627	3.163	74722	1.3245	43428.04
	7	9268	4.682	43389	1.3245	31113.53
	8	17005	4.867	82769	1.3245	60245.88
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	6619	1.324	8761	0.2046	7406.753
	2	4846	1.841	8921	0.2046	7929.508
	3	5155	2.013	10376	0.2046	9321.287
	4	5545	1.094	6064	0.2046	4929.493
	5	5743	1.138	6536	0.2046	5360.982
	6	5709	1.228	7010	0.2046	5841.939
	7	5325	0.762	4055	0.2046	2965.505
	8	6284	0.62	3896	0.2046	2610.294
	9	5700	0.622	3545	0.2046	2378.78
	10	4908	0.628	3081	0.2046	2076.823
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5719	1.674	9571	1.3222	2009.338
	2	4866	1.576	7671	1.3222	1237.175
	3	7206	1.656	11936	1.3222	2408.227
	4	4863	1.895	9213	1.3222	2783.141
	5	8063	1.728	13930	1.3222	3269.101
	6	8308	2.308	19172	1.3222	8187.162
	7	7937	2.624	20826	1.3222	10331.7
	8	4642	2.698	12522	1.3222	6384.348
	9	5181	2.671	13836	1.3222	6985.682
	10	3748	3.108	11647	1.3222	6691.394
10ng/ml of TGFβ1 + 1μM of MK-2206/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	7562	2.827	21381	0.9235	14397.49
	2	7408	3.075	22781	0.9235	15939.71
	3	2275	1.713	3896	0.9235	1795.038
	4	3820	2.222	8487	0.9235	4959.23
	5	1854	1.318	2444	0.9235	731.831

	6	3842	2.507	9631	0.9235	6082.913
	7	2048	6.86	14049	0.9235	12157.67
	8	2136	7.446	15905	0.9235	13932.4
	9	1662	7.554	12554	0.9235	11019.14
	10	2645	4.436	11733	0.9235	9290.343
10ng/ml of TGFβ1 + 1μM of MK-2206/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	2606	0.941	2452	0.8417	258.5298
	2	1359	1.093	1485	0.8417	341.1297
	3	1193	1.409	1681	0.8417	676.8519
	4	2098	1.813	3804	0.8417	2038.113
	5	2241	1.949	4367	0.8417	2480.75
	6	1761	0.945	1664	0.8417	181.7663
	7	1573	0.88	1384	0.8417	60.0059
	8	1761	0.906	1595	0.8417	112.7663
	9	2068	0.904	1869	0.8417	128.3644
	10	1922	0.941	1809	0.8417	191.2526
10ng/ml of TGFβ1 + + 5μM of PD98059/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	9135	4.373	39948	1.302	28054.23
	2	7152	4.038	28877	1.302	19565.1
	3	7748	2.948	22839	1.302	12751.1
	4	8390	1.865	15645	1.302	4721.22
	5	6226	2.343	14588	1.302	6481.748
	6	7920	3.207	25398	1.302	15086.16
	7	6934	3.687	25565	1.302	16536.93
	8	9461	2.618	24768	1.302	12449.78
	9	6675	2.286	15261	1.302	6570.15
	10	8169	4.16	33987	1.302	23350.96
10ng/ml of TGFβ1 + + 5μM of PD98059/ 48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	10359	6.85	70964	4.6312	22989.4
	2	9942	7.233	71911	4.6312	25867.61
	3	14111	5.845	82479	4.6312	17128.14
	4	11035	6.474	71440	4.6312	20334.71
	5	22587	5.411	122209	4.6312	17604.09
	6	14625	6.614	96732	4.6312	29000.7
	7	19150	5.664	108459	4.6312	19771.52
	8	20031	4.595	92038	4.6312	-729.567
	9	12984	4.3	55836	4.6312	-4295.5
10ng/ml of TGFβ1 + 5μM of SB431542/ 24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4794	1.323	6342	0.3115	4848.669
	2	6404	1.386	8875	0.3115	6880.154
	3	5548	1.017	5641	0.3115	3912.798
	4	4003	0.9	3602	0.3115	2355.066
	5	4238	0.798	3381	0.3115	2060.863
	6	3834	0.814	3119	0.3115	1924.709
	7	3895	0.777	3025	0.3115	1811.708
	8	5753	0.777	4472	0.3115	2679.941

	9	7384	0.851	6283	0.3115	3982.884
	10	6737	0.831	5597	0.3115	3498.425
10ng/ml of TGFβ1 + 5μM of SB431542/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	7151	10.069	72004	5.1006	35529.61
	2	6709	9.606	64444	5.1006	30224.07
	3	4610	8.664	39941	5.1006	16427.23
	4	5435	8.367	45473	5.1006	17751.24
	5	4100	7.833	32115	5.1006	11202.54
	6	6637	6.834	45354	5.1006	11501.32
	7	10641	5.708	60740	5.1006	6464.515
	8	8442	5.709	48192	5.1006	5132.735
	9	4314	4.73	20407	5.1006	-1596.99
	10	3713	4.717	17515	5.1006	-1423.53

TYS Cell Line		**ICC for Migration Markers**			**Date:12/10/2021**	
Calculate Correlated total cell fluorescence					**Antibody: Claudin-1**	
SF-MEM/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5798	3.043	17645	2.5981	2581.216
	2	5350	4.271	22850	2.5981	8950.165
	3	9017	3.907	35225	2.5981	11797.93
	4	8020	4.368	35035	2.5981	14198.24
	5	6116	4.947	30256	2.5981	14366.02
	6	4491	4.678	21011	2.5981	9342.933
	7	7868	4.064	31979	2.5981	11537.15
	8	6079	4.455	27083	2.5981	11289.15
	9	5303	4.226	22408	2.5981	8630.276
	10	6720	4.556	30619	2.5981	13159.77
SF-MEM/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5833	4.173	24341	1.2376	17122.08
	2	5217	4.077	21270	1.2376	14813.44
	3	3022	5.126	15490	1.2376	11749.97
	4	3277	3.164	10369	1.2376	6313.385
	5	5180	3.23	16730	1.2376	10319.23
	6	6390	2.118	13536	1.2376	5627.736
	7	4036	2.355	9506	1.2376	4511.046
	8	4924	2.084	10264	1.2376	4170.058
	9	4060	3.151	12793	1.2376	7768.344
	10	5989	2.026	12131	1.2376	4719.014
10ng/ml of TGFβ1 /24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5004	9.109	45579	3.5066	28031.97
	2	9281	8.71	80835	3.5066	48290.25
	3	4496	6.901	31026	3.5066	15260.33
	4	5568	6.455	35939	3.5066	16414.25
	5	5895	5.617	33114	3.5066	12442.59
	6	12367	4.068	50315	3.5066	6948.878

	7	12586	3.608	45405	3.5066	1270.932
	8	6909	6.362	43954	3.5066	19726.9
	9	8701	6.942	60402	3.5066	29891.07
	10	12417	8.29	102939	3.5066	59397.55
10ng/ml of TGFβ1 /48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	9248	1.78	16461	1.1883	5471.602
	2	6188	1.856	11485	1.1883	4131.8
	3	10635	1.887	20064	1.1883	7426.43
	4	10063	2.138	21516	1.1883	9558.137
	5	7196	2.003	14410	1.1883	5858.993
	6	5181	2.147	11124	1.1883	4967.418
	7	4261	2.672	11386	1.1883	6322.654
	8	5512	3.152	17375	1.1883	10825.09
	9	5648	3.633	20521	1.1883	13809.48
	10	7722	2.847	21986	1.1883	12809.95
10ng/ml of EGF/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	6073	7.337	44559	1.9377	32791.35
	2	16192	5.366	86879	1.9377	55503.76
	3	4887	6.559	32052	1.9377	22582.46
	4	8326	5.568	46359	1.9377	30225.71
	5	18913	4.804	90861	1.9377	54213.28
	6	4220	4.168	17591	1.9377	9413.906
	7	7915	3.17	25090	1.9377	9753.105
	8	4125	3.045	12562	1.9377	4568.988
	9	2576	4.125	10626	1.9377	5634.485
	10	4248	4.14	17587	1.9377	9355.65
10ng/ml of EGF/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	3755	1.446	5430	1.4968	-190.484
	2	4136	1.856	7675	1.4968	1484.235
	3	6498	2.18	14164	1.4968	4437.794
	4	6419	2.089	13409	1.4968	3801.041
	5	11137	1.955	21774	1.4968	5104.138
	6	10462	3.167	33137	1.4968	17477.48
	7	3912	2.664	10423	1.4968	4567.518
	8	8568	1.897	16256	1.4968	3431.418
	9	5637	5.526	31149	1.4968	22711.54
	10	35231	6.646	234131	1.4968	181397.2
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	12632	3.836	48458	0.942	36558.66
	2	8552	3.964	33897	0.942	25841.02
	3	4713	4.032	19004	0.942	14564.35
	4	14283	3.067	43800	0.942	30345.41
	5	3123	2.042	6377	0.942	3435.134
	6	3399	1.469	4993	0.942	1791.142
	7	6525	1.302	8494	0.942	2347.45
	8	6704	1.929	12933	0.942	6617.832

	9	5691	2.004	11404	0.942	6043.078
	10	5579	1.869	10429	0.942	5173.582
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4127	7.85	32398	4.6852	13062.18
	2	2849	7.488	21332	4.6852	7983.865
	3	2552	7.324	18691	4.6852	6734.37
	4	2402	6.382	15329	4.6852	4075.15
	5	4987	5.995	29895	4.6852	6529.908
	6	4076	7.569	30853	4.6852	11756.12
	7	1507	6.844	10314	4.6852	3253.404
	8	2237	7.498	16773	4.6852	6292.208
	9	8048	6.256	50348	4.6852	12641.51
	10	5085	6.301	32040	4.6852	8215.758
10ng/ml of TGFβ1 + 1μM of MK-2206/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	6706	4.635	31084	0.7038	26364.32
	2	5853	6.314	36953	0.7038	32833.66
	3	3183	6.422	20442	0.7038	18201.8
	4	3928	5.609	22033	0.7038	19268.47
	5	4277	4.111	17584	0.7038	14573.85
	6	3496	2.836	9914	0.7038	7453.515
	7	4761	1.727	8224	0.7038	4873.208
	8	2846	3.3	9391	0.7038	7387.985
	9	2633	2.149	5659	0.7038	3805.895
	10	4764	1.875	8933	0.7038	5580.097
10ng/ml of TGFβ1 + 1μM of MK-2206/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4122	6.662	27459	4.7981	7681.232
	2	4419	4.464	19727	4.7981	-1475.8
	3	2392	4.868	11644	4.7981	166.9448
	4	1114	4.973	5540	4.7981	194.9166
	5	1690	4.998	8447	4.7981	338.211
	6	5181	4.981	25804	4.7981	945.0439
	7	3205	4.9	15706	4.7981	328.0895
	8	2026	4.793	9711	4.7981	-9.9506
	9	2435	8.536	20784	4.7981	9100.627
	10	2245	8.163	18325	4.7981	7553.266
10ng/ml of TGFβ1 + + 5μM of PD98059/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5237	14.475	75808	13.1262	7066.091
	2	4982	15.196	75705	13.1262	10310.27
	3	5665	15.047	85243	13.1262	10883.08
	4	9554	14.492	138452	13.1262	13044.29
	5	9556	14.43	137897	13.1262	12463.03
	6	9007	13.688	123284	13.1262	5056.317
	7	11777	13.705	161402	13.1262	6814.743
	8	9344	13.439	125573	13.1262	2921.787
	9	8951	13.771	123266	13.1262	5773.384
	10	8268	14.828	122598	13.1262	14070.58

10ng/ml of TGFβ1 + + 5μM of PD98059/ 48 hours	Cell	Area/ pixel	Mean	Integrated intensity	Background mean	CTCF
	1	11818	5.97	70556	4.3317	19363.97
	2	10168	5.986	60864	4.3317	16819.27
	3	7805	6.044	47173	4.3317	13364.08
	4	10292	5.391	55483	4.3317	10901.14
	5	8156	5.293	43170	4.3317	7840.655
	6	10780	5.518	59485	4.3317	12789.27
	7	7848	5.056	39678	4.3317	5682.818
	8	7223	5.002	36126	4.3317	4838.131
	9	10900	5.448	59384	4.3317	12168.47
	10	6309	5.071	31990	4.3317	4661.305
10ng/ml of TGFβ1 + 5μM of SB431542/ 24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5738	2.104	12070	0.2754	10489.75
	2	7577	2.785	21100	0.2754	19013.29
	3	9379	1.782	16717	0.2754	14134.02
	4	4629	1.919	8884	0.2754	7609.173
	5	4113	2.011	8273	0.2754	7140.28
	6	4580	2.117	9698	0.2754	8436.668
	7	7040	2.203	15507	0.2754	13568.18
	8	7677	2.675	20537	0.2754	18422.75
	9	10449	1.543	16122	0.2754	13244.35
	10	12401	0.81	10046	0.2754	6630.765
10ng/ml of TGFβ1 + 5μM of SB431542/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5411	4.304	23291	2.712	8616.368
	2	4347	4.566	19847	2.712	8057.936
	3	5381	4.94	26584	2.712	11990.73
	4	5874	4.734	27810	2.712	11879.71
	5	6186	5.578	34504	2.712	17727.57
	6	5275	5.576	29414	2.712	15108.2
	7	3017	8.046	24276	2.712	16093.9
	8	3283	8.505	27921	2.712	19017.5
	9	3343	8.561	28621	2.712	19554.78
	10	5211	7.088	36938	2.712	22805.77

TYS Cell Line		ICC for Migration Markers			Date:12/10/2021	
Calculate Correlated total cell fluorescence					Antibody: β-Catenin	
SF-MEM/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	20002	18.267	365379	0.2101	361176.6
	2	10884	21.147	230160	0.2101	227873.3
	3	17590	19.481	342669	0.2101	338973.3
	4	12642	18.411	232752	0.2101	230095.9
	5	20430	16.764	342498	0.2101	338205.7
	6	14498	17.309	250951	0.2101	247905
	7	13801	20.076	277067	0.2101	274167.4

	8	13988	18.224	254921	0.2101	251982.1
	9	19569	12.41	242857	0.2101	238745.6
	10	10970	12.762	140001	0.2101	137696.2
SF-MEM/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5338	16.948	90467	3.2684	73020.28
	2	2205	21.885	48257	3.2684	41050.18
	3	5298	13.214	70010	3.2684	52694.02
	4	6812	13.385	91178	3.2684	68913.66
	5	9611	9.711	93333	3.2684	61920.41
	6	15563	9.115	141858	3.2684	90991.89
	7	7625	8.656	66004	3.2684	41082.45
	8	12280	12.665	155522	3.2684	115386
	9	12013	12.578	151099	3.2684	111835.7
	10	16662	11.082	184656	3.2684	130197.9
10ng/ml of TGFβ1 /24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	14241	9.348	133124	0.3484	128162.4
	2	19693	11.294	222407	0.3484	215546
	3	22746	7.584	172508	0.3484	164583.3
	4	20051	10.476	210057	0.3484	203071.2
	5	23596	10.628	250771	0.3484	242550.2
	6	25333	13.255	335794	0.3484	326968
	7	18116	16.405	297185	0.3484	290873.4
	8	17481	14.194	248132	0.3484	242041.6
	9	12419	20.982	260578	0.3484	256251.2
	10	8526	28.437	242458	0.3484	239487.5
10ng/ml of TGFβ1 /48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	8536	11.381	97145	1.941	80576.62
	2	11772	10.485	123428	1.941	100578.5
	3	15342	6.757	103671	1.941	73892.18
	4	8276	8.676	71804	1.941	55740.28
	5	9275	7.146	66276	1.941	48273.23
	6	17896	6.063	108498	1.941	73761.86
	7	15576	8.249	128483	1.941	98249.98
	8	11908	9.441	112429	1.941	89315.57
	9	12479	10.793	134682	1.941	110460.3
	10	25566	16.864	431151	1.941	381527.4
10ng/ml of EGF/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	8971	22.257	199668	0.0836	198918
	2	4359	33.278	145060	0.0836	144695.6
	3	8125	19.286	156701	0.0836	156021.8
	4	3636	30.595	111242	0.0836	110938
	5	11528	28.075	323652	0.0836	322688.3
	6	8249	29.067	239774	0.0836	239084.4
	7	12559	15.761	197947	0.0836	196897.1
	8	4992	14.428	72023	0.0836	71605.67
	9	8610	20.052	172651	0.0836	171931.2

	10	19304	8.311	160428	0.0836	158814.2
10ng/ml of EGF/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	6662	12.901	85949	1.0143	79191.73
	2	13320	11.141	148403	1.0143	134892.5
	3	6619	15.098	99934	1.0143	93220.35
	4	12930	13.298	171945	1.0143	158830.1
	5	10097	11.257	113664	1.0143	103422.6
	6	10199	10.179	103815	1.0143	93470.15
	7	4834	15.028	72643	1.0143	67739.87
	8	9392	10.497	98584	1.0143	89057.69
	9	11197	11.062	123863	1.0143	112505.9
	10	5894	9.58	56467	1.0143	50488.72
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	8897	15.087	134227	0.8446	126712.6
	2	8863	13.123	116310	0.8446	108824.3
	3	9926	14.972	148611	0.8446	140227.5
	4	9502	12.682	120506	0.8446	112480.6
	5	10753	17.288	185894	0.8446	176812
	6	7488	17.417	130422	0.8446	124097.6
	7	13143	16.18	212651	0.8446	201550.4
	8	9541	19.539	186425	0.8446	178366.7
	9	7975	21.247	169444	0.8446	162708.3
	10	8026	21.246	170522	0.8446	163743.2
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	12517	14.403	180278	3.2348	139788
	2	11224	17.956	201534	3.2348	165226.6
	3	17347	14.709	255162	3.2348	199047.9
	4	22080	13.606	300426	3.2348	229001.6
	5	13512	9.65	130393	3.2348	86684.38
	6	11607	15.192	176328	3.2348	138781.7
	7	12019	13.784	165669	3.2348	126789.9
	8	6992	17.751	124118	3.2348	101500.3
	9	14902	11.559	172247	3.2348	124042
	10	16643	14.423	240042	3.2348	186205.2
10ng/ml of TGFβ1 + 1μM of MK-2206/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	9225	16.177	149237	0.7138	142652.2
	2	12807	15.349	196576	0.7138	187434.4
	3	22733	8.53	193909	0.7138	177682.2
	4	4400	15.289	67272	0.7138	64131.28
	5	11290	6.213	70147	0.7138	62088.2
	6	27394	9.132	250162	0.7138	230608.2
	7	5335	8.479	45233	0.7138	41424.88
	8	12057	11.14	134313	0.7138	125706.7
	9	8557	11.21	95926	0.7138	89818.01
	10	15863	8.857	140503	0.7138	129180

10ng/ml of TGFβ1 + 1μM of MK-2206/48 hours	Cell	Area/ pixel	Mean	Integrated intensity	Background mean	CTCF
	1	4788	19.028	91105	2.9421	77018.23
	2	3073	24.839	76329	2.9421	67287.93
	3	2519	26.744	67369	2.9421	59957.85
	4	3658	22.144	81001	2.9421	70238.8
	5	8984	11.886	106785	2.9421	80353.17
	6	2946	21.585	63588	2.9421	54920.57
	7	6231	12.329	76822	2.9421	58489.77
	8	2564	30.102	77181	2.9421	69637.46
	9	5431	23.219	126104	2.9421	110125.5
	10	1620	38.294	62036	2.9421	57269.8
10ng/ml of TGFβ1 + + 5μM of PD98059/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	9865	11.626	114688	0.0518	114177
	2	10790	13.887	149836	0.0518	149277.1
	3	13786	12.616	173930	0.0518	173215.9
	4	10029	12.202	122378	0.0518	121858.5
	5	12548	11.818	148294	0.0518	147644
	6	19845	12.159	241300	0.0518	240272
	7	15014	11.038	165732	0.0518	164954.3
	8	13922	11.345	157949	0.0518	157227.8
	9	10912	13.893	151595	0.0518	151029.8
	10	9865	11.626	114688	0.0518	114177
10ng/ml of TGFβ1 + + 5μM of PD98059/ 48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	16466	11.323	186446	1.5701	160592.7
	2	15409	10.258	158058	1.5701	133864.3
	3	17440	12.041	209997	1.5701	182614.5
	4	17481	11.748	205368	1.5701	177921.1
	5	63668	7.488	476761	1.5701	376795.9
	6	20791	7.675	159575	1.5701	126931.1
	7	24126	10.18	245609	1.5701	207728.8
	8	25469	9.352	238198	1.5701	198209.1
	9	6815	16.204	110427	1.5701	99726.77
	10	12174	9.291	113107	1.5701	93992.6
10ng/ml of TGFβ1 + 5μM of SB431542/ 24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	7148	6.904	49347	0.0798	48776.59
	2	9100	7.006	63754	0.0798	63027.82
	3	6974	9.729	67853	0.0798	67296.47
	4	8044	7.795	62702	0.0798	62060.09
	5	9135	9.201	84050	0.0798	83321.03
	6	11022	8.104	89321	0.0798	88441.44
	7	9618	12.802	123129	0.0798	122361.5
	8	12941	5.386	69701	0.0798	68668.31
	9	8077	6.26	50566	0.0798	49921.46
	10	5988	8.863	53072	0.0798	52594.16
	Cell	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**

10ng/ml of TGFβ1 + 5μM of SB431542/48 hours	**1**	17797	12.231	217684	1.6431	188441.7
	2	11294	11.132	125724	1.6431	107166.8
	3	3808	13.2	50267	1.6431	44010.08
	4	4049	10.589	42875	1.6431	36222.09
	5	5989	13.195	79026	1.6431	69185.47
	6	3936	13.803	54329	1.6431	47861.76
	7	5219	18.395	96002	1.6431	87426.66
	8	11583	12.058	139665	1.6431	120633
	9	11572	14.482	167591	1.6431	148577
	10	17432	12.416	216442	1.6431	187799.5

SAS-H1 Cell Line		**ICC for Migration Markers**			**Date:12/10/2021**	
Calculate Correlated total cell fluorescence					**Antibody: E-cadherin**	
SF-MEM/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	12034	9.26	111437	2.7427	78431.35
	2	8770	9.948	87248	2.7427	63194.52
	3	12549	11.258	141274	2.7427	106855.9
	4	10164	13.716	139414	2.7427	111537.2
	5	8529	17.922	152861	2.7427	129468.5
	6	9515	15.954	151807	2.7427	125710.2
	7	25031	12.402	310431	2.7427	241778.5
	8	8600	10.378	89250	2.7427	65662.78
	9	14363	8.506	122172	2.7427	82778.6
	10	17568	6.572	115464	2.7427	67280.25
SF-MEM/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	25045	8.629	216106	2.0088	165795.6
	2	9446	9.884	93367	2.0088	74391.88
	3	8434	10.425	87925	2.0088	70982.78
	4	10726	7.142	76603	2.0088	55056.61
	5	10307	10.77	111009	2.0088	90304.3
	6	12721	9.978	126936	2.0088	101382.1
	7	7898	10.558	83386	2.0088	67520.5
	8	12457	7.536	93879	2.0088	68855.38
	9	5809	11.183	64963	2.0088	53293.88
	10	7490	11.054	82791	2.0088	67745.09
10ng/ml of TGFβ1 /24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	19622	11.008	215993	4.7748	122301.9
	2	12803	13.984	179032	4.7748	117900.2
	3	22988	13.307	305911	4.7748	196147.9
	4	19146	14.101	269983	4.7748	178564.7
	5	18405	13.921	256223	4.7748	168342.8
	6	12904	15.112	194999	4.7748	133385
	7	11398	16.678	190099	4.7748	135675.8
	8	16600	14.401	239049	4.7748	159787.3

10ng/ml of TGFβ1 /48 hours	Cell	Area/ pixel	Mean	Integrated intensity	Background mean	CTCF
	1	8364	9.55	79874	2.247	61080.09
	2	11989	8.777	105228	2.247	78288.72
	3	13203	7.314	96563	2.247	66895.86
	4	11000	6.94	76345	2.247	51628
	5	13616	5.023	68398	2.247	37802.85
	6	14643	10.131	148347	2.247	115444.2
	7	17630	5.73	101014	2.247	61399.39
	8	14429	6.191	89336	2.247	56914.04
	9	14152	5.186	73389	2.247	41589.46
	10	16149	5.669	91546	2.247	55259.2
10ng/ml of EGF/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	17666	5.052	89247	1.3535	65336.07
	2	16017	6.283	100637	1.3535	78957.99
	3	19839	4.739	94024	1.3535	67171.91
	4	12604	4.954	62438	1.3535	45378.49
	5	17020	4.673	79535	1.3535	56498.43
	6	20548	4.686	96284	1.3535	68472.28
	7	14218	5.518	78456	1.3535	59211.94
	8	20042	7.179	143878	1.3535	116751.2
	9	14836	7.581	112465	1.3535	92384.47
10ng/ml of EGF/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	6900	11.459	79069	2.687	60528.7
	2	9670	6.584	63670	2.687	37686.71
	3	14189	5.36	76046	2.687	37920.16
	4	9748	6.317	61582	2.687	35389.12
	5	19593	6.991	136967	2.687	84320.61
	6	19775	10.036	198466	2.687	145330.6
	7	11233	7.842	88087	2.687	57903.93
	8	10136	10.117	102541	2.687	75305.57
	9	16521	8.923	147414	2.687	103022.1
	10	15614	7.658	119573	2.687	77618.18
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	10476	9.406	98541	2.6588	70687.41
	2	7015	8.295	58192	2.6588	39540.52
	3	20370	8.235	167744	2.6588	113584.2
	4	14646	7.628	111724	2.6588	72783.22
	5	8163	9.042	73806	2.6588	52102.22
	6	12828	8.345	107053	2.6588	72945.91
	7	15339	7.053	108182	2.6588	67398.67
	8	12720	7.533	95822	2.6588	62002.06
	9	6858	9.811	67287	2.6588	49052.95
	10	11025	9.483	104546	2.6588	75232.73
10ng/ml of TGFβ1 + 5μM of TGF-β RI	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5679	14.746	83745	1.4222	75668.33

Kinase Inhibitor VII/48 hours	**2**	5448	13.537	73747	1.4222	65998.85
	3	7803	10.095	78768	1.4222	67670.57
	4	6504	8.1	52683	1.4222	43433.01
	5	7446	7.441	55402	1.4222	44812.3
	6	8778	6.313	55416	1.4222	42931.93
	7	4772	7.783	37142	1.4222	30355.26
	8	9021	9.761	88057	1.4222	75227.33
	9	7560	12.492	94438	1.4222	83686.17
	10	5520	11.314	62451	1.4222	54600.46
10ng/ml of TGFβ1 + 1μM of MK-2206/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5604	5.808	32548	0.8004	28062.56
	2	1440	7.619	10972	0.8004	9819.424
	3	1884	8.287	15613	0.8004	14105.05
	4	4415	8.766	38704	0.8004	35170.23
	5	17565	9.145	160630	0.8004	146571
	6	6224	3.683	22920	0.8004	17938.31
	7	7217	4.647	33539	0.8004	27762.51
	8	7538	4.956	37361	0.8004	31327.58
	9	18454	3.766	69501	0.8004	54730.42
	10	16773	7.374	123688	0.8004	110262.9
10ng/ml of TGFβ1 + 1μM of MK-2206/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4689	8.577	40218	1.2197	34498.83
	2	7810	7.973	62269	1.2197	52743.14
	3	7405	5.733	42451	1.2197	33419.12
	4	5998	7.233	43384	1.2197	36068.24
	5	9228	7.631	70422	1.2197	59166.61
	6	12312	6.219	76565	1.2197	61548.05
	7	4972	6.073	30196	1.2197	24131.65
	8	6519	2.772	18068	1.2197	10116.78
	9	3723	3.173	11813	1.2197	7272.057
	10	7884	4.767	37580	1.2197	27963.89
10ng/ml of TGFβ1 + + 5μM of PD98059/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	12845	9.152	117554	1.2746	101181.8
	2	11787	10.141	119535	1.2746	104511.3
	3	13813	11.642	160811	1.2746	143205
	4	12545	11.503	144308	1.2746	128318.1
	5	16075	8.177	131452	1.2746	110962.8
	6	9083	13.315	120940	1.2746	109362.8
	7	8280	15.711	130083	1.2746	119529.3
	8	8017	12.603	101042	1.2746	90823.53
	9	12650	10.673	135018	1.2746	118894.3
	10	12191	11.59	141294	1.2746	125755.4
10ng/ml of TGFβ1 + + 5μM of PD98059/ 48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	13504	7.046	95149	1.0884	80451.25
	2	16165	4.714	76194	1.0884	58600.01
	3	26223	4.012	105207	1.0884	76665.89

	4	15601	4.983	77732	1.0884	60751.87
	5	18503	4.767	88211	1.0884	68072.33
	6	9651	4.914	47424	1.0884	36919.85
	7	25467	4.801	122267	1.0884	94548.72
	8	17802	4.379	77955	1.0884	58579.3
	9	7971	5.73	45676	1.0884	37000.36
	10	14817	3.84	56904	1.0884	40777.18
10ng/ml of TGFβ1 + 5μM of SB431542/ 24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	17163	11.782	202213	1.1376	182688.4
	2	13070	7.944	103823	1.1376	88954.57
	3	12546	5.217	65454	1.1376	51181.67
	4	12964	5.46	70789	1.1376	56041.15
	5	6815	8.207	55928	1.1376	48175.26
	6	8914	8.508	75842	1.1376	65701.43
	7	7695	7.382	56803	1.1376	48049.17
	8	5050	7.274	36733	1.1376	30988.12
	9	4729	9.421	44553	1.1376	39173.29
	10	13363	5.407	72250	1.1376	57048.25
10ng/ml of TGFβ1 + 5μM of SB431542/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	10599	11.269	119437	1.0836	107951.9
	2	11645	9.008	104898	1.0836	92279.48
	3	14681	6.123	89895	1.0836	73986.67
	4	8226	12.237	100665	1.0836	91751.31
	5	13620	10.743	146315	1.0836	131556.4
	6	16963	6.337	107501	1.0836	89119.89
	7	10346	8.843	91486	1.0836	80275.07
	8	48912	4.756	232619	1.0836	179618
	9	20863	5.627	117400	1.0836	94792.85
	10	26519	4.295	113910	1.0836	85174.01

SAS-H1 Cell Line		**ICC for Migration Markers**			**Date:12/10/2021**	
Calculate Correlated total cell fluorescence					**Antibody: N-cadherin**	
SF-MEM/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5434	3.345	18178	0.2414	16866.23
	2	4145	2.435	10093	0.2414	9092.397
	3	12416	2.166	26892	0.2414	23894.78
	4	4800	2.257	10836	0.2414	9677.28
	5	4340	2.067	8972	0.2414	7924.324
	6	6420	2.33	14957	0.2414	13407.21
	7	4299	1.261	5422	0.2414	4384.221
	8	6740	2.006	13520	0.2414	11892.96
	9	3979	2.517	10017	0.2414	9056.469
	10	9175	0.832	7630	0.2414	5415.155
SF-MEM/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**

	1	7178	1.424	10220	0.9851	3148.952
	2	3512	1.994	7002	0.9851	3542.329
	3	6732	2.62	17637	0.9851	11005.31
	4	4833	1.72	8312	0.9851	3551.012
	5	11432	2.378	27191	0.9851	15929.34
	6	7147	2.074	14822	0.9851	7781.49
	7	7817	2.114	16524	0.9851	8823.473
	8	8899	1.48	13167	0.9851	4400.595
	9	7932	1.252	9932	0.9851	2118.187
	10	13168	4.681	61645	0.9851	48673.2
10ng/ml of TGFβ1 /24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	10086	1.976	19929	1.1732	8096.105
	2	6611	1.871	12372	1.1732	4615.975
	3	6398	2.853	18253	1.1732	10746.87
	4	5399	3.428	18507	1.1732	12172.89
	5	6669	3.844	25636	1.1732	17811.93
	6	11619	2.747	31921	1.1732	18289.59
	7	3574	3.377	12071	1.1732	7877.983
	8	5452	3.851	20997	1.1732	14600.71
	9	7199	3.886	27976	1.1732	19530.13
	10	5912	3.37	19925	1.1732	12989.04
10ng/ml of TGFβ1 /48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	11528	2.348	27069	1.0363	15122.53
	2	12472	1.736	21653	1.0363	8728.266
	3	13337	2.412	32163	1.0363	18341.87
	4	19175	3.037	58240	1.0363	38368.95
	5	14054	3.746	52641	1.0363	38076.84
	6	16738	4.518	75624	1.0363	58278.41
	7	17877	7.752	138585	1.0363	120059.1
	8	13691	5.261	72034	1.0363	57846.02
	9	16577	4.443	73647	1.0363	56468.25
	10	19958	3.908	77989	1.0363	57306.52
10ng/ml of EGF/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	10721	4.283	45916	1.8018	26598.9
	2	14340	4.673	67012	1.8018	41174.19
	3	34683	5.339	185175	1.8018	122683.2
	4	6001	6.123	36745	1.8018	25932.4
	5	9753	6.585	64228	1.8018	46655.04
	6	9622	6.686	64334	1.8018	46997.08
	7	13113	1.843	24171	1.8018	543.9966
	8	5468	1.417	7750	1.8018	-2102.24
	9	5318	1.849	9831	1.8018	249.0276
	10	16358	2.368	38739	1.8018	9265.156
10ng/ml of EGF/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	14942	4.542	67860	0.9637	53460.39
	2	13246	3.962	52487	0.9637	39721.83

	3	9940	2.272	22584	0.9637	13004.82
	4	8459	1.966	16633	0.9637	8481.062
	5	10823	1.572	17009	0.9637	6578.875
	6	7064	1.805	12748	0.9637	5940.423
	7	9897	2.126	21042	0.9637	11504.26
	8	9169	4.349	39874	0.9637	31037.83
	9	12137	1.795	21788	0.9637	10091.57
	10	12095	1.464	17706	0.9637	6050.049
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	12552	2.596	32582	1.8853	8917.714
	2	8646	3.121	26986	1.8853	10685.7
	3	10077	3.789	38184	1.8853	19185.83
	4	4344	4.052	17604	1.8853	9414.257
	5	6183	4.076	25203	1.8853	13546.19
	6	6777	3.736	25319	1.8853	12542.32
	7	10464	3.745	39186	1.8853	19458.22
	8	7095	3.155	22388	1.8853	9011.797
	9	5727	2.691	15414	1.8853	4616.887
	10	8046	3.044	24489	1.8853	9319.876
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4695	2.653	12457	0.9182	8146.051
	2	5289	2.698	14269	0.9182	9412.64
	3	4353	2.875	12513	0.9182	8516.075
	4	3408	2.908	9911	0.9182	6781.774
	5	4509	2.224	10029	0.9182	5888.836
	6	4221	2.434	10276	0.9182	6400.278
	7	4194	2.807	11772	0.9182	7921.069
	8	4077	1.891	7708	0.9182	3964.499
	9	5133	3.145	16144	0.9182	11430.88
	10	4839	3.246	15709	0.9182	11265.83
10ng/ml of TGFβ1 + 1μM of MK-2206/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	6747	2.703	18235	0.9648	11725.49
	2	6747	2.322	15666	0.9648	9156.494
	3	6747	2.296	15489	0.9648	8979.494
	4	6747	1.517	10232	0.9648	3722.494
	5	6747	2.058	13885	0.9648	7375.494
	6	6747	1.793	12100	0.9648	5590.494
	7	6747	1.435	9684	0.9648	3174.494
	8	6747	1.423	9602	0.9648	3092.494
	9	6747	1.234	8325	0.9648	1815.494
	10	6747	1.409	9505	0.9648	2995.494
10ng/ml of TGFβ1 + 1μM of MK-2206/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5817	4.319	25126	0.9487	19607.41
	2	5817	5.084	29574	0.9487	24055.41
	3	5817	3.669	21340	0.9487	15821.41
	4	5817	3.511	20421	0.9487	14902.41

	5	5817	3.628	21102	0.9487	15583.41
	6	5817	3.934	22883	0.9487	17364.41
	7	5817	3.854	22421	0.9487	16902.41
	8	5817	2.424	14098	0.9487	8579.412
	9	5817	3.82	22221	0.9487	16702.41
	10	5817	2.646	15391	0.9487	9872.412
10ng/ml of TGFβ1 + + 5μM of PD98059/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	12162	1.101	13389	0.9403	1953.071
	2	12211	1.137	13887	0.9403	2404.997
	3	9310	4.695	43706	0.9403	34951.81
	4	10053	3.425	34431	0.9403	24978.16
	5	5430	3.487	18936	0.9403	13830.17
	6	7296	3.416	24923	0.9403	18062.57
	7	7228	3.612	26107	0.9403	19310.51
	8	7088	3.647	25851	0.9403	19186.15
10ng/ml of TGFβ1 + + 5μM of PD98059/ 48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	21803	4.878	106358	1.5999	71475.38
	2	24822	4.774	118508	1.5999	78795.28
	3	10618	7.528	79934	1.5999	62946.26
	4	14885	7.278	108335	1.5999	84520.49
	5	11235	5.769	64819	1.5999	46844.12
	6	12629	4.893	61794	1.5999	41588.86
	7	11682	5.146	60114	1.5999	41423.97
	8	13878	3.161	43870	1.5999	21666.59
	9	16782	2.895	48584	1.5999	21734.48
	10	10367	2.193	22740	1.5999	6153.837
10ng/ml of TGFβ1 + 5μM of SB431542/ 24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5866	15.085	88486	12.4901	15219.07
	2	1711	16.343	27963	12.4901	6592.439
	3	1432	16.887	24182	12.4901	6296.177
	4	1912	15.624	29874	12.4901	5992.929
	5	22606	14.533	328530	12.4901	46178.8
	6	21022	13.904	292281	12.4901	29714.12
	7	16314	13.601	221887	12.4901	18123.51
	8	10053	13.473	135442	12.4901	9879.025
	9	4352	13.507	58781	12.4901	4424.085
	10	5719	14.468	82741	12.4901	11310.12
10ng/ml of TGFβ1 + 5μM of SB431542/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	6134	5.986	36720	0.9997	30587.84
	2	9959	4.388	43699	0.9997	33742.99
	3	8422	3.846	32388	0.9997	23968.53
	4	12446	3.066	38159	0.9997	25716.73
	5	11338	1.592	18055	0.9997	6720.401
	6	6232	1.63	10161	0.9997	3930.87
	7	6175	1.885	11638	0.9997	5464.853
	8	13789	3.205	44193	0.9997	30408.14

	9	6398	3.135	20057	0.9997	13660.92
	10	9541	1.457	13902	0.9997	4363.862

SAS-H1 Cell Line		**ICC for Migration Markers**			**Date:12/10/2021**	
Calculate Correlated total cell fluorescence					**Antibody: Claudin-1**	
SF-MEM/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	9925	5.083	50449	2.0246	30354.85
	2	10368	4.761	49357	2.0246	28365.95
	3	10275	4.222	43381	2.0246	22578.24
	4	10179	3.919	39894	2.0246	19285.6
	5	9988	3.596	35919	2.0246	15697.3
	6	7748	3.23	25027	2.0246	9340.399
	7	9130	3.557	32479	2.0246	13994.4
	8	9706	3.773	36618	2.0246	16967.23
	9	10709	4.799	51391	2.0246	29709.56
	10	10904	3.641	39704	2.0246	17627.76
SF-MEM/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	5682	5.434	30875	2.2874	17877.99
	2	5687	5.113	29076	2.2874	16067.56
	3	5482	4.996	27390	2.2874	14850.47
	4	10271	4.531	46543	2.2874	23049.11
	5	8725	4.007	34958	2.2874	15000.44
	6	10631	4.044	42994	2.2874	18676.65
	7	5807	3.723	21621	2.2874	8338.068
	8	6641	3.598	23895	2.2874	8704.377
	9	6773	3.577	24226	2.2874	8733.44
	10	21747	2.592	56366	2.2874	6621.912
10ng/ml of TGFβ1 /24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	18446	3.042	56117	0.8187	41015.26
	2	15808	1.44	22760	0.8187	9817.99
	3	10627	1.871	19883	0.8187	11182.68
	4	8800	1.138	10012	0.8187	2807.44
	5	7928	0.95	7530	0.8187	1039.346
	6	6600	1.102	7275	0.8187	1871.58
	7	13601	1.122	15259	0.8187	4123.861
	8	5481	1.273	6977	0.8187	2489.705
	9	5390	1.326	7148	0.8187	2735.207
	10	7234	1.857	13434	0.8187	7511.524
10ng/ml of TGFβ1 /48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	14496	3.752	54394	1.6358	30681.44
	2	10533	4.011	42243	1.6358	25013.12
	3	8544	3.693	31553	1.6358	17576.72
	4	8413	3.976	33448	1.6358	19686.01
	5	13507	3.481	47018	1.6358	24923.25

	6	8639	4.541	39231	1.6358	25099.32
	7	8255	3.753	30985	1.6358	17481.47
	8	9876	2.608	25757	1.6358	9601.839
	9	10274	3.132	32174	1.6358	15367.79
	10	5100	2.797	14263	1.6358	5920.42
10ng/ml of EGF/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	9314	6.022	56088	2.0297	37183.37
	2	3930	5.434	21354	2.0297	13377.28
	3	7868	5.03	39577	2.0297	23607.32
	4	7985	4.892	39065	2.0297	22857.85
	5	5674	4.789	27172	2.0297	15655.48
	6	10182	4.734	48199	2.0297	27532.59
	7	15366	3.136	48195	2.0297	17006.63
	8	5105	3.35	17102	2.0297	6740.382
	9	10676	3.221	34389	2.0297	12719.92
	10	12018	1.923	23110	2.0297	-1282.93
10ng/ml of EGF/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	7460	5.512	41119	5.5672	-412.312
	2	16032	6.341	101652	5.5672	12398.65
	3	7405	7.574	56084	5.5672	14858.88
	4	14990	5.514	82660	5.5672	-792.328
	5	8997	5.725	51511	5.5672	1422.902
	6	10427	5.734	59785	5.5672	1735.806
	7	10639	8.192	87153	5.5672	27923.56
	8	14642	7.924	116017	5.5672	34502.06
	9	13013	8.445	109898	5.5672	37452.03
	10	12823	8.412	107864	5.5672	36475.79
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4680	2.194	10266	0.9305	5911.26
	2	4746	2.148	10194	0.9305	5777.847
	3	5307	2.557	13569	0.9305	8630.837
	4	5796	2.477	14354	0.9305	8960.822
	5	5250	2.868	15056	0.9305	10170.88
	6	4734	3.811	18039	0.9305	13634.01
	7	6384	4.342	27720	0.9305	21779.69
	8	5043	4.056	20454	0.9305	15761.49
	9	5979	3.739	22356	0.9305	16792.54
	10	6684	3.288	21977	0.9305	15757.54
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	10602	2.105	22322	0.9157	12613.75
	2	7170	1.966	14097	0.9157	7531.431
	3	5496	2.054	11291	0.9157	6258.313
	4	3780	2.23	8428	0.9157	4966.654
	5	5280	2.284	12057	0.9157	7222.104
	6	5607	3.557	19942	0.9157	14807.67
	7	7071	2.472	17478	0.9157	11003.09

	8	5160	2.552	13167	0.9157	8441.988
	9	4644	2.293	10649	0.9157	6396.489
	10	5040	3.312	16693	0.9157	12077.87
10ng/ml of TGFβ1 + 1μM of MK-2206/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	4165	4.614	19217	0.8001	15884.58
	2	4206	3.812	16035	0.8001	12669.78
	3	4404	3.787	16680	0.8001	13156.36
	4	8128	3.528	28677	0.8001	22173.79
	5	7098	2.456	17435	0.8001	11755.89
	6	9762	1.919	18734	0.8001	10923.42
	7	9822	1.411	13862	0.8001	6003.418
	8	7419	0.905	6716	0.8001	780.0581
	9	6213	0.911	5661	0.8001	689.9787
	10	4521	0.853	3857	0.8001	239.7479
10ng/ml of TGFβ1 + 1μM of MK-2206/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	3279	3.041	9971	1.2242	5956.848
	2	3279	2.683	8797	1.2242	4782.848
	3	3279	2.887	9466	1.2242	5451.848
	4	3279	3.261	10692	1.2242	6677.848
	5	3279	2.932	9614	1.2242	5599.848
	6	3279	3.846	12612	1.2242	8597.848
	7	3279	4.067	13336	1.2242	9321.848
	8	3279	4.318	14159	1.2242	10144.85
	9	3279	3.614	11850	1.2242	7835.848
	10	3279	3.276	10743	1.2242	6728.848
10ng/ml of TGFβ1 + + 5μM of PD98059/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	6604	5.06	33418	1.5956	22880.66
	2	6925	5.116	35426	1.5956	24376.47
	3	9057	5.029	45551	1.5956	31099.65
	4	11153	4.238	47271	1.5956	29475.27
	5	4434	4.975	22057	1.5956	14982.11
	6	5642	4.658	26278	1.5956	17275.62
	7	7471	4.432	33110	1.5956	21189.27
	8	7993	3.637	29071	1.5956	16317.37
	9	7098	3.512	24927	1.5956	13601.43
	10	4984	3.404	16965	1.5956	9012.53
10ng/ml of TGFβ1 + + 5μM of PD98059/ 48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	2269	1.276	2895	0.0605	2757.726
	2	2269	1.112	2524	0.0605	2386.726
	3	2269	1.29	2926	0.0605	2788.726
	4	2269	1.23	2791	0.0605	2653.726
	5	11320	1.499	16973	0.0605	16288.14
	6	4715	1.482	6988	0.0605	6702.743
	7	4715	1.318	6215	0.0605	5929.743
	8	4715	1.651	7783	0.0605	7497.743
	9	4715	1.37	6460	0.0605	6174.743

	10	8005	1.178	9431	0.0605	8946.698
10ng/ml of TGFβ1 + 5μM of SB431542/ 24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	17687	5.877	103943	1.6448	74851.42
	2	4522	5.164	23350	1.6448	15912.21
	3	6220	5.488	34135	1.6448	23904.34
	4	5542	5.065	28070	1.6448	18954.52
	5	6690	4.56	30506	1.6448	19502.29
	6	5360	4.147	22227	1.6448	13410.87
	7	6390	4.263	27243	1.6448	16732.73
	8	7948	4.088	32488	1.6448	19415.13
	9	6809	3.93	26760	1.6448	15560.56
	10	5311	3.174	16858	1.6448	8122.467
10ng/ml of TGFβ1 + 5μM of SB431542/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	6601	7.085	46769	5.8243	8322.796
	2	4924	6.906	34007	5.8243	5328.147
	3	4372	6.264	27387	5.8243	1923.16
	4	4411	6.857	30248	5.8243	4557.013
	5	4411	7.337	32363	5.8243	6672.013
	6	4411	6.394	28206	5.8243	2515.013
	7	4411	6.817	30069	5.8243	4378.013
	8	12250	8.492	104028	5.8243	32680.33
	9	9949	9.06	90133	5.8243	32187.04
	10	18163	8.541	155123	5.8243	49336.24

SAS-H1 Cell Line		**ICC for Migration Markers**			**Date:12/10/2021**	
Calculate Correlated total cell fluorescence					**Antibody: β-catenin**	
SF-MEM/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	7566	11.481	86866	0.952	79663.17
	2	11066	10.431	115431	0.952	104896.2
	3	9229	10.984	101375	0.952	92588.99
	4	9909	12.425	123119	0.952	113685.6
	5	11737	13.183	154726	0.952	143552.4
	6	10065	10.657	107258	0.952	97676.12
	7	9565	11.924	114051	0.952	104945.1
	8	8704	14.279	124287	0.952	116000.8
	9	14030	12.183	170921	0.952	157564.4
	10	13158	10.828	142478	0.952	129951.6
SF-MEM/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	27256	10.463	285188	0.4568	272737.5
	2	21466	9.508	204094	0.4568	194288.3
	3	7617	12.47	94985	0.4568	91505.55
	4	10010	11.28	112908	0.4568	108335.4
	5	10109	17.047	172333	0.4568	167715.2

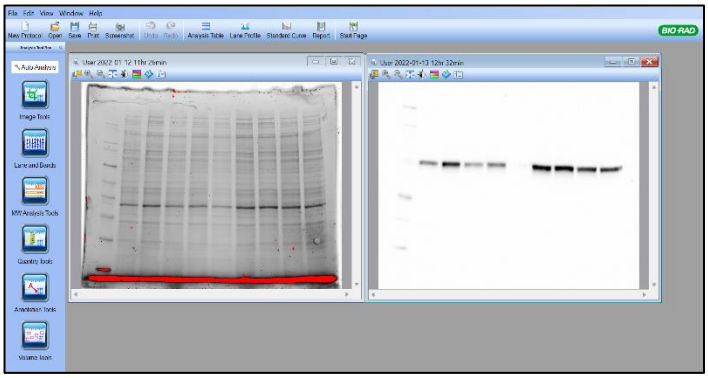
	6	17654	11.767	207731	0.4568	199666.7
	7	16455	7.939	130638	0.4568	123121.4
	8	12415	11.541	143282	0.4568	137610.8
	9	11827	9.708	114813	0.4568	109410.4
	10	15369	6.376	97997	0.4568	90976.44
10ng/ml of TGFβ1 /24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	26960	6.841	184420	0.0194	183897
	2	13924	5.388	75021	0.0194	74750.87
	3	12364	7.916	97873	0.0194	97633.14
	4	19413	8.937	173494	0.0194	173117.4
	5	18321	7.824	143344	0.0194	142988.6
	6	7867	15.687	123408	0.0194	123255.4
	7	20699	12.363	255895	0.0194	255493.4
	8	15432	7.884	121667	0.0194	121367.6
	9	13524	8.257	111668	0.0194	111405.6
10ng/ml of TGFβ1 /48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	13755	5.744	79002	3.324	33280.38
	2	9100	5.976	54380	3.324	24131.6
	3	26764	9.347	250160	3.324	161196.5
	4	22292	12.009	267708	3.324	193609.4
	5	14633	10.958	160347	3.324	111706.9
	6	13552	12.226	165688	3.324	120641.2
	7	10538	12.698	133813	3.324	98784.69
	8	13896	11.891	165234	3.324	119043.7
	9	8098	10.593	85781	3.324	58863.25
	10	8556	11.727	100332	3.324	71891.86
10ng/ml of EGF/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	13847	13.63	188740	1.3167	170507.7
	2	16569	8.545	141582	1.3167	119765.6
	3	11758	8.573	100798	1.3167	85316.24
	4	11560	4.937	57076	1.3167	41854.95
	5	7832	12.502	97913	1.3167	87600.61
	6	20964	5.726	120039	1.3167	92435.7
	7	11166	7.842	87567	1.3167	72864.73
	8	11452	11.615	133019	1.3167	117940.2
	9	14257	10.702	152576	1.3167	133803.8
	10	16305	10.856	177007	1.3167	155538.2
10ng/ml of EGF/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	10378	12.519	129926	3.1069	97682.59
	2	10391	11.182	116191	3.1069	83907.2
	3	13316	9.183	122280	3.1069	80908.52
	4	9861	8.298	81827	3.1069	51189.86
	5	8556	10.191	87193	3.1069	60610.36
	6	14220	9.189	130667	3.1069	86486.88
	7	29074	9.489	275881	3.1069	185551
	8	13295	9.106	121060	3.1069	79753.76

	9	11194	8.616	96449	3.1069	61670.36
	10	12293	13.329	163855	3.1069	125661.9
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	11895	11.955	142199	2.0104	118285.3
	2	7755	17.694	137215	2.0104	121624.3
	3	15112	12.677	191581	2.0104	161199.8
	4	11386	15.87	180698	2.0104	157807.6
	5	6930	10.46	72485	2.0104	58552.93
	6	13381	10.817	144740	2.0104	117838.8
	7	17255	14.677	253249	2.0104	218559.5
	8	10399	13.57	141116	2.0104	120209.9
	9	16029	8.946	143394	2.0104	111169.3
	10	14018	9.317	130604	2.0104	102422.2
10ng/ml of TGFβ1 + 5μM of TGF-β RI Kinase Inhibitor VII/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	14309	10.209	146082	2.1749	114961.4
	2	11794	10.57	124666	2.1749	99015.23
	3	18570	10.477	194556	2.1749	154168.1
	4	14769	9.048	133623	2.1749	101501.9
	5	10676	9.613	102633	2.1749	79413.77
	6	9725	10.735	104397	2.1749	83246.1
	7	14546	9.148	133066	2.1749	101429.9
	8	17845	10.162	181347	2.1749	142535.9
	9	8714	13.584	118373	2.1749	99420.92
	10	10747	12.765	137188	2.1749	113814.3
10ng/ml of TGFβ1 + 1μM of MK-2206/24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	8293	13.049	108216	0.6203	103071.9
	2	12502	11.677	145989	0.6203	138234
	3	6679	10.642	71078	0.6203	66935.02
	4	7491	15.334	114868	0.6203	110221.3
	5	3863	14.259	55083	0.6203	52686.78
	6	11630	15.457	179770	0.6203	172555.9
	7	7536	16.917	127484	0.6203	122809.4
	8	9271	13.075	121215	0.6203	115464.2
	9	7206	14.021	101034	0.6203	96564.12
	10	8887	7.844	69713	0.6203	64200.39
10ng/ml of TGFβ1 + 1μM of MK-2206/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	8126	8.353	67879	0.2323	65991.33
	2	9263	7.649	70851	0.2323	68699.21
	3	7957	9.327	74211	0.2323	72362.59
	4	8973	7.288	65395	0.2323	63310.57
	5	5143	12.493	64254	0.2323	63059.28
	6	5705	7.214	41158	0.2323	39832.73
	7	8089	7.225	58447	0.2323	56567.93
	8	6670	13.121	87514	0.2323	85964.56
	9	8797	11.709	103008	0.2323	100964.5
	10	6466	7.704	49813	0.2323	48310.95

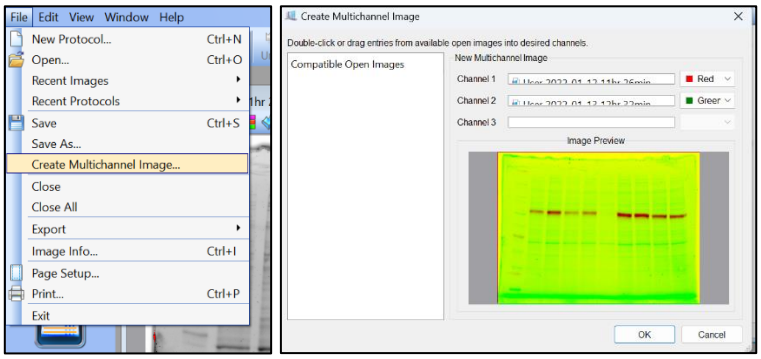
10ng/ml of TGFβ1 + + 5μM of PD98059/24 hours	Cell	Area/ pixel	Mean	Integrated intensity	Background mean	CTCF
	1	9859	8.365	82471	0.0605	81874.53
	2	9227	9.396	86697	0.0605	86138.77
	3	9578	13.79	132084	0.0605	131504.5
	4	11501	9.491	109153	0.0605	108457.2
	5	8201	8.695	71305	0.0605	70808.84
	6	8178	14.704	120253	0.0605	119758.2
	7	12685	15.496	196562	0.0605	195794.6
	8	19663	17.183	337874	0.0605	336684.4
	9	21931	14.643	321140	0.0605	319813.2
	10	8295	14.133	117232	0.0605	116730.2
10ng/ml of TGFβ1 + + 5μM of PD98059/ 48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	8859	17.968	159177	2.3631	138242.3
	2	8368	18.318	153289	2.3631	133514.6
	3	15486	15.141	234472	2.3631	197877
	4	9128	17.3	157918	2.3631	136347.6
	5	17456	13.79	240725	2.3631	199474.7
	6	9728	21.662	210732	2.3631	187743.8
	7	5982	21.535	128822	2.3631	114685.9
	8	5032	26.192	131797	2.3631	119905.9
	9	4982	20.964	104444	2.3631	92671.04
	10	7281	14.653	106685	2.3631	89479.27
10ng/ml of TGFβ1 + 5μM of SB431542/ 24 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	18195	9.081	165227	1.235	142756.2
	2	15078	6.268	94512	1.235	75890.67
	3	14320	7.383	105728	1.235	88042.8
	4	18996	6.089	115674	1.235	92213.94
	5	11320	6.81	77091	1.235	63110.8
	6	17620	6.581	115964	1.235	94203.3
	7	6880	5.615	38632	1.235	30135.2
	8	9932	6.248	62053	1.235	49786.98
	9	15850	7.671	121582	1.235	102007.3
	10	24201	6.224	150632	1.235	120743.8
10ng/ml of TGFβ1 + 5μM of SB431542/48 hours	**Cell**	**Area/ pixel**	**Mean**	**Integrated intensity**	**Background mean**	**CTCF**
	1	10047	9.448	94925	4.0011	54725.95
	2	18414	11.234	206871	4.0011	133194.7
	3	7597	15.091	114648	4.0011	84251.64
	4	9674	12.919	124983	4.0011	86276.36
	5	10861	14.586	158419	4.0011	114963.1
	6	12974	8.716	113081	4.0011	61170.73
	7	19704	9.539	187960	4.0011	109122.3
	8	19422	12.831	249197	4.0011	171487.6
	9	19819	15.183	300920	4.0011	221622.2
	10	9700	10.415	101029	4.0011	62218.33

Appendix 8: Protocol for total protein Normalisation using Image lab from BioRad software.

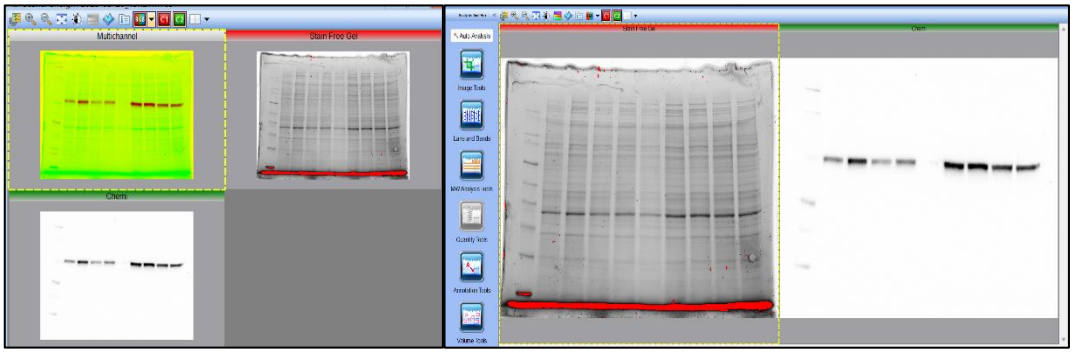
1. Both the gel file and the blot, which were taken by the Doc system, were opened in the software.



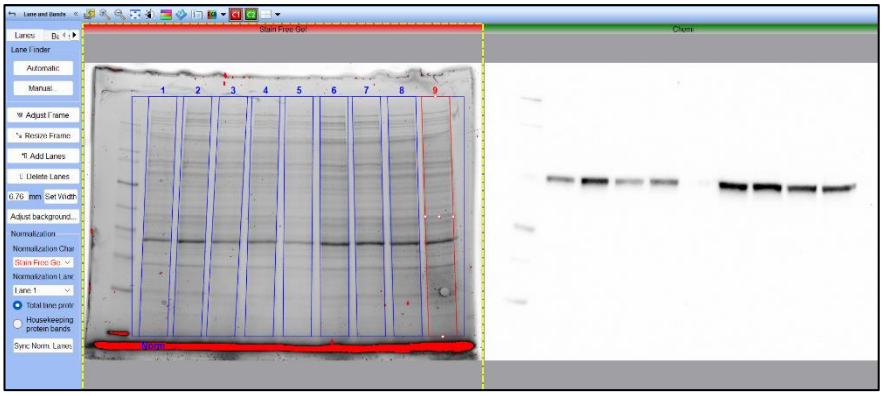
2. From the file list, the option for creating multichannel image was selected. This the image for the gel was selected for channal1 and the image for blot was selected for the channal2.



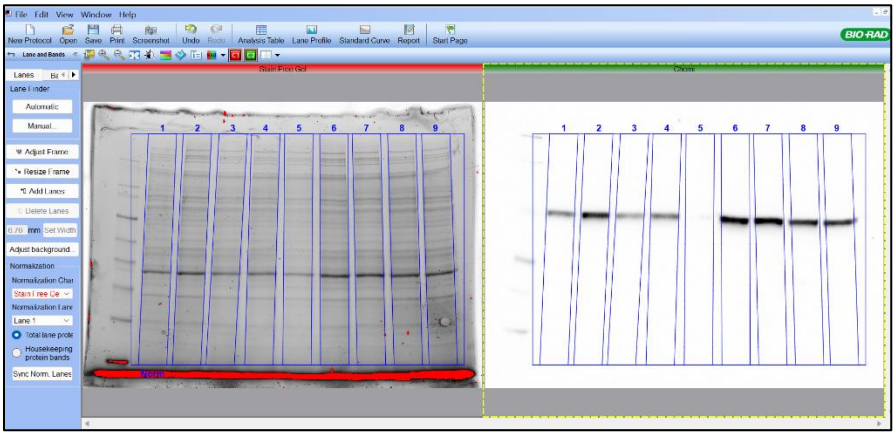
3. The previous step resulted in creating three images. RGB option was selected to generate image for both the gel and the blot.



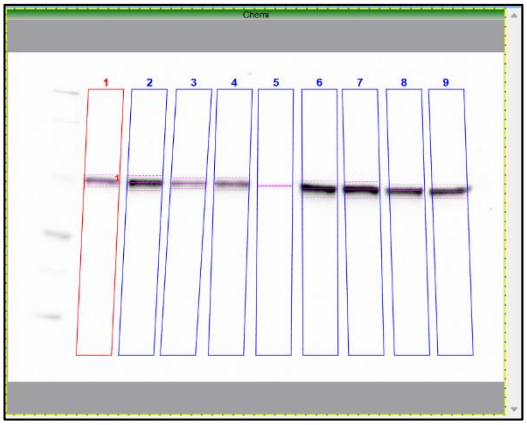
4. By choosing Lane and Bands option, the lane and the bands in the gel image were outline and identified by the software. Also, the normalisation option for the gel was stain free gel and the normalisation lane was the first lane (not the magic marker lane) for total protein.



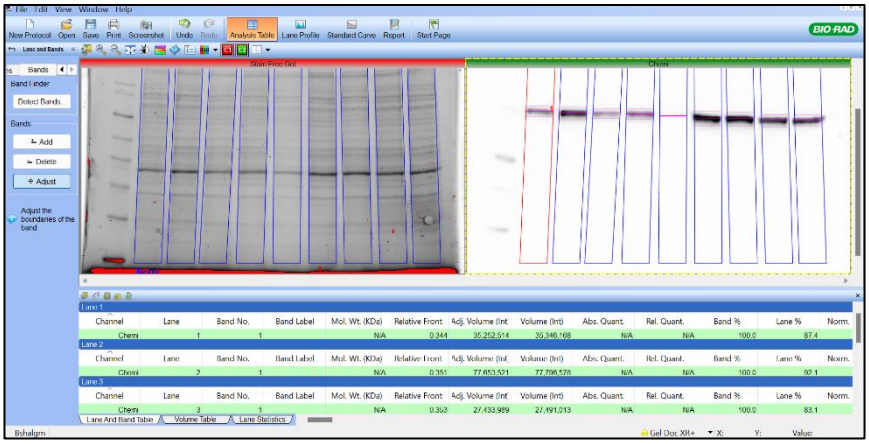
5. All the lanes on the gel were selected, copied and superimpose on the blot.



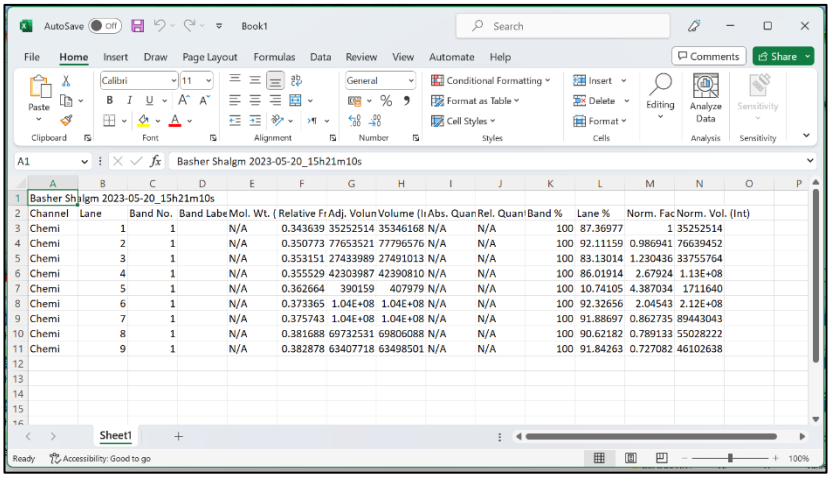
6. The Band option was selected then Detect Bands (Auto detection).



7. For the analysis, the Analysis table from the main bar was selected, which generated the normalisation values.



8. The final step was transferring the date to excel sheet.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Basher Shalgm 2023-05-20_15h21m10s													
2	Channel	Lane	Band No.	Band Labe	Mol. Wt. (	Relative Fr	Adj. Volun	Volume (In	Abs. Quan	Rel. Quant	Band %	Lane %	Norm. Fac	Norm. Vol. (Int)
3	Chemi	1	1		N/A	0.343639	35252514	35346168	N/A	N/A	100	87.36977	1	35252514
4	Chemi	2	1		N/A	0.350773	77653521	77796576	N/A	N/A	100	92.11159	0.986941	76639452
5	Chemi	3	1		N/A	0.353151	27433989	27491013	N/A	N/A	100	83.13014	1.230436	33755764
6	Chemi	4	1		N/A	0.355529	42303987	42390810	N/A	N/A	100	86.01914	2.67924	1.13E+08
7	Chemi	5	1		N/A	0.362664	390159	407979	N/A	N/A	100	10.74105	4.387034	1711640
8	Chemi	6	1		N/A	0.373365	1.04E+08	1.04E+08	N/A	N/A	100	92.32656	2.04543	2.12E+08
9	Chemi	7	1		N/A	0.375743	1.04E+08	1.04E+08	N/A	N/A	100	91.88697	0.862735	89443043
10	Chemi	8	1		N/A	0.381688	69732531	69806088	N/A	N/A	100	90.62182	0.789133	55028222
11	Chemi	9	1		N/A	0.382878	63407718	63498501	N/A	N/A	100	91.84263	0.727082	46102638
12														
13														
14														
15														