DOCTOR OF PHILOSOPHY

Epidermal growth factor and Transforming growth factor motogenic activities are mediated by Epidermal growth factor receptor
Identification of signalling pathways involved in head and neck cancer

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EPIDERMAL GROWTH FACTOR AND TRANSFORMING GROWTH FACTOR α MOTOGENIC ACTIVITIES ARE MEDIATED BY EPIDERMAL GROWTH FACTOR RECEPTOR: IDENTIFICATION OF SIGNALLING PATHWAYS INVOLVED IN HEAD AND NECK CANCER

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Thesis submitted for Doctor of Philosophy

in Dentistry

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DEDICATION

This thesis is dedicated to my parents Aye Mar Oo and Win Naing whose endless love and support make me able to walk this journey. To my brother, Nyi Lynn Htet for being there in time of need.

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Last but not least, I am grateful to my family for their constant support and concern. I cannot be here without their support.

DECLARATION

I declare that I am the author of thesis and I have consulted all the reference cited. The work of which thesis is a record has been accepted for a higher degree. This work has been carried out in the cell and molecular biology laboratory of Dundee Dental School, under supervision of Dr. Ian R Ellis and Prof. Peter A Mossey.

Signature

Date

Aye Myat Thwe

CERTIFICATE

I hereby certify that Aye Myat Thwe has fulfilled the condition of Ordinance of 39 of the University of Dundee and is qualified to submit this thesis for degree of Doctor of Philosophy.

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ABBREVIATION

ADH- Alcohol dehydrogenase enzymes

ALDH- Aldehyde dehydrogenase enzymes

AIDS- Acquired immunodeficiency syndrome

ATP-Adenosine triphosphate

AKT- Protein kinase B

AREG- Amphiregulin

BTC- Betacellulin

CA IX- carbonic anhydrase IX

CDKN2A- Cyclin dependent kinase inhibitor 2A

CDK cyclin dependent kinase

CASP8- Cysteine aspartic acid protease

CCND1-Cyclin D 1

Cox 2 - Cyclo-oxygenase 2

DMSO- Dimethyl Sulphoxide

DNA-Deoxyribonucleic acid

DOI-Depth of invasion

EBV-Epstein Barr Virus

EMT- Epithelial to mesenchymal transition

ENE-Extra nodal extension

EGF-Epidermal growth factor

EGFR-Epidermal growth factor receptor

ECM Extracellular matrix

EPI- Epigen

EREG- Epiregulin

E6- Oncoprotein encoded by HPV

E7- Oncoprotein encoded by HPV

EPOR- Erythropoietin receptor

FHIT- Fragile histidine triad

FCS-Foetal Calf Serum

FGF-Fibroblast growth factor

FSP1- Fibroblast specific protein

FAT1- FAT atypical cadherin 1

FOXC2- Forkhead box C2

Grb2- Growth factor receptor bound protein 2

GLUT-1 glucose transporter 1

HIF1α - hypoxia inducible factor 1α

HPV- Human papilloma virus

HIV-Human immunodeficiency virus

HSV – Herpes simplex virus

HGF -Hepatocyte growth factor

HB EGF - Heparin binding EGF like growth factor

HNSCC- Head and neck squamous cell carcinoma

Insulin-like growth factor 1 receptor (IGF-1R)

JAK-Janus kinaseKi-67 -a protein that in humans is encoded by the MKI67 gene

MAPK-Mitogen activated protein kinase

MDM2-Mouse double minus 2 homolog

MMPs- Matrix metalloprotease enzymes

MET-Mesenchymal to Epithelial transition

mTOR- mammalian target of Rapamycin

MAGE-Melanoma Antigen Gene

NRGs-Neuregulins

NF_{KB}- Nuclear factor kappa light chain enhancer of activated B cells

NSCLC- Non small cell lung carcinoma

NOTCH1- Notch homolog 1, translocation associated (Drosophila)

OSCC-Oral squamous cell carcinoma

OSMR -Oncostain M receptor

OLP-Oral lichen planus

PI- Iso-electric point

pH-Potential hydrogen

PBS-Phosphate buffer saline

pAkt473- Phosphorylated Akt at Serine 473

pAkt308- Phosphorylated Akt at Threonine 308

PDK1-Phosphoinositide biphosphate

PIP2-Phosphoinositide biphosphate

PIP3- Phosphoinositide 3,4,5 triphosphate

PKB-Protein kinase B

PKC-Protein kinase C

PI3K Phosphoinositide 3-kinases

PIK3CA- Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha

PIP2- Phosphatidylinositol 4,5-bisphosphate or PtdIns(4,5)P_2

PIP3- Phosphatidylinositol (3,4,5)-trisphosphate or PtdIns(3,4,5)P_3

PTEN- Phosphatase and tensin homolog

PTB- Phosphotyrosine binding domains

p53- TP53 tumor protein

pRb- Retinoblastoma protein

PDGFR-Platelet direct growth factor receptor

PDGF- Platelet direct growth factor

PCNA -Proliferation cell nuclear antigen

PMD- Potentially malignant disorder

Rb - Retinoblastoma

SH2- Src homology 2

SEA-South East Asia

SF-Serum free

STAT-signal activator and activator of transcription

TBST- Tris buffered saline and Tween 20

TGFα- Transforming growth factor alpha

TGFβ - Transforming growth factor beta

TAMs-Tumour associate macrophage

TKs- Tyrosine kinases

TKIs -Tyrosine kinase inhibitors

VEGF- Vascular endothelial growth factor

VEGFR- Vascular endothelial growth factor receptor

WB -Western blot

Abstract

Background: The alteration of Epidermal Growth Factor Receptor (EGFR) plays a major role in the development of Head and Neck cancer. However, the only FDA approved EGFR target for this neoplasm is the drug Cetuximab. Cetuximab can only be used where there is an over-expression of EGFR. Cetuximab works by competing with the ligands Epidermal Growth Factor (EGF) and Transforming Growth Factor α (TGFα) to bind the extracellular domain of EGFR. Inhibition of this ligand binding blocks the activation of subsequent signalling pathways. It is known that not all Head and Neck cancers over-expressing EGFR respond to Cetuximab. It has been postulated that some of these tumours have additional genetic lesions. Resistance to Cetuximab has been associated with the genetic lesions to EGFR mutation and Ras mutations. These mutations are more common in Asian than Caucasian patients. More recently the Tyrosine kinase inhibitors (Gefitinib and Erlotinib) have been investigated as drugs to target tumours with EGFR mutation. These inhibitors bind to the cytoplasmic domain of the EGFR and compete with ATP and inhibit phosphorylation of the receptor. Another inhibitor of interest is PD98509 which is a selective ERK1/2 inhibitor and may be used in targeting tumours with a Ras mutation. None of these inhibitors has received FDA approval for Head and Neck cancer treatment.

Aim: The main aims of the project were to investigate the effect of the growth factors EGF and TGFα and the inhibitors; Gefitinib, Erlotinib and PD98059 on cancer cell lines

obtained from Asian head and neck cancer patients. The first area of study was to investigate the effect of the growth factors EGF and TGFα on cell proliferation, morphological changes, EMT marker expression, single cell migration and collective cell migration. Once the initial data had been collected a second part was to determine whether the inhibitors; Gefitinib, Erlotinib and PD98059 could block the stimulatory effect of EGF and TGFα in these assays.

Methods: Three tumour cell lines of Asian origin were used in the study and a normal skin keratinocyte as a control. Cell proliferation was measured using an automated cell counting system. Migration was studied using a cell scatter and cell scratch assay. The movement of the cells was documented by using photomicrographs. Epithelial to Mesenchymal Transition (EMT) and protein expression were determined using immunofluorescence and SDS-PAGE and Western blotting.

Results: Cell proliferation data indicated that the growth factors did not stimulate the proliferation of the cells investigated.

Addition of the growth factors for 48 hours induced an EMT like morphological change, individual cell migration in the scatter assay and collective cell migration in the scratch assay. However, the specific markers of EMT (E cadherin and vimentin) as measured by Western blotting and immunofluorescence showed no change in intensity following the addition of the growth factors for 48 hours.

Pre-treatment of the cells for one hour with the tyrosine kinase inhibitors (Gefitinib, Erlotinib) and the MAPK inhibitor (PD98059) inhibited these morphological changes, and individual cell migration. However, only the Tyrosine kinase inhibitors inhibited

collective cell migration. PD98059 partially inhibited collective cell migration of the TYS and HaCaT cell lines and fully inhibited cell migration of the HSG and AZA1 cell lines.

Analysis of the MAPK pathway using the expression of phosphorylated MAPK was investigated by Western Blot and Immunofluorescence. Western blot data indicated an increased expression of phosphorylated MAPK202/204 was observed in cells treated with both EGF and TGFα for 24 hours. At the 24hour time point, tyrosine kinase inhibitors (Gefitinib, Erlotinib) fully inhibited the MAPK202/204 phosphorylation. In contrast, in ERK1/2 inhibitor (PD98059) treated dishes, phosphorylated MAPK 202/204 was still expressed which was likely to be the same level of expression as serum free treated dishes.

Conclusion and Possible Clinical Impact: The data indicates that the growth factors EGF and TGFα both stimulated single and collective cell migration. The data lead us an unexpected answer as to whether single cell migration and collective cell migration utilised the same signalling pathways. The data indicated that these are separate pathways; the MAPK pathway appears to be the only pathway responsible for morphological change and single cell migration in cell lines used in this project. The role of MAPK signalling pathway in collective cell migration appears to be cell line dependent and MAPK alone might not be responsible for collective cell migration. The ability of the inhibitors may in the future lead to a more personalised therapy for patients in Asian countries and more clinical trials across a diverse group of patients is needed.

Chapter 1 Introduction and literature review

1.1 Biology of cancer

Cancer is the disease which at its most basic definition is cells in specific part of body grow uncontrollably (NCI, 2020). These cells can invade the surrounding tissue, organs and eventually spread to other parts of the body which is called metastasis (Guan X, 2015). Cancer is the second leading cause of death worldwide and accounted for 9.6 million deaths in 2018 (WHO, 2018). 90 % of cancer deaths are caused by metastasis (Guan X, 2015, Chaffer & Weinberg, 2011).

There are 6 types of cancer according to the type of the cells that they originated from:

a) Carcinoma- derived from epithelial cells which line internal organ.
b) Sarcomas- derived from mesenchymal origin such as connective and supporting tissues (examples bone, cartilage, fat, and muscle).
c) Leukaemia -derived from hematopoietic cells such as bone marrow.
d) Lymphomas - derived from T or B lymphocytes which are part of immune system.
e) Myeloma-derived from plasma cells which are part of immune system.
f) Brain and spinal cord cancer -which are called central nervous system cancer (CRUK, 2017)

The word tumour simply refers to a mass. This is a general term that can refer to benign or malignant. A benign tumour is slow growing and does not spread to other parts of the body. It they are correctly removed they do not reappear. In contrast, malignant tumours spread to other parts of the body and are likely to recur (CRUK, 2017). All the cancers start with genetic changes which can lead to mutation of proto-oncogenes, loss of function of tumour suppressor genes and eventually acquiring the hallsmark of cancer

(Hanahan & Weinberg, 2000). In 2000, Hanahan and Weinberg published a review article titled “The Hallmarks of Cancer”. It was one of the most downloaded and most cited papers of all time. In 2011, they updated their list by adding four more hallmarks of cancer making a total of 10 hallmarks of cancer.

The Hallmarks of Cancer are (and shown in Figure 1.1):

a) Self-sufficiency in growth signals.

b) Insensitivity to antigrowth signals.

c) Resistance to apoptosis.

d) Limitless replicative potential.

e) Formation of angiogenesis.

f) Activating tissue invasion and metastasis.

g) Genomic instability and mutation.

h) Tumour promoting inflammation.

i) Reprogramming emergency metabolism.

j) Avoiding immune destruction.

All cancers acquire at least some these characteristics in the development of the disease (Hanahan & Weinberg, 2000, Hanahan & Weinberg, 2011).

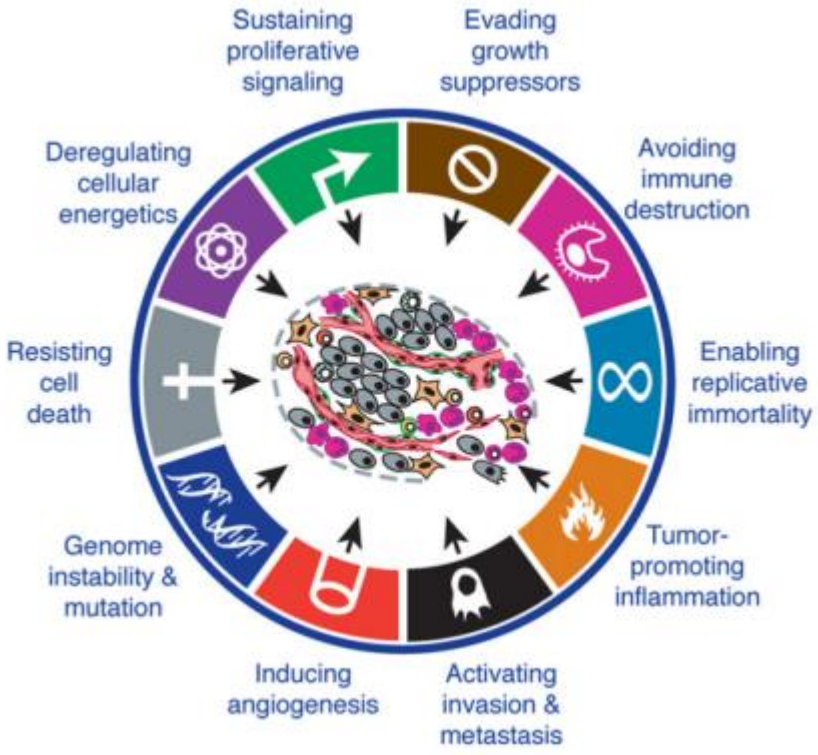


Figure1.1 Hallmarks of cancer: Reproduced and modified from (Hanahan & Weinberg, 2011)

1.2 Head and neck cancer

The UK National Health Service defines head and neck cancer as referring to the group of cancers that develop from the mucosal linings of the upper aerodigestive tract, comprising oral cavity, nasal cavity, lip, tongue, salivary glands, larynx, pharynx (nasopharynx—behind the nose; oropharynx—soft palate, base of the tongue and the tonsils; hypopharynx—the lowest part of the pharynx) and paranasal air sinuses (NHS, 2018). Oral cancer is a subgroup of head and neck cancer. Head and neck cancer are a heterogeneous disease that can be divided into two genetic subclasses: HPV positive and HPV negative which are different in the pathogenesis process.

According to the WHO, Head and neck cancer is the 6th most common cancer worldwide with more than 550,000 cases with around 300,000 deaths per year. The male to female

ratio 2:1 to 4:1. About 90% of all head and neck cancers are reported to be squamous cell carcinomas (HNSCC). Despite the recent developments in medical therapy, the 5 years survival rate of head and neck cancer is still only 40%-50% (WHO, 2014).

The risk of developing head and neck cancer increases with age and most of the cases occur in patients aged 50- years or over (Vigneswaran& Williams, 2014). Development of head and neck cancer is a multistep process which usually starts with potentially malignant disorders (PMD). PMD is the term used to describe potentially malignant lesions and conditions (Warnakulasuriya *et al.*, 2007). A potentially malignant lesion is a morphologically altered mucosa lesion which can transform into malignancy. Any potentially malignant condition arising in the oral cavity will increase the risk of oral cancer (Vigneswaran & Williams, 2014). It is therefore important to detect PMD as early as possible to avoid negative outcome. Therefore understanding the molecular pathogenesis of the disease will help to improve the survival of the patient.

Patients who have been cured from an initial primary tumour have a higher risk of second primary tumour due to field cancerization (Chuang SC *et al.,* 2008, Morris LG *et al.*, 2011, Jones AS *et al*., 1995). Field cancerization can be defined as the exposure of the whole area to carcinogens which leads to development of multiple cancers (Slaughter DP *et al.*, 1953).

Most HNSCC patients have metastatic disease at the time of diagnosis with regional metastasis (Lymph nodal involvement) in 43% and distant metastasis in 10% (Ridge, 2016).

1.3 Aetiology of head and neck cancer

1.3.1 Tobacco

Smoking is the most common use of tobacco. Smoking is a major independent risk factor for Head and neck cancer with 70–80% of new HNSCC diagnoses being related to tobacco and alcohol use (Jethwa & Khariwala, 2017). Incidence of head and neck cancer is related to smoking and varies among the geographic regions of the world. Smokers have higher chance of cancer than non-smokers. The risk increases with quantity and duration of smoking (Radoi & Luce, 2013). Out of 7000 chemicals released from tobacco smoke, 250 are harmful to human body while 69 are known to cause cancer (NCI, 2018). Acetaldehyde, Aromatic amines, Arsenic, Benzene, 1, 3-Butadienc, Cadmium, Chromium, Cumene, Ethylene oxide, Formaldehyde, Polycyclic aromatic hydrocarbon (PAHs), Tobacco-specific nitrosamines, Vinyl chloride are examples of cancer causing chemicals that are found in tobacco smoke (NCI, 2018). It should be noted that smokeless tobacco use is common in South East Asia. Betel quid, Dry snuff, and Zarda are examples of smokeless tobacco (Wynder *et al.*, 1957). Betel quid is the most common smokeless tobacco in South East Asian region. The composition of Betel quid varies from one place to another since different types of tobacco are popular in different regions. However, the core elements remain the same and consists of three components: the betel leaf, the areca nut (fresh or dried) and slaked lime (Xuan Hien & Reichart,2008). Research estimated Betel quid chewing is responsible for half of the oral cancer cases in India **(**Travasso, 2013). The use of electronic cigarettes has risen in popularity since they were first introduced in 2013. Long term use of E-cigarettes has been reported to increase the incidence of oral cancer (Nguyen H *et al*., 2017). The most

common form of E-cigarettes is vaping which is a type of electronic nicotine delivery system utilising a heating of a solution containing liquid nicotine, propylene glycol, glycerol (glycerine), and flavouring agents (Grana R *et al*., 2014).

Liquid nicotine, tobacco-specific nitrosamines, polycyclic aromatic hydrocarbons, cresol, volatile organic compounds, aldehydes, Tobacco alkaloids, metals, flavouring agents, are found in cartridges and aerosols of e-cigarettes (Cheng T, 2014). A study conducted by American chemical society, reported an increased level of DNA damaging substance such as chemicals formaldehyde, acrolein and methylglyoxal were found in lung tissue of e-cigarettes users (Drewett Z, 2018).

1.3.2 Alcohol

Alcohol is another independent risk factor for HNSCC. Alcohol is the by-product of the fermentation of sugar, yeast, and starch. Different type of fruits, grains are used in the fermentation process with the help of yeast to making the final product (alcohol). Different concentrations of ethanol are found in different alcoholic drinks made from different sources (10% in beer, 16% in wine, 40 % in vodka) (Ogden GR, 2018). The exact percentage of alcohol that may cause head and neck cancer varies with different types of alcohol consumption and different regions of the world (Freedman *et al.,* 2007).

It is believed that the higher alcohol consumption over a short period of time might be more harmful than low alcohol consumption over a longer period of time (Lubin *et al.,*2009).

Regular alcohol drinkers have a 2.5 times higher risk of developing head and neck cancer than non-drinkers and occasional drinkers. The risk of head and neck cancer in people with a daily alcohol consumption of 12.5-50g is 12-81% higher than non-drinkers. Daily

alcohol consumption of 50g increases the risk by 5 times compared with non-drinkers. The risk of head and neck cancer is higher in people who use both of alcohol and tobacco (Hashibe *et al., 2009, CRUK* 2018). Ogden GR, 2018 suggested that people need to be aware of their alcohol intake. More public health interventions and the development of alcohol tracking application such as AlcoDriod alcohol tracker should raise awareness and make it easier to track alcohol intake with everyone should be encouraged to use it.

The risk of cancer for an ex-drinker is higher than non-drinker even 10 years after quitting drinking alcohol (Rehm *et al.,* 2007) and only returns to the same level as a non-drinkers 20 years after quitting drinking alcohol (Marron *et al.,* 2010, Rehm *et al.,* 2007, Altieri *et al.,* 2002). Acetaldehyde a by-product of the breakdown of alcohol is a major carcinogen and is much more carcinogenic than alcohol itself (Ogden GR, 2018).

Figure 1.2 describes the break down of alcohol. Acetaldehyde is know to disrupt DNA synthesis and repair and this is a by-product of the breakdown of ethanol in the body and may lead to an increased risk of cancer. Another hypothesis is that alcohol acts as a solvent for carcinogens found in tobacco smoke and this might be the reason for the synergistic effect of alcohol and tobacco which increases the risk of cancer (Kawakita & Matsu, 2017).

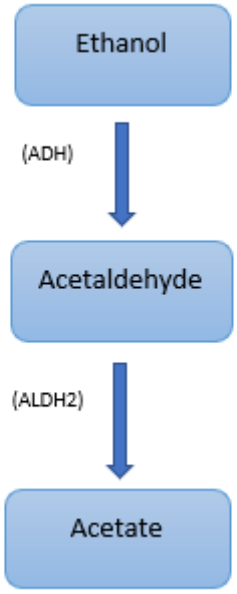


Figure 1.2*:* Alcohol degeneration: shows that ethanol undergoes changes in the body to more carcinogenic substrates *(*Kawakita & Matsuo, 2017).

Conversion of ethanol into acetaldehyde is induced by Alcohol dehydrogenase enzymes (ADH). Acetaldehyde is further broken down by Aldehyde dehydrogenase enzymes (ALDH). It is hypothesized that an active ADH1B *and* ADH1C genotypes are found in Asian population. Active ADH1B and ADH1C have ability to metabolize ethanol quickly and this could lead to an increased risk of head and neck cancer (Brennan *et al*., 2004). Asian populations who have an inactive acetaldehyde dehydrogenase 2 (*ALDH2*) cannot break down acetaldehyde resulting in the accumulation of acetaldehyde in the human body (Brennan *et al.,* 2004). This may explain why light drinking (low alcohol consumption) has a significant risk of oral cancer in Asia (Bagnardi *et al.,* 2015).

1.3.3 Virus

1.3.3.1 Human papilloma virus (HPV)

HPV is a double stranded DNA virus that is known to be the primary aetiology of cervical cancer (Walboomers *et al.,* 1999). There are over 100 types of HPV genotypes and not all of them causes health problems. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66 are known as high risk strains causing cancer while HPV 6 , 11, 40, 42, 43, 44, and 54 are low risk strains and do not cause cancer (Muñoz *et al.,*2003). HPV 16 and HPV 18 are known to cause oral cancer. Incidence of HPV+ head and neck cancer vary by country or region (Mehanna H, 2012). A study found that about 23.5 % of HNSCCs are related with HPV (Kreimer *et al.,* 2005) and that HPV 16 is responsible for 90% to 95% of HPV+ HNSCC (Ram *et al.,* 2011). HPV positive head and neck cancer is related to sexual behaviour such as oral sex and an increased number of sexual partners and is predominantly seen in younger people compared with traditional (or HPV negative) head and neck cancer (Nuyts & Dok, 2016). Oncoproteins (proteins that are coded for by an oncogene) E6 and E7 are expressed in HPV infected cells. E6 binds to tumour suppressor protein p53 and induces degradation of this protein. E7 induces inactivation of pRb tumour suppressor protein (Moody & Laimins, 2010). HPV+ HNSCC are commonly found in the oropharynx as a small primary tumour generally around the lymph nodes, are poorly differentiated, but have better prognosis than classic HNSCC (Fakhry *et al.,* 2008, Ang KK *et al.,* 2010, Lassen *et al.,* 2009, Schwartz *et al.,* 2001, Dayyani *et al.,* 2010). However, prognosis of HPV positive HNSCC patients who are exposed to any form of tobacco may not be as good as HPV+ HNSCC patients who do not smoke (Vigneswaran & Willliams, 2014).

1.3.3.2 Other Viruses

HSV-1 or oral herpes is believed to enhance risk of oral cancer (Schildt *et al.,* 1998, Kassim & Daley, 1988, Starr *et al.,* 2001).

Coinfection of EBV and HPV might increase the risk of oral cancer (Al Moustafa *et al.,* 2009). Hepatitis C virus (HCV) is related with risk of oral cancer (Nagao & Sata, 2009).

1.3.4 Genetic disease

Fanconi anemia is an autosomal recessive disorder that causes genetic instability with congenital malformations leading to bone marrow failure and cancer. There is a clear link between fanconi anemia and increased risk of oral cancer. Several reports have shown that 19 out of 754 fanconi anemia patients developed oral cancer (Kutler *et al.,* 2003, Gasparini *et al.,* 2006).

1.3.5 Candida

Candida infection can increase the risk of patients developing Leukoplakia, dysplasia and malignant transformation (Sankari *et al.,* 2015).

1.3.6 Diet

Certain diets such as salted fish, preserved meat and vegetables, are related with an increased risk of head and neck cancer while a diet rich in fruit and vegetables can protect against cancer (Jia *et al.,* 2010, Yu MC *et al.,* 1986).

1.3.7 Organs transplant patients

Solid organ transplant patients have a higher rate of lip cancer and have poor prognosis compared with the general population (Öhman *et al.,* 2015). This might be due to immunosuppression in organ transplant patients that leads them not being able to stand

up to immune destruction. Although why this would lead to a high rate of lip cancers remains to be resolved.

1.3.8 Family history

As with other cancers, family history of oral cancer is associated with early age onset of cancer (Ankathil *et al.,* 1996, Garavello *et al.,* 2007).

1.3.9 Potentially malignant disorders:

1.3.9.1 Leukoplakia, Erythroleukoplakia, Erythroplakia

Oral Leukoplakia is a white patch which cannot be characterized as any other disease (WHO, 1978). It is more frequently found in smokers than non-smokers. The malignant transformation of leukoplakia ranges from 0.13% to 34.0% showing that leukoplakia has malignant potential (Warnakulasuriya & Ariyawardana, 2015). Overexpression of EGFR in oral leukoplakia patients was reported to increase the chance of transformation into a carcinoma, whilst normal expression of EGFR did not lead to this (Ries *et al.,* 2013). Early detection of transformation could be investigated by testing for EGFR expression with over expression in oral leukoplakia patients being utilised as a diagnostic tool. Leukoplakia can be divided into two subtypes: Homogenous (uniformly flat, uniformly white) and non-homogenous (speckled or nodules, mixture of red and white lesion) which is also known as Erythroleukoplakia (Yardimci *et al.,* 2014). Non-homogenous leukoplakia has higher risk for malignant transformation than homogenous leukoplakia (Vigneswaran & William, 2014). Erythroplakia is red patch which cannot diagnosed as

any other disease is not as common as leukoplakia but is more likely to become dysplastic in nature (Waal, 2009, Reichart & Philipsen, 2005).

1.3.9.2 Oral submucous fibrosis

Oral submucous fibrosis is a premalignant condition common among betel quid chewing south Asian countries. Eating chilies, genetic changes and nutritional deficiencies are also thought to increase the incidence of OSMF. Clinical features of OSMF are limitations of mouth opening, burning sensation, difficulty in mastication, speech and swallowing(Tilakaratne *et al*,2006).The malignant transformation rate of OSMF is in the range from 8% to 12 % (Vigneswaran & William, 2014). OSMF is considered as collagen-metabolic disorder resulting from exposure to areca nuts. Arecoline (component of betel nut) play an important role in stimulating collagen production. Constant irritation of oral mucosa due to direct contact with arecoline leads to chronic inflammation, leading to activation of T cells and macrophages. This activation is followed by an increase in cytokines and growth factors production such as IL-6, TNF and TGFα. TGFα is major inducer of collagen synthesis. Overall increase in collagen production and decrease in collagen degradation leads to collagen deposition and fibrosis (Rajalalitha P *et al* 2005, Tilakaratne *et al* 2006).

1.3.9.3 Oral lichen planus

Lichen planus is a chronic autoimmune, mucocutaneous disease which affects the oral mucosa (oral lichen planus or OLP), skin, genital mucosa, scalp and nails. In the oral mucosa, it presents with white striations (Wickham's striae), white papules, erythema,

erosions or blisters. Malignant transformation of oral lichen planus is in the range from 0% to 5.3% (Ismail *et al.,* 2007).

This means that a routine dental check-up is important to detect these lesions before a transformation into a cancer (discussed detailed in 1.7 clinical feature of head and neck cancer).

1.4 Geographic variation of head and neck cancer rate

The incidence of head and neck cancer has been reported to be higher in southern Asian countries (such as India, Pakistan, Sri Lanka, Taiwan), parts of Europe (France, Hungary), areas of south America (Brazil), and the Pacific region such as Papua New Guinea, Melanesia (Warnakulasuriya, 2009).

1.5 Betel quid chewing in Myanmar and oral cancer statistics

Even though betel quid chewing is uncommon in west, it is practised in South East Asia (SEA) region which has more than 600 million people (10% of total world population) (Cheong *et al.*, 2017). Myanmar is second largest country in SEA with a population of more than 50 million people. About 70% of the population live in rural areas where it is difficult to access health care. Betel quid chewing habit is more common in these rural areas than in the city. It has been reported that more than 50% of Myanmar men use smokeless tobacco (Cheong *et al.,* 2017). The actual number of head and neck cancer incidences in Myanmar is difficult to access, political conflicts, internally displaced persons and struggles with a weak economy are a major challenge in the data collection. According to the official publication of Asia Pacific Journal of Cancer Prevention, oral cancer is the fourth most common cancer among Myanmar males and sixth most

common cancer among Myanmar females (Kimman *et al.,* 2012). A retrospective hospital-based study conducted in Toungoo district in Myanmar from 2012 to 2015, found that head and neck cancer accounted for 19.51% of all cancer patients. Out of these, 5.22% were stage 1, 33.33% were stage 2, 46.4% were stage 3 and 15.03% were stage 4 (Ngwe *et al.,* 2016). The risk of oral cancer is four times higher in areca nut chewers who keep the quid overnight in their mouth (Oo *et al.,* 2011). This might be due to prolonged contact time with betel quid and the oral mucosa. In one study with oral screening targeting betel quid chewers (n=86), 3 patients had already developed carcinoma while a further 16 presented with pre-malignant lesion (unpublished data of Shwe Yaung Hnin Si cancer foundation). These figures are indicating a high incidence of head and neck cancer in Myanmar. In Myanmar, patients usually arrive at Hospital at a very late stage of oral cancer with a large ulceration. Most of these have already tried traditional plant base medicines as a therapy before presenting at the health care centre. The effect of traditional non evidence-based medicine in cancer treatment is questionable. Their molecular pathogenesis might be different from people who never receive traditional plant-based medicine. There are only a few studies of head and neck cancer in Myanmar at molecular level. At present there has been no report of EGFR overexpression/mutation status in Myanmar patients. Further work to investigate EGFR status of betel quid chewing HNSCC patients in Myanmar would be a basis of further work to be discussed later in this thesis.

1.6 Anatomical location of head and neck cancer

The anatomical location of head and neck cancer varies with different geographical location based on difference in aetiology. Carcinoma of tongue is most common in US

with high rate of nodal metastasis (Vigneswaran & Williams, 2014). Hard palate cancer is common in the areas where they practice reverse smoking (Vigneswaran & Williams, 2014). Lower lip cancer is more common in pipe smokers (Vigneswaran & Williams, 2014).

Buccal mucosa and cheek cancer are observed mostly in SEA where the betel quid chewing habit is common (Travasso, 2013).

1.7 Clinical features of head and neck cancer

Clinical features of head and neck cancers are persistent mouth sores, unhealed ulcers that have been present for more than 3 weeks, a lump in oral cavity, difficulty in swallowing and chewing, voice changes, unexplained tooth mobility without periodontal disease, numbness in any area of mouth, Erythroplakia, Leukoplakia, Erythroleukoplakia, and weight loss (Bagan *et al.,*2010).

Since early detection is key to increased survival rate, dental professionals need to understand early signs of head and neck cancer and are able to detect them at very early stage. Unexplained ulceration for more than 3 weeks, persistent and unexplained lumps in the neck or oral cavity, leukoplakia , erythroplakia and erythroleukoplakia are listed in the referral guidelines (NICE, 2017).

1.8 Diagnosis of head and neck cancer

Diagnosis of head and neck cancer is usually done by clinical examination, a biopsy such as scalpel biopsy may be an initial event, other methods such as endoscopy, chest X ray, orthopantomogram, computed tomography (CT), magnetic resonance imaging (MRI),

positron emission tomography combined with computed tomography (PET–CT) (SIGN, 2006).

1.9 Staging of head and neck cancer

In 2017, the American Joint Committee on Cancer published 8th edition of AJCC cancer staging manual. Major changes were introduced around the different staging systems for HPV associated oropharyngeal cancers, and incorporation of depth of invasion and extra nodal extension. Extra nodal extension is used in all head and neck cancers apart from HPV associated p16+ oropharyngeal cancer and nasopharyngeal cancer (Lydiatt *et al.,* 2018, Ettinger *et al.*, 2019).

Table 1.1 HPV negative staging of Head and neck cancer

Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
Stage IV A	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0

	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
Stage IV B	Any T	N3	M0
	T4b	Any N	M0
Stage V C	Any T	Any N	M1

Legend: TNM staging is common system used for many types of *cancer. T stands for Tumour, N stands for Node and M stands for Metastasis. TNM system reports the size of tumour, lymph node metastasis present or not, distant structure metastasis presence or not.*

Tx= Primary tumor cannot be assessed

Tis= Carcinoma in situ = group of abnormal cells or precancer stage

T1, T2, T3, T4 = increasing size and or involvement of primary tumour.

T1 = tumour 2 cm or less than 2cm in greatest dimension, depth of invasion (DOI) is less than 5mm

T2= Tumour 2 cm or less with more than 5mm but less than 10mm DOI or tumour more than 2 cm but not more than 4 cm and DOI not greater than 10mm.

T3= Tumour is bigger than 4 cm in greatest dimension, DOI is larger than 10mm

T4= (Lip) Tumour infiltrated into cortical bone, inferior alveolar nerve, floor of mouth, skin of face

T4a=Tumour infiltrated into cortical bone, muscle of tongue, maxillary sinus or skin of face

T4b= Tumour infiltrated into masticator space, pterygoid plate, skull base and external carotid artery

N_x= Regional lymph node cannot assess.

N_0= no evidence of nodal metastasis

N1, N2, N3= involvement of regional lymph node metastasis.

N1= Metastasis in single ipsilateral lymph node 3 cm or less than 3 cm in greatest dimension and extranodal extension (ENE) negative

N2a= single ipsilateral lymph node more than 3cm but not more than 6cm in greatest dimension and ENE negative

N2b= multiple ipsilateral lymph nodes none more than 6cm in greatest dimension and and ENE negative.

N2c= bilateral contralateral lymph nodes none more than 6cm in greatest dimension and ENE negative.

N3a=lymph node >6cm in dimension, and ENE negative

N3b= metastasis in a single ipsilateral node ENE positive or multiple ipsilateral, bilateral or contralateral nodes ENE positive

ENE now N3b so higher proportion of patients in stage IVb group

M_X= Distant metastasis cannot assess

M_0= No distant metastasis

M_1= Distant metastasis present

TNM staging for HPV positive Oropharyngeal cancer

In HPV positive OPC staging, T classification is largely unchanged apart from Carcinoma *in situ* and T4b are removed. In N classification, there are difference between clinical and pathological staging. Clinical staging is based on laterality and size of nodes while pathological staging is based on number of nodes and for surgical patients only. ENE not included in HPV+ oropharyngeal cancer. M classification is unchanged. Overall stage for HPV positive cancer is changed. Stage IV reserved for M1 disease (Lydiatt *et al.,* 2018, Ettinger *et al.,* 2019).

1.10 Treatment of head and neck cancer

Treatment of head and neck usually starts with a multidisciplinary team which includes medical oncologists, radiation oncologist, surgical oncologist, plastic surgeon, prosthodontist, otolaryngologist, physical therapist, Speech-language pathologist, Audiologist, Psychologist/psychiatrist, Registered dietitian nutritionist (Cancer.Net, 2017). Treatment can be varied depending on cancer stage, size and the location of the tumour, histological grading, patient's medical status, expected side effects, metastasis status (American cancer society, 2018).For example, in carcinoma in situ, the usual treatment is surgical stripping or thin resection. Follow up with the patient is important to check against recurrence and a second primary tumour.

In stage 1 and stage 2, cancer seen in the following places; floor of mouth, tongue, hard palate, buccal mucosa and gum are usually treated by surgery or radiotherapy or surgery followed by radiotherapy or chemoradiation. Radiation alone can be used as main

treatment in some patients who are medically not fit for surgery or patient with cancers in the oropharynx area such as the soft palate, and tonsils. Lip cancer usually has better prognosis.

In stage 3 and 4 cancer, treatment is combination of surgery (include nodal dissection), radiotherapy and chemotherapy (CRUK 2014, American cancer society 2018). Early-stage tumours have a more favourable prognosis.

Patients who were cured from initial primary tumor have higher risk of second primary tumour due to field cancerization (Jones AS *et al.,* 1995, Chuang *et al.,* 2008, Morris *et al.,* 2011). Field cancerization means the exposure of whole area to carcinogens which leads to development of multiple cancers (Slaughter *et al.,* 1953).

Common drugs used in oral cancer are cisplatin, bleomycin, Carboplatin, 5-fluorouracil, Paclitaxel, Methotrexate, Docetaxel, Hydroxyurea (CRUK, 2014) which are cell cycle target therapeutic agents.

Other drugs such as Cetuximab and Nivolumab have FDA approval to treat head and neck cancer in 2006 and 2016 respectively (Bonner *et al.,* 2010, Bonner *et al.,* 2006, NCI, 2016).

1.11 Overview of cell signalling

Cell signalling is one of the most fundamental processes in biological life. The human body is composed of organs and systems. Organs are composed of tissues. Tissues are composed of cells. Cells need to communicate with each other in order to perform their normal function and that communication is based on a phenomenon called cell

signalling. Dysregulation of cell signalling leads to cells performing abnormal functions which could eventually lead to cancer. Cancer in fact is dysregulation of cells signalling (Weinberg, 2006).

There are three basic steps in cell signalling:

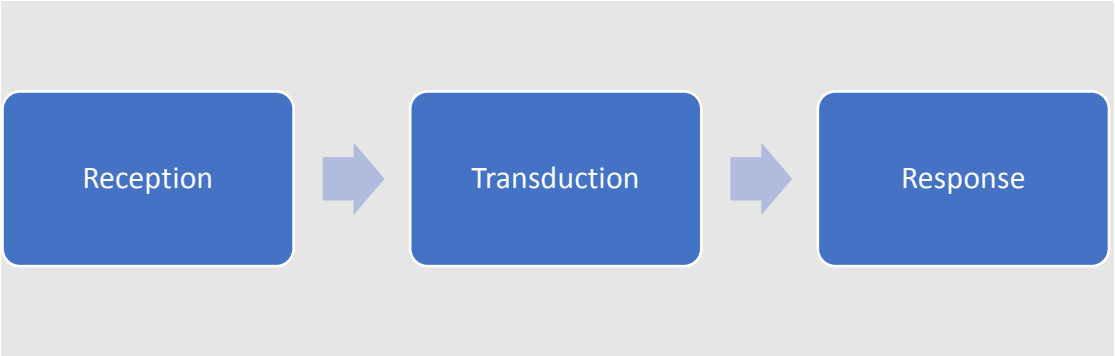


Figure 1.3 Three stages of cell signalling

Legend: Reception- When a signal (also known as a ligand) from outside of the cell binds to a receptor which is located on the cell membrane.

Transduction -When ligand binding leads to receptor shape change. These changes leading to the activation of signalling process known as cascades. Each molecule in cascade activates the next molecule in correct sequence.

*Response -When cellular response occurs as the result of signalling (*Alberts *et al.,* 1983).

Membrane receptors transfer information from the environment to the cell's interior. Once signal molecules (often called ligands) bind to a receptor which is located on the membrane, information enters the cells usually without the ligands themselves entering the cell. Ligand binding leads to a structural change of receptor which in turn activates the second messenger system within the cell. Secondary messengers relay information from the ligand receptor complex into the cell nucleus. It should be noted that the signal may be amplified significantly as it crosses the cell and into the nucleus.

There are three types of cell communications:

- Autocrine signalling: the cells produce its own molecules and receptor.
- Paracrine signalling: the cells receive signals from adjacent cells.
- Endocrine signalling: hormones are produced by endocrine gland and sent through the blood stream to distant cells.

Protein phosphorylation is a common way of transferring information. Phosphorylation is the process of introducing a phosphate group onto a molecular protein at the amino acid level. Only three amino acids can be phosphorylated; serine, tyrosine and threonine. Dephosphorylation is the process of removing the phosphate group from these amino acids. Phosphorylation and dephosphorylation can mean activation or deactivation of the protein depending on the specific protein involved. Protein kinases are enzymes which transfer phosphate groups on to a protein. Protein phosphatase are enzymes that remove phosphate groups from amino acids on proteins. Phosphorylation and dephosphorylation play a critical role in the regulation of cellular processes such as the cell cycle, proliferation, growth, apoptosis, and signal transduction pathways. It is the most common means of regulating protein function and the transmission of signals throughout a cell (Ardito *et al.,* 2017).

1.12 Genomic alterations in Head and Neck cancer

At least 20 genes are known to be mutated in head and neck cancer (Sarode *et al.,* 2018). A vast amount of literature links the following genes; p53, PIK3CA, FAT1, NOTCH1, CASP8, CDKN2A as the most mutated genes in head and neck cancer (Iglesias Bartolome *et al.,* 2013, Pickering *et al.,* 2013). In contrast however, other genes, such as EGFR and CCND1, were not include in highly mutated gene lists, but their alteration also play a

significant role in head and neck carcinogenesis (Stransky *et al.,* 2011, Leemans *et al.,* 2011).

1.12.1 p53

p53 is a tumour suppressor gene and it has been cited as the most common tumour suppressor gene to undergo mutation in head and neck cancer. A mutation of p53 is found in more than 70% of traditional head and neck cancer while HPV positive head and neck cancer does not harbour a p53 mutation (Zhou G *et al.,* 2016). Smokers and alcohol drinkers have higher chance of having a p53 mutation (Brennan *et al.,* 1995). In HPV positive head and neck cancer, p53 is inactivated by Oncoprotein E6. The p53 level within the cell is regulated by MDM2 which binds to p53 and induces its degradation (Pant & Lozano, 2014). It is known that p53 plays an important role in the activation and transcription of genes important in cell-cycle control, DNA repair, senescence, metabolism, and cell death (Brady & Attardi, 2010).

1.12.2 Ras

The vast majority of the literature does not list Ras as a highly mutated gene. Mutation of Ras is low in the west (Europe and North America). In fact, only 5% of head and neck cancer patients in UK harboured the H-Ras mutation (Chang *et al.,* 1991). Another study conducted in USA also found low occurrence of H-Ras mutation (Xu J *et al.,* 1998). In contrast, 35% of betel quid chewing related oral SCC in western India have the H-ras mutation (Saranath *et al.,* 1991). Further studies conducted in Eastern India, reported that H- and K-ras genes mutations in head and neck cancer patients have a frequency of 28 % and 33%, respectively (Das N *et al.,* 2000). A study conducted in Taiwan (which is another betel quid chewing region) found that 18% tumour specimens contained K-*ras*

mutation (Kuo *et al.,* 1994). This leads to questions of whether Ras mutation is caused by a specific carcinogen in areca nuts or mutations that are found in certain ethnicities.

1.12.3 PIK3CA

About one third of HNSCC have reported a PI3K pathway mutation and on that pathway the protein *PIK3CA* shows the highest level of mutation (HNSCC-PI3K mutational profile (Lui *et al.,* 2013). Multiple PI3K pathway mutations are only found in the advanced stage of the disease (Lui *et al.,* 2013). A detailed analysis of the PI3K pathway mutational events showed that PIK3CA is mutated in a range between 12.6% (Lui *et al.,* 2013) to 17.5% of all head and neck cancer (Cai *et al.,* 2017). PIK3CA mutation is more frequent in HPV positive cancer than HPV negative (Nichols *et al.,* 2013). PIK3CA mutation is found in 30% of HPV positive head and neck cancer while PI3KCA amplification and loss of PTEN were found in 20% and 33% of the case respectively (Chiosea *et al.,* 2013). One study believed that even though PIK3CA overexpression alone is not sufficient for initiation of HNSCC, it increases invasion and metastasis by inducing the Epithelial to mesenchymal transition and cancer stem cell properties (Du *et al.,* 2016).

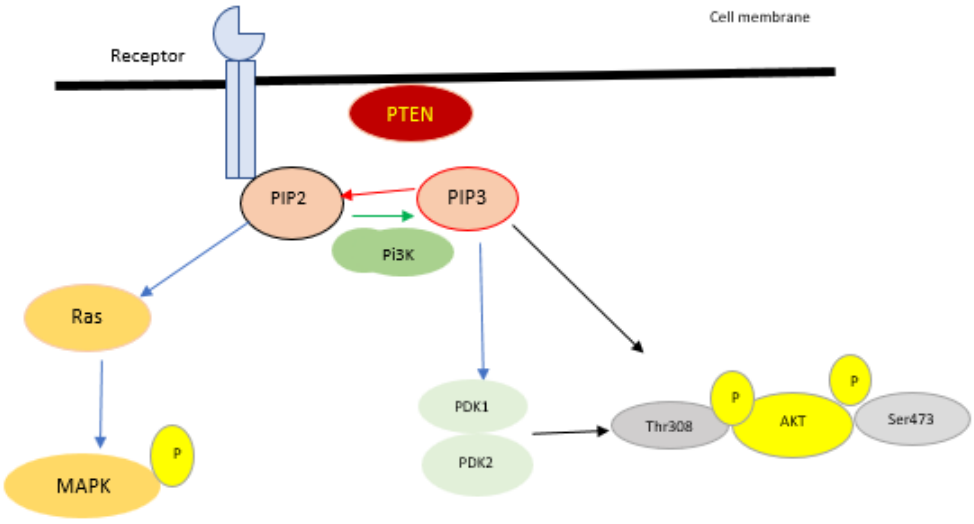


Figure 1.4 PI3K pathway: Reproduced and modified from (Giudice, 2013).

1.12.4 PRAD-1 (CCND1)

PRAD-1 (CCND1) is a proto-oncogene localized on chromosome 11q13 which encodes the protein cyclin D1. Cyclin D1 plays an important role in controlling the cell cycle. Overproduction of CyclinD1 leads to uncontrolled cell division and proliferation. Both CCND1 amplification and overexpression of the protein cyclin D1 was found in head and neck cancer patients and could be related with poor prognosis. One study reported more than 30 % of head and neck cancer patients have CCND1 amplification (Callender *et al.,* 1994). Co-amplification of EGFR and CCND1 was also found in head and neck cancer patient samples (Sheu *et al.,* 2009). Another study reported oral cancer patients have higher Cyclin D1 expression than normal control group and related with poor outcome (Swaminathan *et al.,* 2012, Zhao *et al.,* 2014, Mineta H *et al.,* 2000).

1.12.5 Notch

The Notch family is a family of highly conserved cell signalling molecules and has 4 receptors (Notch 1-4). Notch 1 mutation is found in 10 to 15% of the HNSCC tumours (Fukusumi & Califano, 2018, Agrawal *et al.,* 2011, Stransky *et al.,* 2011). Notch 1 is a transmembrane receptor which plays an importance role in cell to cell interaction, differentiation and embryogenesis. Notch 1 acting as tumour suppressor or activator is dependent on cellular context and very complex (Yap *et al.,* 2015). Whether the mutation of Notch1 is related to ethnicity is questionable. One study found that 43.1 % of Chinese oral squamous cancer patients harboured Notch mutation and these patients had shorter overall and disease-free survival rate (Song *et al.,* 2014). Another study

conducted in Japan reported completely different findings where only 9.5% of Japanese oral squamous cell cancer patients had the notch mutation, interestingly, Notch mutated Japanese patients had longer disease-free survival than patients without the notch mutation (Aoyama *et al.,* 2014). This leads to the question of whether tumour suppressive or oncogenic role of Notch is dependent on ethnicity.

1.12.6 Caspase 8

CASP8 mutation was reported in 9% of HNSCC patients and appeared to be related to tumour migration, invasion and resistance to death receptor-mediated apoptosis (Li *et al.,* 2014).

1.12.7 FAT1

FAT1 mutation in head and neck cancer patient studies varies in the range from 6.7% to 26.9% (Morris *et al*., 2013) and seems dependent on HPV status. FAT 1 mutation is higher in HPV-negative HNSCC (26.9%) than HPV-positive HNSCC (7.9%) (Kim *et al.,* 2016). FAT proteins belong to the Cadherin superfamily of proteins. The function of FAT1 in cancer can be either tumour suppressive or oncogenic dependant on the context (Katoh, 2012). In oral cancer, FAT 1 mutation or knockdown is related with disease progression and recurrence (Lin *et al.,* 2018). However, FAT1 inhibits tumour growth via Hippo signalling while FAT1 has been reported to upregulate tumour migration through actin polymerization at lamellipodia and filopodia (Katoh, 2012).

1.12.8 Cyclin-dependent kinase inhibitor 2A (*CDKN2A*)

Cyclin dependent kinase inhibitor 2A is located on chromosome 9p21, it encodes two tumour suppressor genes p16INK4a and p14 ARF which control the cell cycle. Alteration of CDKN2A expression due to mutation, loss of heterozygosity or DNA hypermethylation

is commonly found in head and neck cancer (Pérez-Sayáns *et al.,* 2011, Lim *et al.,* 2014, Zhou C, 2018). The loss of CDKN2A leads to the loss of function of p16 and p14. p16 inhibits CDK4 and CDK6 activity and prevents Rb phosphorylation a feedback loop exists between p16 and Rb, by which p16 expression is controlled by Rb. p14ARF activates p53 by inhibiting MDM2. Loss of p14 lead to loss of p53 function (Bates *et al.,* 1998).

1.13 Biomarkers of head and neck cancer

Biomarker are a measurable indicator of the presence of disease and whether they can be used in the pathology service either diagnostically or prognostically. Biomarkers are always gene products which can be translated or untranslated products. Ideal biomarkers need to be easily detected in body fluids such saliva or serum and useful for either early detection, diagnosis or prognosis.

There are some controversial literature which produce different lists of the biomarkers for head and neck cancer. (Dahiya & Dhankhar, 2016) stated that Microsatellite instability, HPV, Chemokine receptors, Methylation markers, Interleukins, MMPs, miRNA, MAGE, Centrosome abnormalities, actin and myosin, Cytokeratins, p53, Eukaryotic translation factor 4 E, Loss of heterozygosity (LOH) in chromosome 3p, 9q,13q,17p are useful biomarkers of head and neck cancer. However, (Tsuda & Ohba, 2012) reported that EGFRs, cyclin D1, cyclin B1, Ki-67, PCNA, Akt1. p53/p63, p21/p27, Bcl-2 family members, pRb, Survivin, HIF-1α, CA IX, GLUT-1, EPOR, VEGF, CD105, CD34, Eph receptor tyrosine kinase /Eph A2, MMP-7,-9,-13,-14, CD44, E-cadherin, N-cadherin, β and γ-catenin, versican, Parathyroid hormone related protein, Endothelins and their receptor, inflammatory cytokines and chemokines, and receptor activator of NF-$_{KB}$ ligand as functional biomarkers of OSCC. Therefore one set of markers are very specific

at a molecular and protein level and the former are more molecular indicators. Thomas GR *et al.,* 2005 argues that p16, p53, Cyclo-oxygenase 2 (Cox-2), CCND1 (gene encode Cyclin D1), EGFR, VEGF, MMPs, FHIT genes are also molecular markers of head and neck cancer.(Quon *et al.,* 2001) reported that EGFR, TGF-α, cyclin D1, p53 protein expression are useful biomarkers.

The majority of the literature indicates that EGFR, TGF-α, cyclin D1, p53, HPV and p16 are prognostic markers of head and neck cancer. Previously the role of cyclin D1 and p53 were discussed. EGFR and TGFα will be discussed in the next section. HPV and p16 will be discussed below.

HPV status is an independent prognostic factor for survival of HNSCC patients. Oral cancer patients who are HPV positive have a better prognosis than patients who are HPV negative. The 3 years overall survival rate for the HPV positive group is 82.4% compared with 57.1% in the HPV negative group (Ang KK *et al.,* 2010). Tonsil and oropharynx are the most common anatomical location to detect HPV virus in the oral cavity (Hobbs, 2006). In HPV positive HNSCC patients the oncoproteins E6 and E7 inactivate p53 and pRb, respectively. In HPV positive cancers, the inactivation of pRb by HPV E7 protein may lead to p16 over-expression since Rb normally represses p16 transcription (Ang KK *et al.,* 2010).

In HPV+ HNSCC, p16 was expressed as a consequence of pRb inactivation by the E7 but is minimally detected in classic HNSCC (Ang KK *et al.,* 2010, Pérez-Sayáns *et al.,* 2011).There is a strong correlation between HPV16 DNA detection and $p16^{INK4A}$ expression (Lassen P *et al.,* 2009).

p16 expression is found in HPV positive HNSCC and related with favourable prognosis (Sedghizadeh *et al. ,*2016). The 3-year rate of overall survival was 83.6% in the group with p16 positive group and 51.3% in the group negative for p16 expression showing that p16 positive with HPV positive HNSCC has better prognosis (Ang KK *et al.,* 2010, Lassen P *et al.,* 2009). This leads to the potential use of p16 as a prognosis marker in the future. It should be noted that expression of $p16^{INK4A}$ is not limited to HPV-positive tumours(Lassen P *et al.,* 2009) .One study argued that testing p16 alone is sufficient and HPV testing may be unnecessary since they found p16+ HPV- HNSCC has survival comparable to p16+ HPV+ HNSCC and statistically significantly better than p16 - HPV- HNSCC (Lewis *et al.,* 2010).

1.14 EGF like ligands

Growth factors are naturally occurring substances such as protein or hormone which have ability to stimulate cell growth. Epidermal growth factor (EGF), Transforming growth factor α (TGFα), Amphiregulin (AR), Betacellulin (BTC), Epiregulin, Heparin-binding EGF like growth factor (HB-EGF), Epigen are all ligands that bind to Epidermal growth factor receptor. Some ligands are more potent than other. In here, Epidermal growth factor (EGF), Transforming growth factor α (TGFα) will mainly discussed since they are most widespread ligands for EGFR (Roskoski, 2014).

1.15 Epidermal growth factor (EGF) and Transforming growth factor alpha (TGFα)

EGF is 6-kDa protein with 53 amino acid and three disulphide bonds. EGF was discovered as a contaminant when isolating nerve growth factor. EGF was originally isolated from

submaxillary gland of mouse and human urine (Carpenter & Cohan, 1979). Precocious eyelid opening and incisor eruption were found when EGF was injected into newborn mice (Carpenter & Cohan, 1979).

EGF is heat stable, low molecular weight and accounted for 0.5% submaxillary gland protein (Carpenter & Cohan, 1979).

TGFα gene is known to be associated with cleft lip and palate (Ardinger *et al.,*1989). Precocious eyelid opening was also found in newborn mice when TGFα was injected into the developing foetus (Smith *et al.,* 1985). TGFα was first isolated from retro virus transformed cells (De Larco & Todaro, 1980). TGFα is widely expressed in both normal epithelium and tumour cells, and it is considered as normal physiologic ligand for EGFR (Ebner & Derynck ,1991). TGFα is structurally similar with EGF and showed similar effects in a number of assays. TGFα is more potent than EGF which might be related with receptor recycling (Ceresa & McClintock 2010, Ebner & Derynck, 1991). Even though both EGF and TGFα induce DNA synthesis, wound healing (Schultz et al, 1991) and angiogenesis, the angiogenic effect of TGFα is more potent than EGF (Schreiber *et al*., 1986). TGFα also induces more bone resorption than EGF does (Stern *et al*., 1985).

EGF and TGFα display around 35% sequence homology with 6 cysteine residues in similar positions (Winkler *et al.,* 1987). The location of the 3 disulfide bridges in both EGF and TGFα allows them bind to same receptor, EGFR (Schreiber *et al.,* 1986).

Knock-out mice of each ligand showed mild defects, EGF knock-out mice had abnormal mammary gland development (Luetteke *et al.,* 1999). TGFα knock-out mice had hair follicle and eye abnormalities (Luetteke *et al.,* 1993). In contrast, knock-out of 3 of the

ligands for EGFR (amphiregulin, EGF and TGF-alpha) together lead to growth retardation (Luetteke *et al.,* 1993).

TGFα, EGFR mRNAs and protein levels are elevated in oral cancer patients compared with disease free control and its elevation might be related with poor prognosis (Grandis *et al.,* 1996, Grandis *et al.,* 1998). Another study reported that TGFα mRNA and EGFR mRNA are elevated in histologically normal mucosa of oral cancer patients compared with disease free patients. This suggests that TGF α/EGFR gene transcription is an early event during carcinogenesis and could be a precursor to a tumour (Grandis &Tweardy, 1993, Grandis *et al.,* 1996). This hypothesis could lead to an assay for early detection of oral cancer being developed in the future especially in a subset of the population who have strong history of smoking and alcohol use. A number of studies reported that tobacco smoke induces the production of amphiregulin which is ligand that binds to EGFR (Du *et al.,* 2005, Grandis *et al.,* 1998). It has also been reported that TGF-α and EGFR play an important role in the proliferation of oral cancer cells but not for normal oral epithelium cell growth (Grandis *et al.,*1997).

There is also a changing pattern of expression of at least one of these factors during the different stages of cancer. For example, in early stages of prostate cancer, TGFα is produced as a paracrine signal while in late stage of the diseases it is believed to be a localised autocrine signal (Scher *et al.,* 1995).

Stability of ligand receptor complex is different between EGF and TGF-α. The iso-electric point (PI) of EGF is 4.6 while the PI value of TGFα is 5.9. The difference in PI value between EGF and TGFα suggests that TGFα dissociates from EGFR at higher pH than EGF (Ebner & Derynck, 1991). Half maximum dissociation value pH for TGFα is 6.9 while pH

value for EGF is 5.6 (Ebner & Derynck, 1991). After receptor internalization, intracellular TGF-α is more rapidly dissociated from EGFR and cleared than EGF. TGF-α induces receptor recycling whilst EGF induces receptor down regulation (Ebner & Derynck, 1991).

Studies also indicate a difference between TGFα ligand binding to EGFR is different from EGF binding to EGFR. The 13A9 monoclonal antibody raised against EGFR blocked binding of TGFα ligand but not EGF ligand to the receptor (Winkler *et al.,*1987).

1.16 ErbB receptor family

The ErbB receptor are a group of receptor tyrosine kinases (RTKs) involved in cellular function. ErbB receptor family has 4 receptors, EGFR (ErbB-1), HER2 (ErbB-2), (HER3/ErbB-3) and (HER4/ErbB-4). Several different ligands bind to each of ErbB receptors and overexpression of these receptor are found in cancers. In this section EGFR (ErbB-1) will be mainly discussed.

ErbB receptor and their corresponding ligands are represented in the Table below

Table 1.2 ErbB receptor and their corresponding ligands

EGFR / ErbB1 (HER1)	ErbB2 (HER2)	ErbB3(HER3)	ErbB4(HER4)
EGF TGFα Amphiregulin (AREG) Betacellulin (BTC) Epiregulin (EREG) Heparin- binding EGF like growth factor (HB-EGF) Epigen(EPI)	No ligands	Neuregulins 1 and 2 (NRGs)	Neuregulins 1 Neuregulins 2 Neuregulin 3 Neuregulin 4 Heparin binding EGF like growth factor Betacellulin Epiregulin

(Roskoski, 2014).

Type of ErbB receptors and their relationship in different types of cancer are represented in the Table below:

Table 1.3 Type of ErbB receptors and their relationship in different type of cancer

Name of receptor	Ligand	Type of alteration	Cancer types
EGFR/ErbB1	EGF,TGFα and various	Overexpression	Non small cell lung cancer, ovarian cancer, head and neck cancer, renal, pancreatic colorectal, breast cancer, bladder cancer, stomach cancer, esophageal cancer, prostate
ErbB2/HER2	No ligand	Overexpression	Breast Adenocarcinoma, oral HNSCC
ErbB3/HER3	Various	Overexpression	Oral HNSCC
ErbB4/HER4	Various	Overexpression	Oral HNSCC

(Salomon *et al.,* 1995, Grandis & Sok, 2004).

1.17 Epidermal growth factor receptor

The epidermal growth factor receptor (EGFR) is a member of ErbB family. EGFR is a transmembrane protein receptor which has tyrosine kinase activity. It has extra-cellular domain, which is important for ligand binding, a single hydrophobic transmembrane domain, and an intra cellular domain which has 3 portions: juxtamembrane region, tyrosine kinase domain and carboxy terminal tail (Ullrich *et al* 1984, Lemmon *et al* 2010). EGFR was the first receptor tyrosine kinase discovered by Carpenter and co-workers in 1978. It was also the first cloned transmembrane receptor and has since been shown to have a clear connection with cancer. Most breast cancer diagnostic screening in UK

involves investigation of EGFR expression (Milanezi *et al.,* 2008). EGFR is involved in various cellular process such as cell proliferation, differentiation, migration, cell death. It is expressed in both epithelial and mesenchymal cells. Cellular processes mediated by EGFR family and their ligands are very complex (Carpenter & Cohan, 1979).

Mice with mutated EGFR gene die at early age because of multi organ failure indicating a role for EGFR in development (Sibilia *et al.,* 2007).

In a normal cell, 40,000 to 100,000 EGFR protein molecules are seen while cancer cells have been shown to have up to $2x10^6$ receptor molecules per cell being expressed (Carpenter & Cohan, 1979, Herbst, 2004).

1.18 EGFR overexpression

Overexpression of EGFR is seen in a variety of cancers such as non-small cell lung carcinoma (NSCLC), central nervous system, head and neck, bladder, pancreas, and breast (Salomon *et al.,* 1995). EGFR overexpression has been reported in 80%- 90% of head and neck cancers (Kalyankrishna & Grandis, 2006) (Reuter *et al.,* 2007). Over-expression of EGFR can hypersensitize malignant cells to low concentrations of growth factors (Salomon *et al.,* 1995). EGFR over-expression is also related with drug resistance, poor prognosis and shorter survival rate (Ang KK *et al.,* 2002, Maurizi *et al.,* 1996). EGFR overexpression was found in both HPV+ and HPV- HNSCC (Romanitan *et al.,* 2013)

EGFR upregulation has been reported as a two steps process. Firstly, the intensity of EGFR level in histologically normal epithelium adjacent to tumour is higher than mucosa of disease-free controls (Shin *et al.,* 1994, Ang KK *et al.,* 2002). Secondly, the EGFR level increases dramatically from dysplasia to carcinoma in HNSCC patients (Shin *et al.,* 1994).

However, it has also been reported that EGFR level are different at different sites of head and neck cancer (Takes *et al.,* 1998).

1.19 Co-expression with other ErbB family members

Other ErbB family members are also overexpressed in cancer. For example, HER2 overexpression/amplification is common in breast cancer (Burstein HJ, 2005). In HNSCC, the individual expression of either ErbB2 or ErbB3 or ErbB4, was rarely observed while coexpression with EGFR is much more common (Bei *et al.,* 2001). *EGFR* is more important than the other ErbB members for head and neck cancer development (Sheu *et al.,* 2009). A study stated HER2/ ErbB2 expression is less common than HER3 and HER4 overexpression (Pannone *et al.,* 2013) while results from another study found that HER2/ErbB2 expression higher than ErbB3 and ErbB4 expression (Bei *et al.,*2001). (Xia *et al.,* 1997) reported that HER2 overexpression is limited to oral SCC but not other head and neck cancers. They also reported that HER2 overexpression in oral SCC is related with high nodal metastasis and poor survival rate.

EGFR has been reported to crosstalk with other cell surface receptors such as G protein couple receptor, insulin like growth factor I receptor, c-Met/ hepatocyte growth factor receptor and reported to induce signalling pathways (Kalyankrishna & Grandis, 2006, Adams *et al.,* 2004, Jo *et al.,* 2000).

EGFR is 170 kDa protein containing 20% carbohydrate and being heavily N glycosylated. Glycosylation is important in ligand to receptor interaction and also determines the protein structure. Out of 11 possible sites for glycosylation, 8 sites are fully glycosylated while 1 site is glycosylated a fraction (Zhen *et al.,* 2003). When the ligand does not bind

to EGFR, it stays as the inactive form. When the ligand binds to receptor it changes shape and leads to activation (Goodsell, 2003).

Some reports in the literature suggest that an increase in EGFR gene copy number is related with poor outcome in HNSCC patients (Chung *et al.,* 2006, Temam *et al.,* 2007). EGFR amplification occurs in about 30% of HNSCCs, and often overlaps with EGFR overexpression. Fifty percent of head and neck cancer specimens carried genetic alterations which promote the activation of EGFR signalling (Sheu *et al.,* 2009).

1.20 Architecture of EGFR

The extra-cellular region of EGFR has four sub-domains which are arranged in sequence of L1-CR1-L2-CR2. Domain I and III is important for ligand binding. However, the majority of energy which is important for ligand binding comes from domain III (about 400nM for EGF). Domain I and III are called L domain which represent leucine rich family and they are 37% similar in structure (Ward & Garrett 2001, Lax *et al.,* 1991, Lemmon *et al.,* 1997). Domain II and IV are called Cysteine rich domains and contain multiple disulfide bonds. Binding of ligands to domain I and III creates the whole EGFR shape change and domain II then participates in either homo-dimerization or hetero-dimerization with other receptors (mostly with ErbB2 / HER2). Hetero-dimerization is more potent signaling than homo-dimerization. When the ligand binds to EGFR, a dimerization loop (beta hairpin) is protruded from the receptor and interacts with other ErbB molecules. ErbB 2/HER 2 have no known ligands but its protrusion loop enhances hetero-dimerization with other ErbB receptors. Dimerization with HER2 is common in many cancer and has the worst prognosis than single receptor dimerization (Normanno *et al.,* 2006). Dimerization leads to activation of intracellular tyrosine kinase domain and the receptor undergoes

phosphorylation of internal tyrosine residues. About 6 EGFR tyrosine residues from carboxyl terminal are phosphorylated during ligand mediated EGFR dimerization (Schulze *et al.,* 2005). These phosphorylation sites serve as docking site for intra-cellular proteins containing Src homology 2 (SH2) or Phosphotyrosine binding (PTB) domains to become activated which then leading to activation of multiple downstream pathways.

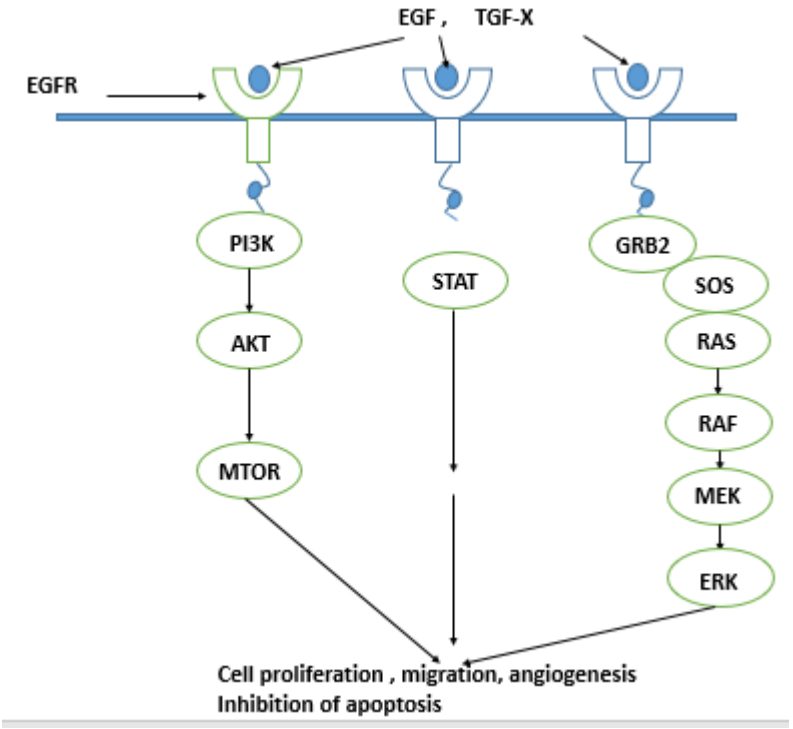


Figure 1.5 EGFR signalling pathway

1.21 Activation of specific tyrosine kinase residues

Once ligand induced dimerization has occurred activation of intracellular tyrosine kinase domains happens and the receptor undergoes phosphorylation of specific tyrosine residues. The phosphorylated tyrosine kinase residues act as docking site for adaptor proteins and enzymes and provide a crucial connection between the external stimuli and internal signalling pathways. There are 6 tyrosine residues phosphorylated by EGF and TGFα induce EGFR activation (Guo *et al.,* 2003). They are Y1068, Y1148, Y1173 (Downward *et al.,* 1984), Y1086 (Hsuan *et al.,* 1989, Margolis *et al.,* 1989), Y992 (Walton

et al., 1990) Y1045 (Levkowitz *et al.,* 1999). At low concentrations of EGF and TGFα ligands produce the highest phosphorylation of Y992 while other sites did not reach maximum phosphorylation even at highest concentration of ligands (Guo *et al.,* 2003). There is only a slight difference between EGF and TGFα induced phosphorylation (Guo *et al.,* 2003).

1.22 Inhibition of tyrosine kinase residues

Some phosphorylation sites are sensitive to inhibitors while others are not (Guo *et al.,* 2003). In human epidermoid cancer cell lines with high EGFR overexpression, tyrosine kinase inhibitors blocked all ligand phosphorylation sites except for Y992 and Y1068 (Guo *et al.,* 2003). Research conducted at the University of Dundee discovered phosphorylation of EGFR Tyr-residue 974 triggers EGFR endocytosis while phosphorylation of Tyr-residue 1045 causes Cbl dependent ubiquitination (Coachrane, master thesis). This indicates that different phosphorylation sites lead to different downstream pathways. Different ligand binding can cause activation of different tyrosine kinase activity. EGF and TGFα use different proteins that initiate signaling pathways. $p70^{S6K}$ and CD44 are required for EGF to induce signaling while the integrin αVβ3 is needed for TGFα ligand (Ellis *et al.,*2007).

1.23 EGFR internalization

Prior to ligand binding, EGFR is located in caveolae (which is a specialized plasma membrane micro-domain). Ligand binding leads to induction of EGFR and the departure of EGFR from caveolae (Mineo *et al.,* 1999). Ligand induction followed by activation of the tyrosine kinase on the receptor leads to EGFR being internalized into an intracellular

endosomal compartment. From there, the receptors are either degraded via lysosomes or recycled back to the cell surface depending on which ligands are bound to the receptor (Henriksen *et al.,* 2013, Carpenter, 2000).

It has been reported that EGF induced binding induces the majority of receptor down a degradation pathway while TGFα induced receptor leads to recycling (Decker, 1990). This is likely to be caused by difference in pH sensitivity making TGFα to be able to rapidly dissociate from EGFR allowing recycling. Longer binding by EGF would send most of the receptor down the degradation pathway. Similarly, HB-EGF- and BTC stimulation leads to receptor internalization followed by degradation of large portion of internalized receptor. TGF-*α* and EPI ligands, lead to an intermediate internalization followed by the complete recycling of internalized receptor. AREG also induce receptor internalization, but the receptor did not readily return to the surface and it was not broken down. It was suggested that this was probably because of slow recycling (Henriksen *et al.,*2013) (Roepstorff etal.2009).

Table 1.4 EGFR status after ligands binding on whether degraded or recycled.

Receptor degradation	**Receptor recycling**
Majority of EGF, HB-EGF- and BTC	Small amount of EGF, TGF-*α* , EPI ligands AREG

1.23.1 EGFR endocytosis

Once the receptor has been internalized there are two pathways that endocytosis can follow. The Clathrin dependent pathway is the main route of EGFR endocytosis while the clathrin independent pathway is less common. One study reported that EGF-, TGF-*α*-, AR- and EPI-induced receptor internalization was almost completely inhibited by clathrin knockdown, whereas HB-EGF- and BTC-induced internalization was only partly inhibited

(Henriksen *et al.,*2013). This could means EGF, TGF-*α*, AR and EPI use a clathrin dependent receptor endocytosis while HB-EGF and BTC use clathrin independent pathway. In contrast, one study reported that the dose of ligand is major factor deciding on whether cells use clathrin or non clathrin pathway. They stated that clathrin dependent endocytosis was reported to be induced by low dose EGF while high EGF induce clathrin independent endocytosis (Sigismund *et al.,* 2005). However, how this occurs is secondary to the effect this has on the cell. Receptor degradation leads to the temporary down regulation of EGFR on the cell surface and decrease EGFR signaling (Sorkin & Waters, 1993).

When talking about EGFR internalization, HER2 should not ignored since co-expression of EGFR-HER2 is common. HER2 also has an effect on EGFR endocytosis. Dimerization with ErbB2 increases the stability of EGFR by decreasing the rate of dissociation of the ligand receptor complex to prolong activation of signaling. EGF ligand induced heterodimerization with ErbB2 decreases receptor endocytosis and increases receptor recycling (Lenferink *et al.,* 1998). TGFα directs EGFR recycling regardless of presence or absence of any co-receptor (Lenferink *et al.,* 1998).

It should be noted that EGFR has the potential to be translocated to the nucleus. Nuclear internalization of EGFR has been reported to acts as a transcription factor (Lin *et al.,* 2001). It should be noted that the transcription factors Cyclin D1 and STAT 3 interact with nuclear EGFR (Lo *et al.,* 2005, Lin *et al.,* 2001). A recent study reported that EGFR could also be internalized into mitochondria but it was assumed that it was not related with receptor endocytosis (Yao *et al.,* 2010).

Patients with a high nuclear EGFR content were shown to have a high rate of disease recurrence and poor survival (Psyrri *et al.,*2005). Cetuximab (see later) resistant cells also have high levels of nuclear EGFR expression and src family kinase which are known to mediate ligand induced translocation of EGFR (Li *et al.,* 2009).

1.24 EGFR variant III or ΔEGFR, or de2–7EGFR

EGFR variant III which is commonly found in glioblastoma has also been found in head and neck cancer. EGFR variant III mutant is 145kDa protein that is missing the extracellular domain I and domain II which are encoded by the segment in exon 2 to exon7. These missing two domains of EGFR mean that the receptor cannot bind any ligands. This leads to the weak constitutive activation of any ligand independent signalling pathway and as such might not be strongly oncogenic (Gan *et al.,* 2013, Sok *et al.,* 2006).

Sok *et al., (*2006) stated that EGFR variant III expression was only detected in the presence of EGFR wild type. The truncated form of EGFR type III mutant is known to coexist with EGFR overexpression. This EGFR variant III mutant does not activate MAPK pathway and only activates the PI3Kpathway (Moscatello *et al.,* 1998, Chang *et al.,* 2013). The EGFR variant III expression is unique to cancer and not observed in normal tissues (Sok *et al.,* 2006). This could lead to its potential use as a diagnosis marker.

There is some controversy about the prevalence of EGFR variant III. One study found EGFR variant III mutation in 42% of HNSCC tumour samples conjunction with EGFR

wildtype while in another study stated EGFR variant III mutation is less than 1%. (Sok *et al.,* 2006, Khattri *et al.,* 2015).

Even though there is limited information about the communication between EGFRvIII and other EGFR family members. There is some evidence that heterodimerization between EGFRvIII and either wildtype EGFR or HER2 is possible. Ligand independent signalling of EGFRvIII induce down-regulation and internalization of both wtEGFR and erbB2 (Zeineldin *et al.,* 2010)

EGFRvIII also interacts with other receptors such as c -Met receptor and enhances the production of HGF and in turn enhances HGF signalling (Garnett *et al.,* 2013, Gan *et al.,* 2013). EGFRvIII also activates STAT3 pathway. STAT3 was found to be important in c-Met-mediated HGF expression in EGFRvIII/ ΔEGFR expressing cells (Garnett *et al.,*2013).

There is an evidence of a direct interaction between the EGFRvIII and OSMR (Oncostain M receptor) protein. Knock-down of OSMR in EGFRvIII-expressing mouse can reduce EGFRvIII expression with the loss of STAT3 activity (Greenall & Johns, 2016). Some studies concluded that EGFR vIII might be responsible for cetuximab resistance (Sok *et al.,* 2006) while it is not related with resistance to TKIs. EGFRvIII enhanced proliferation of head and neck cancer cells and induce resistance to chemotherapy induce apoptosis (Sok *et al.,* 2006).

1.25 EGFR mutation

EGFR mutation is more prevalent in non-small cell lung cancer than head and neck cancer, and a lot of research had been done in NSCLC patients. A number of mutations in EGFR have been reported. One of the most studied is known as the kinase domain

mutation of EGFR. It is known as an activating mutation since they are independent of ligands and signal even when no ligand is available. All somatic activating EGFR mutations involve an ATP binding pocket in the tyrosine kinase domain which is the binding site for the inhibitors gefitinib and erlotinib (Gazdar AF, 2009). The activating mutation of EGFR has been isolated to the first 4 exons (18 to 21) of the tyrosine kinase domain. This EGFR mutation has be studied extensively in a lung cancer model.

There are three classes of mutation of EGFR. Class I mutations are in-frame deletions in exon 19, class I in frame deletions include the amino acid leucine residue at 747 to glutamic acid at 749 and are located in N-terminal region of kinase domain C-helix. These class I mutations are responsible for about 44% of tyrosine kinase domain mutations (Kumar *et al.,* 2008).

Class II mutations are single nucleotide substitutions that cause alteration in the amino acid sequence. The most frequent single point mutation is in exon 21 in which there is an exchange of an arginine for a leucine at codon 858 (L858R) and this is responsible for about 41% of tyrosine kinase mutations. Glycine mutation to either serine, alanine or cysteine was responsible for 4% of TKs mutations while the result 6% responsible for other missense mutation (Kumar *et al.,* 2008).

Class III mutations are in-frame duplications and/or insertions in exon 20 and are responsible for the remaining 5% of TKs domain mutations (Kumar *et al.,* 2008).

In lung cancer, Exon 19 deletion mutations and the single-point substitution mutation L858R in exon 21 are the most frequent and termed classical mutation (Gazdar AF, 2009). There is difference between EGFR mutation of HNSCC and that of Non-small cell

lung carcinoma. In head and neck cancer, a large amount of the EGFR mutation are scattered throughout exons 18 to 21 (Perisanidis *et al.,* 2017). There is no report of EGFR mutation in HPV positive HNSCC patients (Na *et al.,* 2007).

Mutation in head and neck cancer appears to be less common than overexpression (Lee *et al.,* 2005). However, EGFR tyrosine kinase domain mutation varies with geographic location. The EGFR mutation had not been studied world-wide. Limited data has been produced from Africa, the Middle East, Central Asia, and Australia. Reports suggest that the EGFR mutation is uncommon in the West (Loeffler-Ragga *et al.,* 2006) whilst its mutation is found more frequently in South Korea and South East Asia region (Perisanidis, 2017, Vatte *et al.,*2017). The mechanisms behind the different rates of *EGFR* mutation in different ethnicities remains to be determined. The South East Asia region is densely population area of the world with a population of around 600 million people and it definitely needs attention in terms of more research. It is a worry since none of TKIs targeting EGFR mutations has received FDA approval for HNSCC. Although EGFR mutation is associated with sensitivity to gefitinib and erlotinib treatment, not all the tumours with an activating EGFR mutation respond to these drugs. It is believed that these tumours might have an additional genetic lesion (Wakeling *et al.,* 2002, Massarelli *et al.,* 2007).

1.26 Secondary EGFR mutation

Secondary mutations of EGFR or EGFR T790M mutation have been reported in lung cancer patients who at first responded to gefitinib and erlotinib treatment (Xu S *et al.,* 2017). The T790M mutation is the substitution of threonine 790 with methionine. T790M mutation is responsible for about half of all patients who develop resistance to

gefitinib and erlotinib (Kosaka *et al.,* 2006, Kobayashi *et al.,* 2005). The first time that the EGFR T790M mutation was reported in research conducted in HNSCC patients was in Saudi Arabia (Vatte *et al.,* 2017).

The secondary mutation of EGFR results in an alteration of the topology of the ATP-binding pocket or increasing ATP affinity. Since TKIs compete with ATP for binding to the kinase site an increase in ATP affinity is expected to decrease the potency of TKIs (Yun CH *et al.,* 2008). Since T790M reduces the efficacy of ATP-competitive inhibitors, it might be good idea to develop distinct, non-ATP competitive inhibitors or irreversibly binding inhibitors to the tyrosine kinase domain (Gazdar AF, 2009). A more recent study reported that EMT is a common mechanism underlying the resistance to different generations of EGFR TKIs (Weng *et al.,* 2019). It leads to question that T790M mutation is related with EMT in HNSCC patients.

1.27 Activation of signalling pathways

The activation of EGFR leads to a variety of signaling pathways such as ERK MAPK, PI3K AKT mTOR, PLC-γ1-PKC, JNK, and STAT pathways being activated. These signalling pathways are connected (by cross talk) with each other and lead to a wide variety of cellular responses such as proliferation, migration, differentiation, inhibition of apoptosis, invasion and metastasis, angiogenesis (Molinolo *et al.,* 2010).

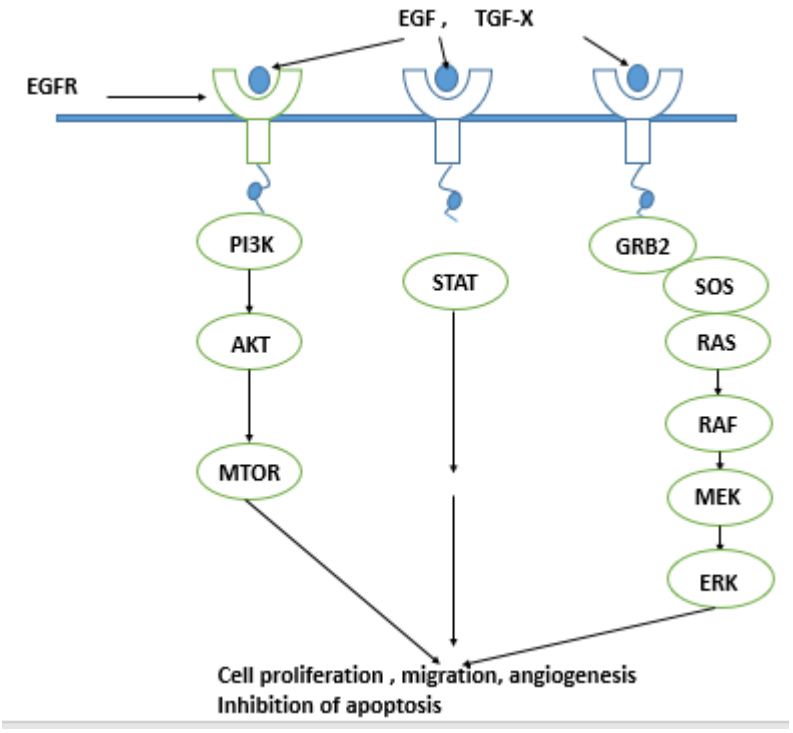


Figure 1.5 EGFR signalling pathway

1.28 RAS-RAF-MAPK pathway

MAPKs are serine/threonine protein kinases which are involved in cell proliferation, migration, differentiation, and apoptosis. Signals from ligands such as cytokines, growth factors, and mitogens all stimulate the activation of these MAPKs kinases. MAPK is one of the major pathways initiated by EGFR activation (Peng *et al.,* 2018). In mammals, there are more than a dozen MAPK genes. The best known MAPKs cascades are four sub-pathways: extracellular signal-regulated kinase 1 and 2(ERK1/2), c Jun N terminal kinase (JNK)/ stress-activated protein kinase SAPK, p38 isoforms (p38α, p38β, p38γ, p38δ) and ERK5 (Plotnikov *et al.,* 2011). Each cascade has three enzymes which activate in series: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAP kinase (MAPK). MAPKKKs is the first phosphorylated which in turn activates downstream MAPK

kinase (MAPKK). Then MAPK kinase (MAPKK) in turn phosphorylates and activates MAPKs (Plotnikov *et al.,* 2011). MAPKs phosphorylation leads to a change in gene expression and protein function and results in specific biological responses such as EMT, increased cell proliferation, invasion, and resistance to apoptosis, and angiogenesis (Plotnikov *et al.,* 2011).

The Erk1/2 cascade has received most attention because of it has 3 important components Ras, Raf and MEK (Peng *et al.,* 2018). Phosphorylated tyrosine residues serve as binding sites for proteins which contain Src homology 2 (SH2) domain or phosphotyrosine-binding (PTB) domains, such as growth factor receptor bound protein 2 (Grb2). Grb2 is key factor in the activation of Ras. Following the auto-phosphorylation, the SH2 domain of Grb2 binds to EGFR directly via Y1068 and Y1086 or indirectly via Shc. Following ligand induce EGFR activation, Shc is phosphorylated and associated with EGFR via the SH2 domain. Shc binds in turn to the SH2 domain of Grb2 protein leading to relocation of Grb2 SOS complex from the cytosol to the plasma membrane (where SOS stimulates the exchange of GDP for GTP on Ras, converting to active stage; Walker, 1998).

Ras, is a proto oncogene and is a small, monomeric G protein with very low GTPase activity on its own. All three family genes (KRAS, NRAS, HRAS) have been reported to be mutated in cancer patients. The mutation of the Ras family is more common in Asia when compared with the West (Saranath *et al.,* 1991, Chang *et al.,* 1991, Xu J *et al.,* 1998, Das N *et al.,* 2000). Ras activates the MAPK and PI3K pathways. When Ras is bound to GDP it is in an inactive state. Upon binding to GTP, it is converted into the active form. GTP bound Ras directly binds and recruits Raf to the plasma membrane. Then Raf is phosphorylated and activates MEK which induces phosphorylation and activation of

ERK. Activated ERK1/ERK2 induce expression of genes responsible for cell proliferation, migration, angiogenesis, and cell death (Plotnikov *et al.,* 2011, Peng *et al.,* 2018). According to a study conducted in Germany, the B raf mutation is rare seen in about 3% of HNSCC patients (Weber *et al.,* 2003). Unlike Ras, B-Raf mutation can only activate the PI3K pathway (Peng *et al.,* 2018). It has been reported that Ras mutation is related with resistance to Cetuximab (Rampias *et a.,l* 2014, Misale *et al.,* 2012).

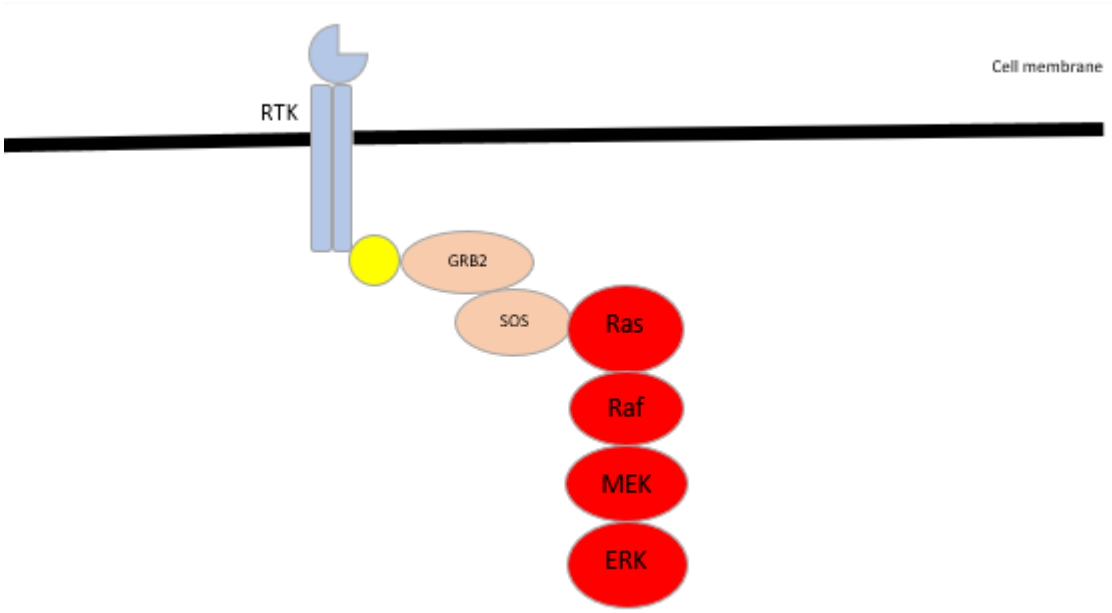


Figure 1.6 MAPK pathway Reproduced and modified from (Plotnikov *et al.,* 2011) (Peng *et al.,* 2018).

1.29 PI3K -AKT-MTOR pathway

The PI3K/Akt pathway is important for cell growth, differentiation, development and survival (Philippon *et al.,* 2015). The PI3K/Akt pathway is the most commonly mutated pathway in head and neck cancer patients, with approximately 3 times higher levels than RAS/MAPK and JAK/STAT pathway The PI3K pathway appears to play an important role in HPV positive HNSCC as well (Lui *et al.,* 2013).

PI3K are classified into classes I, II, and III. Class I is sub divided into 1A and 1B and they consist of regulatory subunit (p85 for class IA and p101 and p87 for class IB) and P110 catalytic units (p110α, p110β, and p110δ). Class IA which will be described here is activated by receptor tyrosine kinases such as EGFR, Met receptor, PDGFR, VEGF, and insulin-like growth factor 1 receptor (IGF-1R) while class IB is activated primarily by G protein couple receptor. Class IA consist of P85 regulatory subunit and P110 catalytic units (p110α, p110β, and p110δ). p110α, p110β, and p110δ are encoded by *PIK3CA, PIK3CB*, and *PIK3CD* genes, respectively (Cai *et al.,* 2017). EGFR signalling activates PI3K by recruitment of the p85 regulatory sub-unit to the activated receptor. Ras activates the PI3K pathway via p110α. Activated PI3K converts PIP2 to PIP3 by adding a phosphate group. PIP3 then act as a second messenger and subsequently activates AKT. Phosphatase and tensin homolog (PTEN), is a tumour suppressor protein and is a negative regulator of PI3K. Activation of the PI3K pathway is a potential mechanism of resistance to anti-EGFR therapy (Rebucci *et al.,* 2011). Patients with a mutated PI3K pathway are sensitive to BEZ-235 which is dual inhibitor of PI3K and mTOR (Lui *et al.,* 2013).

EGFR is overexpressed in cell lines with mutated PTEN are less sensitive to Gefitinib (Bianco *et al.,* 2003). Reduced PTEN expression was found in about 30% of HNSCC (Squarize *et al.,* 2013). Loss of *PTEN* expression is related with poor prognosis (Lee *et al.,* 2001).

AKT or protein kinase B is an important downstream effector of PI3K. Following PI3K activation, AKT is recruited into the membrane and activated by the phosphorylation of PDK1 at threonine 308 and the mTORC2 complex at serine 473(Alessi *et al.,* 1997, Vanhaesebroeck & Alessi, 2000 , Sarbassov *et al.,* 2005). Akt-308 is important for cell

proliferation, cell shape and protein synthesis, whereas Akt-473 is important for the phosphorylation of pro-apoptotic protein Foxo3a and inhibits apoptosis (Liu and Dean, 2010).

AKT activation stimulates cell proliferation by controlling CyclinD1 and p27Kip1 which are cell cycle protein and cell cycle inhibitor respectively (El-Naggar *et al.,* 2009). The phosphorylation of Akt on Thr308 is a more reliable biomarker than phosphorylation of Akt on Ser473 in non small cell lung carcinoma (Vincent *et al.,* 2011). The mutation of retinoblastoma Rb1 leads to phosphorylation of AKT473 but not Akt-308 (Liu and Dean, 2010). And also to the overexpression of mTOR, constitutive phosphorylation of Akt at Serine 473, resistance to anoikis, a blocking in c-Raf activation, and the blocking of the phosphorylation of Erk1/2(El-Naggar *et al.,* 2009). Activated Akt, in turn, phosphorylates a series of molecules including Mechanistic target of rapamycin (mTOR) which is an important downstream effector of the PI3K pathway (Amornphimoltham *et al.,* 2017) (Molinolo *et al.,* 2012). The mTOR pathway can be inhibited by Rapamycin (Amornphimoltham *et al.,* 2005). As discussed above, PIK3CA mutation is more common in HPV positive HNSCC than HPV negative. Unlike traditional HNSCC, HPV positive HNSCC showed p16 activation which is regarded as having good prognosis. p16 positive HNSCC have activates the mTOR pathway. This could lead to potential use of mTOR inhibitors in HPV positive HNSCC (Molinolo *et al.,* 2012).

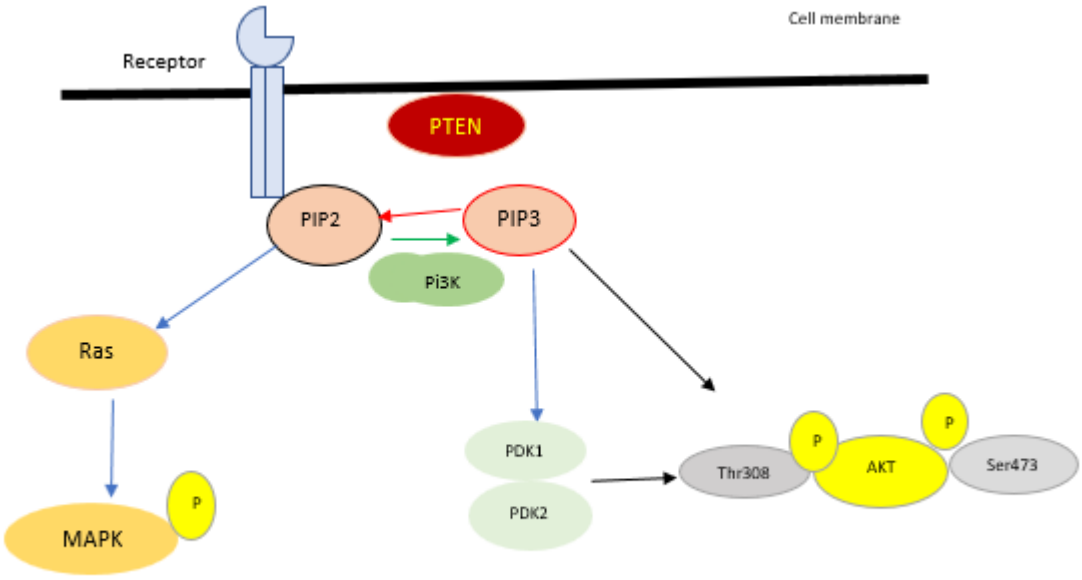


Figure 1.4 PI3K pathway: Reproduced and modified from (Giudice, 2013).

The PI3K pathway activates CDK1 and can also cross talk with TGFβ pathways suggesting a potential role of CDK1 and TGFβ in Head and Neck cancer (Du *et al.,* 2016). A study reported that dual inhibition of PI3K and mTOR can overcome radio resistance in HNSCC patients (Yu CC *et al.,* 2017).

Activation of the PI3K pathway is a potential mechanism of resistance to anti EGFR therapy. Cetuximab resistance was found in tumour with PI3K pathway activation and inhibition of PI3K pathway restored cetuximab sensitivity in a cetuximab resistant cell line (Rebucci *et al.,* 2011, Keysar *et al.,* 2013, Simpson *et al.,* 2015).

1.30 STAT signal transducer and activator of transcription

STAT is another downstream effector of EGFR signalling. STAT can also be activated via EGFR independent mechanisms (Sriuranpong *et al.,* 2003). Activation of the STAT pathway occurs via one of two mechanisms; JAK dependent or independent. STATs are

downstream transcription factors of JAK and other kinases. Signal transducers and activators transcription (STAT) proteins family consists of STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. Out of these proteins, STAT1, STAT3, STAT5a, STAT5b have a role in certain cancers (Quesnelle *et al.,* 2007). In general, STAT3 and STAT5a/b promote carcinogenesis, in contrast STAT1 activation has an opposing effect (Thomas SJ *et al.,* 2015). EGF and TGFα activate STAT1 and STAT3 in EGFR overexpressed cells. Ras stimulated cells also see an activation of STAT3 and that stimulation is blocked by EGFR specific kinase inhibitors (Song &Grandis, 2000). Upregulation of STAT3 has been found in variety of cancers. STAT3 activation has been shown to be an early event of HNSCC (Song &Grandis, 2000).

EGF and TGFα induces EGFR dimerization leading to phosphorylation of tyrosine residue and through the JAK dependent pathway, activation of (STAT) occurs when tyrosine residues are phosphorylated by JAK which initiates formation of the dimer. This is transferred into the cell nucleus and activates c-myc and cyclin D (Reich &,Liu, 2006).

JAK dependent STAT pathway signalling could be abolished by using JAK inhibitors (Sen *et al.,* 2015). In JAK independent pathway, STAT was directly activated by EGFR kinase. Tyrosine residues Y1068 and Y1086 are critical for STAT activation (Coffer &Kruijer , 1995.) After activation, STAT dimerizes, and translocates into the nucleus and regulates the expression of genes involved in proliferation, differentiation, cell survival. Even through STAT is involved in carcinogenesis, there has been no drug specifically targeting of the STAT pathway (Quesnelle *et al.,* 2007). Co-localization of STAT3 and EGFR was found in the cell nucleus (Lo *et al.,* 2005). Intrinsic EGFR kinase activity is necessary for

STAT activation while autophosphorylation of EGFR is not required for STAT activation (David *et al.,* 1996).

There are some controversial findings in related with nuclear STAT3 and prognosis. (Macha *et al.,* 2011) reported nuclear accumulation of STAT3 is found in premalignant stage and related with poor prognosis while (Masuda *et al.,* 2002) and (Pectasides *et al.,* 2010) stated that high nuclear level of STAT3 is predictor of better prognosis. Abolishing STAT3 function in oral carcinogenesis leads to an increase in apoptosis and downregulation of Bcl-x_L (Song &Grandis, 2000).

There is a strong association between high STAT3 and cyclin D1 overexpression in HNSCC (Masuda *et al.,* 2002). This study found that EGFR overexpression in oesophageal keratinocytes activates STAT in a JAK dependent manner leading to increase cell migration, which can be blocked by JAK inhibitor (Sen *et al.,* 2015, Andl *et al.,* 2004). JAK1 and JAK2 are needed for STAT 1 and STAT3 activation in response to EGFR overexpression which leads to cell migration which might be mediated by MMP1(Andl *et al.,* 2004). EGFR collaborates with STAT3 to induce EMT in cancer cells via up-regulation of TWIST (Lo *et al.,* 2007). Erlotinib was found to inhibit oral cancer in mouse model via EGFR-STAT3 signaling pathway (Leeman *et al.,* 2011, Grandis *et al.,* 1998, Psyrri *et al.,* 2013).

1.31 JNK pathway or c-Jun N-terminal kinases

JNK is member of MAPK family. JNK pathway is associated with activation of MAPKKK. JNK pathway is commonly activated by cytokines, DNA damaging substances, UV

radiation and less frequently activated by growth factors (Gkouveris & Nikitakis, 2017). The role of JNK in cancer is controversial. First, it was thought to be oncosuppressive but more recently was described as having a tumorigenic role (Gkouveris & Nikitakis, 2017). Now oncogenic or tumour suppressive effect of JNK signalling is believed to be tissue specific and cell type dependent (Gkouveris & Nikitakis, 2017). In head and neck cancer, it is believed that JNK is acting alone or may cross talk with other signalling molecules such as STAT pathway (Gkouveris *et al.,* 2016).

1.32 Phospholipase C gamma (PCLγ) pathway

PCLγ is another downstream target of EGFR signalling. High levels of PCLγ 1 was reported in tumour samples when compared with adjacent normal mucosa. A study reported that inhibition of PCLγ blocked cell migration but not cell proliferation (Thomas SM *et al.,* 2003).

1.33 Cell proliferation

Cell proliferation is an essential component of carcinogenesis. Cell proliferation is tightly regulated in normal healthy person. In contrast, cancer is the over proliferation of cells leading to big mass or tumour which can either benign or malignant. One of the hallmarks of cancer is the overproduction of growth factors. The overproduction of growth factors or overexpression of receptors lead to the activation of signalling pathways and this may then lead to dysregulation of the cell cycle proteins such as cyclin D which are involved in cell proliferation (Poch B, 2001).

The classic eukaryotic cell cycle has two major stages which are interphase and M phase. Interphase can be divided into three stages, Gap1 (G_1), Synthesis(S) and Gap2 (G_2). Cells grows and prepare for cell division in interphase. DNA replication occurs in S phase (Romar *et al.,* 2016).

Cell division occurs in M phase. M phase is the phase where mitosis and cytokinesis occurs. Mitosis is the division of nucleus with equal chromosomes separation. Mitosis has prophase, metaphase, anaphase and telophase. Division of cell membrane and cytoplasm occurs in cytokinesis (Romar *et al.,* 2016).

Progression through each of cell cycle phases and transition from one phase to the next is monitored by three checkpoints. They are G1/S check point, G2/M check point and M phase spindle checkpoint. Function of the check point is to detect the DNA damage and not letting the cells enter into next phase until repair has been done G_0 is called resting phase of cell cycle where cell has left the cell cycle and stopped growing.

Cell cycle proteins such as cyclin dependent kinases (CDK) and cyclin control the cell division and DNA replication. For example, Cyclin D is stimulated by growth factors (Poch B, 2001). Activity of CDK are dependent on specific cyclins and are regulated by CDK inhibitors, phosphorylation and dephosphorylation events (Malumbres & Barbacid, 2009).

Cyclins, CDKs, inhibitory enzymes, Rb protein, p21, p27 and p53 regulate cell cycle progression. Overexpression of Cyclin and CDKs and loss of CDK is related with cancer.

Cyclin D drives the cell cycle from the G1 to the S phase by following mechanism. Cyclin D is the first cyclin increase in cell cycle. G1 is the first phase of the cell cycle. In G1 phase, cells are grows in size, synthesizes mRNA and protein and prepare for mitosis. G1 phase lasts about 18 hours in human somatic cells. The transition to G1 to S phase is controlled

by Cyclin D-CDK4/6 and Cyclin E-CDK2. Cyclin D bind to CDK4/6 resulting Cyclin D-CDK4/6 complex. CyclinD/CDK4 and/or CDK6 complexs phosphorylates the retinoblastoma protein. Phosphorylation of pRb leading to dissociation of pRb/E2F complex, released E2F transcription factor which bind to DNA, regulate gene expression and cell enter into S phase (Sherr CJ, 1994).

Deregulated expression of cyclin D 1 is associated with resistance to EGFR inhibitors in head and neck cancer (Dhingra *et al.,* 2017, Kalish *et al.,* 2004). Cell proliferation marker Ki-67 and Cyclin B1 are also higher in HNSCC (Watanabe *et al.,* 2010).

In a healthy individual the $P16^{INK4a}$, tumour suppressor protein prevents phosphorylation of pRb by inhibiting cyclin D-CDK 4/6 to phosphorylate pRb (Serrano *et al*., 1993). pRb is mainly hypophosphorylated and is found in complexes with E2F transcription factor, blocking cells enter into S phase (Pande et al., 1998). Down regulation of p16 is commonly found in classic head and neck cancer (Perez Sayans *et al.,* 2011). E2F1 transcriptionally up-regulates expression of p14ARF (Bates *et al.,*1998). p14ARF promotes p53 by inhibiting MDM2 which binds and degrades p53. Loss of $P14^{ARF}$ will lead to elevated level of MDM2 and then to loss of p53 function and cell cycle control (Bates *et al.,* 1998). p53 is called the 'guardian of the genome' and is responsible for determining whether the cell should enter S phase where DNA replication occurs.

INK4a/AFR is one of mechanism involved in cellular senescence (Collado *et al.,* 2007). Normal cells will divide between 50-70 times before cell death. Each time when a cell undergoes mitosis, the telomeres which is located at the ends of each chromosome shorten slightly. Once they reached certain length then apoptosis will be invoked in the cell. Cellular senescence is a phenomenon in which normal cells cease to divide and was

first reported by Hayflick in 1961. However, cancer cells can overcome senescence by using an enzyme called telomerase which maintains the telomere length (Hanahan & Weinberg, 2011). One of the hallmarks of cancer includes resistance to apoptosis by producing excess telomerase and gaining limitless replicative potential (Hanahan & Weinberg, 2011). Cellular senescence can be triggered by multiple mechanisms including telomere shortening, the epigenetic derepression of the INK4a/ARF locus, DNA damage and aging (Collado *et al.,* 2007).

It is expected that EGF and TGFα ligands might use two discontinuous pulses for cell proliferation like PDGF: initial pulse from resting cells into G_1 (after 8 hrs introduction with ligands) and is followed by a second pulse (7 to 9 h later) which drives the cells into S phase (Pennock & Wang , 2003).

1.34 Epithelial to mesenchymal transition (EMT) in HNSCC

Epithelial to mesenchymal transition (EMT) is an important process in cancer since it facilitates cells migration, invasion and metastasis. There are a lot of signalling pathways that initiate or regulate EMT. EMT can be triggered by different signalling molecules, such as by epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), transforming growth factor β (TGFβ), hypoxia-inducible factor (HIF), bone morphogenetic proteins (BMPs), WNTs and Notch (Thiery & Sleeman, 2006). EMT is the transdifferentiation of epithelial cells into mesenchymal cells. Epithelial cells are cuboidal in shape, have an apical basal polarity and are closely connected to each other by specialized membrane structures, such as tight junctions, gap junction, adherens junctions, and desmosomes (Thiery & Sleeman, 2006). During the EMT process, epithelial cells lose their cuboidal shape, cell to cell adhesion, apical basal

polarity and then undergo cytoskeleton remodelling, forming a mesenchymal or elongated shape, front-to-back leading edge polarity, motile and therapy resistance (Thiery & Sleeman, 2006).In vitro, mesenchymal cells have a spindle-shaped fibroblast-like morphology, whereas epithelial cells grow as cell clusters (Thiery & Sleeman, 2006). Growth factors such as EGF, TGFα, FGF, HGF, bone morphogenesis proteins (BMPs), and extracellular matrix components such as collagen, hyaluronic acid, and cellular processes such as chronic inflammation and hypoxia are known to induce EMT. There are three types of EMT. Type I EMT is found in embryogenesis, type II EMT is found in wound healing, tissue regeneration, and organ fibrosis. Type III EMT is found in carcinogenesis (Kalluri &, Weinberg, 2009). EMT is a reversible process and conversion into MET is frequently seen in cancer (Neilson & Zeisberg, 2009, Thiery & Sleeman, 2006). In EMT, epithelial markers such as E cadherin are downregulated with upregulation of mesenchymal markers such as Vimentin. The following are examples of epithelial and mesenchymal markers.

Table 1.5 Epithelial and mesenchymal markers associated with EMT

Epithelial markers	Mesenchymal markers
E cadherin	Vimentin
Z0-1	N cadherin
Desmoplakin	integrin αvβ6
cytokeratin	Fibronectin
occudin	FOXC2
miR- 200	Sox10
	smooth muscle actin
	Fibroblast specific protein(FSP1)
	Snail1
	Snail2(slug)
	Goosecoid
	Twist
	miR- 21
	MMP2, MMP3, MMP9

From (Neilson & Zeisberg 2009, Lee JM 2006)

a) E-cadherin is a calcium dependent cell adhesion molecule (CAMs). It is the most famous epithelial marker. The cytoplasmic domain of E-cadherin is linked to the actin cytoskeleton through interactions with catenins, and cadherins-catenin complexes localized in cell to cell contact area known as the adherens junction (Pećina-Slaus, 2003). The loss of E-cadherin in cancer means loss of the epithelial

morphology and the acquisition of mesenchymal phenotype. Reduced expression of E-cadherin is one of the main molecular events in EMT and cell migration. E-cadherin is regarded as tumour suppressor gene (Pećina-Slaus, 2003). Down regulation of E-cadherin and upregulation of N-cadherin which is called cadherin switching can be seen in EMT (Wheelock *et al.,* 2008). In cancer, E cadherin is downregulated by a number of mechanisms, including mutation, and transcriptional repression. In cancer, transcription factors such as snail, slug, ZEB1 and FOXC2 bind to the E box of E-cadherin promoter and repress its transcription (Batlle *et al.,* 2000).The relationship between E-cadherin and EGFR is quite controversial. One study reported that E-cadherin-mediated-cell–cell adhesion can trigger a ligand-independent activation of the EGFR, followed by activation of PI3-K and ERK while another study reported that E cadherin inhibits ligand dependent activation of EGFR (Reddy *et al.,* 2005, Qian X 2004). E-cadherin-mediated cell-cell adhesion promotes anoikis resistance via ligand-independent activation of EGFR and downstream MAPK pathway (Shen & Kramer, 2004).The methylation of E-cadherin and the degradation of membranous β-Catenin can also induce cell migration (Kudo, 2004).

b) Vimentin is intermediate filament (IF) protein expressed in mesenchymal cells. Vimentin is important for maintaining the integrity of the cells in cytoskeleton, orienting microtubules for directional migration, morphological changes and the migration of cells (Liu *et al.,* 2015, Mendez *et al.,* 2010). Loss of E-cadherin and acquired vimentin is correlated with metastasis (Nijkamp *et al.,* 2011).

c) Snail is transcription factor elevated in EMT cells (Summary: Figure 1.6). Signalling pathways such as PI3K, MAPK, TGFβ, Notch, BMP and Wnt pathway

activate snail. Snail downregulates epithelial markers, upregulates mesenchymal markers, participates in cell morphology changes, cell proliferation and cell survival (Barrallo & Nieto, 2005).

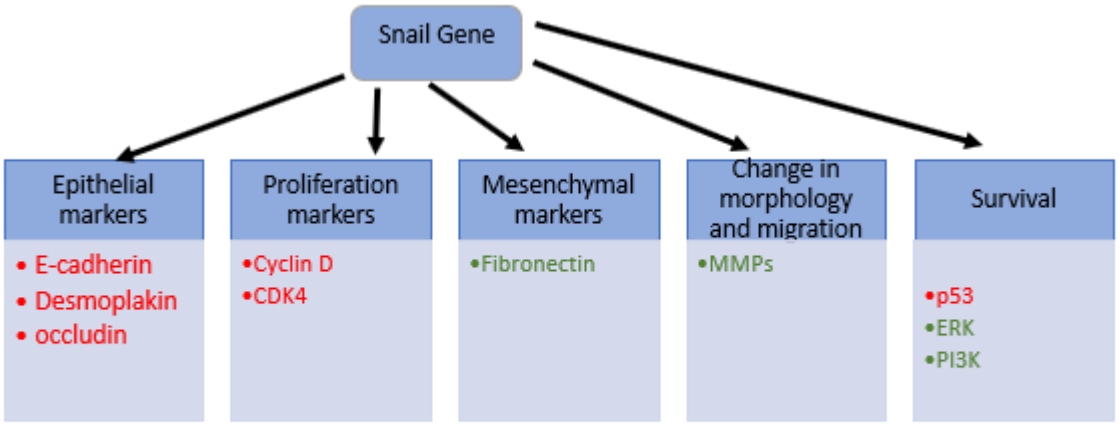


Figure 1.7: Role of snail in EMT (Barrallo & Nieto, 2005) (Yokoyama *et al.,* 2003).

d) Micro RNA play an important role in controlling EMT. Some miRNA induce EMT while others supress EMT (Ding XM, 2014).

It has been suggested that EMT promotes the generation of cancer stem cells (Mani *et al.,* 2008). To corroborate this it was also shown that cells undergoing EMT have stem cells like characteristic such as self- renewal and resistance to cancer therapeutic drugs (Singh & Settleman, 2010).

As discussed in above, EGFR collaborates with STAT3 to induce EMT in cancer cells via an up-regulation of TWIST (Lo *et al.,* 2007). However, it has also been suggested that EMT is related to the resistance to TKIs which is going to discuss in next sections (Weng *et al.,* 2019).

1.35 Cell migration

One of the other important hallmarks of cancer is invasion and metastasis. Cancer cells degrade the ECM and basement membrane, pass through surrounding tissue, travel into blood or lymphatic stream, attach at new site and grow into secondary tumour mass. Cells that are closely attached to each other under normal condition (homeostasis) by cell to cell adhesion and cell to extracellular matrix adhesion have these adhesions broken down by the secretion of substances which degrade the ECM and basement membrane (Guan X, 2015). Loss of cell to cell adhesion allows cancer cells to dissociate from the primary tumour. Loss of cell to matrix adhesion allow cells to invade surrounding stroma (Manzanares & Horwitz, 2011).

Cell to cell adhesions are stabilized by catenins which link cadherins to the cytoskeleton. Down-regulation of E-cadherin breaks down this link, disassembly of the adherent junctions and translocation of membrane bound β-catenin into the cell nucleus where it acts as a transcription factor (Hajra & Fearon, 2002). It is known that cell surface receptors called integrin are responsible for cell to ECM adhesion (Guan X, 2015). They are members of a glycoprotein family which allow the cell surface to contact with ECM molecules such as fibronectin, laminin, and collagen (Manzanares & Horwitz, 2011).

Normal cells undergo anoikis (apoptosis caused by detachment from the ECM) once they detached from ECM. In contrast, cancer cells have ability to resist anoikis (Guan X, 2015). The loss of contact inhibition not only leads to cell migration but also uncontrolled cell growth allowing for the spread and survival of tumour cells (Roycroft & Mayor, 2016) (Buchheit *et al.,* 2014).

There are two types of cell migration in cancer. Individual/single cell migration and collective cell migration. In individual/single cell migration, cell to cell contacts are lost.

1.35.1 Single cell migration

Cells which used mechanism of single cell migration have different phenotype- ameboid or mesenchymal phenotype (Figure 1.7). In amoeboid-like migration, cells are rounded shape and come in different variants: 1) they change their shape very fast, have short thin protrusions, show no blebbing, are fast moving 0.4–5 µm/min. 2) Slow and disorganize migration with a blebbing morphology. 3) Cells with short protrusion which are slow moving ~0.1 µm/min (Clark & Vignjevic, 2018, Friedl & Gilmour, 2009). In mesenchymal migration, cells are elongated spindle shape morphology with slow moving with cells pulling or pushing themselves across a surface.

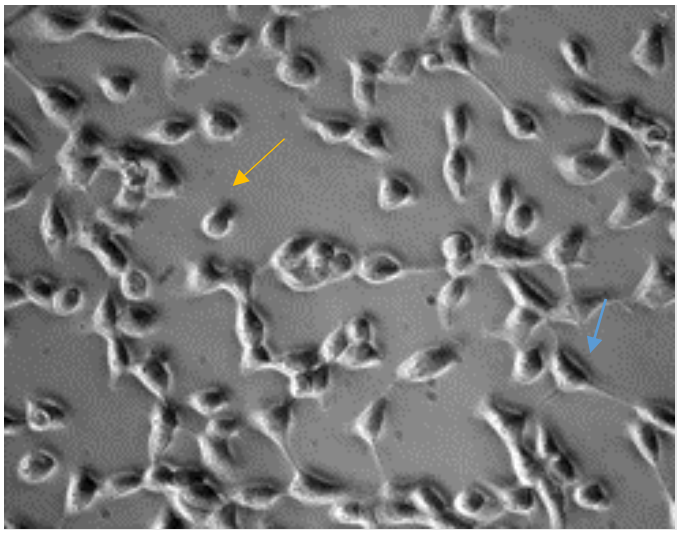


Figure 1.8 Example of single cell migration (Yellow arrow indicate ameboid, Blue indicate mesenchymal).

1.35.2 Collective cell migration:

In collective cell migration, cell to cell adhesion is maintained. Cells that are migrating to distant site do so as a group or sheets. This requires force generation of pulling cells a

large group of cells together. In collective cell migration, the free end of the cell group forms a dynamic cytoskeleton leading to multiple ruffled borders resulting in traction. Tensile stress created by these contractile forces is responsible for direction of the cell movement. Moving cells sheets modify the tissue along the route and secondary remodelling of ECM and rearrangement of basement membrane takes place (Friedl & Gilmour, 2009, Friedl & Illina, 2009).

There are two cell types involved in collective cell migration. First is the leader cells or the pioneer cells which lead. The second cell type is known either as the follower cells or rear cells. The phenotype may be different between the leader cells and the follower cells. Collective cell migration is slowest mode of migration in cancer 0.01–0.05 µm/min while it is faster during embryonic development 0.2-1 µm/min. In cancer, remodelling of the cellular junctions, reorganization of actin cytoskeleton and the formation of F-actin rich membrane protrusions are formed during collective cell migration (Ganjre *et al.,* 2017).

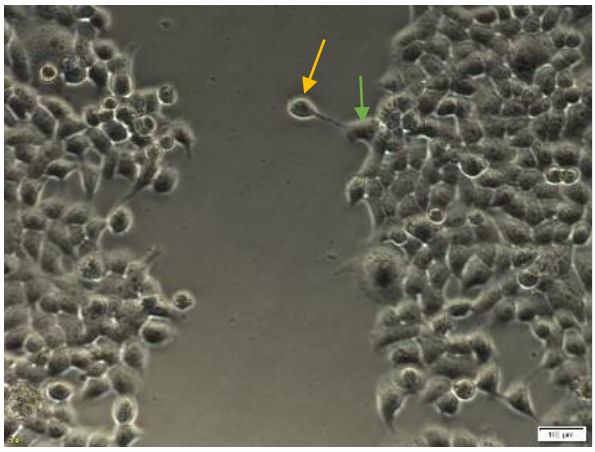


Figure 1.9 Example of collective cell migration (yellow indicate leader cells and green indicate follower cells).

The protrusion of the cell membrane is an initial step in cell migration. These dendritic and spiky protrusion are formed at the leading edge of the migratory cells and they are called; filopodia, lamellipodia, podosomes and invadopodia. Formation of these structures is controlled by actin polymerization. Lamellipodia are flat broad membranous protrusions. These are located at the leading edge of the migrating cells and control the direction of the cell movement. Filopodia are a thin finger like projection at the leading edge of the cells. It also controls the direction of the cell movement. Lamellipodia and filopodia are also present in normal epithelial cells (Guan X, 2015). Podosomes are found in normal highly motile cell. They have a shorter life span usually minutes while invadopodia (similar to lamellipodia) are mostly found in cancer and have a longer life span which vary from minutes to hours (Guan X, 2015). Both invadopodia and podosomes are rich in actin and able to degrade the ECM (Yamaguchi *et al.,* 2005). WASP (Wiskott-Aldrich Syndrome protein) and N-WASP (neutral WASP) protein play an important role in formation of cellular protrusion (Yamaguchi *et al.,* 2005).

The tumour microenvironment plays an important role in carcinogenesis. Chemokines, growth factors, cancer associated fibroblast, tumour associate macrophage (TAMs), Rho, Rac, Cdc42 play an important role in cell migration (Alizadeh *et al.,* 2014).

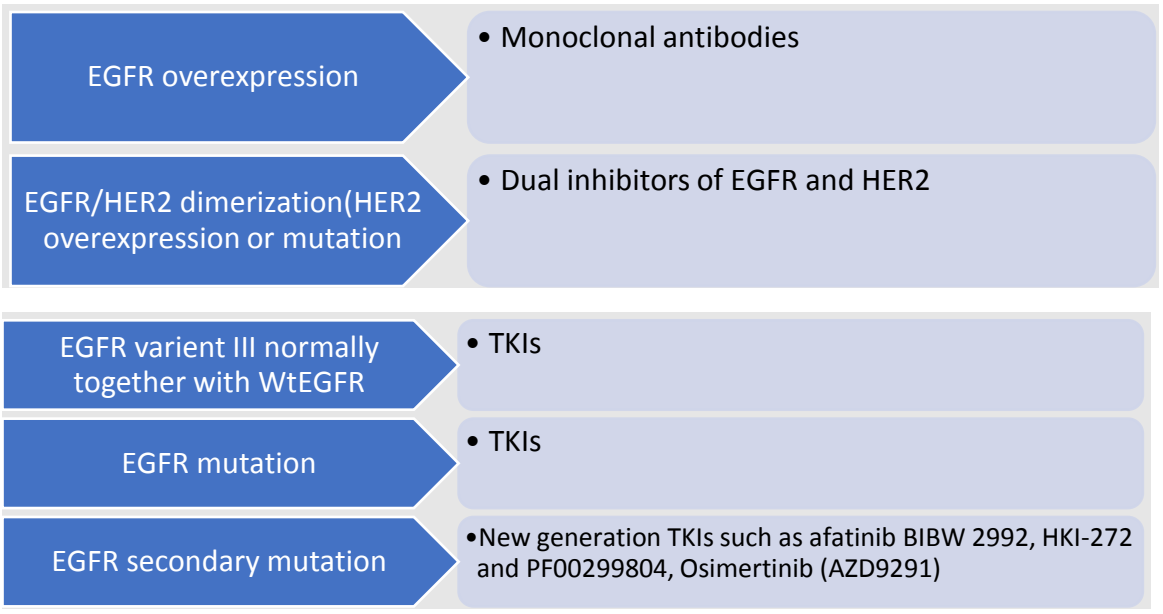
The secretions of tumour associate macrophage from primary tumours increase metastasis (Pollard, 2004). Macrophages can express colony stimulating factor receptor 1 and secrete EGF whilst cancer cells have EGFR and secrete CSF-1. Therefore these two cell types reciprocally induce each other to migrate in the tumour microenvironment allowing tumour cells to be able to metastasize (Wyckoff *et al.,* 2004).

Matrix metalloproteinase (MMPs) are group of enzymes which cleave/degrade the extracellular matrix and enhance the migration process. Head and neck cancer is composed of multiple cell types each with different types of MMP expression. MMP-1, MMP-2, MMP-9, and Membrane-type matrix metalloproteinase MT-1 MMP are most commonly found in HNSCC and are associated with disease progression. MMP and tissue inhibitors of MMP (TIMPs) have been reported as being related with invasion and nodal metastasis (Kurahara *et al.,* 1999).

In general, HPV+ head and neck cancer patients have lower MMP expression than HPV negative head and neck cancer patients (Hauff *et al.,* 2014). Hepatocyte growth factors are also reported to upregulate MMP genes (Hanzawa *et al.,* 2000).

1.36 Drug target EGFR signalling

A number of drugs have been reported to affect EGFR signalling. They are summarised in the Figure 1.10.



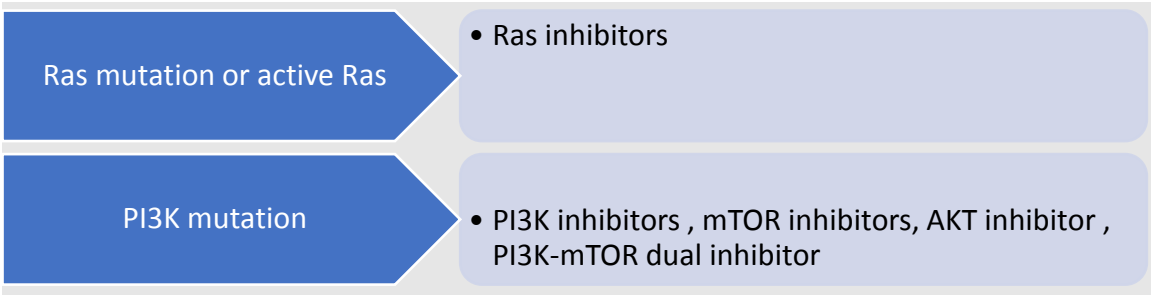


Figure 1.10 Drugs targeting EGFR pathway

There are two types of drug specifically targeting EGFR. The first group are monoclonal antibodies which are for targeting EGFR overexpression. The second group are small molecules tyrosine kinase inhibitors which target EGFR mutations.

The monoclonal antibodies bind to the extra-cellular domain of EGFR and block ligand binding. FDA has approved only two MoAbs for clinical use, cetuximab (for HNSCC and colorectal cancer) and panitumumab (for colorectal cancer, Martinelli *et al.,* 2009).

1.36.1 Cetuximab

Cetuximab is the only EGFR target drug which has received FDA approval to treat head and neck cancer (NCI, 2019). Cetuximab is chimeric mouse/human monoclonal antibody which targets domain III of the extracellular region of EGFR. Once cetuximab binds to EGFR, ligands cannot bind to the receptor, the receptor is internalized and then degraded leading to downregulation of EGFR on the cell surface. Autophosphorylation and activation of the EGFR was inhibited using this therapy (Dutta & Maity, 2007). (Mandic, 2006) proposed that the cetuximab might transiently stimulate EGFR phosphorylation prior to downregulation. Cetuximab may also induce antibody-dependent cellular cytotoxicity mediated by the host immune cells (Bleeker, 2004).

Cetuximab which got FDA approval in 2004 for use in EGFR expressing colorectal cancer resistant to irinotecan based chemotherapy (Wong, 2005).

In 2016, FDA approved Cetuximab in combinantion with radiotherapy to treat locally invasive head and neck cancer. Studies found that head and neck patients receiving radiation plus cetuximab show better overall survival than patients with radiation therapy alone (Bonner *et al.,* 2006,Bonner *et al.,* 2010).

Inhibition of EGFR can improve the efficacy of radiotherapy. There appears to be a connection between radiation-induced EGFR activation and DNA repair. Radiation induces translocation of EGFR into the nucleus while C225 (cetuximab) plus radiation prevents this EGFR translocation to the nucleus, and inhibits DNA damage repair by decreasing the nuclear level of the DNA repair enzyme DNA-PK (Bandyopadhyay *et al.,* 1998, Dittmann *et al.,* 2005, Huang & Harari, 2000). Cetuximab plus radiation also induced cells cycle arrest at G_1, G_2-M which are radiosensitive phases of cell cycle instead of the radioresistant S phase (Huang & Harari, 2000).

Vermorken *et al., (*2008) reported that Cetuximab plus platinum fluorouracil-based chemotherapy improved overall survival compared with compared with platinum–fluorouracil chemotherapy alone in metastasis or recurrent HNSCC.

1.36.2 Resistance to Cetuximab/ monoclonal antibodies

Although, EGFR overexpression is observed in more than 90% of HNSCC only 10% of HNSCC patients respond to cetuximab and often only for a short period of time(Matta & Ralhan,2009). There are conditions where cetuximab cannot be effective. First of all, NIH press released that Cetuximab with radiotherapy was inferior to standard treatment

in HPV positive HNSCC (NIH, 2018). (Mirghani *et al.,* 2015) also concluded that anti-EGFR is not effective in HPV positive HNSCC. Further research needs to be carried out to find out why cetuximab is not effective in HPV+ HNSCC. Mirghani *et al., (*2015) suggested it is important to analyse the Patient HPV status before cetuximab treatment and that use alternative drugs should be prescribed in HPV positive patients.

Secondly, co-expression of EGFR with other members ErbB family receptors such as HER2, ErbB3, ErbB4 are common in head and neck cancer (Bei *et al.,* 2001). Therefore targeting of EGFR alone may not be effective in those cases. A series of papers has supported the suggestion that HER2 activation is related with cetuximab resistance (Yonesaka *et al.,* 2011, Wheeler *et al.,* 2008).

Currently, there are no available drugs targeting ErbB3 and ErbB4. However, drugs targeting EGFR-HER2 are on the way to the clinic. More research has been done for EGFR- HER2 overexpression or EGFR/HER2 heterodimerization. In the past, HER2 overexpression/ or dimerization was thought to be rare in head and neck cancer. However, these reports were focusing on tumour specimens from specific areas such as larynx and pharynx. HER2 overexpression is characteristic of oral SCC but not other forms of head and neck cancer (Xia *et al.,* 1997). They also found HER2 overexpression in oral cancer is related with high nodal metastasis and poor survival rate (Xia *et al.,* 1997). They hypothesised that failure to target HER2 might be the reason of poor survival in oral SCC cases.

In this case, dual inhibitors of EGFR-HER2 should be used to block both receptors. Lapatinib is dual inhibitor of EGFR-HER2 which is commonly used in breast cancer patients (Burstein HJ, 2005) .However, Lapatinib produced controversial results in

clinical trial in HNSCC patients. One study reported that Lapatinib was only effective in HPV positive head and neck cell lines (Fumagalli *et al.,* 2014). Dual inhibitor targeting for HPV negative head and neck cancer should be developed in the future. One study reported that the dual kinase inhibitors of EGFR and HER2 could overcome cetuximab resistance (Quesnelle & Grandis, 2011).

Thirdly, in EGFR variant III mutation which was found in 42% of HNSCC to coexist with Wild type EGFR overexpression. This variant is missing extracellular domain I and II of EGFR meaning ligands could not bind to receptor, leading to constantly weak activating of tyrosine kinase. EGFR variant III mutation is related with cetuximab resistance (Tinhofer *et al.*, 2011).In this case, treatment with TKIs which inhibit the tyrosine kinase domain could overcome the problem (Sok JC *et al.,*2006).

Fourthly, EGFR mutation is more common in south East Asia region compared with the West. Like EGFRvIII, EGFR activating mutation leads to the activation of the tyrosine kinase. This could also be overcome by using TKIs (Gazdar AF, 2009).

Fifthly, a secondary mutation of EGFR or EGFR T790M mutation. This secondary mutation of EGFR is found in patients who were initially responsive to TKIs. For the first time secondary mutation of EGFR was found in Saudi head and neck cancer patients (Vatte *et al.,* 2017). Treatment of patients with this secondary mutation of EGFR might be overcome by using non-ATP competitive inhibitors or irreversibly inhibitors or second or third generation TKIs (Gazdar AF, 2009, Li *et al.,* 2008, Engelman, 2007).

Sixthly, mutation or constitutive activation of downstream signalling pathways such as; mutation or constitutive activation of the Ras/Raf/MAPK, STAT3 and PI3-K/AKT/mTOR are not uncommon in head and neck cancer patients. As discussed above about 30% of

areca nut related head and neck cancer patients in India have a Ras mutation (Saranath *et al.,* 1991, Das N *et al.,* 2000). PIK3CA mutation has also been found in both HPV- (12.6% to 17.5%) and HPV+ (30%) head and neck cancer patients (Lui *et al.,* 2013, Cai *et a.,l* 2017, Chiosea *et al.,* 2013). Mutation of these downstream pathways has been suggested to be related with resistance to anti-EGFR drugs (De Roock *et al.,* 2010).

1.36.3 Cross talking within signaling pathways

A number of studies have found that inhibition of one pathway can lead to the activation of another pathway (Turke *et al,* 2012, Serra *et al.,* 2011).Therefore multiple inhibitors have been used to good effect in the laboratory. The combination of therapy of PI3K and MEK pathway inhibitors enhanced the efficacy in both sensitive and resistant cell lines (Engelman *et al.,*2008).

1.36.4 Other monoclonal antibodies

Fully humanized monoclonal antibodies might be less prone to stimulate neutralizing antibodies or hypersensitive reactions. Panitumumab is a fully humanized monoclonal antibody which might inhibit ligand binding leading to internalization of EGFR but not EGFR degradation (Yang *et al.,* 2001, Dutta & Maity, 2007). In 2006, Panitumumab got FDA approval to treat colorectal cancer with wildtype KRAS. In clinical trials of recurrent or metastatic HNSCC patient, Panitumumab did not increase overall survival while it increased progression free survival in p16 negative patients (Vermorken *et al.,* 2013).

Zalutumumab is a fully humanized monoclonal antibody which has had controversial results. One study showed encouraging result with zalutumumab (Schick *et al.,* 2012) while another study reported that zalutumumab plus the radiotherapy did not show any significant improvement in HNSCC patient compared with control(Eriksen *et al.,* 2014).

Nimotuzumab, a humanized monoclonal antibody has been granted approval for use in HNSCC in some countries such as India, Cuba, Argentina, Colombia, Ivory Coast, Gabon, Ukraine, Peru and Sri Lanka (Ramakrishnan *et al.,* 2009). Nimotuzumab has lesser affinity to the receptor and has a transient binding interaction, it spares healthy tissues, and has no toxic cutaneous effect and showed promising results in clinical trials (Ramakrishnan *et al.,* 2009, Subramanium *et al.,* 2015). The receptor binding mechanism is reportedly different from cetuximab (Talavera *et al.,* 2009).

1.36.5 Tyrosine kinase inhibitors

Tyrosine kinase inhibitors bind to the cytoplasmic region of EGFR by competing with ATP and inhibit phosphorylation of the receptor. Example of tyrosine kinase inhibitors are Erlotinib, Gefitinib which both selectively and reversibly inhibit tyrosine kinase activity (Herbst, 2004). None of these TKIs has been approved to treat HNSCC. Some of them are currently under clinical trials (Chapman *et al.,* 2016).

The advantages of tyrosine kinase inhibitors is they can be orally administered, they can block EGFR overexpression, EGFRvIII and EGFR mutation. The main disadvantages of tyrosine kinase inhibitors is their lack of specificity or cross-inhibition of other receptor or non-receptor tyrosine kinase activity (Dutta & Maity, 2007).

Gefitinib got FDA approval in 2003 and is used to treat metastatic non-small cell lung carcinoma .Gefitinib is a reversible TKI which prevents EGFR auto-phosphorylation by competing with adenosine triphosphate for its binding site on the intracellular domain of EGFR. Gefitinib induces neither receptor internalization nor degradation and thus did not reduce EGFR protein level on the cell surface(Dutta & Maity, 2007).Gefitinib has undergone clinical trials in head and neck cancer patients and did not produce

encouraging results(Chua *et al.,* 2008) .One side effect reported in the clinical trials was skin toxicity when the Gefitinib dose was escalated, while it failed to show significance improvement in patient outcome*(*Perez *et al., 2012)* .Patients with the *EGFRvIII* mutation did respond to gefitinib treatment (Argiris *et al.,* 2013).

Erlotinib got FDA approval to treat NSCLC in 2004. Erlotinib is also reversible TKI of EGFR wildtype and the EGFRvIII mutant. Like Gefitinib, Erlotinib did not downregulate EGFR protein level. In head and neck cancers Erlotinib has already undergone clinical trials for HNSCC and known to have some toxic effect such as skin toxicity (Li & Perez-Soler, 2009, Soulieres *et al.,* 2004). Patients receiving erlotinib with cisplatin and radiotherapy failed to show significant increase in complete response rate and progression free survival (Martin *et al.,* 2013). Erlotinib did however inhibit carcinogenesis via EGFR-STAT 3 pathway in a mouse model (Leeman-Neill *et al.,* 2011).

The reason of why these two TKIs are not producing encouraging results in those trials might be activation of downstream signalling pathways such as ERK (Wakeling *et al.,* 2002). Other research has shown that the K-ras mutation is related with resistance to tyrosine kinase inhibitors in non-small cell lung cancer (Massarelli *et al.,* 2007).

Another possible reason of drug resistance to Tyrosine kinase inhibitors is EMT. EMT is believed to be underlying mechanism of drug resistance to TKIs (Weng *et al.,* 2019, Maseki *et al.,* 2012, Frederick BA *et al.,* 2007).

Second generation TKIs targeting EGFR secondary mutation are under clinical trials. Irreversible EGFR inhibitors such as Afatinib /BIBW 2992, HKI-272 and dacomitinib /PF00299804 inhibit EGFR secondary mutation or EGFRT790M mutation in preclinical models with Afatinib also targeting HER2 (Li DM *et al.,* 2008, Engelman *et al.,* 2007).

Osimertinib (AZD9291) was found to be more effective than Afatinib in clinical trial with EGFR T790M mutated lung cancer patients (Jänne PA *et al.,* 2015).

1.36.6 Pathway inhibitors under clinical trials for head and neck cancer

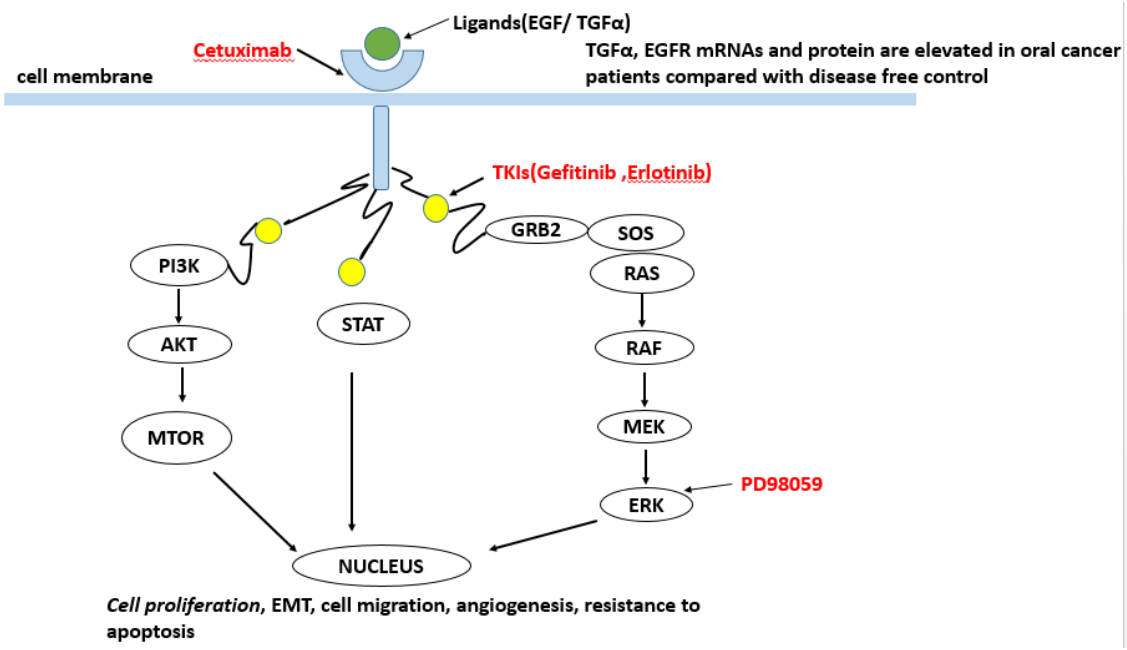
Table 1.6 Pathway inhibitors under clinical trials for head and neck cancer

MAPK inhibitor	Binimetinib
PI3K inhibitor	Buparlisib (BKM120), Alpelisib (BYL719), PX-866, Copanlisib
AKT inhibitor	MK2206
mTOR inhbitor	rapamycin (sirolimus), Temsirolimus (CCI-779), Everolimus (RAD001)
PI3K/mTOR dual inhibitor	SF1126, Gedatolisib, Dactolisib (BEZ235)

From: (Cai *et al.,* 2017) (Wang *et al.,* 2017) (Jung *et al.,* 2018).

1.37 The need for development of drugs targeting EGFR mutation and Ras mutation

As discussed detail in literature review, Cetuximab is targeting to EGFR overexpression and not effective in patients with EGFR variant III mutation,EGFR tyrosine kinase mutation and Ras mutation. So, it is important to develop target drugs for people with these kinds of mutation. Currently, Tyrosine kinase inhibitors Gefitinib and Erlotinib are used to treat EGFR mutated non small cell lung cancer patients. PD98059 have ability to target the down stream of Ras by inhibiting ERK phosphorylation. So, it is important to see the effect of Tyrosine kinase inhibitors (Gefitinib, Erlotinib) and selective MAPK inhibitor (PD98059) in EGFR signalling pathway. Data we have produced during this study will backup/contradict the current and ongoing research with Gefitinib, Erlotinib and PD98059.


Ligands(EGF/ TGFα)
Cetuximab
TGFα, EGFR mRNAs and protein are elevated in oral cancer patients compared with disease free control
cell membrane
TKIs(Gefitinib ,Erlotinib)
GRB2
SOS
PI3K
RAS
STAT
AKT
RAF
MTOR
MEK
PD98059
ERK
NUCLEUS
Cell proliferation, EMT, cell migration, angiogenesis, resistance to apoptosis

Chapter 2 Aim and Hypothesis

This chapter will set out the aims and a hypothesis for the project. It is easier to do this as a list as this give a greater scope for understanding of the project.

2.1 Aims of the project

1) To investigate the effect of EGF and TGFα on proliferation of cancer and control cell lines.
2) To investigate effect on EGF and TGFα on cell morphology.
3) To investigate the status of EMT marker in response to EGF and TGFα.
4) To investigate the effect of EGF and TGFα on migration of cancer and control cell lines.
5) To investigate the phosphorylation of MAPK 202/204 and AKT473 in response to EGF and TGFα.
6) If EGF and TGFα has an effect, to investigate whether Tyrosine kinase inhibitors (Gefitinib, Erlotinib) and the selective ERK1/2 inhibitor (PD98059) inhibit these effect of EGF and TGFα.

The specific studies carried out towards these objectives include:

a) To observe the effect of EGF and TGFα on cell proliferation of HSG, AZA1, TYS and HaCaT cell lines by using automated cell counter.
b) To observe the EMT markers E cadherin and Vimentin on the HSG, AZA1, TYS and HaCaT cell lines treated with EGF and TGFα by using immunofluorescence and Western blot.
c) To observe the localization of total EGFR in response to EGF and TGFα treatment by using immunofluorescence.

d) To observe the effect of EGF and TGFα on morphology of HSG, AZA1, TYS and HaCaT cell line by using photograph as different time point. To investigate whether an hour pre-treatment with tyrosine kinase inhibitor (Gefitinib, Erlotinib) and ERK1/2 inhibitor (PD98059) inhibit the effect of EGF and TGFα.

e) To observe the effect of EGF and TGFα on migration of HSG, AZA1, TYS and HaCaT cell lines by using scratch assay. To investigate whether Gefitinib, Erlotinib and PD98059 inhibit the effect of EGF and TGFα.

f) To observe effect of EGF and TGFα on phosphorylation of EGFR1068, MAPK 202/204 and AKT473 on HSG, AZA1, TYS and HaCaT cell lines. To investigate whether Gefitinib, Erlotinib and PD98059 inhibit the effect of EGF and TGFα.

g) To observe localization of MAPK202/204 and AKT473 on HSG, AZA1, TYS and HaCaT cell line by using immunofluorescence. To investigate whether Gefitinib, Erlotinib and PD98059 inhibit the effect of EGF and TGFα.

2.2 Hypothesis of the study

The hypothesis is that EGF and TGFα would induce the cell proliferation, EMT like morphological change, migration, phosphorylation of tyrosine kinase residues, activation of MAPK and AKT pathway and EGFR internalization.

There was also a hypothesis that Tyrosine kinase inhibitor and downstream inhibitor inhibit the effect of EGF and TGFα.

Null hypothesis would be that growth factors have no effect on cell proliferation, EMT, migration, phosphorylation of tyrosine kinase residues, activation of MAPK and AKT pathway and EGFR internalization.

Pilot experiments were performed by the author during her master thesis to test the suitability of assays.

Chapter 3 Materials and methods

This chapter will be mainly the general methods used in the project. More detailed and specific experimental methods are found in the results chapters.

3.1 The cell lines used in the study and source

Table3.1 The cell lines used in the study and source

Name	Cell type	Derived / originated from	Secondary source	Primary source
HacaT	Normal adult keratinocyte	skin	Prof.S.L. Schor(Late) DDS	(Boukamp *et al., 1988)*
TYS	Oral adeno squamous cell carcinoma	isolated from a well-differentiated squamous cell carcinoma Originated from floor of the mouth and derived from the minor salivary gland	Dr Koji Harada University of Tokushima, Japan	(Yanagawa *et al., 1986)*
HSG	Human salivary tumour cell line	Epithelial duct cell line derived from irradiated Human salivary gland	Prof-Mitsunobu Sato, University of Toushima, Japan	(Shirasuna K *et al.,1981*)
AZA1	HSG cells treated with 5-Azacytidine	Epithelial duct cell line derived from irradiated Human salivary gland.	Prof-Mitsunobu Sato, University of Toushima, Japan	(Shirasuna K *et al.,1981*)

Justification of the cell lines

The cell lines were chosen upon ethnicity of the patients(asian) rather than location of tumour. HaCaT cell was kind gift from Dr Norbett Fusenig. HaCaT cell line was selected as control cell line due to unavailabbility of normal oral cell line. The morphology and function is similar to oral squamous epithelial cells. TYS cell line was a kind gift from Prof. Mitsunobu Sato from University of Tokushima. The cells were obtained from biopsy of primary oral cancer from 81 years old japanese female. Despite isolating from well differentiated squamous cell carcinoma located from flooe of the mouth, it was histopathologically interpreted as acinic cell carcinoma with squamoid lesion.

HSG cell line was also a kind gift from Prof. Mitsunobu Sato from University of Tokushima. The cells were obtained from 54 years old japanese man who received therapeutic radiation for cancer at the floor of the mouth.

AZA1 cells are HSG cells treated with 5-Azacytidine. It is a potent growth inhibitor and cytotoxic gagent. It has ability to inhibits DNA methyltransferase causing DNA demethylation and allow transcription factors to bind DNA and reactivate tumour suppressor genes. In clinic, Azacytine is mainly used to treat myelodysplastic syndrome and some case of acute myeloid leukemia as hypomethylation agent. In vitro, it can be used to remove methyl group. In research 5-azacitidine is used to promote cardiomyocyte differentiation of stem cells. In contract, it promote transdifferentiation of cardiac cells into skeletal myocyte.5-Azacytidine inhibit T cell proliferation, block the cell cycle, inhibit the production of proinflammatory cytokines.(Sanchez-Abarca etal,2010).

3.2 List of equipment

Table 3.2 List of equipment

Name	Make/Origin
Class II biological safety cabinet	Medical Air Technology, Manchester, Uk
Incubator	Thermo Scientific, Waltham, MA,USA
Water bath	Grant Instruments, Cambridge, UK
Centrifuge machine (Mistral 1000)	MSE,London,UK
Pipette	Thermo Scientific, Waltham, MA,USA
Pipette boy	Integra bioscience, Zizers, Switzerland
Nunclone cell culture dish	Thermo Fisher Scientific, Denmark
Light microscope, IX50	Olympus, Tokyo,Japan
Automated cell counter TC10	Bio-Rad, Hercules, CA,USA
Orbital shaker	Stuart scientific, Staffordshire, UK
Centrifuge (Sigma 1-13)	Sigma, Osterode am Hartz, Germany
Inverted fluorescence microscope (IX70)	Olympus, Tokyo, Japan
Plastic pipette	Alpha laboratotories Catalogue LW4061
Cryovial	Fisher brand Catalogue 12-567-501

3.3 List of reagents and antibodies

Table 3.3 List of reagents and antibodies

Cell culture reagents	Company	Catalogue no.
Foetal Calf Serum (FCS)	Sigma-Aldrich, St.Louis,MO,USA	F-2442
Minimum essential Medium Eagle (MEM)	Sigma-Aldrich, St.Louis,MO,USA	M-0275
DMEM – Dulbecco's Modified Eagle Medium (GlutaMAX™ Supplement)	Thermo Fisher Scientific, MA, USA	61965026
EGTA	Sigma-Aldrich, St.Louis,MO,USA	E-8145
Trypsin	Sigma-Aldrich, St.Louis,MO,USA	T-4549
L-Glutamine	Sigma-Aldrich, St.Louis,MO,USA	G-7513
PBS	Sigma-Aldrich, St.Louis,MO,USA	P-4417
HBSS (HANK'S balanced salt solution)	Sigma-Aldrich, St.Louis,MO,USA	H-4641
Methanol	VWR BDH,PA, USA	101586B
Epidermal growth factor	insight biotechnology	Cat No: 10-1033-B,
Transforming growth factor α	insight biotechnology	Cat No: 10-1033
DMSO	Sigma-Aldrich, St. Louis, MO, USA	Cat No:D-5859
Gefitinib	Cell signalling technology	4765
Erlotinib	Cell signalling technology	5083
PD98059	Cell signalling technology	9900
E cadherin	Cell signalling technology	3195-135KDA
Vimentin	Cell signalling technology	3932-57KDA
Anti EGFR Rabbit monoclonal antibody(EP38Y)	Abcam	Ab 52894
Phospho EGF receptor pathway antibody sampler kit (Phospho-EGF Receptor (Tyr1068) (D7A5) XP® Rabbit mAb #3777	Cell signalling technology	9789
Phospho P44/42 MAPK(Erk1/2) (Thr202/tyr204)	Cell signalling technology	4370 (1:2000) WB (1:50) immunoprecipitation (1:100) Immunohistochemistry
AKT473	Cell signalling technology	4060
Antirabbit IgG, HRP linked antibody	Cell signalling technology	7074

3.4 Measuring cell proliferation

Cell proliferation can be measured by a number of methods. These can include everything from the humble Haemocytometer to the labelling of cells with radioactive thymidine (Romar *et al.,* 2016). Work in our laboratory has in the past investigated these other methods and found that the most simple method for studying was to use an automated cell counter. In the last year a major workshop has concluded that for consistency in this area a logical system should be devised (Lin-Gibson *et al.,* 2018). Using our own system which is a fully automated system (TC10 from Biorad) allows us the investigated cell growth in this manner. Automated cell counter is also one of the easiest ways to measure the cell proliferation giving both accurate counting and quick and reproducible results. Comparison studies have shown that they are quicker and easier than Haemocytometer (Cadena- Herrera *et al.,* 2015). In this chapter, 4 different cell lines are used three from various Head and neck tumours being epithelial in character and one “normal” epithelial cell line.

3.5 Cell culture

All the cells were cultured at 37° Celsius (°C)and 5% CO_2 in MEM with 10% Foetal calf serum (FCS) and 200mM glutamine. All the tasks such as sub culturing, passaging, cryopreservation and resuscitation were done according to standard laboratory protocol set up by the Unit of Cellular and Molecular Biology, the Dental School, University of Dundee, UK.The cells were sub-cultured 1 to 2 times a week depend on confluency of the cells. Growth medium was changed every 3 to 4 days the spent medium was aspirated off and replaced with fresh medium. All the stock cells were

grown on 90mm dishes. Once the dish (35mm or 60mm) was decided upon would ascertain how the dishes were prepared. A 90mm stock dish was taken out of the incubator. Media was aspirated off washed twice with HBSS and replace with 2 ml of trypsin. Then the dish was placed into incubator for 5 mins. The cells need to be scrapped off if they are firmly attached to dish. 4mls of 10% FCS was added into dishes to neutralised the trypsin and suspension were then transferred into a universal tube using a plastic pastette and centrifuge for 5 mins at 900rpm. The trypsin/media mixture was aspirated off and then cell pellet was re- suspended in the appropriate volume of 10% FCS either 2mls for 60mm dishes or and 1ml for 35mm. Fresh dishes were prepared, the number and size would depend on the intention of the experiments, adding 2mls of 10% FCS to each of 60mm dish or 1ml to each of 35mm dishes. The cells suspension was then added into the prepared dishes and the cultures incubated at 37°C. This is described as day 0 and dishes were placed in the incubator overnight. The next day, the cells were checked for their confluency, healthiness and sign of any bacterial and fungal contamination.

3.6 General cell culture for maintenance of stock cells

For sub-culturing, 90mm culture dish was taken from incubator and check for confluency and heathiness and sign of bacteria and fungal contamination. Once they became confluence, spent medium was aspirated off. The dishes were washed twice with Hank balanced salt solution (4mls) and then 2mls Trypsin/EGTA was added. The dishes were transferred onto 37°C hot plate or in incubator for 5minutes. The cells were normally float off from the dish.The cells need to be scrapped off if they are firmly attached to dish. 4mls of 10% FCS was added into dishes to neutralised the trypsin and suspension

were then transferred into a universal tube using a plastic pastette. The cells were counted using a cell counter and then centrifuged at 900rpm for 5 minutes. The trypsin/media mixture was aspirated off and then cell pellet was re- suspended in the appropriate volume of 10% FCS 2mls for each 90mm dish. Fresh dishes were prepared and 5mls of 10% FCS were added to each of 90mm dish. The cells suspension was then added into the prepared dishes and the cultures incubated at 37°C and 5% CO2. On the next day, cells were checked for confluency and heathiness. Media (10%FCS) was changed every 2 to 3 days.

3.7 Cryopreservation and resurrection

The dishes were washed twice with Hank balanced salt solution (4mls) and then 2mls Trypsin/EGTA was added. The dishes were transferred onto 37°C hot plate or in incubator for 5minutes. The cells were normally float off from the dish.The cells need to be scrapped off if they are firmly attached to dish. 4mls of 10% FCS was added into dishes to neutralised the trypsin and suspension were then transferred into a universal tube using a plastic pastette. The cells were then centrifuged at 900rpm for 5 minutes. The trysin/media mixture was aspirated off and then the pellets were re-suspended in 1ml freeze mix (10% DMSO/40% FCS/50% media) for each of cryovials. The vials were then stored in -80°C.

For resuscitation of cells, cryovials were put in 37°C water baths to defrost. At the same time 90mm dishes were prepared by adding 5mls of 10% FCS. Cryovial became warm within few mins in the water bath. Once the cells defrosted, the cells were poured into cluture dishes (1 dish for 1 vial) and then incubated overnight. Next day, when the cells

are fully attached, the medium was changed. All the procedure were written in cell culture log book.

3.8 Scatter assay

Scatter assay is a simple low-cost method used to investigate the cell morphology and individual migration of the cells. The cells were first passaged and plated at 1x 10^5 per dish and allowed to attach until it reach to mid confluency. The cells were serum starve overnight. Next day, different concentration of growth factors and inhibitors were added into the dishes. The photographs were taken at appropriate time out to monitor the cell morphology and migration of the cells. Detail step by step procedure for each of the experiments were described in 4.3.2

3.9 Definition of epithelial characteristic characteristics

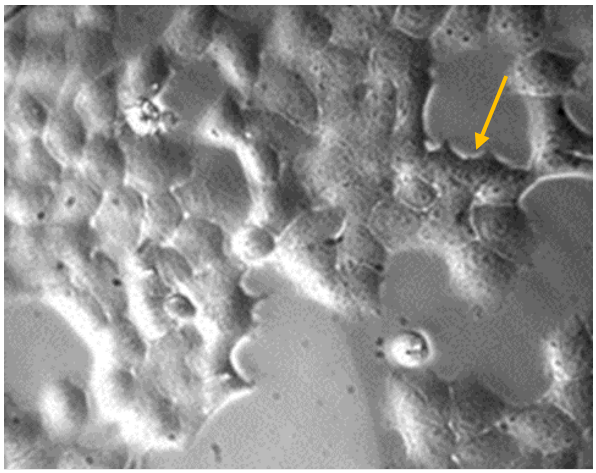


Figure 3.1 Epithelial shape cells- note the closely connected cells with cuboidal shape

3.10 Definition of mesenchymal characteristic characteristics

As seen in Figure 3.2:

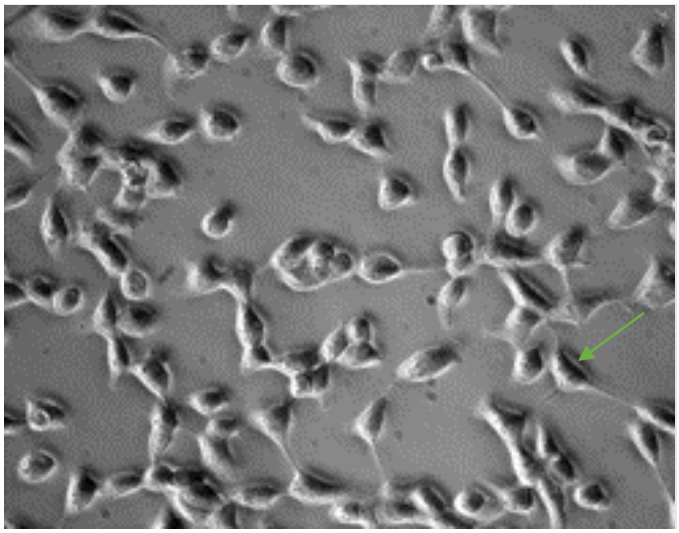


Figure 3.2 Mesenchymal shape cells- note the green line indicates loosely connected cells with and elongated shape.

3.11 Scratch assay

Scratch assay is a simple low-cost method which is used to measure the differences in cell migration rates between treated and untreated cells. The cells were seeded at $1x10^5$per dish. Once the cells reached 90% to 100% confluence. They were put into serum free and left overnight. Next day, the scratch line was made by using white pipette tip. The dishes were washed with serum free for two times to remove the debris. Different concentration of growth factors and inhibitors were added into the dishes. Photographs were taken at appropriate time point. Detail step by step procedure for experiment was describes in 4.3.3.

3.12 Immunofluorescence

Immunofluorescence is a technique using specific antibodies and antigens conjugated to fluorescent dyes which allows visualization of the distribution of the target molecule inside the cell or tissue (Cell signal, 2019).

Cells are grown on plastic tissue culture dishes and incubated in test conditions. The first step is cell fixation. After the pre-requisite time the cells to be analysed need to undergo fixation. The dishes containing the test cells were washed with PBS and then fixed with ice-cold methanol for 20 minutes. After that, the dishes were washed with PBS 3 times for 5 minutes during which time each dish was placed on an orbital shaker. Then each dish was washed with 0.2% Triton X-100 in PBS for 5 minutes (placing dish on orbital shaker). The dishes were washed a further 3 times with PBS for 5 minutes and each time placed on an orbital shaker. After that a number of circles were drawn onto an area of the dish using an immuno pen.

The second step is known as blocking. The circles had a few hundred µl of 5% Normal serum/PBS-T (TBS+ 0.1% Tween 20) placed onto them (enough to cover the circle and for it to not dry out) at room temperature for 60 minutes. After 60 minutes the blocking buffer was removed and circled area was incubated with primary antibody in a humidified chamber (in the fridge, 4°C) overnight. The best temperature was set 4°C. Note the primary and secondary antibody is diluent was 5% normal serum in PBS-T (PBS+0.1% Tween 20). The next morning, the dishes were equilibrated to room temperature for 60 minutes. The dishes were then rinsed with PBS gently and then washed with PBS. Secondary antibody was added to the circled areas and dishes were kept in a humidified chamber for 1 to 2 hours. After that dishes were then washed gently

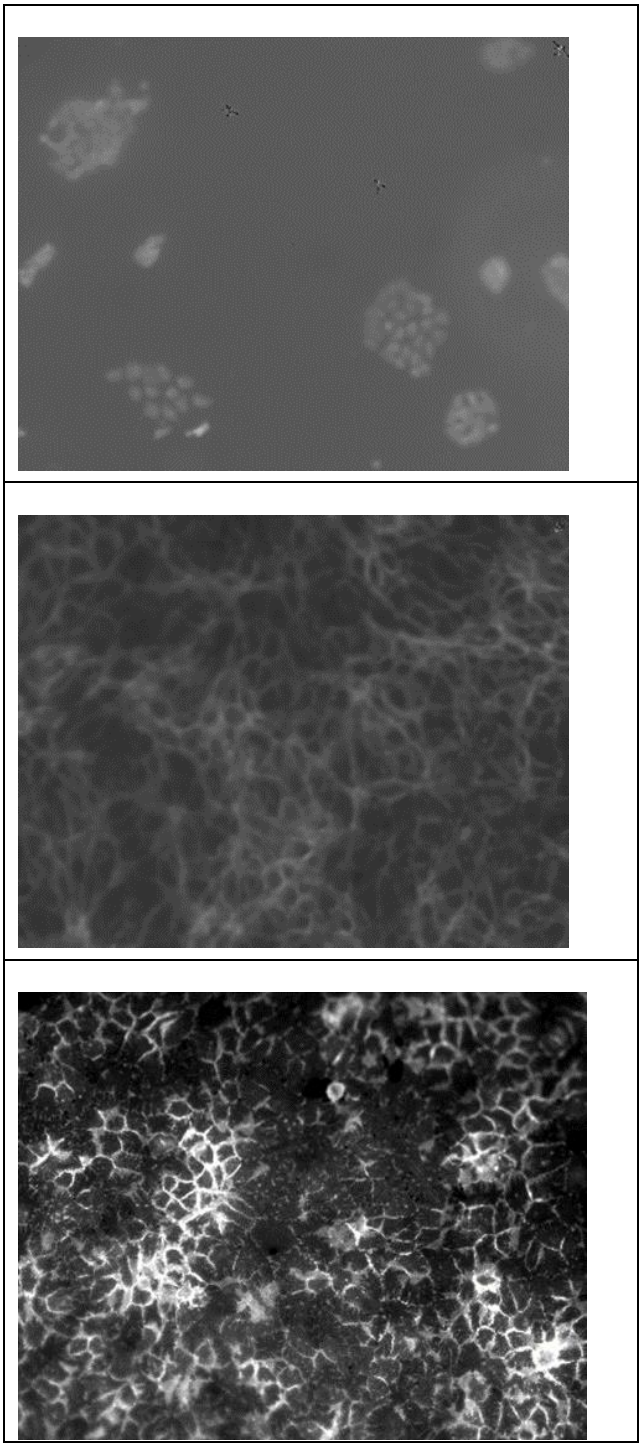
with PBS. And then washed again with PBS. Dishes were mounted with DPX and coverslips before examination under microscope and stored in the dark cold room at 4°C.

3.13 Example of weak, moderate and strong staining (Figure 3.3)

The three photomicrographs below are examples of the staining described in the results chapters. The upper picture is weak staining, the middle picture is moderate and the bottom picture is strong staining.

Figure 3.3: Example of staining patterns

Top is weak staining, Middle panel is medium staining and bottom panel is strong staining.



3.14 Cell lysis

In order to lyse the dishes, ice trays were prepared. One tablet of Protease and one tablet of phosphatase inhibitor were put into 10ml RIPA buffer and put it on the ice tray for few minutes until it dissolved. Placed the cell culture dishes on the ice. Dishes were washed with ice cold PBS. Then aspirate PBS and add ice cold lysis buffer (0.5ml per dish) for 10 minutes on ice tray. The cells were than scraped with cell scraper and transfer the cell lysate into label microcentrifuge tube. The tubes were stored in the fridge.

3.15 Protein assay -Pierce BCA protein Assay Kit (23225)

It is used to determine the concentration of protein in solution. Copper ($Cu2^{+}$) salt was utilized in this method to cuprous state by proteins. The $Cu2^{+}$ion form an intense coloured complex with bicinchninic acid reagent. Intensity of colour is proportionate with amount of protein in the sample. 96 well plate was used in this experiment with Standards were in triplicate and samples were either duplicate or triplicate. Lysate samples were defrosted and Needle and syringe were used to break down the gelation of lysate samples. Lysates samples were spun for 5minutes (1200rpm) in bench top microfuge and supernatants were used. Standards were made up as follows and Diluent was RIPA buffer.

Preparation of Diluted Albumin (BSA) Standards

Table3.4 Preparation of Diluted Albumin (BSA) Standards

Vial	Volume of Diluent	Volume and source of BSA(µL)	Final BSA concentration(µg/ml)
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of viral E dilution	250
G	325	325 of viral F dilution	125
H	400	100 of viral G dilution	25
I	400	0	0= Blank

Dilution scheme for Standard Test Tube Protocol and microplate procedure (working range= 20-2,000µg/ml).

25µl of standards and samples were pipetted into appropriate wells according to plan.

BCA working reagent was made by mixing 50 parts BCA reagent A with 1 part of reagent B.

Total volume WR required= (no of standard+ no of unknown) x (number of replicate) x (volume of WR sample).

200µl of working reagent were added into wells. The plate was put on the shaker for 30 seconds. The plate was wrapped in cellophane and then incubate for 2hrs at 37'C. The plate was cool to room temperature for 10-20 minutes before reading on OPTIMA plate reader.

3.16 SDS PAGE

SDS PAGE is a technique used to separate proteins present in cell lysates according to their sizes. Prior to SDS PAGE, the cell lysates and loading buffer which contain SDS and 2 Mercaptoethanol were denatured by heating at 95 degree Celsius water bath. Heating allows denaturing of the proteins and loss its 3D structures. SDS is anionic detergent which bind to denatured protein. The proteins are migrated according to the size. Magic marker XP from Invitrogen were run for reference to estimate the protein fraction. The separated proteins were transferred into nitrocellulose membrane or PVDF by western blot. Different antibodies were used to probe the specific proteins.

The procedures were described as follows:

Mini-Protean Precast Gel (TGX) set up overview

10 % gels which fractionate proteins between 30 and 150kDa Biorad TGX precast gels.

First is to remove the comb by pushing the ridges of the comb with two thumbs. Then the orientation is of the wells were checked and correct with disposable pipette if it has dis alignment. Then remove the Green tape at the bottom of cassette. The wells were then rinse with TGS running buffer. Cassette were then assembling into the running module of mini PROTEAN system. TGS running buffer were prepared by diluting 10x stock to 1x (100ml with 900 ml of distilled water). Then TGF running buffer was added to inner and outer chambers of the electrophoresis tank.

For SDS-PAGE, cell lysates were thawed and spun at 13,000 rpm for 5 minutes. Suitable amount of sample were aliquot into labelled 500µl Eppendorf tube. Samples were then mixed with appropriate volume of loading buffer including 5% (v/v) 2 – mercaptoethanol. Samples were then heated at 95'C in a water bath for 5 minutes then centrifuge briefly. Samples were loaded onto wells which fractionate proteins between 30 and 150kDa Biorad TGX precast gels. Gels were then connected to a power pack set at the voltage of 150V (which may be increase to 180-200V in the middle of running depend on the current) and run for approximately 40-45 minutes. The electrophoresis was continued until the blue line reach line reaches the black line at the bottom of the cassette. Then power was turn off, disconnect the gel rig, remove the cassettes and the running buffer was pour into the sink.

After removing the gels gently from the cassette they were placed into distilled water and viewed under the UV of gel doc machine to know whether it is transfer properly. Then gels were placed into transfer buffer.

3.17 Western blot

Western blot is a method used to transfer the proteins bands from SDS-PAGE onto a membrane using an electric current. After transfer, the non-specific binding sites on the blot were blocked by incubation in blocking buffer. The immobilised bands were then probed with specific antibodies against the target proteins (primary antibodies). The primary antibody was then added and labelled with the enzymes horse radish peroxide for visualisation. The substrate was added to the enzyme HRP generating chemiluminescene in in order to locate the antibody. Protocol for western blotting are described as follow.

After SDS-PAGE, the low fluorescence PVDF membrane was cut into appropriate size enough to cover the gel and marked with pencil for orientation. Then PVDF was soaked into methanol for 30 seconds then placed in transfer buffer. BioRad extra thick paper was cut into two pieces, slightly bigger than gel size. Then placed into transfer buffer. BioRad extra thick paper was placed on the centre of the electrode. Then PVDF was placed on top of think paper. Acrylamide gel was placed carefully on the top of PVDF. Then covered with remaining BioRad thick paper. Roller was used to eliminate air bubbles between layers. Then run at a constant voltage of 15V for 42 minutes. Blocking buffer (1% w/v dried milk or 5% BSA depend on primary antibody in 1x TBST) was prepared and incubated with the blots for 10 to 30 minutes. Blots were then incubated with primary antibody anti E-cadherin(1:1000), anti-Vimentin(1:1000), anti-

EGFR(1:1000), anti-P44/42MAPK(Erk1/2)Antibody(1:2000), anti-phospho p44/42 MAPK(Erk1/2)(Thr202/Tyr204)(1:2000), anti-phospho -akt(Ser473)(1:2000) for overnight. Maintained the temperature according to manufactures instructions. Antibody solutions were rinsed off with 1xTBST. Then blots were washed three times in 1x TBST, 20 minutes per wash. The blots were incubated with goat anti-rabbit secondary antibody (1:2000) in blocking buffer on orbital shaker at room temperature for an hour. Blots were then washed 2 times in 1xTBST 20 minutes per wash. Rinsed the blot thoroughly with 1xTBST and then 1xTBS for 5 minutes and transferred into clean vessel. Developed the blot with 3.5ml of each reagent of the chemiluminescent substrate: BioRad Clarity Western ECL Substrate. Incubate for 5 minutes. Chemiluminescence was detect by using GelDoc system.

Chapter 4: To study the effect of the growth factors EGF and TGFα on proliferation, morphology and the migration of head and neck cancer cell lines using changes in cell shape as a marker of Epithelial Mesenchymal Transition

4.1 Introduction

In this chapter, we investigated cell proliferation, morphology changes (EMT), and cell migration. Each of these was measured by using an automated cell counter, a scatter assay, and scratch assay respectively in the presence and absence of treatment with EGF and TGFα.

4.1.1 Proliferation

One of the hallmarks of cancer is the overproduction of growth factors (Hanahan & Weinberg, 2000). Cell proliferation is a normal process in which an increase in cell numbers is the result of cell growth and cell division. In a normal healthy individual cell proliferation is balanced. This means that as cells become worn out they are replaced and there is no overall increase in the total. In contrast, the cell proliferation rate can be increased in cancer leading to an overall increase in the number of cells that are present in tissues. This has implications on cell blood supply and the amount of nutrients within a tissue and also the removal of waste products. One of the major factors in any tumour growth is interaction of growth factors with cellular receptors. The EGFR family of receptors has been implicated in a large number of tumour situations (Salomon *et al.,* 1995). In fact, in breast cancer EGFR status is part of the diagnosis of the cancer (Milanezi *et al.,* 2008). It has been reported that EGFR could be used either as a prognostic factor in HNSCC (Bossi *et al.,* 2016). Data in this paper also suggested that ligands of the receptor may also be factors that can be used as prognostic markers. TGFα and EGF are two of a family of ligands that bind to EGFR (Roskoski, 2014). Experimental data indicates that these growth factors have an effect on cellular proliferation. However, this data is cell and tissue dependent.

Cell proliferation is a major important part of cancer development and progression (Schafer, 1998). Assessing cell proliferation is one of the fundamental research areas in medicine. There are various ways to determine the cell proliferation such as measuring cell number, DNA synthesis (BrdU, EdU), cellular metabolism (MTT, WST01), proliferation proteins (Ki-67, PCNA, MCM-2).

4.1.2 EMT and migration

The majority of cancer deaths are caused by metastasis of tumour cells to secondary sites (Chaffer & Weinberg, 2011). Metastasis is defined as the spread of a cancer from the primary site to other parts of the body. Cell migration is a pivotal process in metastasis. Cancer cells degrade the extracellular matrix and basement membrane, break through surrounding tissue, enter blood or lymphatic stream, attach at a new site and grow into a secondary tumour mass(Guan X, 2015). Cancer cells have the ability to breakdown cell to cell adhesion and cell to extracellular matrix adhesion. These adhesions are broken down by secretion of substances collectively known as matrix degrading enzymes (usually ascribed to a family of proteins known as Matrix Metalloproteases MMP'S) which degrade both the ECM and the basement membrane. Loss of cell to cell adhesion allows the cancer cells to dissociate from the primary tumour. Therefore these cells become individual cells so they can change shape and migrate by the loss of cell to matrix adhesion the cells will be able to invade the surrounding stroma(Manzanares & Horwitz, 2011).For this to occur there is a down-regulation of E-cadherin leading to a break down in cadherin- catenin link(Hajra& Fearon, 2002).

Epithelial cell shape plays an important role in a tissue. In the oral mucosa they form a barrier to keep important molecules in the body and invasive bacteria and other things

out. However, when it all goes wrong this barrier can break down and the cells can invade into the outlying area. To migrate away from this area the cells need to break down the tight cell-cell junctions and be able to move to distant sites. One theory is that this occurs by epithelial to mesenchymal transition (EMT). Normal epithelial cells grown in tissue culture grow as tight colonies but once the cells undergo EMT they have a more fibroblastic phenotype (Figure 4.1).

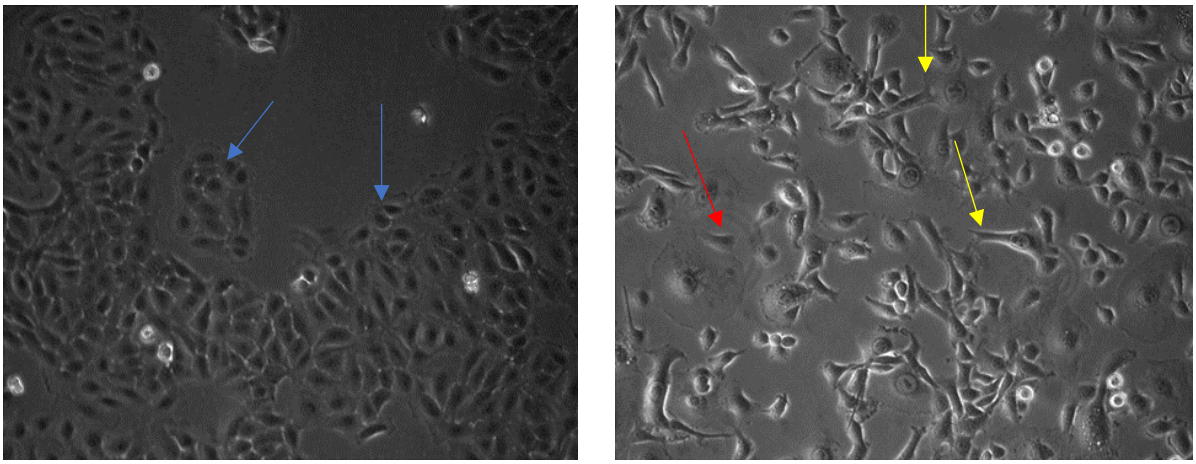


Figure 4.1 EMT a) *Epithelial cells- HaCaT are typical epithelial cells when grown in culture as tight colonies- blue arrow. They are a regular shape. b) Epithelial cell line (EMT6-picture provided by Dr I Ellis) a cell line that has undergone EMT- cells do not grow in tight colonies and have* irregular shape (red arrow) and different projections (yellow arrows). 80X magnification

EMT is the process by which cells transform from closely connected cuboidal shaped epithelial colony to a more elongated motile mesenchymal shape (Thiery & Sleeman, 2006). EMT is reversible process and can be found in both normal and disease conditions (Kalluri & Weinberg, 2009). EMT is the initial first step in cancer cell migration, invasion and metastasis. Invasion and metastasis is another hall mark of cancer (Hanahan & Weinberg, 2000). Growth factors such as EGF, TGFα, FGF, HGF, bone morphogenesis proteins (BMPs), and extracellular matrix such as, collagen, hyaluronic acid (HA), as well

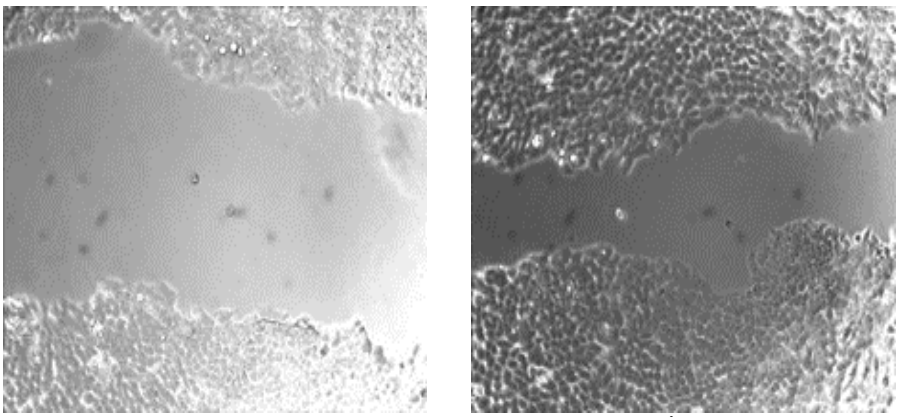
as chronic inflammation and hypoxia are known to induce EMT (Thiery & Sleeman, 2006). It has also been reported that cells undergoing EMT have stem cell like characteristics and are resistant to therapy. The restoration of E-cadherin can increase the sensitivity to EGFR-inhibitors (Mani *et al.,* 2008, Singh & Settleman, 2010).

One of the indications of EMT are the changes in the so-called markers of EMT. These markers are described in detail in the introduction (Chapter 1.34). EMT induces the down-regulation of epithelial markers such as E-Cadherin, Z0-1, Desmoplakin, cytokeratins, occudin and the upregulation of mesenchymal markers such as vimentin, N-cadherin, integrins αvβ6, Fibronectin, FOXC2 (Neilson& Zeisberg, 2009, Lee JM, 2006).

One of the most studied areas is the down regulation of the epithelial marker E-cadherin and the subsequent up regulation of the mesenchymal marker vimentin which have been reported in both EMT and cancer metastasis (Neilson & Zeisberg, 2009, Nijkamp *et al.,* 2011). E-cadherin is a calcium dependent adhesion molecule which links actin cytoskeleton through interaction with catenins. The cadherin-catenin complex plays an important role in the adherens junction which are essential for the homeostasis of epithelial tissues (Pećina-Slaus, 2003). In contrast, vimentin is an intermediate filament (IF) protein expressed primarily by mesenchymal cells. Vimentin is important for maintaining the cells integrity via the cytoskeleton, orienting microtubules, morphological changes and the migration of cells (Liu *et al.,* 2015, Mendez *et al.,* 2010).

Dysregulation in EGFR signalling has been reviewed with cancer cell proliferation, migration, invasion and metastasis, resistance to apoptosis (Wee & Wang 2017, Ohnishi *et al.,* 2017, Lu *et al.,* 2001, Andl *et al.,* 2004).

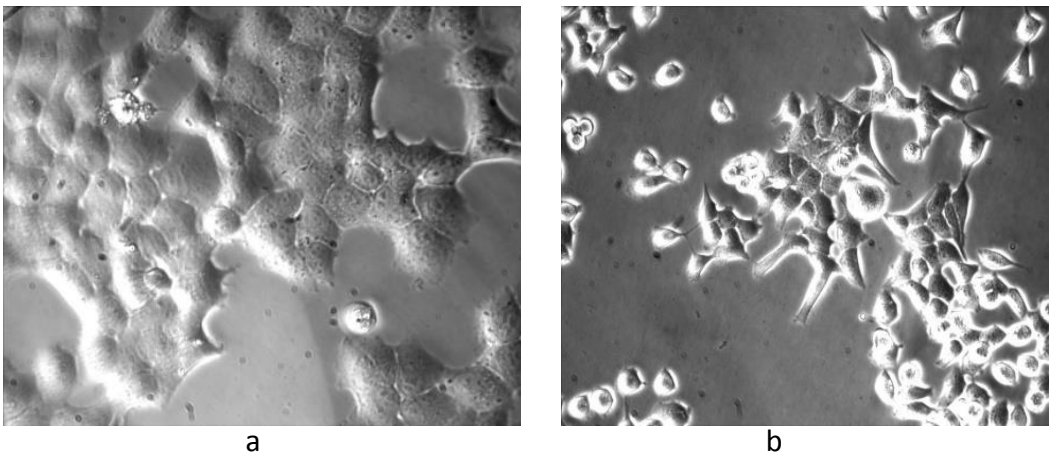
There are two types of cancer cell migration. Individual cell migration and collective cell migration. The contrast between collective cell migration where cells move as groups or sheets (Figure 4.2 a, b) and individual cell movement where cells need to break contacts and move individually is quite stark (Figure 4.3 a, b) (Friedl, 2009). Collective cells movement follows three principles in that cells remain: 1) attached both physically and functionally. 2) The polarity causes the actin filaments to generate traction. 3) The cells are able to modify the tissue in their path (Friedl, 2009).



a b

Figure 4.2 collective cell migration: *HSG cells plated onto the surface of a plastic tissue culture dish were grown to confluency. They surface was scratched and the migration noted after 24 hours. Note the gap has narrowed No single cell are visible in the gap so we surmise that the cells have moved as a collective a) HSG cells in time 0, b) HSG cells after 24 hours (Magnification:200x)*

In individual cell migration, cells to cell contacts are lost with and there is the formation of ameboid or mesenchymal phenotype (Figure 4.3 a. and b). Cell produce different types of extensions when they migrate: Filopodia (finger like projection), lamellipodia (broad like protrusion), podosomes and invadopodia are cellular protrusion which forms at the edge of migrating cells (Yamaguchi *et al.,* 2005).



a b

Figure 4.3 Cell scattering : *HSG cells plated onto the surface of a plastic tissue culture dish were grown to 50% confluency. EGF 50ng/ml was added into the dish. After 48 hours, the cells became scattering with some projection appeared. a) HSG cells in serum free, b) HSG cells in EGF 50ng/ml (200X magnification)*

Using the Scatter assay it should be important to reflect on the different types of cell movement. In this assay the cells start as a colony and appear to reduce the cell-cell contacts as they move. They move as individual cells moving away from the colony.

4.2 Aim and hypothesis

The study in this Chapter of the thesis will investigate the addition of the growth factors EGF and TGFα to head and neck cell lines and measure their response of cells in terms of proliferation, EMT like morphology changes, and migration.

Pilot experiments were done in author master thesis to test suitability of assays(Thwe A,2015).

Previous work in this laboratory using a limited number of concentrations of EGF and TGFα (1 and 10ng/ml) indicated that they had no effect on the proliferation of a cancer cell line (TYS) and a normal oral mucosa stromal cell line (MM1). The aim of this part of the study was to investigate the role of a wider range of concentrations of the growth factors on cell proliferation. The working hypothesis was that neither growth factor would stimulate the proliferation of these cell lines.

The literature has suggested that EGF can stimulate the morphological change in cell shape that is consistent with EMT. Our aim is investigate changes in morphology and migration in response to EGF and TGFα. Our hypothesis is that both growth factors will stimulate morphology changes and migration.

4.3 Materials and methods

4.3.1 Experimental details using Automated cell counter

Cells were sub-cultured as described in chapter3 (3.5). Once the dish (35mm or 60mm) was decided upon would ascertain how the dishes were prepared. The trypsin/media mixture was aspirated off and then cell pellet was re- suspended in the appropriate volume of 10% FCS either 2mls for 60mm dishes or and 1ml for 35mm. Fresh dishes were prepared, the number and size would depend on the intention of the experiments, adding 2mls of 10% FCS to each of 60mm dish or 1ml to each of 35mm dishes. The cells suspension was then added into the prepared dishes and the cultures incubated at 37°C.

In the initial pilot experiment the cells were first passaged and then plated at an initial density of $5x10^4$ density. However, due to wanting to investigate cell proliferation over a greater number of days an initial pilot study was performed using the AZA1 cell line. This involved plating cells at an initial density of $5x10^3$, $1x10^4$, $2x10^4$, $2.5x\ 10^4$ and $5x\ 10^4$ and counting the cells on Day 1-5. In later experiments, it was changed into $2.5x10^4$ (Figure 4.4) indicates that this density was the best for cell proliferation assays due to normal growth phases (lag phase, log phase and saturation density). Cells were passaged and then plated onto 35 mm dishes and this is described as day 0 and dishes were placed in the incubator overnight. The next day, the cells were checked for their confluency, healthiness and sign of any bacterial and fungal contamination. Medium was prepared for each of the test condition (1ng/ml, 10ng/ml, and 50ng/ml) of both EGF and TGFα in serum free medium.

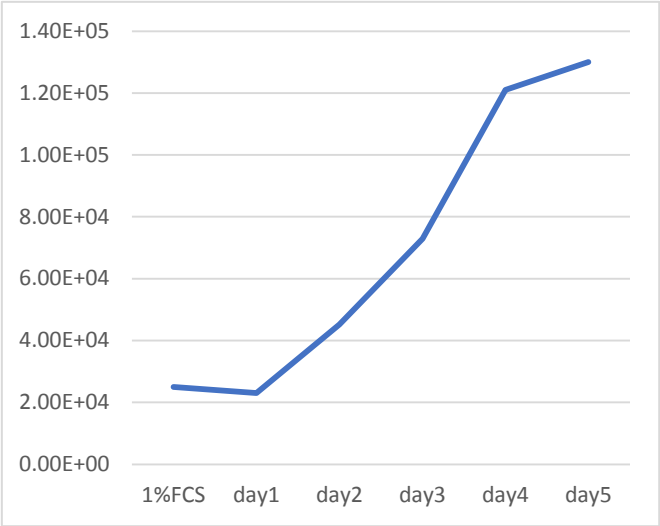


Figure 4.4 *The AZA1 cell line was plated at different densities ($5x10^3$, $1x10^4$, $2x10^4$, $2.5x\ 10^4$ and $5x\ 10^4$) onto 35mm dishes in 10% FCS. The cells were allowed to attach overnight and transferred to test conditions (1% FCS) the following day. Cell number was determined by cell counting by using automated cell counter. The data shown is from a plating density of $2.5x10^4$ cells. This shows a typical growth curve to a saturation density of around $1.3x10^5$ cells. Separate experiments were performed for each plating density data not shown here.*

The control was 1% FCS. Medium was changed into the test condition on day 1 and a base line count was performed on day 1. Overlay was changed day 3 or day 4. At first the experiment was set for 3 days. However, data from the pilot experiment enabled us to change to 5 days cell count. In order to count the cells, the cells were first trypsinized for 5 mins. Then pipette 10µl of the cell suspension into chamber of cell counting slide. While loading the sample, the pipet tip was placed 45˚ angle at the bottom of loading area. And then simply inserting the slide into slide slot of instrument. Within 30 seconds cells number was be appear on screen. After recording the cells number, slides could be taken out.

4.3.2 Experimental details for scatter assay

Cells were sub-cultured as described in the General method Chapter 3 (3.5, 3.6)

Once the dish (35mm or 60mm) was decided upon would ascertain how the dishes were prepared. The trypsin/media mixture was aspirated off and then cell pellet was re-suspended in the appropriate volume of 10% FCS either 1.5mls for 60mm dishes or and 1ml for 35mm. Fresh dishes were prepared, the number and size would depend on the intention of the experiments, adding 2mls of 10% FCS to each of 60mm dish or 1ml to each of 35mm dishes. The cells suspension was then added into the prepared dishes and the cultures incubated at 37°C and 5% CO_2. On the next day, the cells were serum sterve for 24 hours before addition of (1ng/ml,10ng/ml,50ng/ml) of EGF and (1ng/ml,10ng/ml,50ng/ml) of TGFα. The cells were monitored for 48hours. The photomicrographs were taken at appropriate time. Results were summerized in section 4.5.2 (figure 4.13).

4.3.3 Experimental detail for scratch assay

The cells were seeded at $1x10^{5}$ per dish. Once the cells reached 90% to 100% confluence, they were put into serum free and left overnight. After 24 hours, the scratch line was made by using white pipette tips (which is the smallest one). The dishes were washed with serum free for two times to remove the debris and then added test condition of EGF(1ng/ml,10ng/ml,50ng/ml) and TGFα(1ng/ml,10ng/ml,50ng/ml). Serum free is used as control. The cells were monitored for 48 hours. Photographs were taken at appropriate time points.

4.3.4 Experimental detail for Immunofluorescence

After the scatter assay, dished used in the scatter assay were fixed, washed and then blocked with Ecadherin and Vimentin antibodies with dilution 1:200 and 1:50 respectively as described in chapter3 (3.12). Once it finished photographs were captured the areas inside circled areas and recorded.

4.3.5 Experimental detail for Protein assay, SDS PAGE and Western blot

The four cell lines were grown to confluence, treated with the respective growth factors for 24 hours and then the cells lysed (as described in Chapter 3(3.14). Protein assay, SDS PAGE and Western blot were performed with Ecadherin and Vimentin antibodies as described in Chapter 3(3.15,3.16,3.17).

4.4 Results

The results part of the chapter are split into the effect of the growth factors EGF/TGFα on proliferation, scattering/morphology changes, migration/gap closure, localisation of markers/immunofluorescence/SDS-PAGE/Western blotting into a number of different sections:

4.4.1 Proliferation rates of the 4 cells lines as determined by automated cell counter

Cells proliferate at different rates depending on their origin, growth factor receptor expression and availability of resources within the cell. The cell lines used in this study were from a number of different sources. They all had characteristics of being epithelial in nature. However, they obtained from different sites of the oral cavity. The HaCaT cell

line is from skin and so not directly from the oral cavity but they come from a stratified squamous phenotype origin and can grow this way in culture.

The experimental pilot indicated that the AZA1 cell line grew in such a way as to follow classic cell biological standard in that Figure 4.4 has an initial plating density, a lag phase, log phase and a saturation density. It is interesting to note that the 3 cancer cell lines used have a very standard saturation density whilst the HaCaT cell line was lower.

Table 4.1 saturation density for each cell line

Cell lines	Saturation density for 1%FCS
HSG	$2x10^5$
AZA1	$2x10^5$
TYS	$1.8x10^5$
HaCaT	$1x10^5$

Cell proliferation studies monitored the effect of the growth factors EGF and TGFα upon each. As described in the methods section we originally determined a plating density of 2.5 $x10^4$ cells per dish was best for a 5 days growth curve. Cells were plated at this density and allowed to attach overnight. After an initial count the medium was changed into the test conditions. Cell number was determined over the next four days using an automated cell counter. For ease of understanding the data is presented in two different formats. The first Figure breaks down the graphs into each growth factor (*e.g*. Figure 4.5) across the days and the second Figure puts all the data on two graph so that we can compare the effect each factor has on that proliferation (*e.g*. Figure 4.6 A and B).

Data is presented in Figure 4.5 for the cancer cell line HSG. This cell was originally isolated from the collecting duct of an irradiated human salivary gland. The data shows each individual growth conditions and Figure 4.6 A and B then grouped on two graphs. The data indicates that for the HSG cell line the cells reach a saturation density at around

$2x10^5$ cells for 1% FCS. However, addition of the growth factors indicates lower growth and a lower saturation density (Figure 4.6). This finding is inconsistent over a number of experiments.

Data is presented in Figure 4.7 for AZA1 cells. This cell was originally HSG cells treated with 5-Azacytidine. The data shows each individual growth condition and Figure 4.8 (A and B) then grouped on two graphs. The data indicates that for AZA1 cells, the cells also reach a saturation density at around $2x10^5$ cells for 1% FCS. In contrast, addition of growth factors reduce growth and a lower saturation density (Figure.4.8).

Data is presented in Figure 4.9 for TYS cells. This cell was derived from floor of the mouth. The data shows each individual growth condition and Figure 4.10 (A and B) then grouped on two graphs. The data indicates that for TYS cells, the cells also reach a saturation density at around $1.8x10^5$ cells for 1% FCS. However, addition of growth factors reduce growth and lower saturation density (Figure 4.10).

Data is presented in figure 4.11 for HaCaT cells. HaCaT cell was derived from skin. The data shows each individual growth condition and figure 4.12(A and B) then grouped on two graphs. The data indicates that for HaCaT cells, the cells reach a saturation density at around $1.5x\ 10^5$ for 50ng/ml EGF and 50ng/ml TGFα. In contrast, 1%FCS, 1ng/ml EGF, 10ng/ml EGF, 1ng/ml TGFα, 10ng/ml TGFα reduce growth and a lower saturation density $1.3x10^5$(Figure 4.12).

Figure 4.5: HSG cell proliferation data

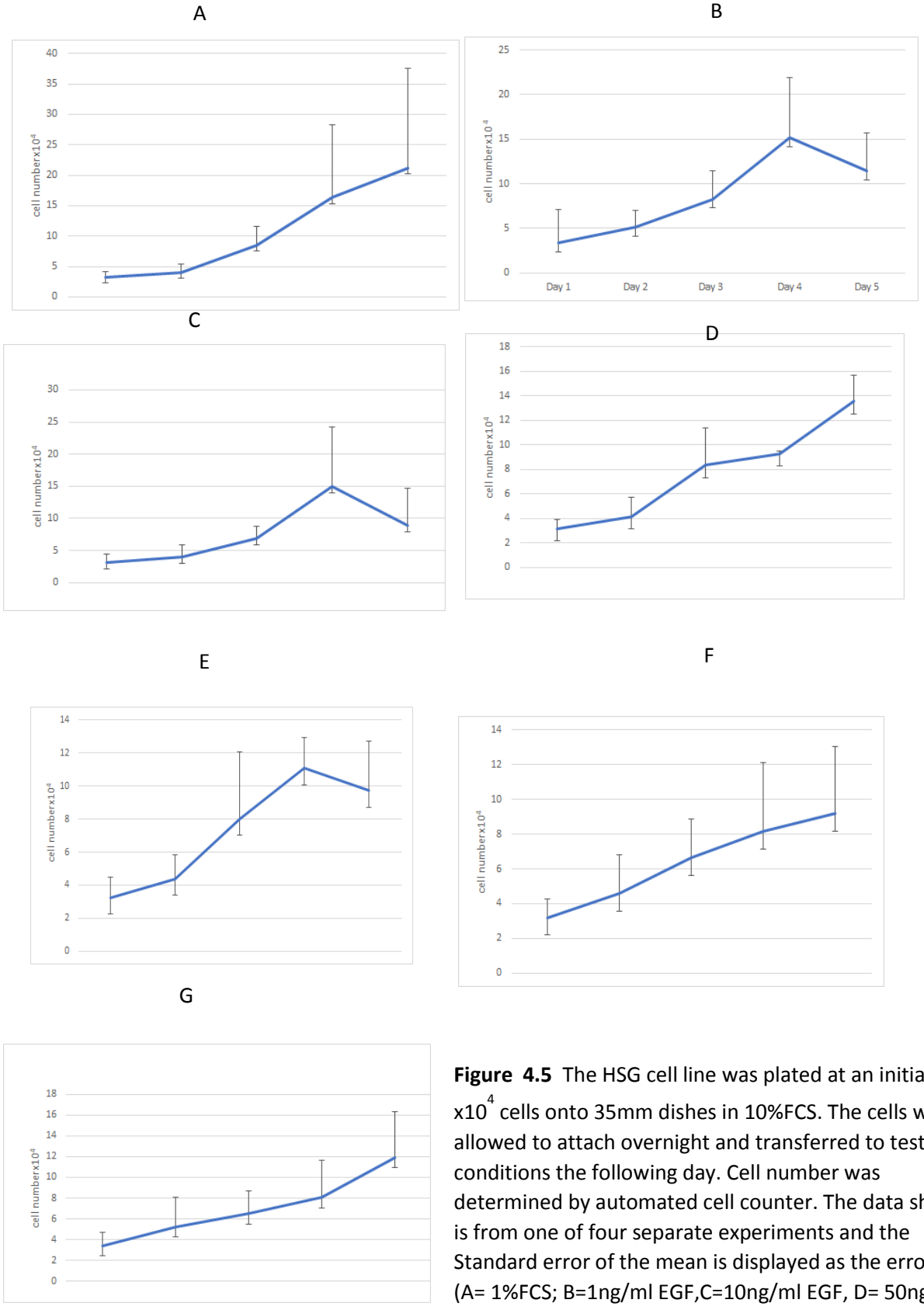


Figure 4.5 The HSG cell line was plated at an initial 2.5 x10^4 cells onto 35mm dishes in 10%FCS. The cells were allowed to attach overnight and transferred to test conditions the following day. Cell number was determined by automated cell counter. The data shown is from one of four separate experiments and the Standard error of the mean is displayed as the error bars. (A= 1%FCS; B=1ng/ml EGF,C=10ng/ml EGF, D= 50ng/ml EGF,E=1ng/ml TGFα,F=10ng/ml TGFα, G=50ng/ml TGFα)

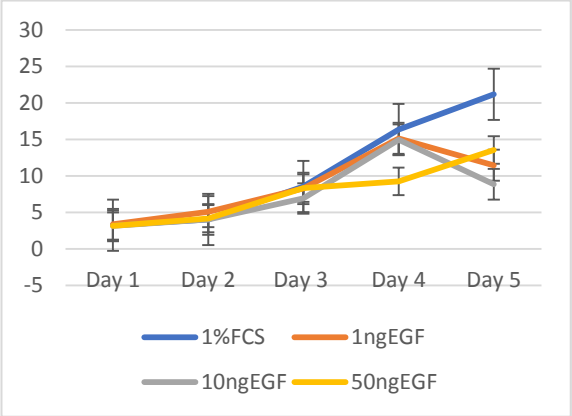


Figure 4.6 A HSG cell proliferation data group for EGF:

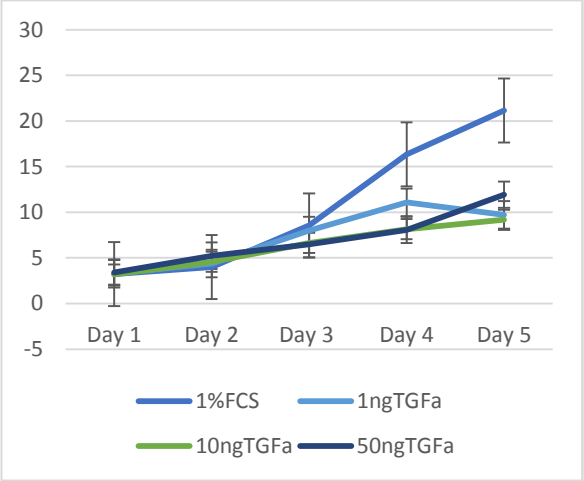


Figure 4.6 B HSG proliferation data grouped for TGFα:

The HSG cell line was plated at 2.5x10^4 onto 35mm dishes in 10% FCS etc. The cells were allowed to attach overnight and transferred to test conditions the following day. Cell number was determined by automated cell counter. The data shown is from one of four separate experiments and the Standard error of the mean is displayed as the error bars.

Figure 4.7 AZA1 proliferation data

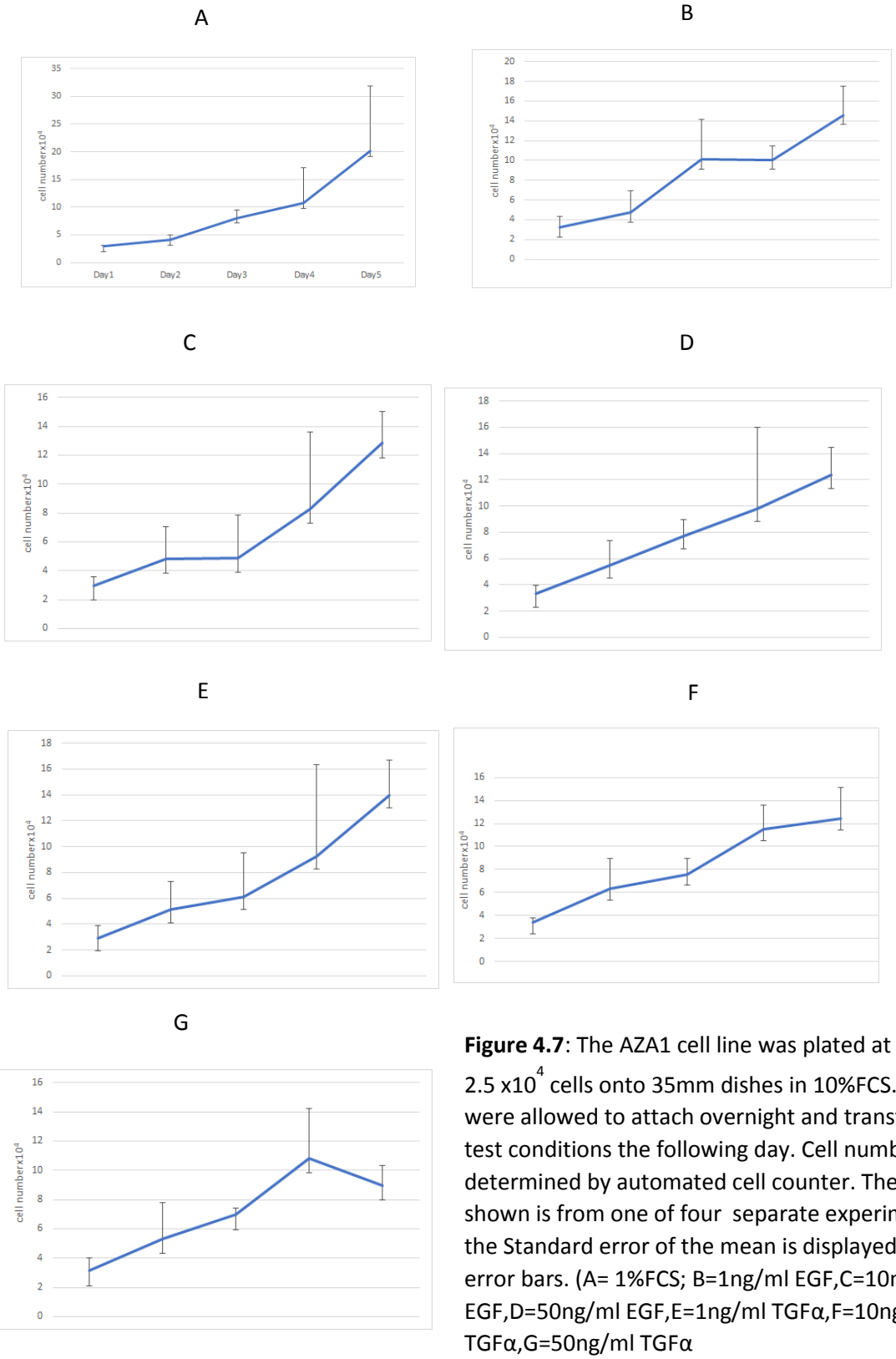


Figure 4.7: The AZA1 cell line was plated at an initial 2.5 x10^4 cells onto 35mm dishes in 10%FCS. The cells were allowed to attach overnight and transferred to test conditions the following day. Cell number was determined by automated cell counter. The data shown is from one of four separate experiments and the Standard error of the mean is displayed as the error bars. (A= 1%FCS; B=1ng/ml EGF,C=10ng/ml EGF,D=50ng/ml EGF,E=1ng/ml TGFα,F=10ng/ml TGFα,G=50ng/ml TGFα

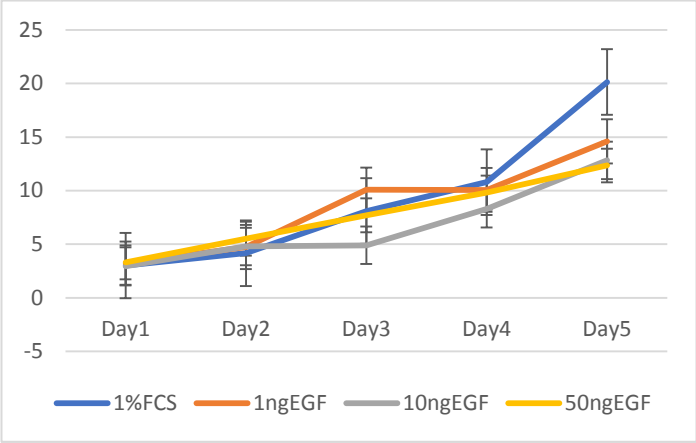


Figure 4.8 A AZA1 proliferation data grouped for EGF:

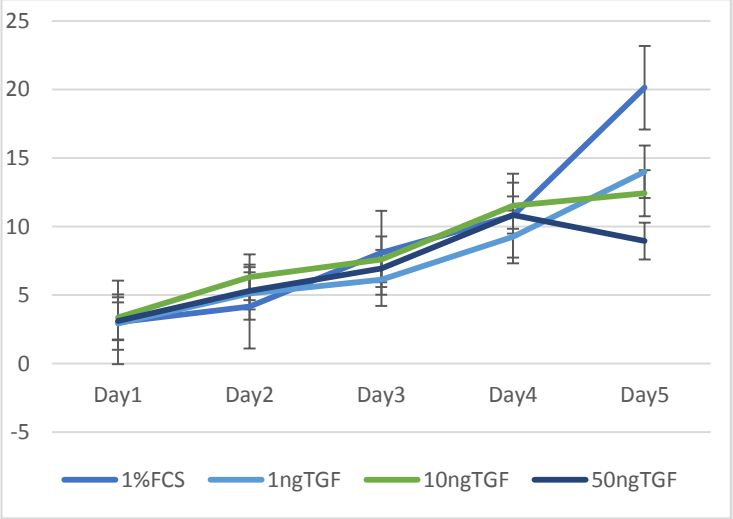


Figure 4.8 B AZA1 proliferation data grouped for TGFα:

The AZA1 cell line was plated at 2.5x10^4 onto 35mm dishes in 10% FCS etc. The cells were allowed to attach overnight and transferred to test conditions (1%FCS, 1ng/ml EGF, 10ng/ml EGF, 50ng/ml EGF, 1ng/ml TGFα, 10ng/ml TGFα, 50ng/ml TGFα) the following day. Cell number was determined by automated cell counter. The data shown is from one of four separate experiments and the Standard error of the mean is displayed as the error bars.

Figure 4.9 TYS proliferation data

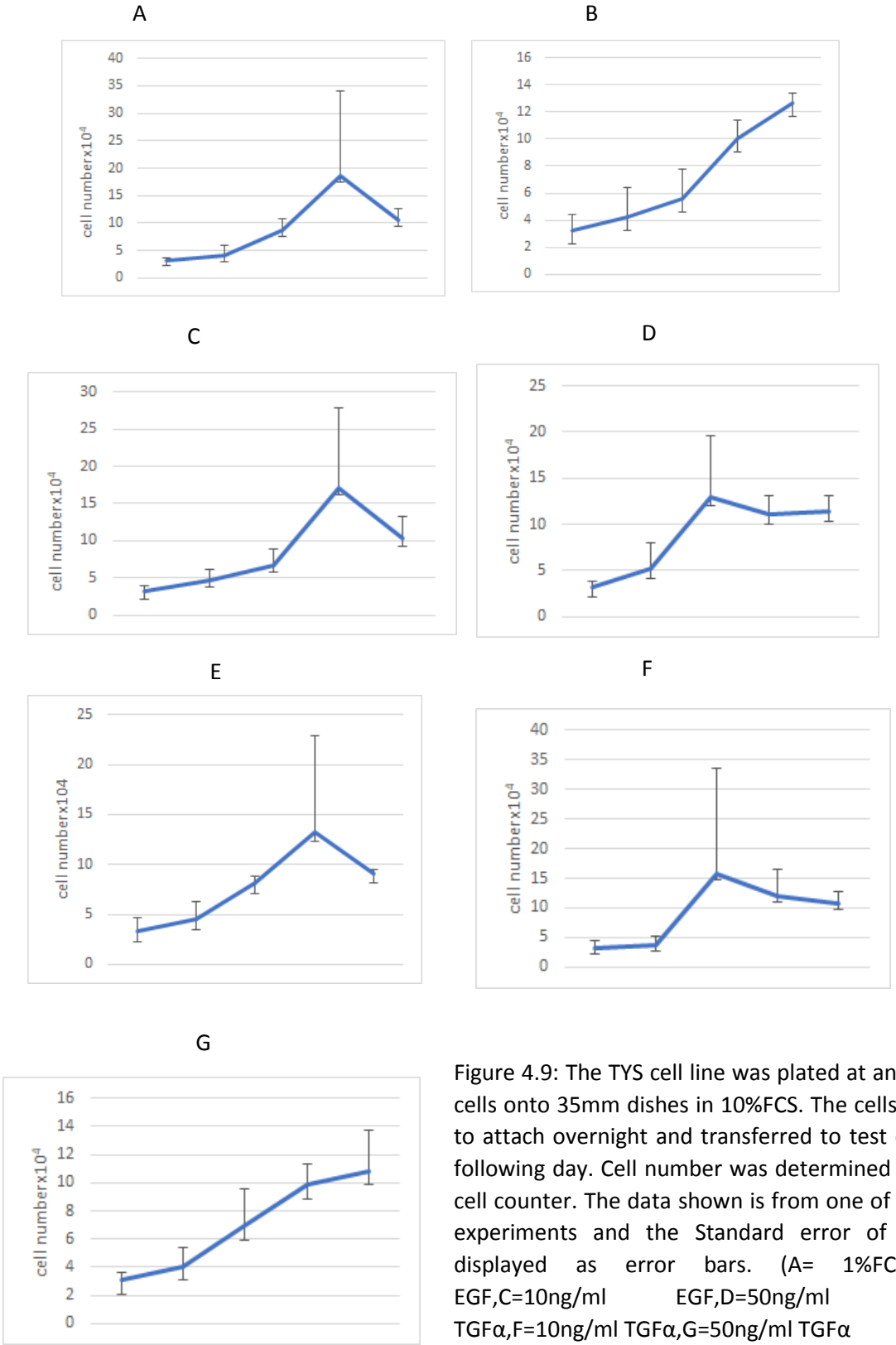


Figure 4.9: The TYS cell line was plated at an initial $2.5x10^4$ cells onto 35mm dishes in 10%FCS. The cells were allowed to attach overnight and transferred to test conditions the following day. Cell number was determined by automated cell counter. The data shown is from one of four separate experiments and the Standard error of the mean is displayed as error bars. (A= 1%FCS; B=1ng/ml EGF,C=10ng/ml EGF,D=50ng/ml EGF,E=1ng/ml TGFα,F=10ng/ml TGFα,G=50ng/ml TGFα

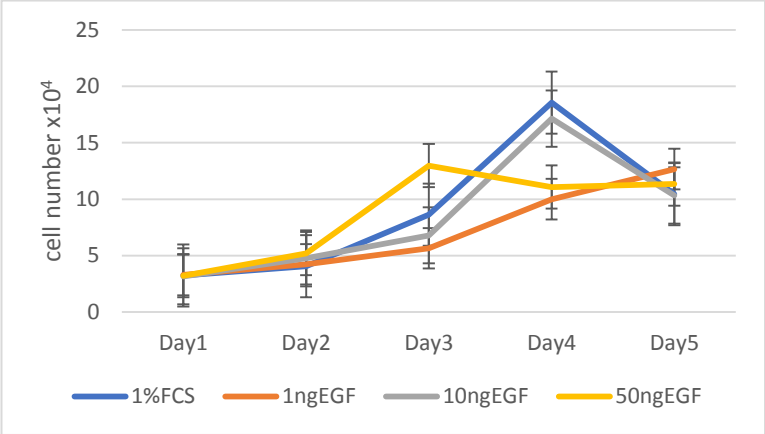


Figure 4.10 A TYS proliferation data grouped for EGF:

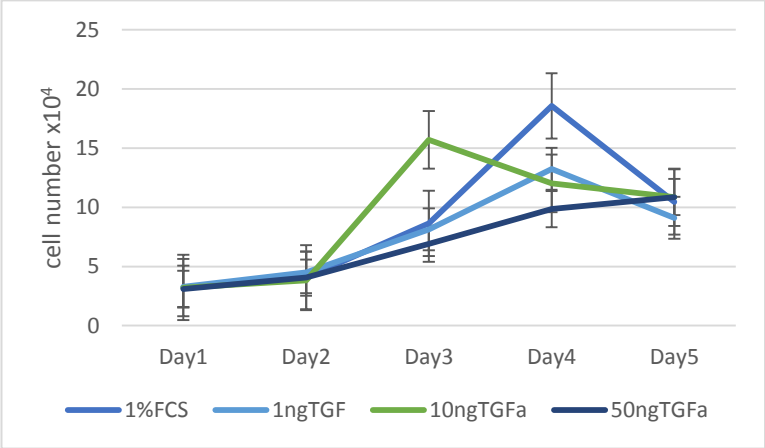


Figure 4.10 B TYS proliferation data grouped for TGFα:

The TYS cell line was plated at 2.5x10^4 onto 35mm dishes in 10% FCS etc. The cells were allowed to attach overnight and transferred to test conditions (1%FCS, 1ng/ml EGF, 10ng/ml EGF,50ng/ml EGF, 1ng/ml TGFα,10ng/ml TGFα, 50ng/ml TGFα) the following day. Cell number was determined by automated cell counter. The data shown is from one of four separate experiments and the Standard error of the mean is displayed as the error bars.

Figure 4.11 HaCaT proliferation data:

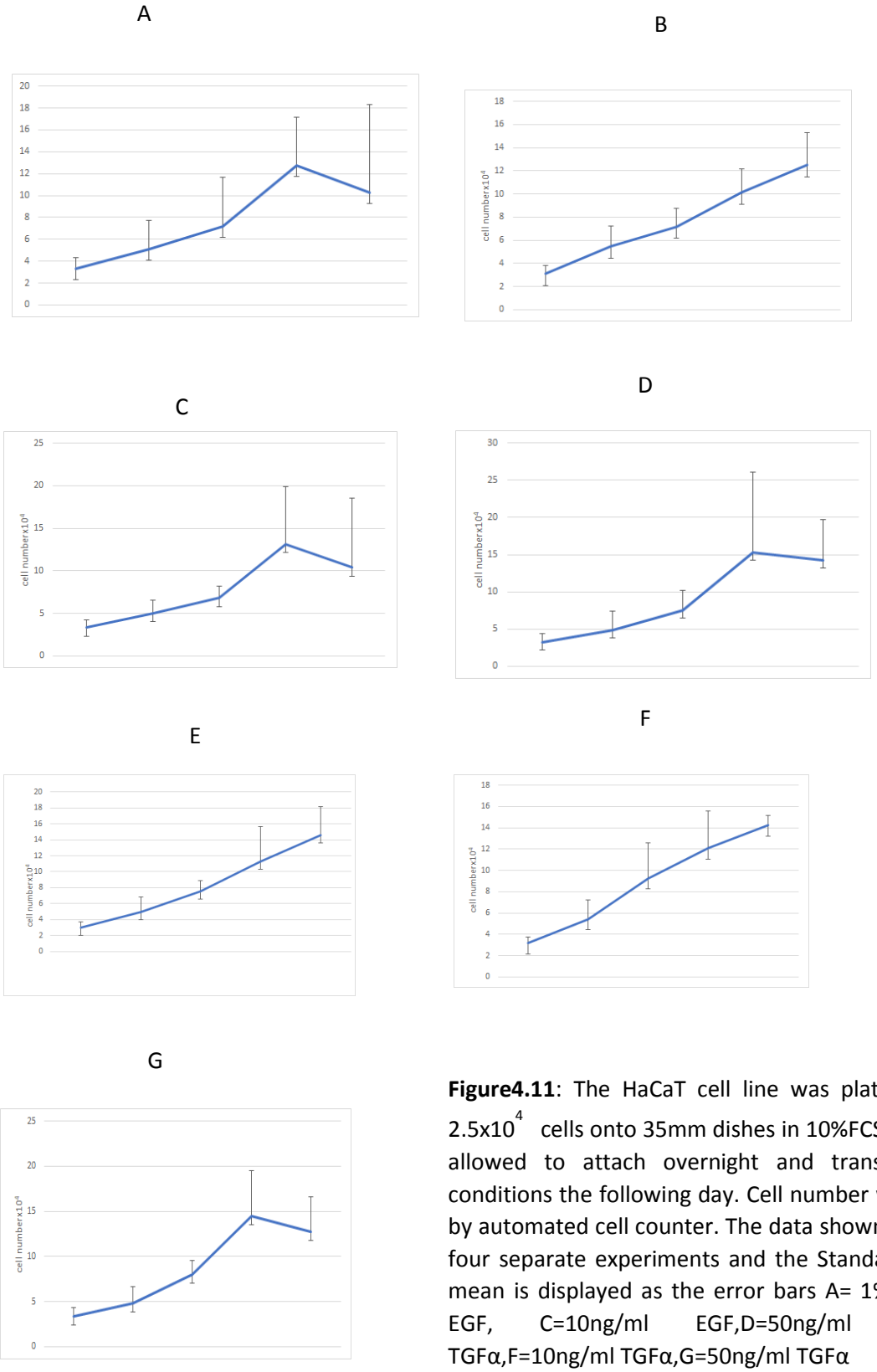


Figure4.11: The HaCaT cell line was plated at an initial $2.5x10^4$ cells onto 35mm dishes in 10%FCS. The cells were allowed to attach overnight and transferred to test conditions the following day. Cell number was determined by automated cell counter. The data shown is from one of four separate experiments and the Standard error of the mean is displayed as the error bars A= 1%FCS; B=1ng/ml EGF, C=10ng/ml EGF,D=50ng/ml EGF,E=1ng/ml TGFα,F=10ng/ml TGFα,G=50ng/ml TGFα

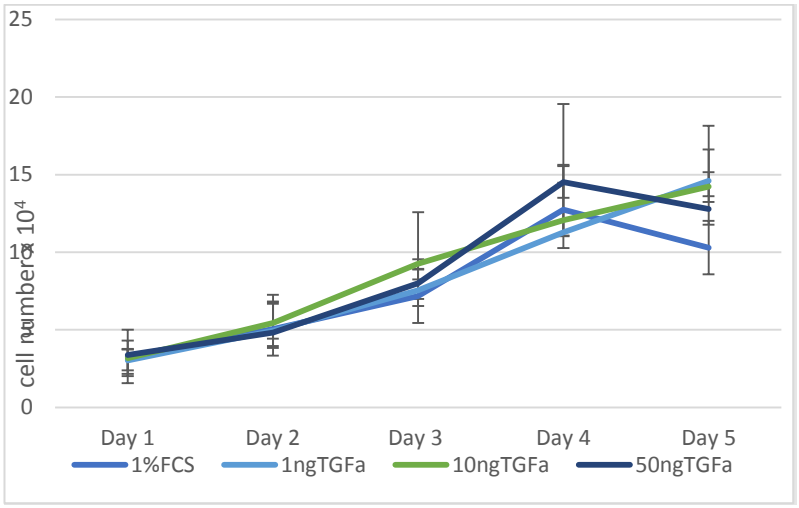


Figure 4.12 A HaCaT proliferation data grouped for EGF:

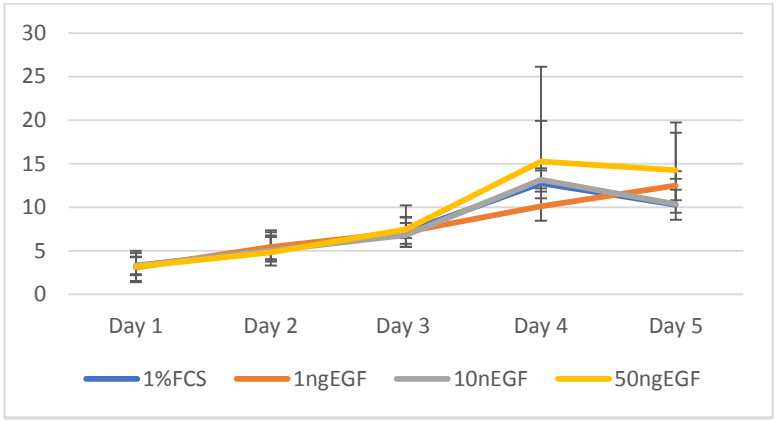


Figure 4.12 B HaCaT proliferation data grouped for TGFα:

The HaCaT cell line was plated at 2.5x10^4 onto 35mm dishes in 10% FCS etc. The cells were allowed to attach overnight and transferred to test conditions (1%FCS, 1ng/ml EGF, 10ng/ml EGF, 50ng/ml EGF, 1ng/ml TGFα, 10ng/ml TGFα, 50ng/ml TGFα) the following day. Cell number was determined by automated cell counter. The data shown is from one of fours separate experiments and the Standard error of the mean is displayed as the error bars.

4.4.2 Cell morphology changes

4.4.2.1 Pilot study to determine optimal plating density, optimal growth factor concentration and time

4.4.2.1.1 Plating density

An initial pilot study using three cell densities was performed (2.5 $x10^4$, 1x 10^5 and 5x 10^5) the cells plated at low density 2.5 $x10^4$ cell dishes (less than 20% confluency on 35mm dishes), required 4 to 6 days after the addition of EGF and TGFα to display a morphological change (Data not show). Cells plated at higher density 1x 10^5 per dish (30%-50% confluency), only 24 hour to 48 hours were needed to acquire this morphological change. 1x10^5 per dish was chosen as the best density to do the experiments since it took faster to see the cells change. Cells plated at 5x 10^5 per dish (90% to 100 % confluence) there was no or very little changes in cell morphology after 48 hours when the growth factors were added (Figure 4.13).

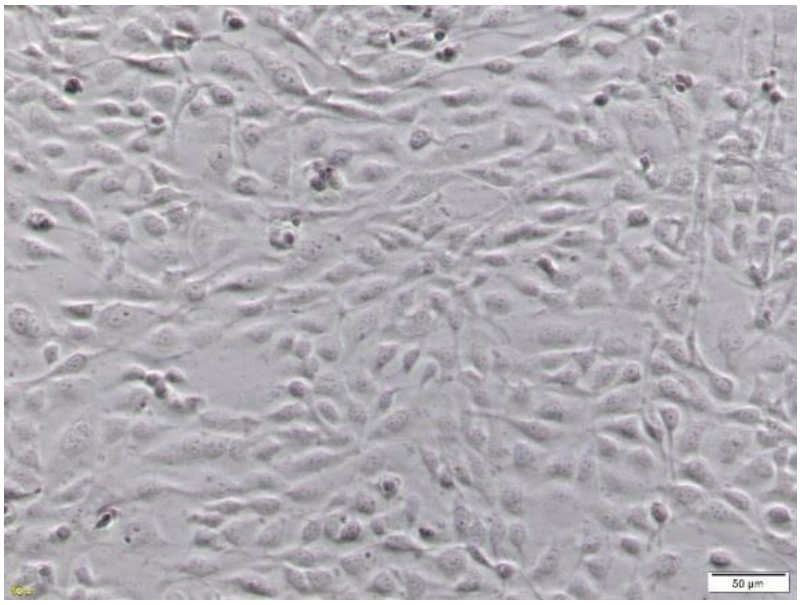


Figure 4.13: The *TYS cell line was plated at* 5x 10^5 per dish *(90% confluence) and EGF was added. The photomicrograph was taken after 24 hrs of addition of EGF 50ng/ml. The cells have little or no morphological change. In 100% confluence dishes, the cells float off after 48hours.*

4.4.2.1.2 Growth factor concentration

A second pilot study using different concentrations of the growth factors was performed. Cells were passaged and plated onto dishes, the dishes were kept in incubator for 24 hours. After 24 hours the medium was aspirated off the dish and serum free-MEM was added for 24 hours. The following day different concentrations of the two growth factors was added (1ng/ml, 10ng/ml and 50ng/ml) with serum free media. 2 ml of media was added to each dish. Results from preliminary experiments found that 1ng/ml (EGF and TGFα) showed little or no changes in cell morphology. 10ng/ml (EGF and TGFα) showed some changes in cell morphology but results were not consistent across a number of dishes. However, 50ng/ml (EGF and TGFα) showed prominent and consistent changes in cell morphology. It was decided that 50ng/ml concentration of each factor was selected to use in the further experiments. Since it induces prominent morphological change in a consistent manner (Figure 4.14). Some of the cells have become rounded and others have projections indicating a possible shift to a mesenchymal phenotype.

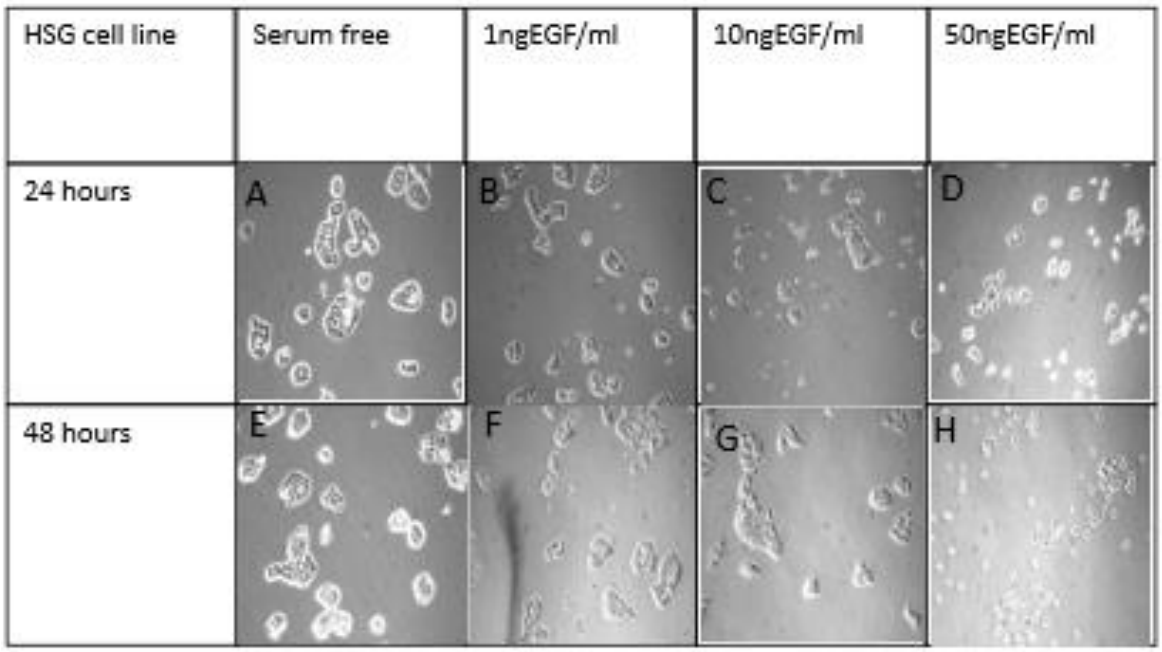


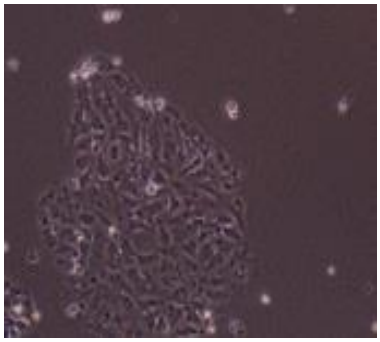
Figure 4.14 HSG cell line and effect of growth factor EGF with different dilutions (Serum free, 1ng/ml, 10ng/ml, and 50ng/ml) on two different incubation time (24hours and 48hours). On 24hours time point, 50ng/ml (picture D) cell scattering were observed while rest have similarity to control in term of scattering effect. On 48hours time point, 50ng/ml (picture H) groups showed prominent scattering compared with 24hours. In contrast, morphological changes in 1ng/ml and 10ng/ml were minimum. Pictures were taken at 40x magnification.

4.4.2.2 Study to investigate the effect of the growth factor EGF and TGFα on morphological change HaCaT cells

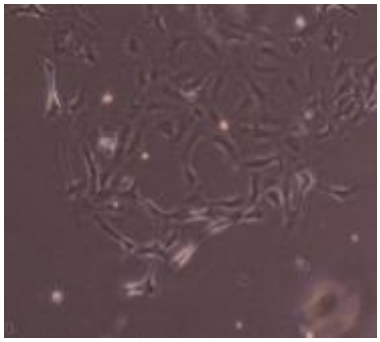
HaCaT cells are characterised and reported as a normal epithelial cell line, although they do have some peculiarities, in that they produce e-cadherin and no vimentin (Boukamp etal., 1988). In the following experiments the cells were plated onto dishes at $1x10^5$cells per dish and allowed to attach. The medium was changed to SF-MEM and the cells left overnight. The following day the medium was changed into test conditions and photomicrographs taken at a time point of 48 hours. The test conditions are described in the Figure legend and it should be noted that the photomicrographs of the serum free, EGF treated and TGFα treated cells in all parts of Figure 4.15. The cells show loosely connected cells with cellular protrusion which is growth factor dependant.

Figure 4.15: HaCaT cells treated with different growth factors for 48 hours

i) serum free



ii) 50ngEGF/ml



iii) 50ng TGFα/ml

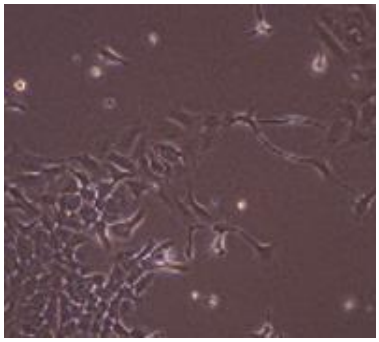


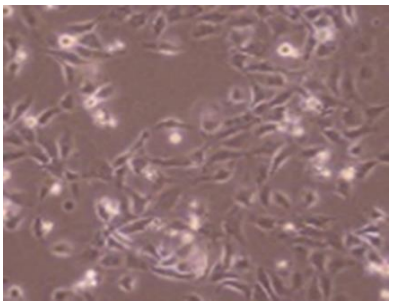
Figure 4.15: The *HaCaT cell line was plated at* 1x 10^5 per dish *(30% confluence) EGF and TGFα were added after colonies had started to form (usually 24 hours). The photomicrographs were taken after 48 hrs of addition of the different growth factors. The cells show loosely connected cells with cellular protrusion which is growth factor dependant.*

4.4.2.3 Study to investigate the effect of EGF and TGFα on morphological change TYS cells

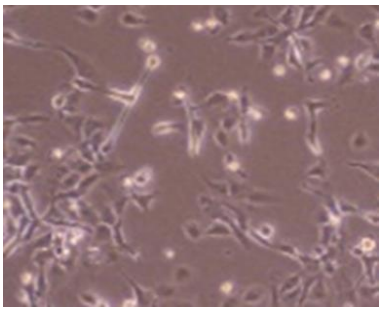
TYS cells are characterised and reported as an oral squamous cell carcinoma, although there is some ambiguity in the original report (Yanagawa *et al.,* 1986). In the following experiments the cells were plated onto dishes at $1x10^5$cells per dish and allowed to attach. The medium was changed to SF-MEM and the cells left overnight. The following day the medium was changed into test conditions and photomicrographs taken at a time point of 48 hours. The test conditions are described in the Figure legend and it should be noted that the photomicrographs of the serum free, EGF treated and TGFα treated cells in all parts of Figure 4.16. The cells show cellular protrusion and cell scattering which are growth factor dependant.

Figure 4.16: TYS cells treated with different growth factors for 48 hours

serum free



50ngEGF/ml



50ngTGFα/ml

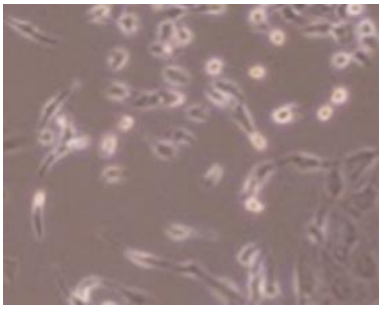


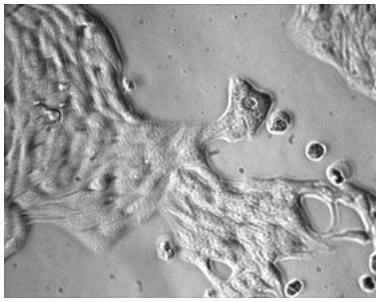
Figure 4.16: The *TYS cell line was plated at* 1x 10^5 per dish *(30% confluence) EGF and TGFα were added. The photomicrographs were taken after 48 hrs of addition of the different growth factors. The cells show cellular protrusion and cell scattering which are growth factor dependant.*

4.4.2.4 Study to investigate the effect of the growth factors EGF and TGFα on morphological change of AZA1 cells

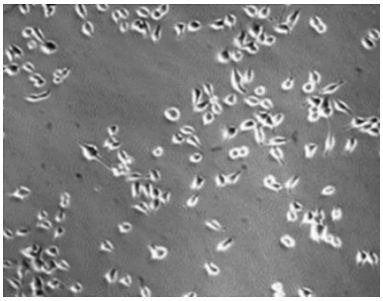
AZA1 cells are HSG cells treated with 5-Azacytidine (Shirasuna K *et al.,* 1981).In the following experiments the cells were plated onto dishes at $1x10^5$cells per dish and allowed to attach. The medium was changed to SF-MEM and the cells left overnight. The following day the medium was changed into test conditions and photomicrographs taken at a time point of 48 hours. The test conditions are described in the Figure legend and it should be noted that the photomicrographs of the serum free, EGF treated and TGFα treated cells in all parts of Figure 4.17. The cells show cell scattering which is growth factor dependant.

Figure 4.17: AZA1 cells treated with different growth factors for 48 hours

i) serum free



ii)50ngEGF/ml



iii)50ng TGFα/ml

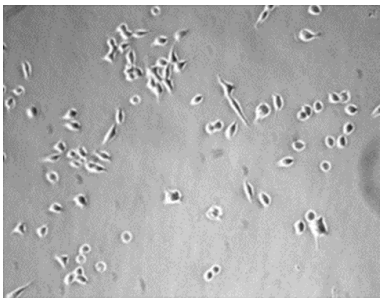


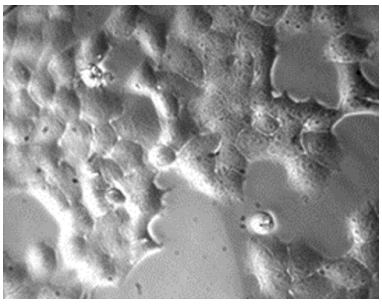
Figure 4.17: The *AZA1 cell line was plated at* 1x 10^5 per dish *(30% confluence) EGF and TGFα were added. The photomicrographs were taken after 48 hrs of addition of the different growth factors. The cells show cell scattering which is growth factor dependant.*

4.4.2.5 Study to investigate the effect of the growth factor EGF and TGFα on morphological change HSG cells

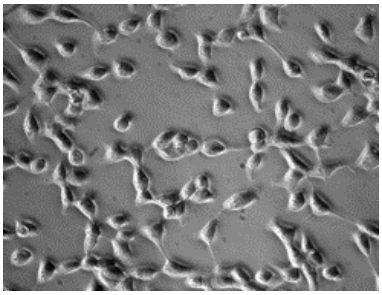
HSG cells are characterised and reported as an epithelial duct cell line derived from human salivary gland (Adenocarcinoma of submandibular gland) (Shirasuna K *et al.,* 1981) .In the following experiments the cells were plated onto dishes at $1x10^5$cells per dish and allowed to attach. The medium was changed to SF-MEM and the cells left overnight. The following day the medium was changed into test conditions and photomicrographs taken at a time point of 48 hours. The test conditions are described in the Figure legend and it should be noted that the photomicrographs of the serum free, EGF treated and TGFα treated cells in all parts of Figure 4.18. The cells show cell scattering which is growth factor dependant.

Figure 4.18: HSG cells treated with different growth factors for 48 hours

i) Serum free



ii)50ngEGF/ml



iii)50ngTGFα/ml

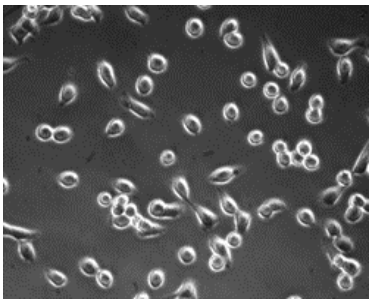


Figure 4.18: The HSG cell line was plated at 1x 10^5 per dish (30% confluence) EGF and TGFα were added. The photomicrographs were taken after 48 hrs of addition of the different growth factors. The cells show cell scattering which is growth factor dependant.

4.4.3 Scratch assay

4.4.3.1 Pilot experiment to investigate the best time point in the scratch migration assay

The scratch migration assay was used to investigate the difference in cell motility. Early experiments had indicated that the four cell lines had different methods of migrating, either as single cells or as sheets. The HSG cell line and the growth factor EGF were chosen to investigate the best time point for cell migration.

A pilot study using two concentrations of the growth factor (EGF- 1ng and 10ng/ml) was performed(Thwe A,2015). Cells were passaged and plated onto dishes, the dishes kept in the incubator and allowed to grow to confluence. After they were confluent the medium was aspirated off the dish and serum free-MEM was added for 24 hours. The following day the monolayer was scratched using a plastic white tip. After an hour the medium was changed to the test condition different concentrations of the growth factors was added.

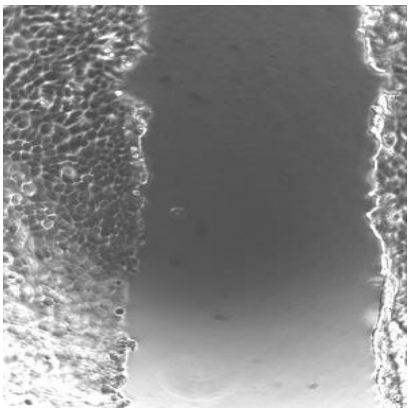
Data shown in Figure 4.19 are for different time points of 2, 4, 6 and 24 hours. The results suggested that the best time point was 24 hours.

Scratch assay images showed variable migratory behaviour of different cell lines. Scratch assays of AZA1 cell line showed very little or no migratory response upon treatment with EGF and TGFα. Whereas HaCaT , TYS and HSG were stimulated to migrate in response to EGF and TGFα.

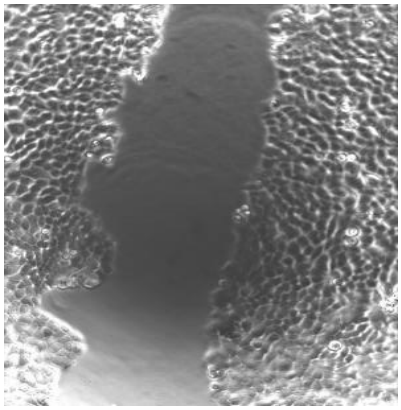
Figure 4.19 Investigation of different time points in the scratch migration assay

A) 2 hours

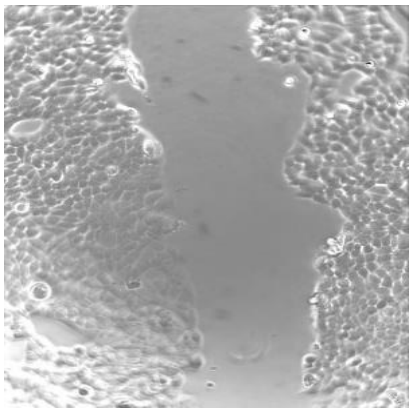
i)serum free



ii)1ngEGF/ml

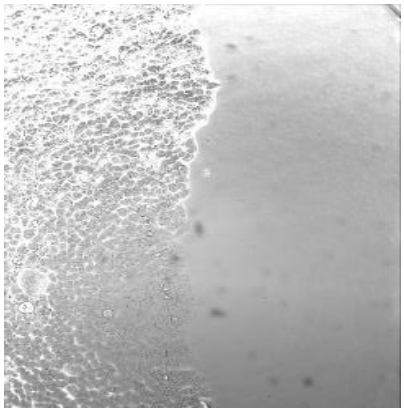


iii)10ngEGF/ml

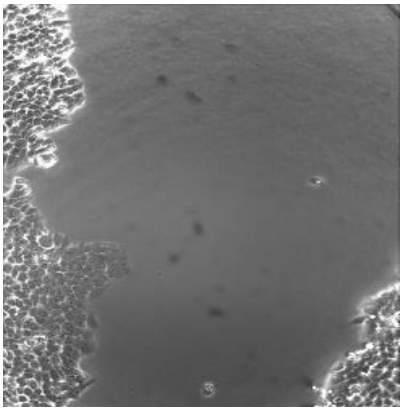


B) 4 hours

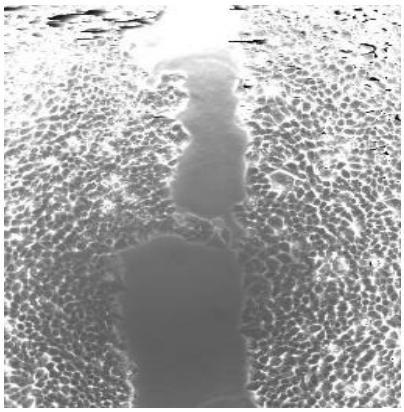
i)serum free



ii)1ngEGF/ml

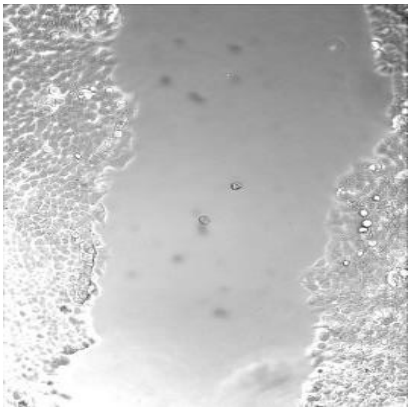


iii)10ngEGF/ml

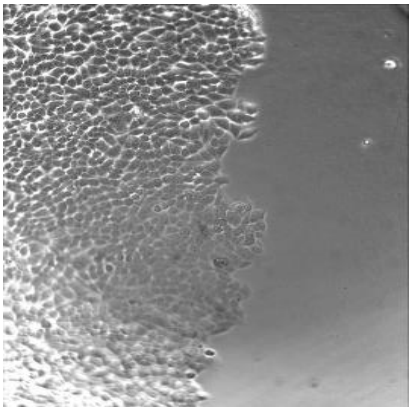


C) 6 hours

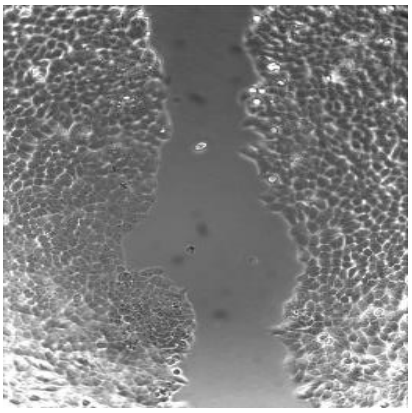
i) serum free



ii) 1ngEGF/ml

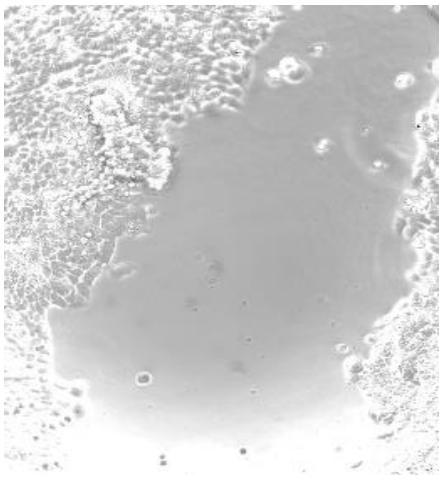


iii)10ngEGF/ml

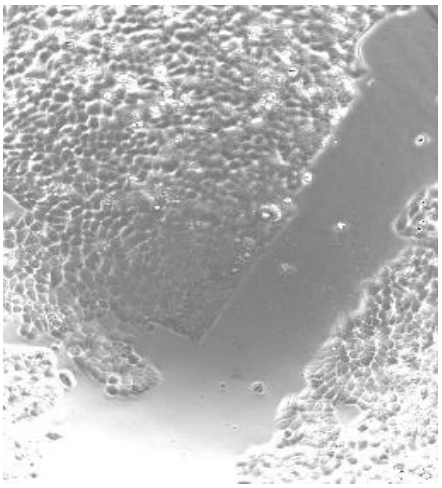


D) 24 hours

i) Serum free



ii)1ngEGF/ml



iii)10ngEGF/ml

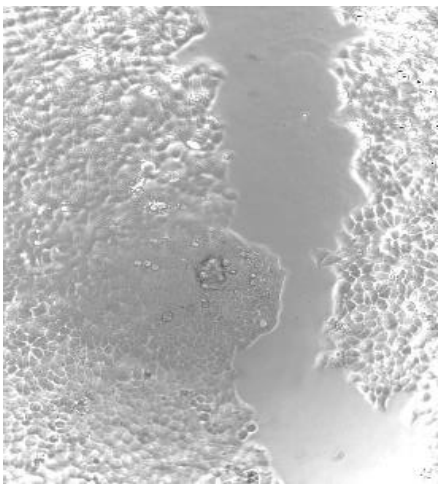
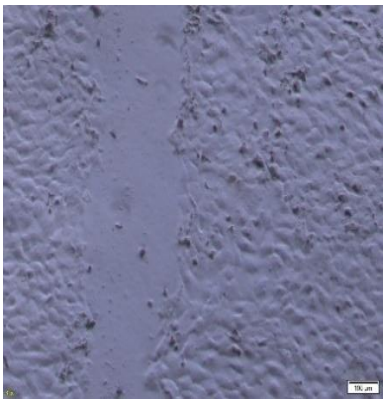


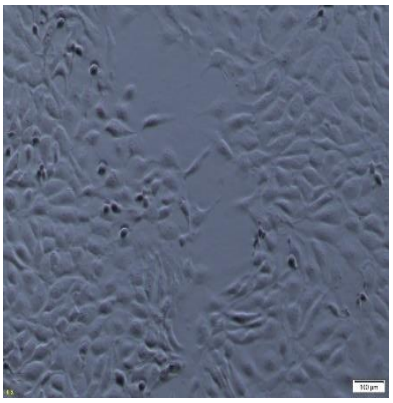
Figure 4.19: The *HSG cell line was plated at* 3x 10^5 per dish *(60% confluence) and grown until confluent changing the medium (10%FCS-MEM) every 48 hours. Once confluent the medium was changed to SF-MEM overnight. A scratch was made from one side of the dish to the other. The medium was changed into test condition (SF-MEM, 1ng/ml EGF and 10ng/ml EGF). They were then incubated and the photomicrographs were taken after the time points above a) is 2 hours, b) is 4 hours, c) is 6 hours and d) is 24 hours.*

Figure 4.20: Scratch assay using HaCaT cells treated with different growth factors for 24 hours

i)serum free



ii)50ngEGF/ml



iii)50ngTGFα/

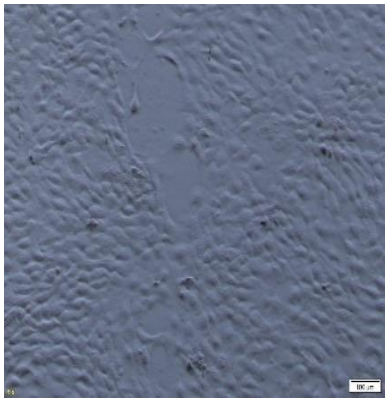
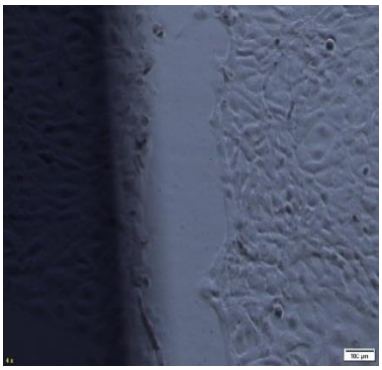


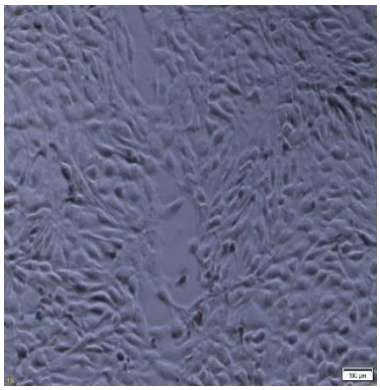
Figure 4.20: The *HaCaT cell line was plated at* 3x 10^5 per dish *(60% confluence) and grown until confluent changing the medium (10%FCS-MEM) every 48 hours. Once confluent the medium was changed to SF-MEM overnight. A scratch was made from one side of the dish to the other. The medium was changed into test condition (SF-MEM, 50ng/ml EGF and 50ng/ml TGFα). They were then incubated and the photomicrographs were taken after 24 hours. Photograph showed that HaCaT cell line was stimulated to migrate in response to EGF and TGFα*

Figure 4.21: Scratch assay using TYS cells treated with different growth factors for 24 hours

i)serum free



ii)50ngEGF/ml



iii)50ngTGFα/ml

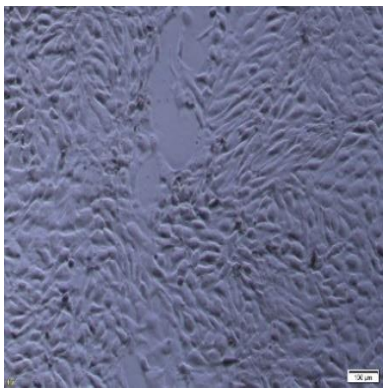
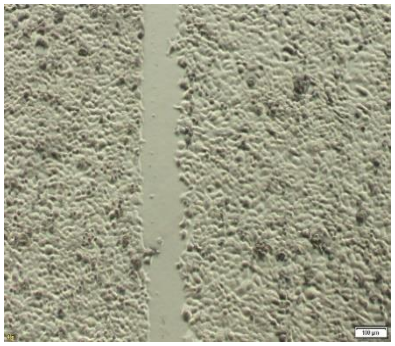


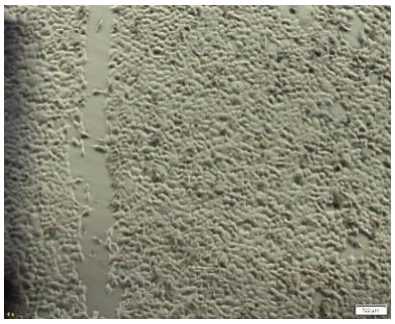
Figure 4.21: The *TYS cell line was plated at* 3x 10^5 per dish *(60% confluence) and grown until confluent changing the medium (10%FCS-MEM) every 48 hours. Once confluent the medium was changed to SF-MEM overnight. A scratch was made from one side of the dish to the other. The medium was changed into test condition (SF-MEM, 50ng/ml EGF and 50ng/ml TGFα). They were then incubated and the photomicrographs were taken after 24 hours. Photograph showed that TYS cell line was stimulated to migrate in response to EGF and TGFα.*

Figure 4.22: Scratch assay using HSG cells treated with different growth factors for 24 hours

i)serum free



ii)50ngEGF/ml

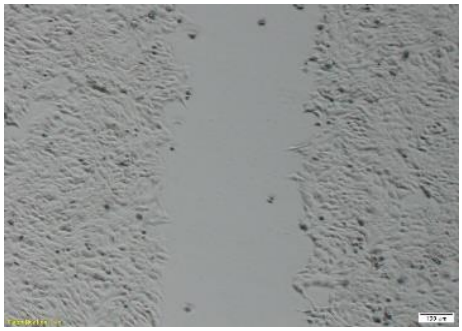


iii)50ngTGFα/ml

Figure 4.22: The *HSG cell line was plated at* 3x 10^5 per dish *(60% confluence) and grown until confluent changing the medium (10%FCS-MEM) every 48 hours. Once confluent the medium was changed to SF-MEM overnight. A scratch was made from one side of the dish to the other. The medium was changed into test condition (SF-MEM, 50ng/ml EGF and 50ng/ml TGFα). They were then incubated and the photomicrographs were taken after 24 hours. Photograph showed that HSG cell line was stimulated to migrate in response to EGF and TGFα.*

Figure 4.23: Scratch assay using AZA1 cells treated with different growth factors for 24 hours

i)serum free



ii)50ngEGF/ml

iii)50ngTGFα/

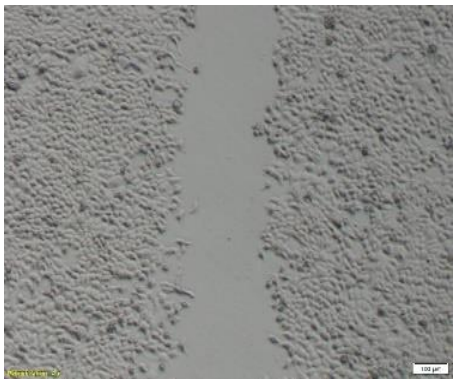


Figure 4.23: The *AZA1 cell line was plated at* 3x 10^5 per dish *(60% confluence) and grown until confluent changing the medium (10%FCS-MEM) every 48 hours. Once confluent the medium was changed to SF-MEM overnight. A scratch was made from one side of the dish to the other. The medium was changed into test condition (SF-MEM, 50ng/ml EGF and 50ng/ml TGFα). They were then incubated and the photomicrographs were taken after 24 hours. Photograph showed that AZA1 cell line was not stimulated to migrate in response to EGF and TGFα.*

4.4.4 Changes in Protein expression by cancer cell lines in response to growth factors

The four cell lines were investigated in respect of the expression of being epithelium-like (E-Cadherin) or mesenchyme-like (vimentin) and how the growth factors EGF and TGFα may change this in terms of EMT. The experiments undertaken to investigate this were immunoflourescence or SDS-PAGE/Western blotting. In the following experiments only changes in expression in response to the growth factors were investigated.

Data presented in Figure 4.24 for the HaCaT cell line indicated that all the cells expressed strong level (as described in chapter3)of E-cadherin and it was mainly visualised on the cell membrane. The response to the growth factors indicated that with EGF some cells appeared to stain less well for E-cadherin. However, less change was noted with TGFα. Vimentin staining did not appear to be present in the HaCaT cell line even when the growth factors were added.

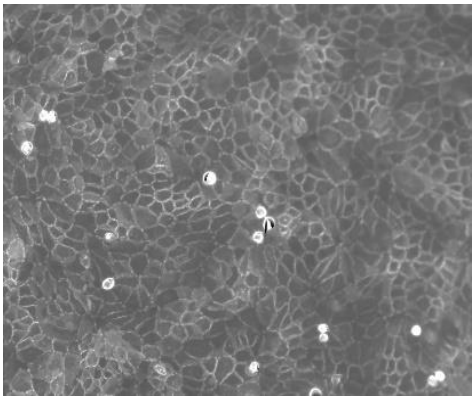
Data presented in Figure 4.25 for the TYS cell line indicated that all the cells expressed both E-cadherin and vimentin at moderate level. E-cadherin was mainly visualised on the cell membrane while Vimentin was visualised in the whole cells. There was no obvious difference between serum free and growth factor treated conditions

Dat presented for Figure 4.26 for the AZA1 cell line indicated that little or no E-cadherin expression while Vimentin was highly expressed in certain area. For vimentin, both membranous expression and diffuse expression was found in photographs.

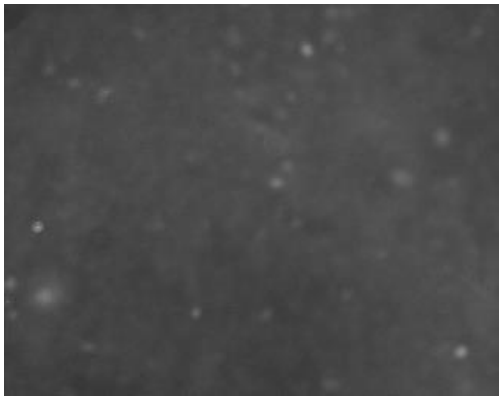
Data presented in Figure 4.27 for the HSG cells line also indicated that little or no E-cadherin expression while Vimentin was highly expressed in certain area. In compared with previous cell lines, only diffuse vimentin expression was found in HSG cells.

Figure 4.24: HaCaT cell line: Expression of markers of EMT (E-cadherin and vimentin) and visualised with immunoflourescent secondary antibody. The cells response to growth factors.

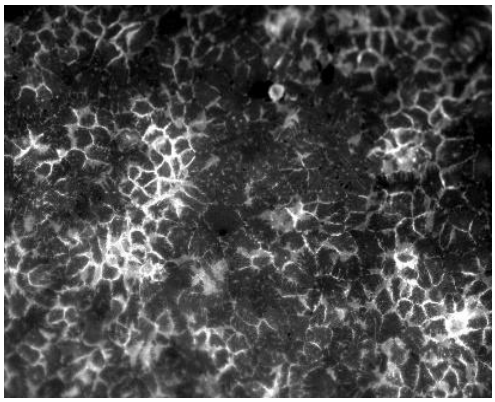
i)serum free: Ecadherin



ii) seum free:Vimentin

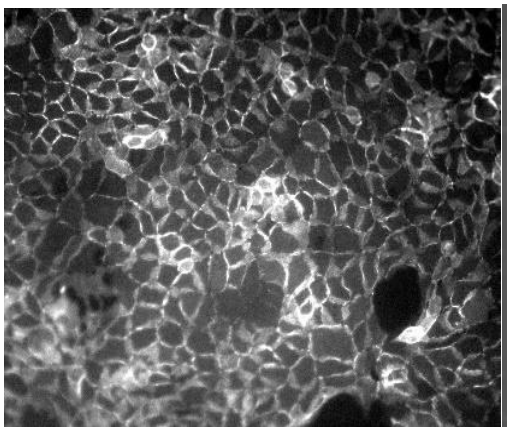


iii)50ngEGF/ml:E cadherin



iv)50ngTGFα/ml:Vimentin

v) TGFα50ng/ml: E-Cadherin



vi) TGFα50ng/ml:Vimentin

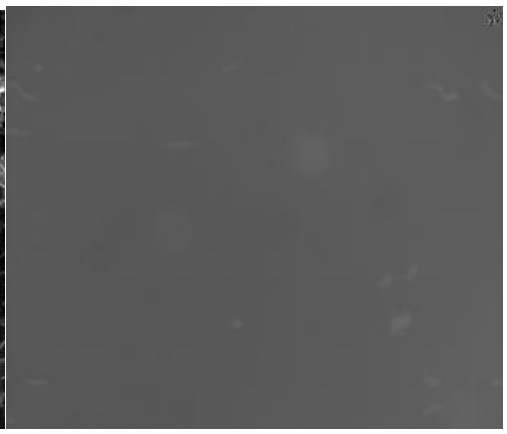
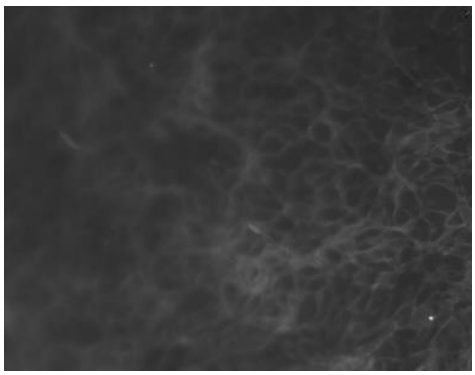


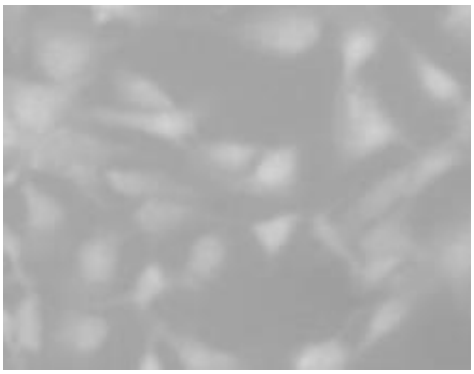
Figure 4.24: The *HaCaT cell line was plated at* 1x 10^5 per dish *(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added. After 48 hours in test conditions the cells were fixed and stained as described in the materials and Methods. Photomicrographs were taken at 200x magnification.*

Figure 4.25: TYS cell line: Expression of markers of EMT (E-cadherin and vimentin) and visualised with immunoflourescent secondary antibody. The cells response to growth factors

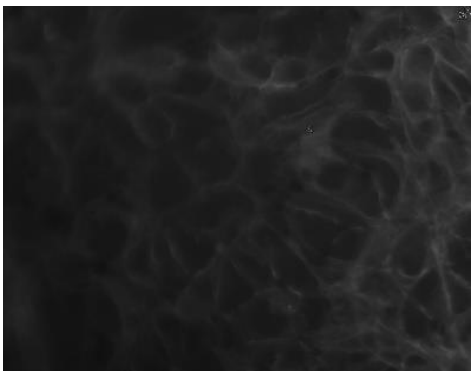
i)serum free: Ecadherin



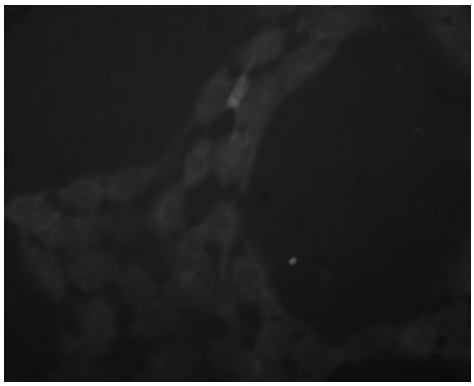
ii)serum free:Vimentin



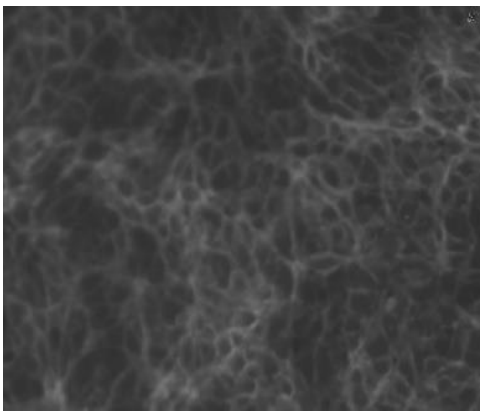
iii) 50ngEGF: Ecadherin



iv) 50ngEGF:Vimentin



v)50ngTGFα:Ecadherin



vi)50ng TGFα:Vimentin

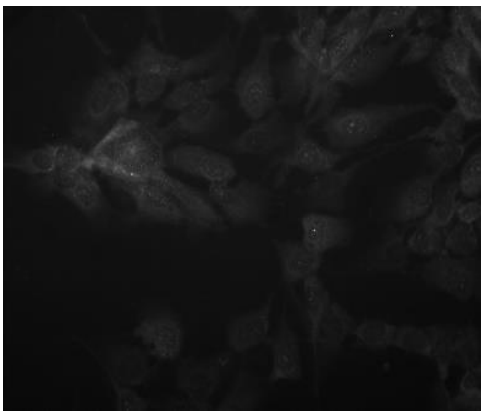
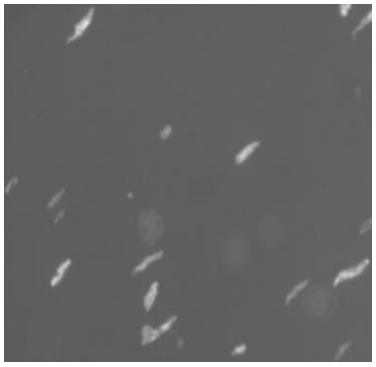


Figure 4.25: The *TYS cell line was plated at* 1x 10^5 per dish *(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added. After 48 hours in test conditions the cells were fixed and stained as described in the materials and Methods.*

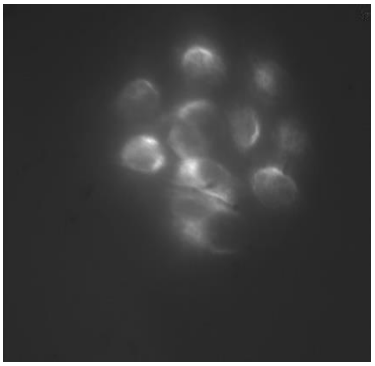
*Photomicrographs were taken at 200x magnification***.**

Figure 4.26: AZA1 cell line: Expression of markers of EMT (E-cadherin and vimentin) and visualised with immunoflourescent secondary antibody. The cells response to growth factors.

i)serum free:E cadherin

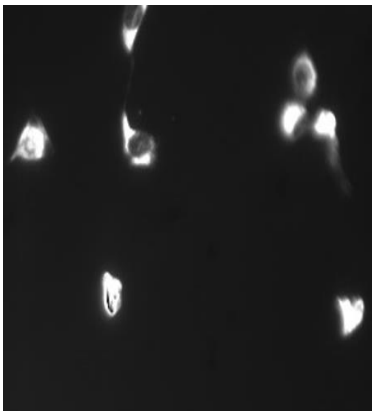


ii)serum free:Vimentin



iii)50ngEGF:Ecadherin

iv)50ng EGF:Vimentin



v)50ng TGFα:Ecadherin

vi)50ngTGFα/ml

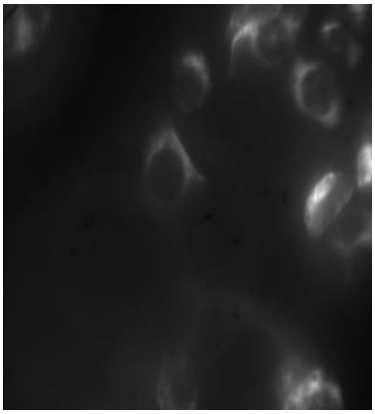
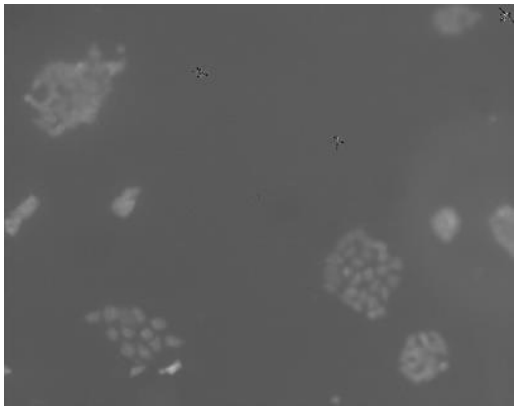


Figure 4.26: The *AZA1 cell line was plated at* 1x 10^5 per dish *(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added. After 48 hours in test conditions the cells were fixed and stained as described in the materials and Methods.*

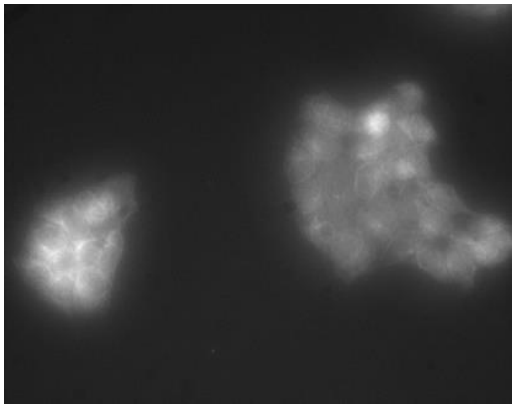
Photomicrographs were taken at 200x magnification.

Figure 4.27 HSG cell line: Expression of markers of EMT (E-cadherin and vimentin) and visualised with immunoflourescent secondary antibody. The cells response to growth factors.

i) serum free:Ecadherin

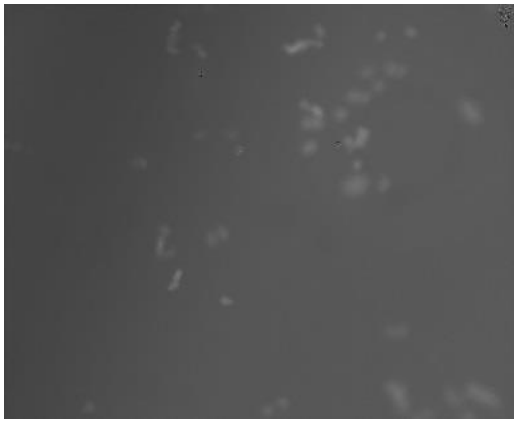


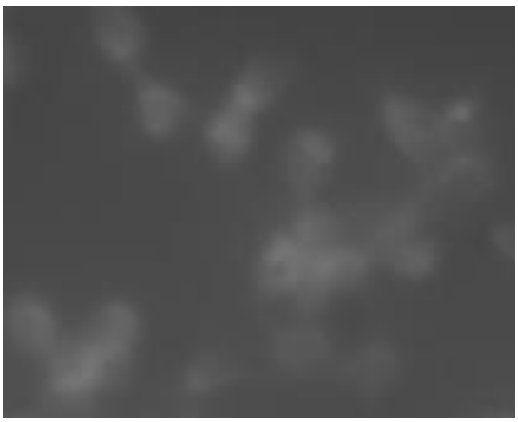
ii) serum free: Vimentin



iii) 50ng EGF:Ecadherin

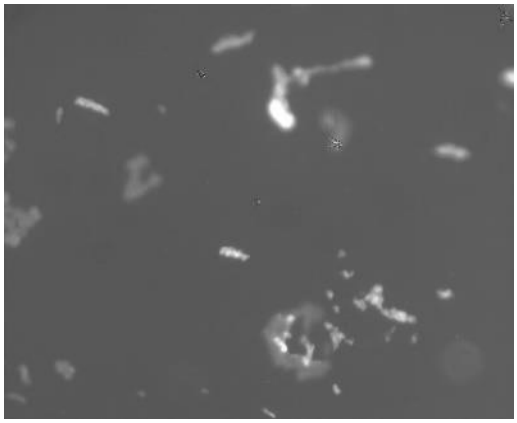
iv)50ng EGF:vimentin





v) 50ng TGFα: Ecadherin

vi) 50ngTGFα: Vimentin



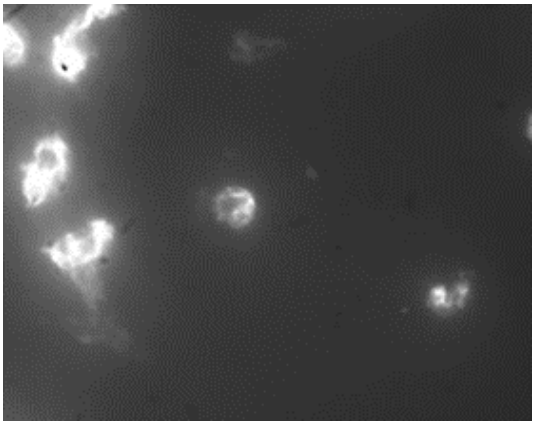
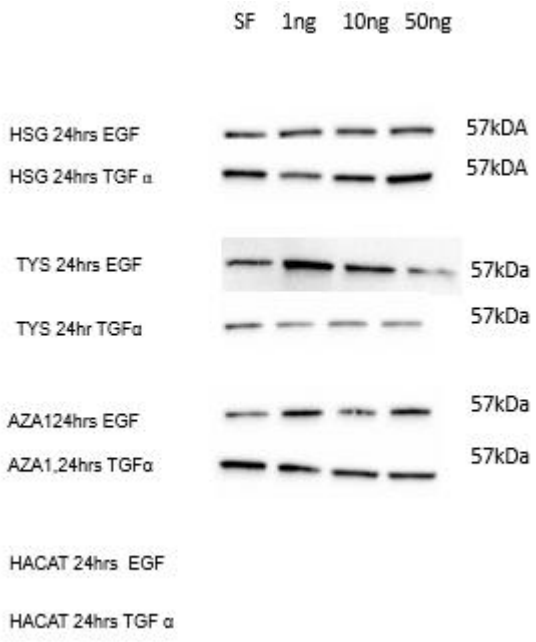


Figure 4.27: The *HSG cell line was plated at* 1x 10^5 per dish *(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added. After 48 hours in test conditions the cells were fixed and stained as described in the materials and Methods. Photomicrographs were taken at 200x magnification***.**

Data presented in Figure 4.28 is for the expression of different proteins associated with EMT. Vimentin is a marker associated with mesenchymal cells. E cadherin is a marker associate with Epithelial cells.The four cell lines were grown to confluence, treated with the respective growth factors for 24 hours and then the cells lysed (as described in Chapter 3(3.14). Protein assay, SDS and Western blot were performed with Ecadherin and Vimentin antibodies as described in Chapter 3(3.15,3.16,3.17).

Figure 4 .28: SDS-Page and Western blotting of Head and Neck cancer samples

i) Vimentin



a) E cadherin

Figure 4.28: Western blot experiments for Vimentin (a) and E-Cadherin (b) in the 4 different cell lines with a spectrum of EGF and TGFα concentrations. (a) Vimentin expression in HaCaT, TYS, HSG and AZA1 EGF and TGFα concentrations of 1 ng/ml, 10 ng/ml and 50 ng/ml. All the cells were treated with the growth factors for 24 hours. (b) E-Cadherin expression in the same cell lines with the same growth concentrations and time as (a).

4.5. Result Summary

4.5.1 Cell proliferation

Table 4.2 Summary for cell proliferation

Cell lines	Serum free	1ng EGF	10ng EGF	50ng EGF	1ng TGFα	10ng TGFα	50ng TGFα
HSG		Little or no effect	Little or no effect	Little or no effect	Little or no effect	Little or no effect	Little or no effect
AZA1		Little or no effect	Little or no effect	Little or no effect	Little or no effect	Little or no effect	Little or no effect
TYS		Little or no effect	Little or no effect	Little or no effect	Little or no effect	Little or no effect	Little or no effect
HacaT		Little or no effect	Little or no effect	Little or no effect	Little or no effect	Little or no effect	Little or no effect

(Little or no effect can be defined as + or – 10% of the serum free result. This leads to our understanding that these growth factors have no proliferative effects.)

4.5.2 Cell morphology / single cell migration

Table 4.3 Summary for cell morphology / single cell migration

Cell lines	Serum free	EGF50ng/ml	TGFα50ng/ml
HacaT	Tightly packed colonies, Complete epithelial characteristic.	loosely connected cells with dendritic or spiky projection in some area	loosely connected cells with dendritic or spiky projection in some area
TYS	partially transformed into mesenchymal shape.	More mesenchymal shape some with dendritic or spiky projection	More mesenchymal shape dendritic or spiky projection
HSG	Epithelial shape colonies	cell scattering, mixture of rounded cells and elongated cell, dendritic or spiky projection in some cells	cell scattering, smaller cells, mixture of rounded cell and elongated cells,dendritic or spiky projection in some cells

AZA1	Epithelial shape colonies	cell scattering, mixture of rounded cells and elongated cells, dendritic or spiky projection in some cells	cell scattering, mixture of rounded cell and elongated dendritic or spiky projection in some cells

4.5.3 Collective Cell migration

Table 4.4. Summary for collective cell migration

Cell lines	Serum free	EGF50ng/ml	TGFα 50ng/ml
HaCaT	Visible scratch line	Fully migration, gap closure in most area	Fully migration, gap closure in most area
TYS	Visible scratch line	Fully migration, gap closure in most area	Fully migration, gap closure in most area
HSG	Visible scratch line	Moderate migration, visible, gap still visible	Moderate migration, visible, gap still visible
AZA1	Visible scratch line	Very little migration	Very little migration

4.5.4 Immunofluorescence

Table 4.5 Summary for Immunofluorescence

Cell lines	Condition treated	E-cadherin	Vimentin
HaCaT	Serum free	Strong expression	Very little or no expression
	EGF50ng/ml	Strong expression	Very little or no expression
	TGFα 50ng/ml	Strong expression	Very little or no expression
TYS	Serum free	Moderate expression	Moderate expression
	EGF50ng/ml	Moderate expression	Moderate expression
	TGFα 50ng/ml	Moderate expression	Moderate expression
AZA1	Serum free	Very little or no expression	Strong expression
	EGF50ng/ml	Very little or no expression	Strong expression

	TGFα 50ng/ml	Very little or no expression	Strong expression
HSG	Serum free	Very little or no expression	Strong expression
	EGF50ng/ml	Very little or no expression	Strong expression
	TGFα 50ng/ml	Very little or no expression	Strong expression

4.6 Discussion

In this chapter, effects of EGF and TGFα on proliferation, morphology changes, migration were investigated by a variety of technique.

For proliferation, EGF and TGFα did not increase the cell proliferation.

In contrast, both EGF and TGFα induce EMT like morphological changes and cell migration. Expression of EMT markers E-cadherin and Vimentin were neither increased nor decreased followed by growth factors treatment.

4.6. 1 Proliferation

We have discussed in the introduction that some growth factors induce cellular proliferation. We wanted to investigate whether EGF or TGFα could have similar effect.

Growth curve data in response to both EGF and TGFα indicated that both growth factors had no effect or a negative effect on cell proliferation of epithelial cell lines from the Head and Neck region used in this study. This is an unusual finding. This is an unusual finding in that the established literature suggests that the growth factors both increase the proliferation of epithelial cells (Anchan RM, 1991). The data for the cell growth curve used were in the normal range, 1ng/ml, 10ng/ml, 50ng/ml concentration of both EGF

and TGFα were added into cells plated at 30mm dishes. Cells were counted by using automated cell counter. Data from these experiments found that the results are inconsistent and there is little or no effect of EGF and TGFα on proliferation of HSG, AZA1, HacaT, and TYS cell lines. Earlier work from our laboratory indicated that these growth factors stimulated the proliferation of fibroblast cell lines (Banyard, 1997 PhD thesis). However, very few papers are produced where either no effect or a negative effect of growth factors are reported. One of the few that was found by (Aladib *et al.,*1990) used two of the cell lines used in this Chapter (HSG and AZA1). Their reported findings are consistent with ours. Other projects in the laboratory have also indicated that these growth factors do not stimulate the proliferation of these 4 cell lines. However, these projects used a smaller range of concentrations (1 and 10ng/ml). Here we widened the range but found the same results. Literature also confirm that myoepithelial cells from pleomorphic adenoma (Navarini, 2015) reported that both 5 and 10ng/ml of EGF had no effect on proliferation.

In contrast, TGFα reported to upregulate proliferation of granulosa cell tumour (Cheng Wang, 2012). These results are different from my results. However, these cells are from a different area of the body and under hormonal control which may explain the difference in the findings. Other factors that may make the response to growth factors are the matrix cells are grown on. HSG cells respond differently to growth factors depending on their differentiation state. By growing cells on Matrigel they were found to respond to nerve Growth Factor (NGF) (Zheng *et al.,* 1998, Lam K *et al*., 2005). It may be that the matrix cells are grown on will influence how they proliferate. Future experiments could investigate the effect of the ECM on the cells response to EGF and TGFα.

Other elements that could influence the effect of growth factors on the cells have been reported by (Johnson *et al.,* 1993). They noted that in certain cell lines the growth factors EGF and TGFα have an inhibitory effect on proliferation due to the presence of endogenous levels of growth factors and EGFR over expression (Johnson *et al.,* 1993). In this study we did not investigate this and it may be an area for future research

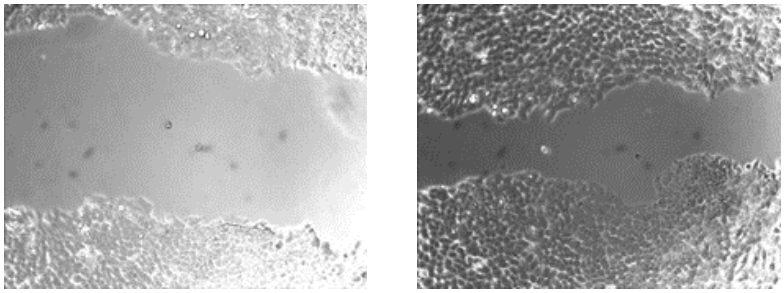
This leads to the question that do neither EGF nor TGFα stimulate cell proliferation in tested cell lines or could it be the wrong choice of assay. It was decide in the long run that proliferation of cells was not the long-term goal of the project and that we would concentrate efforts on the motility of the cells used. For proliferation it would be better to confirm using alternative techniques to measure cell proliferation such as MTT or BrDU assays as to whether the growth factors would affect it.

Although the data could be viewed as a negative in terms of proliferation data they provided some useful insight into both the plating and saturation densities of each cell type and the level of growth factor that could be added to the cells in culture.

4.6.2: EMT, single cell migration and collective cell migration

Experimental data from this project support hypothesis of the study. We discovered two types of cancer cell migration. Individual cell migration and collective cell migration. The contrast between collective cell migration where cells move as groups or sheets (Figure 4.29 a, b) and individual cell movement where cells need to break contacts and move individually is quite stark(Figure 4.30 a, b) (Friedl, 2009).Collective cells movement follows three principles in that cells remain: 1) attached both physically and functionally.

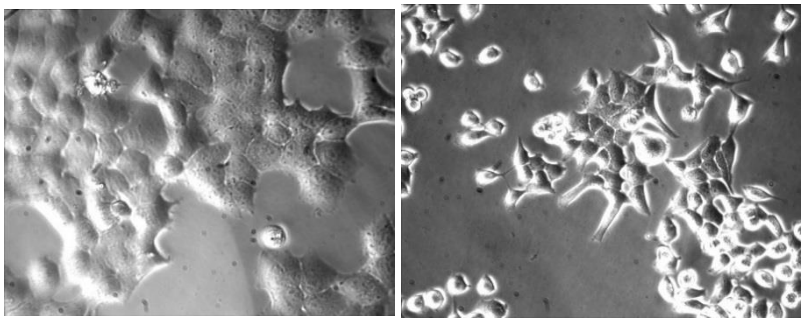
2) The polarity causes the actin filaments to generate traction. 3) The cells are able to modify the tissue in their path (Friedl,2009).



a b

Figure 4.29 collective cell migration*: HSG cells plated onto the surface of a plastic tissue culture dish were grown to confluency. They surface was scratched and the migration noted after 24 hours. Note the gap has narrowed No single cell are visible in the gap so we surmise that the cells have moved as a collective a) HSG cells in time 0, b) HSG cells after 24 hours (Magnification:200x).*

In individual cell migration, cells to cell contacts are lost with and there is the formation of ameboid or mesenchymal phenotype (Figure 4.30 a and b). Cell produce different types of extensions when they migrate: Filopodia (finger like projection), lamellipodia (broad like protrusion), podosomes and invadopodia are cellular protrusion which forms at the edge of migrating cells (Yamaguchi *et al.,* 2005).



a b

Figure 4.30 single cell migration: *HSG cells plated onto the surface of a plastic tissue culture dish were grown to 50% confluency. EGF 50ng/ml was added into the dish. After 48 hours, the cells became scattering with some projection appeared. a) HSG cells in serum free, b) HSG cells in EGF 50ng/ml (200X magnification).*

EGF and TGF α induce morphological changes in all the cell lines but intensity of changes were different with each cell line. In HaCaT cell line, that morphology changes induced by EGF and TGFα treatment is minimum compared with HSG and AZA1 cells line. Cell scattering was not found in HaCaT. There was no complete transformation of EMT in response to EGF and TGFα. In TYS cell line, Introduction of EGF and TGFα induced scattering of some cells, more mesenchymal shape and dendritic projection.

In HSG and AZA1 cell line, scattering of cells was observed after EGF and TGFα treatment.

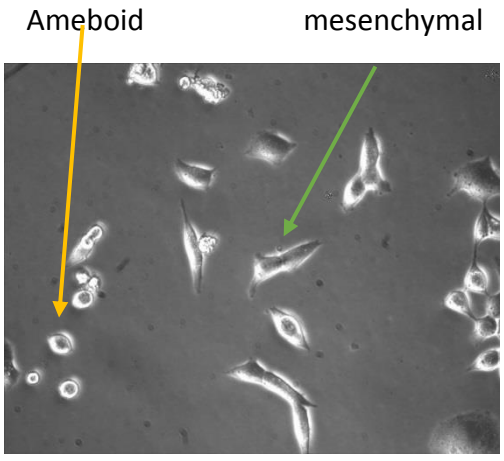


Figure 4.31 ameboid shape and mesenchymal shape in cell scattering

Cell scattering is the process in which epithelial cells colonies are scatter into an individual cells with mesenchymal like morphology with dendritic or spiky projection. Cell scattering could also regarded as individual cell migration. Cell scatter can be used to detect EMT. Dendritic and spiky projection are also sign of migration

One study found out cell scattering in MCF-7 cells followed by introduction of EGF (KimJ, 2016). Another study also reported cell scattering induced by TGFα treatment (Hasina R, 1999). Results from this project was matched with their finding.

Migration of cells in scratch assay is believed to be collective cell migration. Full closure of scratch line was found in TYS and HacaT cells line. HSG cells migrate across the scratch line while there is no obvious migration of AZA1 cell lines. Combination of two techniques give an idea of cells might use both single cell migration and collective cell migration.

Data from this project support our hypothesis of EGF and TGFα induce EMT and cell migration.

This data also matched with other literatures which stated that EGF and TGFα induce EMT, cell scattering and migration (Thiery & Sleeman, 2006, KimJ, 2016, Hasina R, 1999 Ohnishi *et al.,*l 2017).

4.6.3 Discussion for EMT markers

Theoretically, EGF and TGFα induced EMT in both normal and cancer cell lines. E cadherin (most common epithelial marker) is supposed to be lower in EMT while vimentin (mesenchymal markers) is expected to high (Thiery & Sleeman 2006, Neilson & Zeisberg 2009, Lee JM 2006). Upregulation of E-cadherin expression in cancer is

associated with increase disease free survival (Ren X,2016). In contrast, EMT (down regulation of Ecadherin and upregulation of vimentin) is related with cancer progression (Myong H 2012, Nijkamp et al 2011, Schipper J *et al* 1991). In fact, EMT is reported to be related with resistance to TKIs (Weng *et al.*, 2019). So, it is important to know E cadherin and Vimentin status of the cells in this project. All the cells lines used in this project were tested with E-cadherin and vimentin antibodies to understand whether the cells are epithelial or mesenchymal characteristic by using western blot and immunofluorescence. Western blot measured the total amount of protein while immunofluorescence localized protein in specific area of the cells. In western blot, we found out HacaT cells line has strong E-cadherin and no vimentin expression making complete epithelial characteristic. In contrast,TYS, HSG and AZA1 showed EMT(no Ecadherin and strong vimentin). This is quite surprising since HSG and AZA1 look morphologically similar to epithelial cells. However, this might be because of these cells were grown in 2D condition. (Royce *et al*., 1993) reported differentiation of HSG cells on Matrigel. In western blot, there was no change in intensity of E-cadherin and vimentin expression between growth factor treated dishes and control dishes even though EMT like phenotypic changes appeared in growth factor treated dishes. Experimental data like that did not appear to support hypothesis of the study. However, one thing to consider is that cells in this writer's project were lysed after 48 hours when they changed their morphology. Results from other study observed E cadherin level is decreased after 73 hours followed in EGF treated dishes (Kim J, 2016). So, it might be good idea to wait at least 72hrs to measure E-cadherin and vimentin expression in future experiments.

In immunofluorescence, we also found out transformed cell lines are low Ecadherin and high vimentin while epithelial cell line has stong Ecadherin with low vimentin expression.

This data is supported by a series of literatures (Thiery & Sleeman 2006,Neilson & Zeisberg 2009, Lee JM 2006). In addition, we also found out, some HaCaT cells in EGF treated dish appeared to stain less well for E-cadherin. There was also Ecadherin internalization in some of the HaCaT cells followed by EGF treatment. These data also matched with other literatures(Kim J 2016, Lu *et al* 2003).

Chapter 5 To investigate the effects of inhibitors on cell scattering, migration and the localisation and expression of signalling molecules in growth factor stimulated head and neck cell lines.

5.1 Introduction

Results in the previous Chapter established that both growth factors stimulate the migration of the tumour cells and appear to work via an EMT-like mechanism. However, it is may not be the only mechanism that the cells use. How that mechanism is initiated and controlled is the essence of the work in this following chapter. It is known that the inhibition of certain pathways can be used in cancer therapies. Work in this Chapter will focus around the receptor for both growth factors EGFR and will investigate using "off the shelf" inhibitors.

5.1.1 Background

The receptor EGFR is a transmembrane tyrosine kinase receptor which belongs to EGFR/ErbB family (Roskoski 2014, Salomon *et al.,* 1995). It has been reported that EGFR is overexpressed in 80% to 90% of head and neck cancer (Kalyankrishna & Grandis, 2006) (Reuter *et al.,*2007). The growth factors EGF and TGFα are ligands that bind to EGFR. Ligand binding results in the dimerization of EGFR, the phosphorylation and activation of a number of downstream signaling pathways. About 6 tyrosine residues are phosphorylated at the COOH-terminal tail of EGFR: they are Y1068, Y1148, Y1173(Downward *et al.,*1984) ,Y1086 (Hsuan *et al.,* 1989, Margolis *et al.,* 1989), Y992(Walton *et al.,*1990) Y1045 (Levkowitz *et al.,*1999).

Phosphorylation of EGFR at Y1068 and Y1086 creates binding sites for Grb2 adaptor protein, leading to activation of the MAPK pathway and a binding site for Gab1, which recruits the p85 subunit of PI3-kinase, which in turn can and activate the AKT pathway(Rodrigues *et al.,* 2000).Phosphorylation of tyrosine residue Y1068 has been

suggested to be a predictive biomarker for tyrosine kinase inhibitors treatment for patient with wildtype EGFR in lung cancer patients(Wang *et al.,* 2012, Sette *et al.,* 2015). In this chapter, phosphorylation of Y1068 EGFR was analysed by Western blot while localization of total EGFR was examined by immunofluorescence.

MAPKs is a family of serine/threonine kinase proteins which play an important role in a variety of cellular process such as cell proliferation, migration and apoptosis. One of the constituents of the MAPK pathway (p44/42 ERK1/2) is activated by growth factors and cytokines (Peng *et al.,* 2018). Targeting Ras-Raf-MEK-ERK pathway is important in treatment of cancer. MEK1 and MEK2 activate P44 and P42 through phosphorylation at Thr202/Tyr204 and Thr185/Tyr187, respectively. In this chapter, phosphorylation of MAPK202/204 was measured by Western blot and localisation of MAPK202/204 was examined by immunofluorescence. PD98059 which is a selective inhibitor of MAPK extracellular signaling-regulated kinase (ERK) kinase (MEK), was used in the experiment.

PI3K- AKT pathway is another pathway that plays an important role in carcinogenesis and activation of PI3K is induced by introduction of growth factors. In this chapter, phosphorylation of Akt-473 is measured by Western blot and localization of Akt-473 is observed by immunofluorescence. As described in introduction. Phosphorylation of Akt-473 is related with inhibition of apoptosis (Hemmings, 1997, Itoh *et al.,* 2002) and mutation of Rb1 is causative factor of Akt-473 phosphorylation (El-Naggar *et al.,* 2009).

There are two types of drug specifically targeting EGFR. The first type of drugs are Monoclonal antibodies used to target EGFR overexpression. Monoclonal antibodies compete with ligands to bind EGFR. (Martinelli *et al.,* 2009). The second type are the small molecules tyrosine kinase inhibitors targeting EGFR mutation (Ciardiello & Tortora,

2008, Modjtahedi & Essapen, 2009). Tyrosine kinase inhibitors bind to the cytoplasmic region of EGFR by competing with ATP and inhibit the phosphorylation of the receptor. Gefitinib and Erlotinib are example of TKIs which have been used to treat Non-Small Cell Lung Cancer. None of TKIs has yet received FDA approval to treat head and neck cancer, they are undergoing clinical trials but early reports are not encouraging (Chua *et al*., 2008, Li & Perez-Soler, 2009, Soulieres *et al*., 2004). One of the possible reason for this is the activation of downstream signalling pathways such as ERK (Wakeling *et al.,* 2002). K-ras mutation has also been reported to be related with resistance to tyrosine kinase inhibitors in non-small cell lung cancer (Massarelli *et al.,* 2007). Previous studies reported that EMT is believed to be the underlying mechanism of drug resistance to TKIs in both NSCLC and HNSCC (Weng *et al.,* 2019, Maseki *et al.,* 2012, Frederick *et al.,* 2007 Uramoto *et al.,* 2010).

In this study, we are going to investigate whether EGF and TGFα induce EMT or not and whether EMT is reversed with drugs which are TKIs.

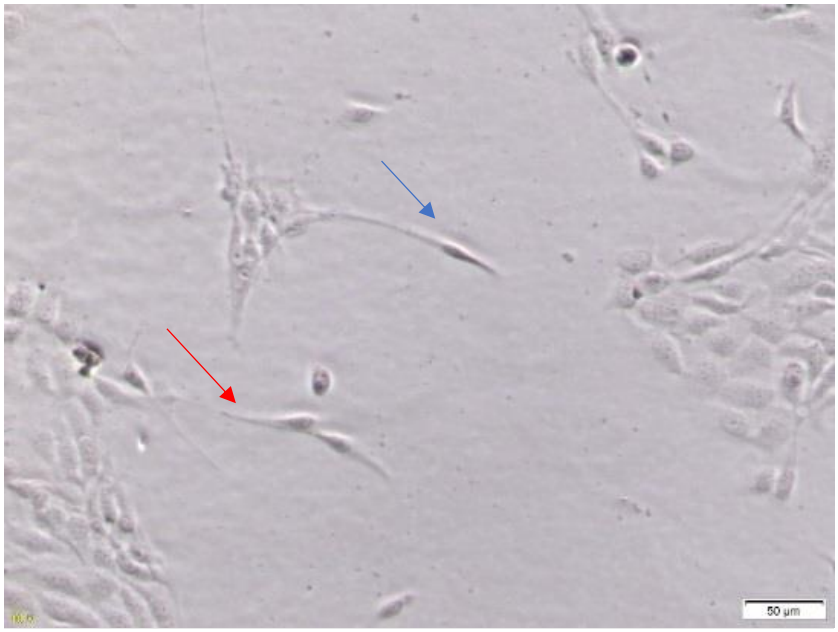


Figure 5.1 Example of cellular projection*: TYS cells plated onto the surface of a plastic tissue culture dish grown in colonies. However, in response to serum or individual growth factors TYS cells start by forming projections (blue arrow) and then migrate across the dish (40x magnification) (Red arrow).*

5.2 Aim and hypothesis

Studies in this chapter of thesis will explore the effect of Tyrosine kinase inhibitors (Gefitinib, Erlotinib) and ERK1/2 inhibitor (PD98059) by pre-treatment of these inhibitors on migration and morphological changes before addition of EGF and TGFα and investigate whether these inhibitors inhibit the effect of EGF and TGFα.

It has also been reported in the literature that EGFR TKIs can reverse these changes *in vitro* for other cancers (La Monica *et al* 2013, Fichter *et al* 2014). In the previous chapter, we reported that EGF and TGFα have an effect on cell morphology and migration. This chapter will involve the investigation of whether the tyrosine kinase inhibitors (Gefitinib, Erlotinib) and ERK1/2 inhibitor (PD98059) inhibit these changes. Experiments will aim to observe the localization and the expression and distribution of EGFR and the phosphorylation of one of its tyrosine residues (P-Tyr 1068). The aim is also to observe the expression of signalling molecules MAPK202/204 and AKT473 as markers of change in response to both the growth factors and inhibitors.

The working hypothesis is changes caused in previous chapter can be inhibited by blocking the receptor signalling pathways.

The null- hypothesis of this Chapter is that the inhibitors of EGFR and MAPK have no effect on EGF and TGF α stimulation of the migration of the different cell types.

5.3 Experimental Methods

The methods used in this Chapter are found in Chapter 3. The following parts are the specific experiments which deal with the role of the inhibitors.

5.3.1 General Tissue Culture for Scratch assay

Cells were sub-cultured as described in 3.5. Once the experiment outlines were decided either 35mm or 60mm would determine how the dishes were prepared. The trypsin/media mixture was aspirated off and then cell pellet was re- suspended in the appropriate volume of 10% FCS 1ml for 35mm or 2ml for 60mm. Fresh dishes were prepared, the number and size would depend on the intention of the experiments, adding 1mls of 10% FCS to each of 35mm dishes (or 2ml for 60mm). The cells suspension of $1x10^{5}$ (or $3 x10^{5}$ for 60mm) was then added into the prepared dishes and the cultures incubated at 37°C. Once the cell reach the desired confluency, the cells were changed into serum free media for 24 hours. On the next day, the medium were refreshed with Gefitinib, Erlotinib, and PD98059 before addition of growth factors an hour later.

5.3.2 Scratch assay

The cells were seeded at $1x10^{5}$per dish. Once the cells reached 90% to 100% confluence. They were put into serum free and left overnight. After 24 hours, a uniform scratch line was made by using white pipette tips. The dishes were washed with serum free two times to remove the debris and then 5µM Gefitinib, 5µM Erlotinib and 25µM PD98059 were added into the dishes one-hour prior addition of EGF and TGFα. Photographs were taken at appropriated times.

5.3.3 Immunofluorescence

As described in detail in Chapter 3 section 11, immunofluorescence technique were performed with Anti EGFR antibody with 1:300 dilution, Phospho P44/42 MAPKErk1/2)(Thr202/tyr204) and Phospho-473 Akt with 1:200 dilution respectively.

5.3.4 SDS and Western blot

As described in Chapter 3, SDS and Western blot were performed with phosphor- EGFR (Tyr1068) antibody with 1:1000 dilution, Phospho- P44/42 MAPK (Erk1/2) (Thr202/tyr204) and Phospho-473 Akt antibodies with 1:2000 respectively. Cells treated with serum free control and tested conditions for 5 minutes and 24 hours were put into the lane as table below.

Table 5.1 Western blot lanes and test condition

Lane 1	Serum free
Lane 2	EGF 50ng/ml
Lane 3	TGFα 50ng/ml
Lane 4	5μM Gefitinib+EGF 50ng/ml
Lane 5	5μM Gefitinib+TGFα 50ng/ml
Lane 6	5μM Erlotinib+EGF 50ng/ml
Lane 7	5μM Erlotinib+TGFα 50ng/ml
Lane 8	25μM PD98059+EGF 50ng/ml
Lane 9	25μM PD98059+TGFα 50ng/ml

5.4 Results

The results part of the chapter are split into the effect of the inhibitors on scattering/morphology changes, migration/gap closure, localisation of markers/immunofluorescence/SDS-PAGE/Western blotting into a number of different sections:

5.4.1: Inhibitor concentrations of EMT and scattering assay

Inhibitors were incubated on the cells for an hour before the addition of EGF (50ng/ml) and TGFα (50ng/ml). Photomicrographs were taken at two time points 24 and 48 hours. In growth factor treated dishes, there was no significant change in cell morphology at the 24hours time point in most of the experiments (Discussed in chapter 4).

For the inhibitor treated dishes, the recommended dosage for Gefitinib and Erlotinib are 1-10 µM for 0.5-2hr prior to treating with EGF and TGFα. It has been reported that it can be used up to 24 hr. The recommended dose for PD98059 is 5 - 50µM for one hour prior to treating with a stimulator. There was no suggested time limit for PD98059.

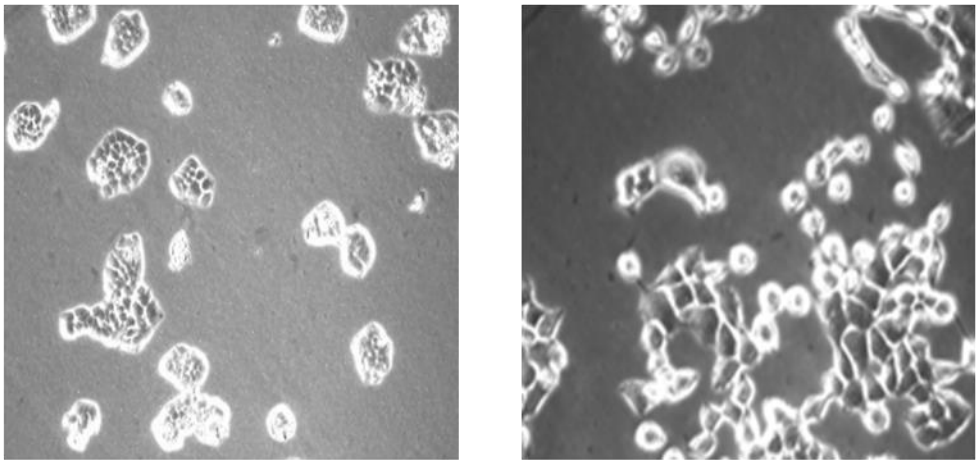
In this pilot project, 3µM, 5µM and 10µM of (Gefitinib and Erlotinib) and 5µM, 25µM and 50µM of PD98059 were used as preliminary experiments. Later, 5µM of (Gefitinib and Erlotinib) and 25µM of PD98059 was chosen to do experiments because these are the concentrations which fully block the effect of EGF and TGFα without being toxic to the cells. In this project inhibitors were used up to 48 hour for monitoring of the morphology changes which is beyond the recommend time point from the manufacturer. Usually, the cells became unhealthy (full of debris-data not shown) by the end of experiment. We deduce from the photographic evidence that this is caused

by the effect of inhibitors since serum free (negative control) and growth factors (positive effects) treated dishes have no unhealthy cells. Photographs were also taken at 48 hour time point. Changes in cell morphology usually occurred around 48 hours' time point. Once the photomicrographs were taken, cells were fixed for immunofluorescence.

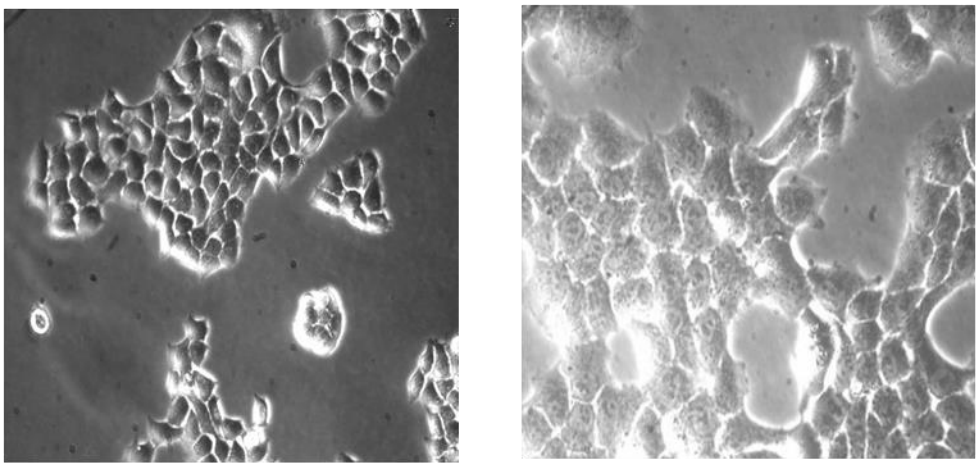
An example of preliminary experiment is shown in Figure 5.2: The HSG cell line was plated at 1x 10^5 per dish (30% confluence) and TGFα (50ng/ml) and the inhibitor Erlotinib was added at different concentrations (3μM, 5μM and 10μM). The photomicrographs show tight colonies in the serum free conditions and that the cells round and migrate apart in the presence of TGFα (Figure 5.2.i and ii). With the addition of the different concentrations of the inhibitor Erlotinib the cells morphology was restored to similar to serum free with all concentrations. The data indicates that the cells morphology was restored to being similar to cells in Serum free conditions at all concentrations of Erlotinib but was decided to use 5μM of Erlotinib in the following experiments (similar results were seen with Gefitinib and cells required 25μM of PD98059 data not shown). It should also be noted that different magnifications were used here to emphasise the change in shape. The serum free being at a lower power to show that small tight colonies.

Figure 5.2: Different doses of the inhibitor Erlotinib of HSG cell line.

i) Serum free **ii) TGFα 50ng/ml**



iii) Erlotinib 3µM+ TGFα50ng **iv) Erlotinib 5µM +TGFα50ng**



v) Erlotinib 10µM + TGFα 50ng/ml

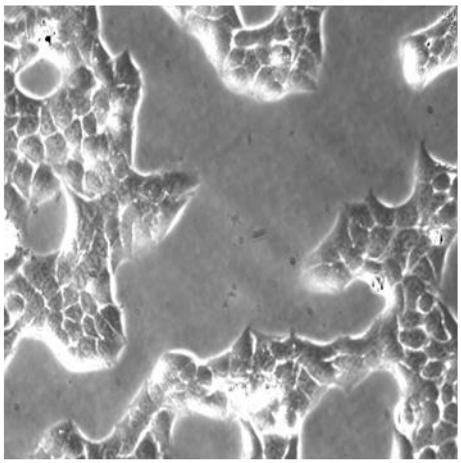


Figure 5.2: The *HSG cell line was plated at* 1x 10^5 per dish *(30% confluence) and TGFα and the inhibitor Erlotinib was added. The photomicrograph was taken after 48 hrs of addition of 50ng/ml TGFα different and concentrations of Erlotinib (3µM, 5µM and 10µM) at 40x magnification.*

5.4.2 HaCaT cell line in response to growth factors and inhibitors

HaCaT cells are characterised and reported as a normal epithelial cell line, although they do have some peculiarities, in that they produce e-cadherin and no vimentin. In the following experiments the cells were plated onto dishes at $1x10^5$ cells per dish and allowed to attach. The medium was changed to SF-MEM and the cells left overnight. The following day the medium was changed into test conditions and photomicrographs taken at various time-points (not all data shown). The test conditions are described in the Figure legend and it should be noted that the photomicrographs of the serum free, EGF treated and TGFα treated cells in all parts of Figure 5.3 are the same- they are shown like this for comparison to the inhibitors.

Figure 5.3a shows the effect of the EGFR inhibitor Gefitinib (5μM) on EGF and TGFα stimulated cells. The photographs indicate that both EGF and TGFα induced reduced cell-cell contact and a spindle shaped morphology in the cells Figure 5.3a.iii and v (Coloured arrows- yellow arrows indicate normal epithelial cell morphology and red arrows indicate mesenchymal morphology). Treatment with the EGFR inhibitor Gefitinib inhibits these changes (Figure 5.3a.iv and vi). Note that the cell-cell contacts are intact and the cells retain their tightly formed colony status.

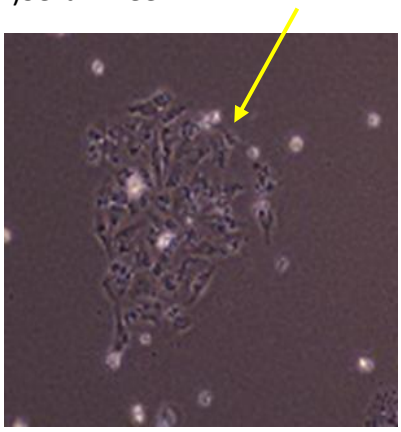
Figure 5.3b shows the effect of the EGFR inhibitor Erlotinib (5μM) on EGF and TGFα stimulated cells. The photomicrographs indicate that both EGF and TGFα induced reduced cell-cell contact and a spindle shaped morphology in the cells Figure 5.5.5.1b.iii and v (Coloured arrows- yellow arrows indicate normal epithelial cell morphology and red arrows indicate mesenchymal morphology). Treatment with the EGFR inhibitor

Erlotinib inhibits these changes (Figure 5.5.5.1b.iv and vi). Note that the cell-cell contacts are intact and the cells retain their tightly formed colony status.

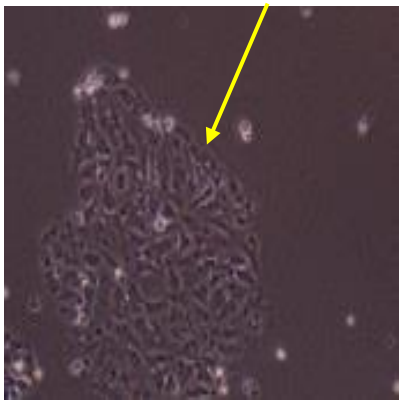
Figure 5.3.c shows the effect of the MAPKinase inhibitor PD98059 (25μM) on EGF and TGFα stimulated cells. The photographs indicate that both EGF and TGFα induced reduced cell-cell contact and a spindle shaped morphology in the cells Figure 5.5.5.1c.iii and v (Coloured arrows- yellow arrows indicate normal epithelial cell morphology and red arrows indicate mesenchymal morphology). Treatment with the MAPkinase inhibitor inhibits these changes (Figure 5.5.5.1c.iv and vi). Note that the cell-cell contacts are intact and the cells retain their tightly formed colony status.

Figure 5.3a:HaCaT cell line in response to growth factors and Gefitinib:

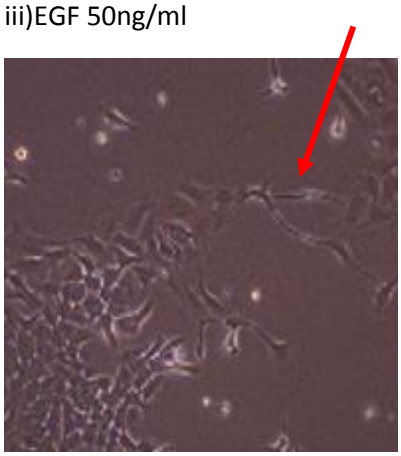
i)Serumfree



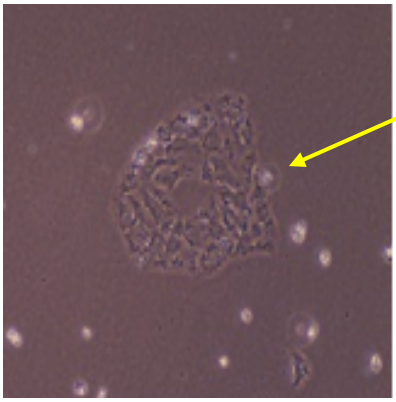
ii)Gefitinib5µM



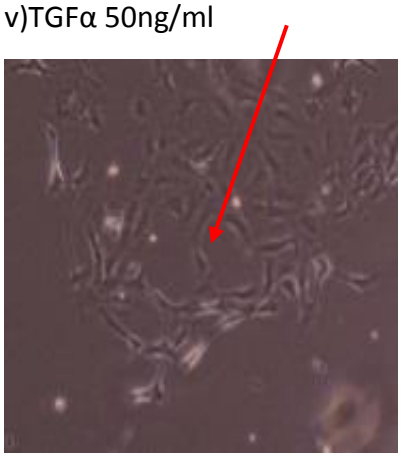
iii)EGF 50ng/ml



iv)Gefitinib5µM+ EGF 50ng/ml



v)TGFα 50ng/ml



vi)Gefitinib5µM+TGFα50ng/ml

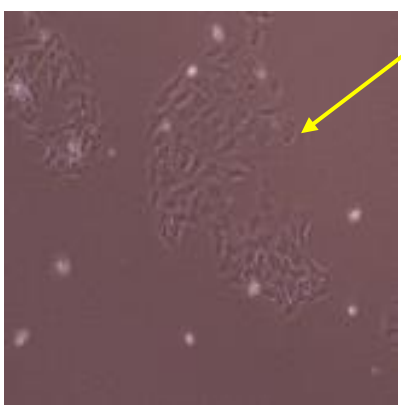


Figure 5.3a: The HaCaT cell line was plated at 1x 10^5 per dish (30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Gefitinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 40x magnification. Treatments: i) SF-MEM, ii) 5µM Gefitinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Gefitinib, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Gefitinib.(yellow arrow indicates epithelial phenotype and red arrow indicates mesenchymal phenotype)

Figure 5.3.b: HaCaT cell line in response to growth factors and Erlotinib:

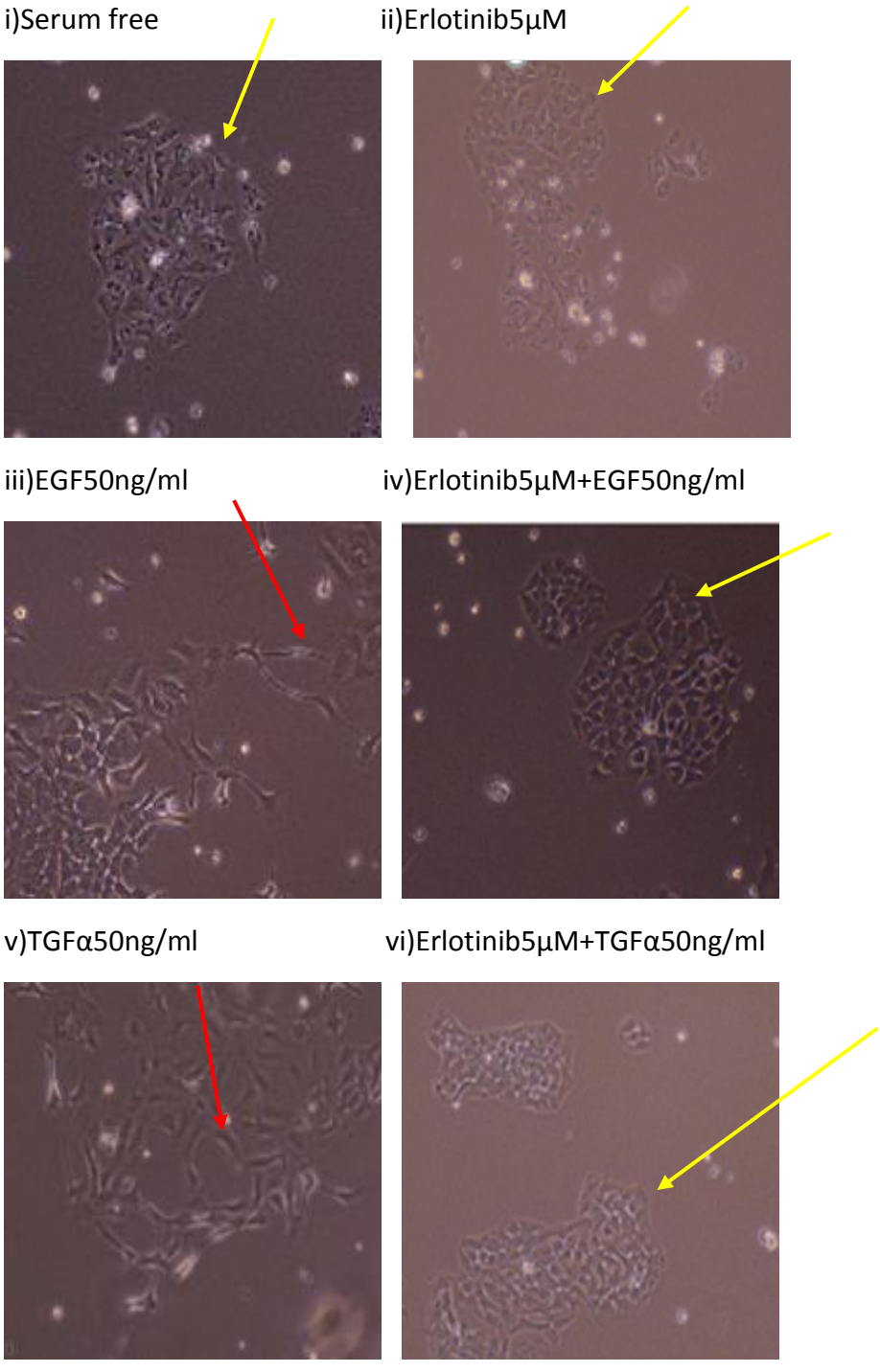


Figure5.3b The HaCaT cell line was plated at 1x10^5 per dish(30% confluence) and EGF(50ng/ml) and TGFα(50ng/ml) were added with 5µM Erlotinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 100x magnification. Treatments: i)SF-MEM, ii) 5µM Erlotinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF / 5µM Erlotinib v) 50ng/ml TGFα, vi) 50ng/ml TGFα/ 5µM Erlotinib(yellow arrow indicates epithelial phenotype and red arrow indicates mesenchymal phenotype.

Figure 5.3c: HaCaT cell line in response to growth factors and PD98059:

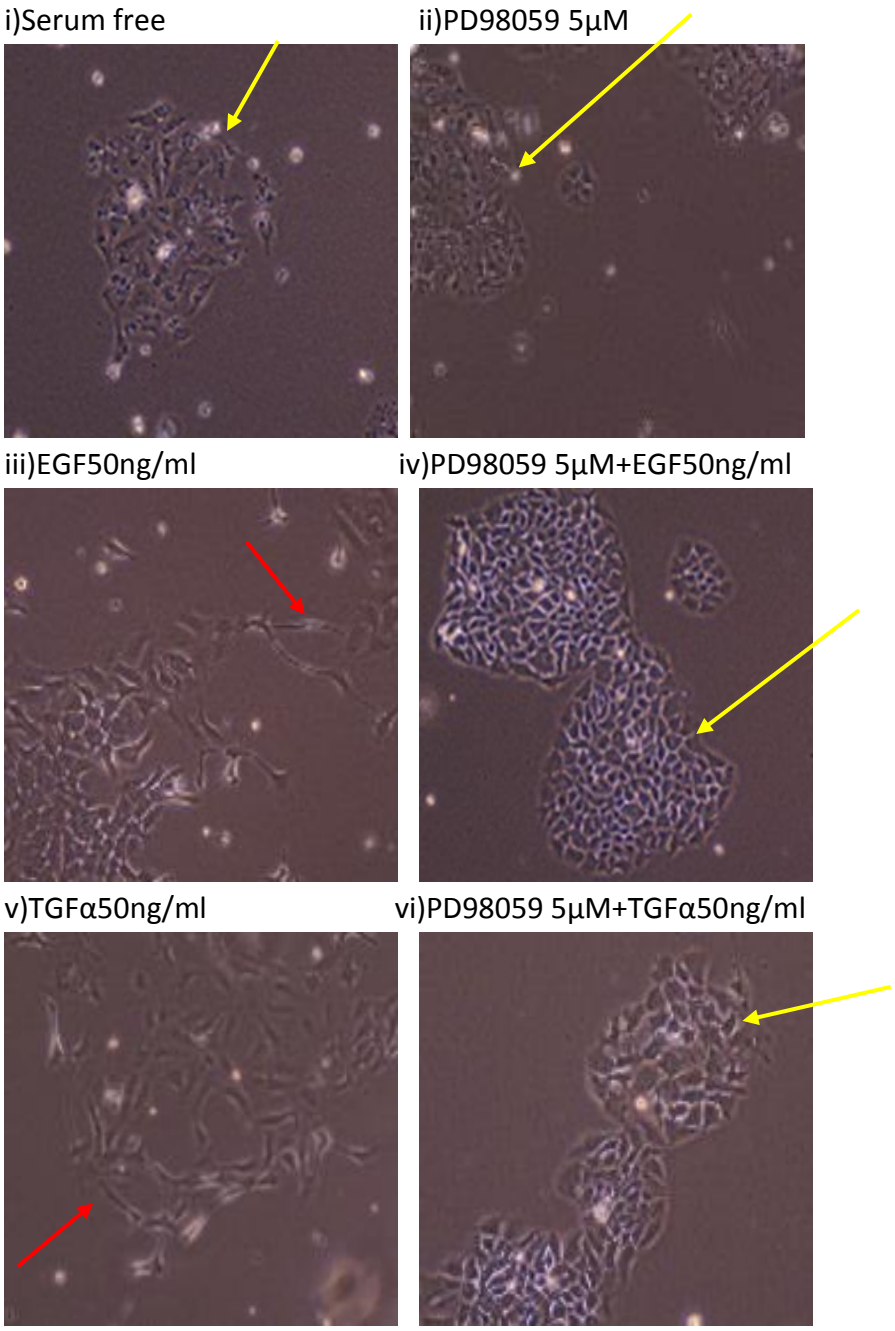


Figure 5.3c: The HaCaT cell line was plated at 1x 10^5 per dish (30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 25µM PD98059. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 40x magnification. Treatments: i) SF-MEM, ii) 25µM PD98059, iii) 50ng/ml EGF, iv) 50ng/ml EGF/25µM PD98059, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/25µM PD98059. (yellow arrow indicates epithelial phenotype and red arrow indicates mesenchymal phenotype)

5.4.3 TYS cell line in response to growth factors and inhibitors

TYS cells are characterised and reported as an oral squamous cell carcinoma, although there is some ambiguity in the original report (see Material and Methods). In the following experiments the cells were plated onto dishes at $1x10^5$ cells per dish and allowed to attach. The medium was changed to SF-MEM and the cells left overnight. The following day the medium was changed into test conditions and photomicrographs taken at various time-points (not all data shown). The test conditions are described in the Figure legend and it should be noted that the photomicrographs of the serum free, EGF treated and TGFα treated cells in all parts of Figure 5.4 are the same- they are shown like this for comparison to the inhibitors.

Figure 5.4a shows the effect of the EGFR inhibitor Gefitinib (5μM) on EGF and TGFα stimulated cells. The photographs indicate that both EGF and TGFα induced reduced cell-cell contact and a spindle shaped morphology in the cells Figure 5.4a.iii and v (Coloured arrows- yellow arrows indicate normal epithelial cell morphology and red arrows indicate mesenchymal morphology). Treatment with the EGFR inhibitor Gefitinib inhibits these changes (Figure 5.4a.iv and vi). Note that the cell-cell contacts are intact and the cells retain their tightly formed colony status.

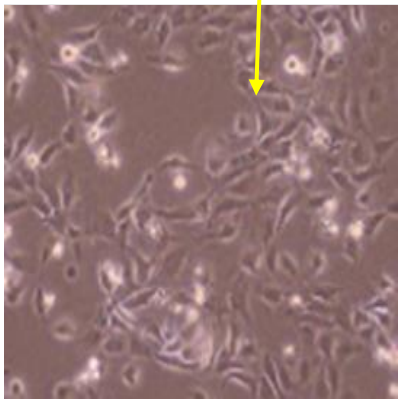
Figure 5.4b shows the effect of the EGFR inhibitor Erlotinib (5μM) on EGF and TGFα stimulated cells. The photographs indicate that both EGF and TGFα induced reduced cell-cell contact and a spindle shaped morphology in the cells Figure 5.4.iii and v (Coloured arrows- yellow arrows indicate normal epithelial cell morphology and red arrows indicate mesenchymal morphology). Treatment with the EGFR inhibitor Erlotinib

inhibits these changes (Figure 5.4b.iv and vi). Note that the cell-cell contacts are intact and the cells retain their tightly formed colony status.

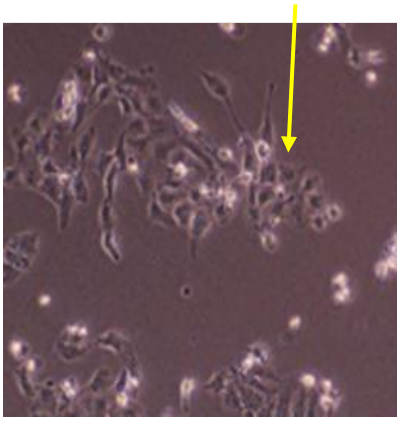
Figure 5.4c shows the effect of the MAPKinase inhibitor PD98059 (25μM) on EGF and TGFα stimulated cells. The photographs indicate that both EGF and TGFα induced reduced cell-cell contact and a spindle shaped morphology in the cells Figure 5.4c.iii and v (Coloured arrows- yellow arrows indicate normal epithelial cell morphology and red arrows indicate mesenchymal morphology). Treatment with the MAPkinase inhibitor inhibits these changes (Figure 5.4c.iv and vi). Note that the cell-cell contacts are intact and the cells retain their tightly formed colony status.

Figure5.4a: TYS cell line in response to growth factors and Gefitinib:

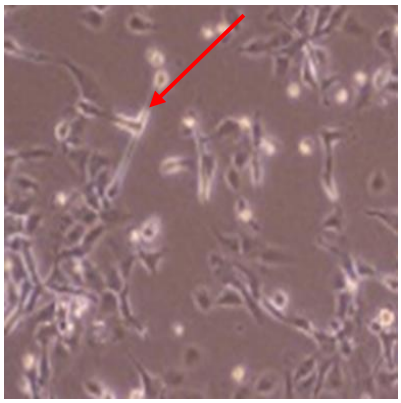
i)Serum free



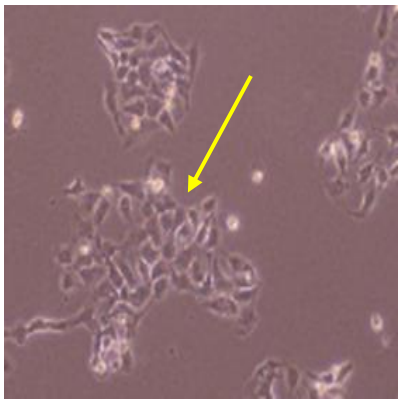
ii)Gefitinib5µM



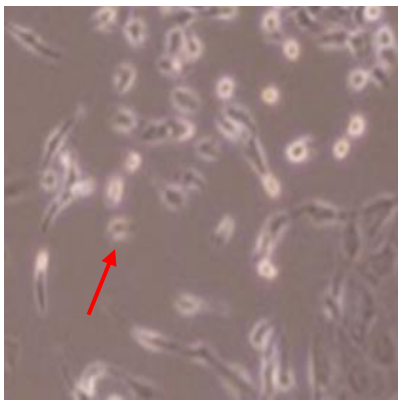
iii)EGF50ng/ml



iv)Gefitinib 5µM+EGF50ng/ml



v)TGFα 50ng/ml



vi) Gefitinib 5µM+ TGFα 50ng/ml

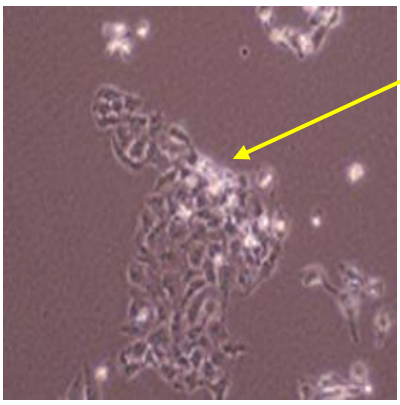
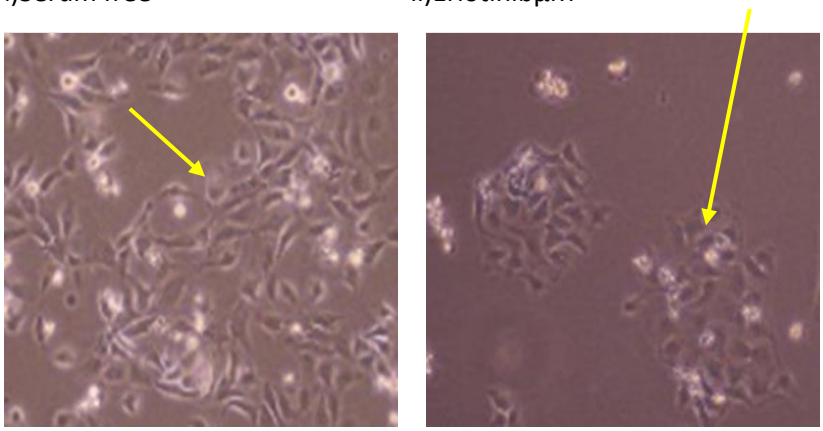


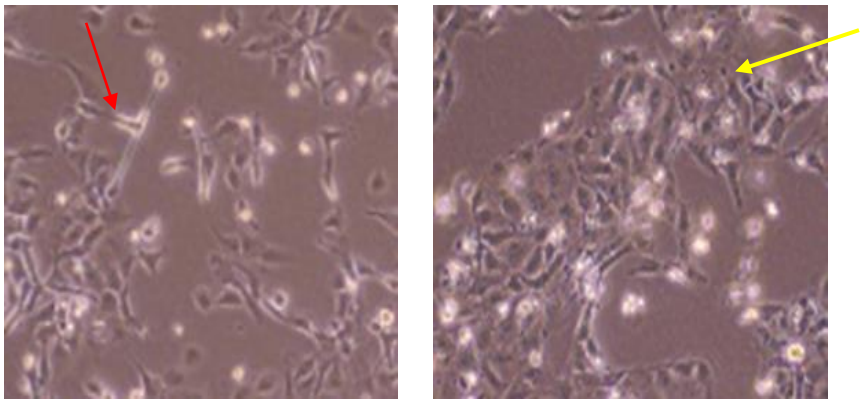
Figure 5.4a: The TYS cell line was plated at 1x 10^5 per dish (30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Gefitinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 40x magnification. Treatments: i) SF-MEM, ii) 5µM Gefitinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Gefitnib, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Gefitnib (yellow arrow indicates epithelial phenotype and red arrow indicates mesenchymal phenotype)

Figure 5.4b: TYS cell line in response to growth factors and Erlotinib:

i)Serum free ii)ErlotinibµM



iii)EGF50ng/ml iv)Erlotinib5µM+EGF50ng/ml



v)TGFα50ng/ml vi)Erlotinib 5µM+ TGFα50ng/ml

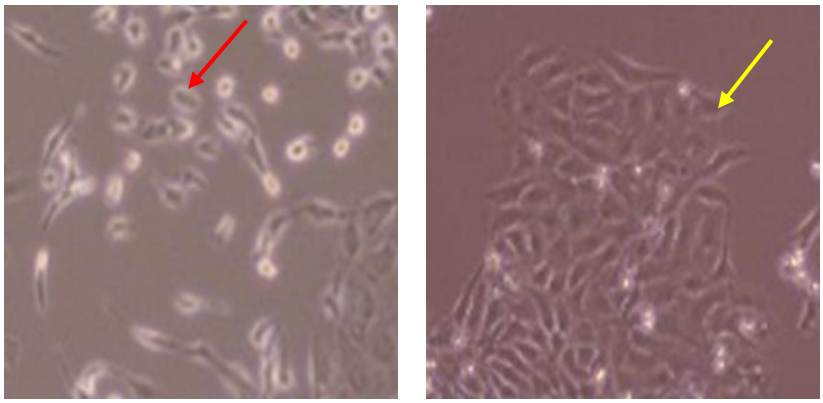


Figure5.4b The TYS cell line was plated at 1x10^5 per dish(30% confluence) and EGF(50ng/ml) and TGFα(50ng/ml) were added with 5µM Erlotinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 100x magnification. Treatments: i) SF-MEM, ii) 5µM Erlotinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF / 5µM Erlotinib v) 50ng/ml TGFα, vi) 50ng/ml TGFα/ 5µM Erlotinib(yellow arrow indicates epithelial phenotype and red arrow indicates mesenchymal phenotype.

Figure 5.4c: TYS cell line in response to growth factors and PD98059:

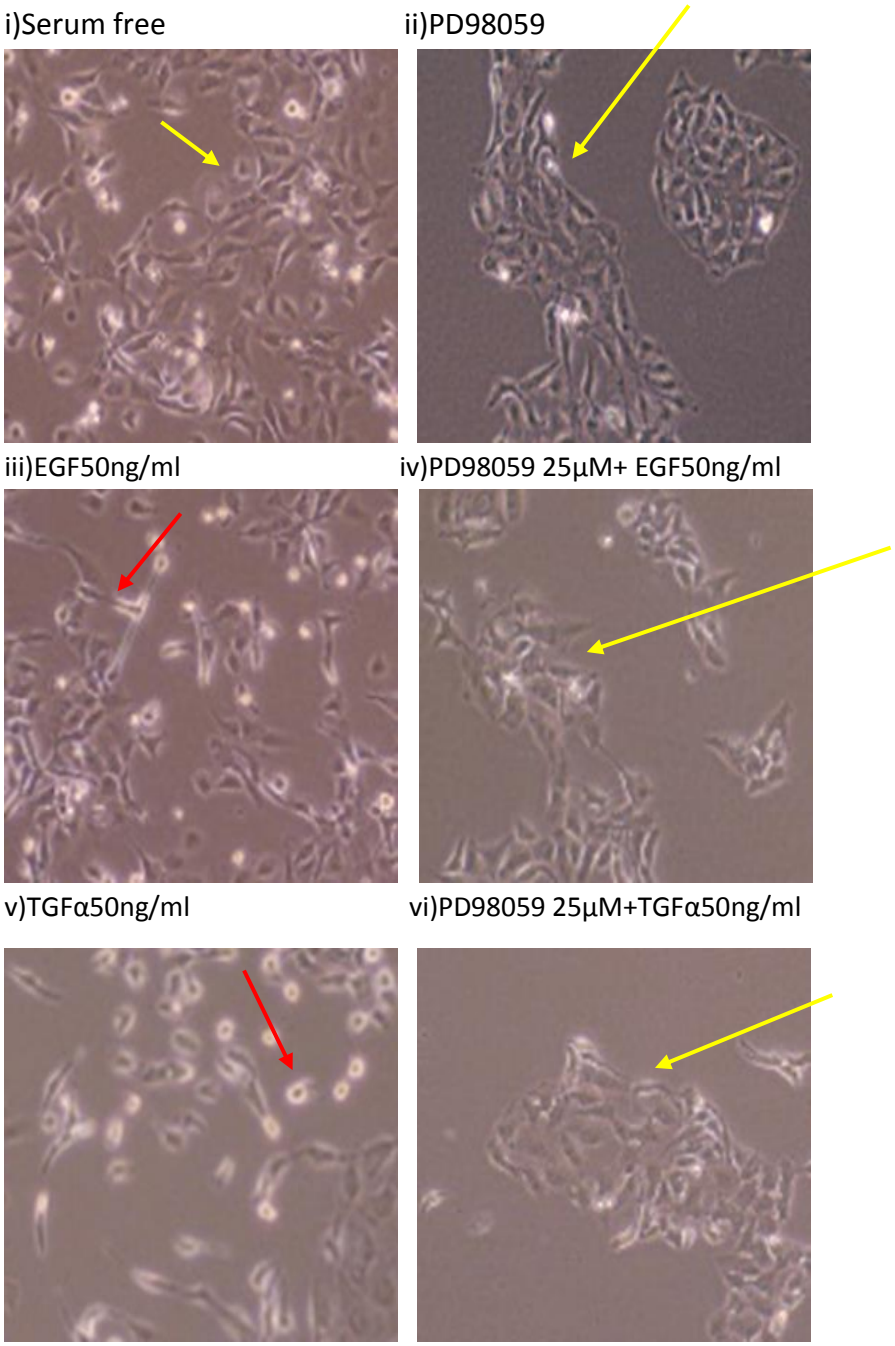


Figure5.4c: The TYS cell line was plated at 1x10^5 per dish(30% confluence) and EGF(50ng/ml) and TGFα(50ng/ml) were added with 25µM PD98059. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 100x magnification. Treatments: i) SF-MEM, ii) 25µM PD98059, iii) 50ng/ml EGF, iv) 50ng/ml EGF / 25µM PD98059 v) 50ng/ml TGFα, vi) 50ng/ml TGFα/ 25µM PD98059 (yellow arrow indicates epithelial phenotype and red arrow indicates mesenchymal phenotype.

5.4.4 AZA1 cell line in response to growth factors and inhibitors

AZA1 cells are characterised and reported HSG cell line treated with 5 Azacytidine (see Materials ad Methods for description). In the following experiments the cells were plated onto dishes at $1x10^5$ cells per dish and allowed to attach. The medium was changed to SF-MEM and the cells left overnight. The following day the medium was changed into test conditions and photomicrographs taken at various time-points (not all data shown). The test conditions are described in the Figure legend and it should be noted that the photomicrographs of the serum free, EGF treated and TGFα treated cells in all parts of Figure 5.5 are the same- they are shown like this for comparison to the inhibitors.

Figure 5.5a shows the effect of the EGFR inhibitor Gefitinib (5μM) on EGF and TGFα stimulated cells. The photographs indicate that both EGF and TGFα induced reduced cell-cell contact and a spindle shaped morphology in the cells Figure 5.5a.iii and v (Coloured arrows- yellow arrows indicate normal epithelial cell morphology and red arrows indicate mesenchymal morphology). Treatment with the EGFR inhibitor Gefitinib inhibits these changes (Figure 5.5a.iv and vi). Note that the cell-cell contacts are intact and the cells retain their tightly formed colony status.

Figure 5.5b shows the effect of the EGFR inhibitor Erlotinib (5μM) on EGF and TGFα stimulated cells. The photographs indicate that both EGF and TGFα induced no cell-cell contact and produced a rounded shaped morphology in the cells Figure 5.5b.iii and v (Coloured arrows- yellow arrows indicate normal epithelial cell morphology and red arrows indicate mesenchymal morphology). It should also be noted that a small number of the cells had a fibroblast morphology. Treatment with the EGFR inhibitor Erlotinib

inhibits these changes (Figure 5.5b.iv and vi). Note that the cell-cell contacts are intact and the cells retain their tightly formed colony status.

Figure 5.5c shows the effect of the MAPKinase inhibitor PD98059 (25μM) on EGF and TGFα stimulated cells. The photographs indicate that both EGF and TGFα induced reduced cell-cell contact and a spindle shaped morphology in the cells Figure 5.5c.iii and v (Coloured arrows- yellow arrows indicate normal epithelial cell morphology and red arrows indicate mesenchymal morphology). Treatment with the MAPkinase inhibitor inhibits these changes (Figure 5.5c.iv and vi). Note that the cell-cell contacts are intact and the cells retain their tightly formed colony status.

Figure 5.5a: AZA1 cell line in response to growth factors and Gefitinib:

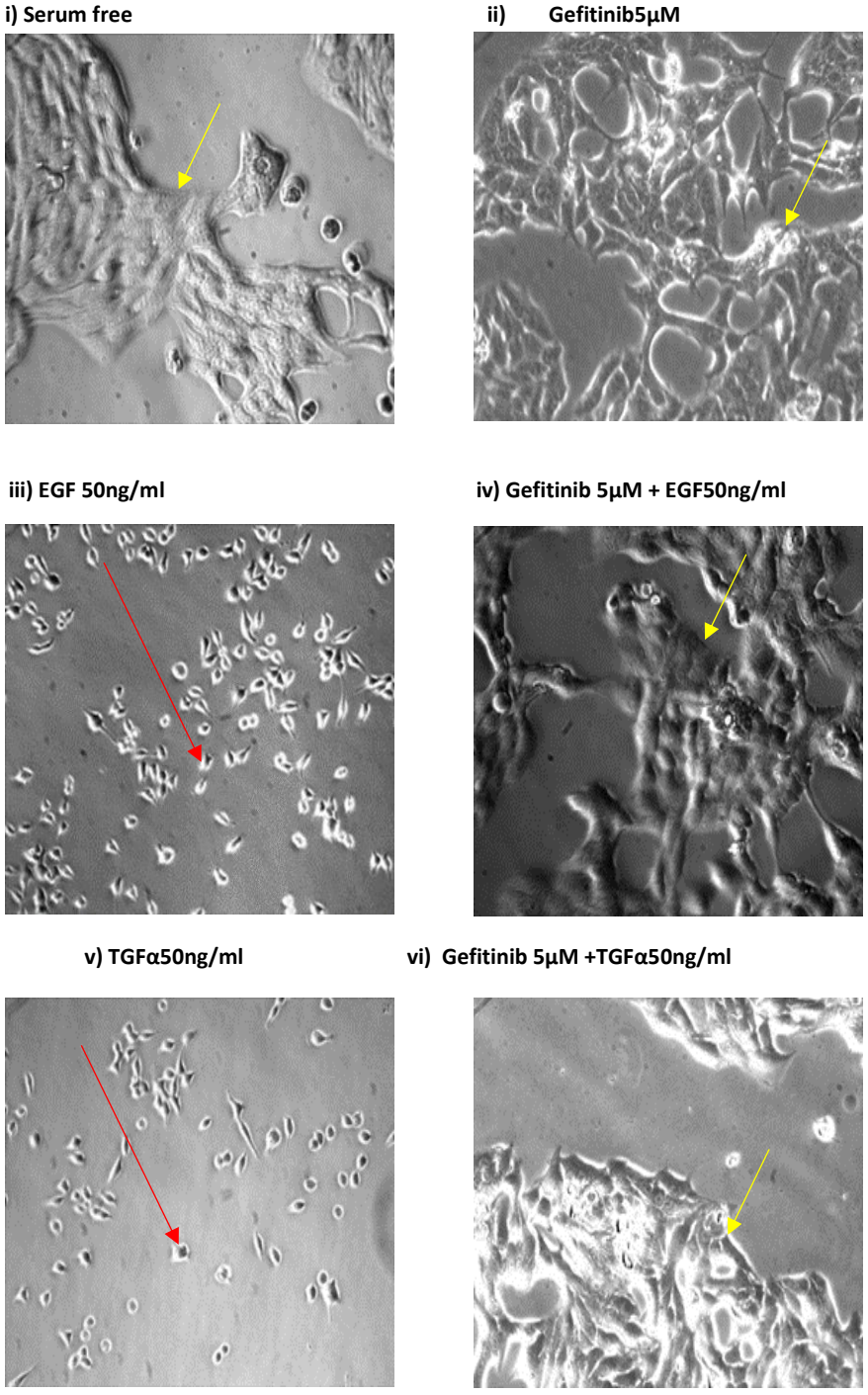
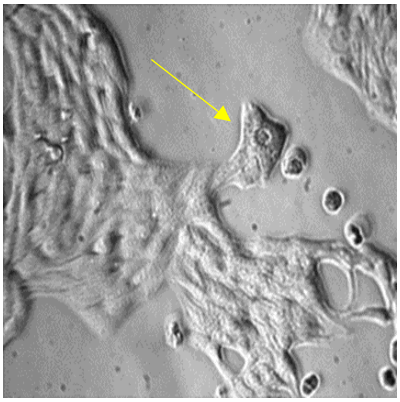
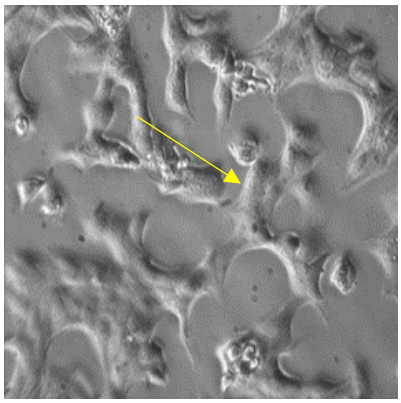


Figure 5.5a: The AZA1 cell line was plated at 1x 10^5 per dish (30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Gefitinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 40x magnification. Treatments: i) SF-MEM, ii) 5µM Gefitinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Gefitinib, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Gefitinib. (yellow arrow indicate epithelial phenotype and red arrow indicate mesenchymal phenotype)

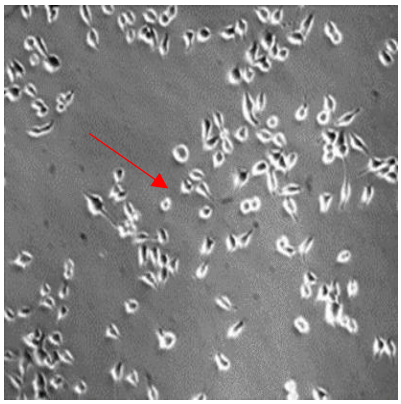
Figure 5.5b: AZA1 cell line in response to growth factors and Erlotinib:

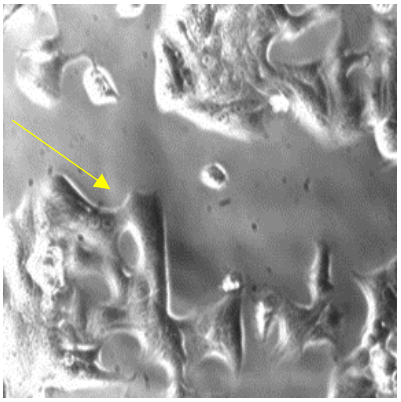
i) Serum free **ii) Erlotinib5µM**



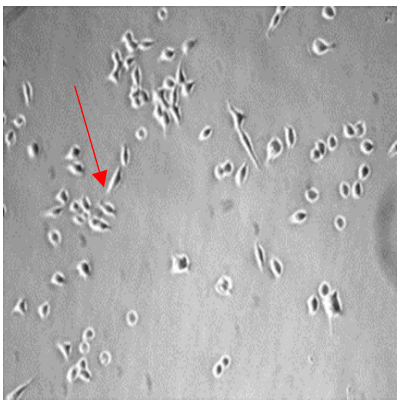


iii) EGF 50ng/ml **iv) Erlotinib 5µM + EGF50ng/ml**





v) **TGFα50ng/ml** **vi) Erlotinib 5µM +TGFα50ng/ml**



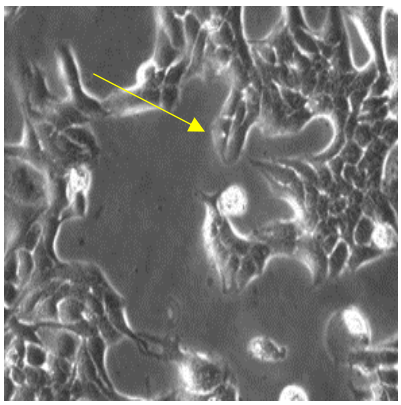


Figure 5.5b: AZA1 ***cell line was plated at*** **1x 10^5 per dish** ***(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Erlotinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 100x magnification. Treatments: i) SF-MEM, ii) 5µM Erlotinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Erlotinib,v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Erlotinib. (yellow arrow indicate epithelial phenotype and red arrow indicate mesenchymal phenotype)***

Figure 5.5c: AZA1 cell line in response to growth factors and PD98059:

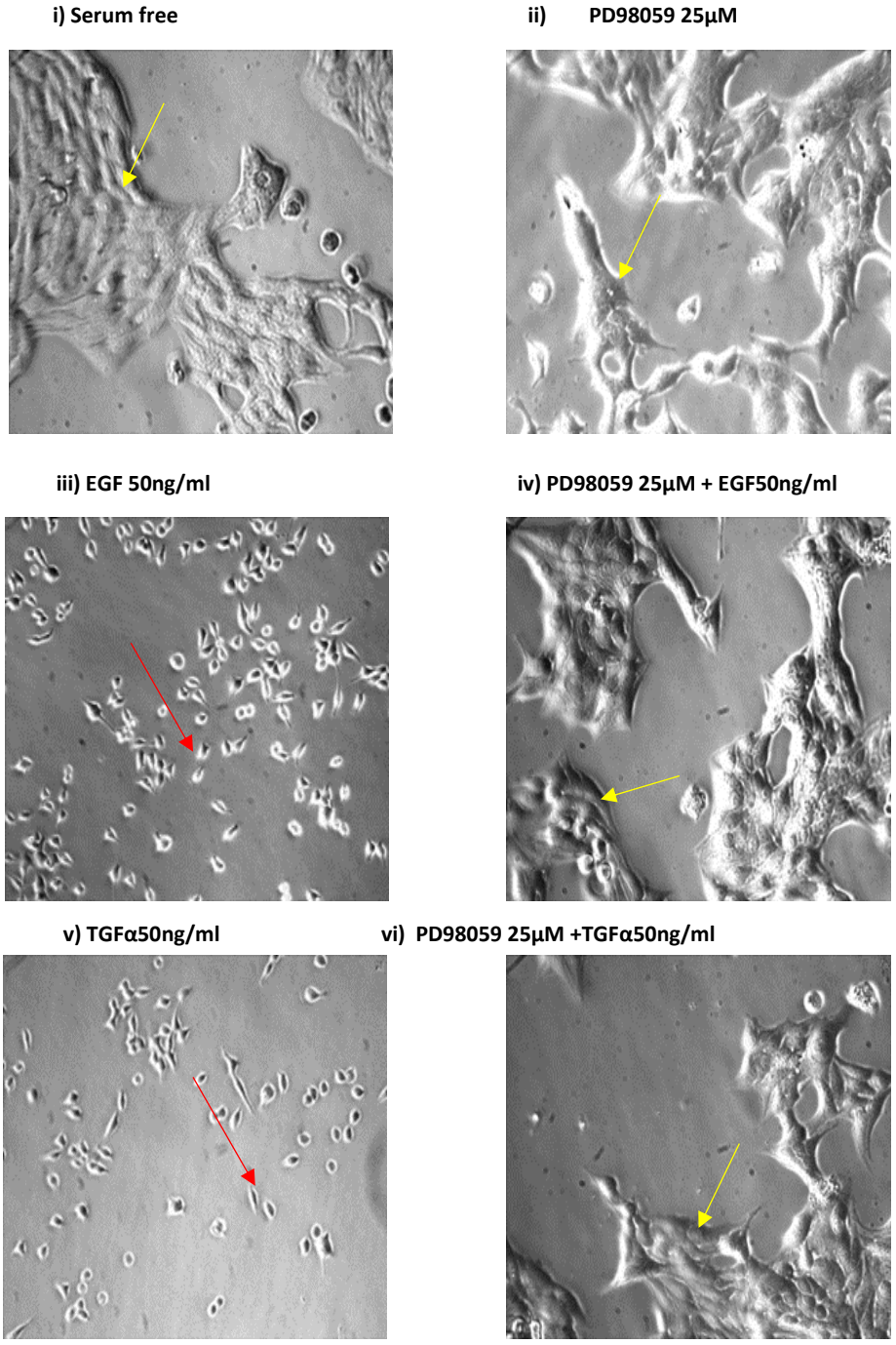


Figure 5.5c: AZA1 cell line was plated at 1x 10^5 per dish (30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 25µM PD98059. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 40x magnification. Treatments: i) SF-MEM, ii) 25µM PD98059, iii) 50ng/ml EGF, iv) 50ng/ml EGF/25µM PD98059, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/25µM PD98059. (yellow arrow indicate epithelial phenotype and red arrow indicate mesenchymal phenotype).

5.4.5 HSG cell line in response to growth factors and inhibitors

HSG cells are characterised and reported as an epithelial duct cell line derived from human salivary gland (Adenocarcinoma of submandibular gland). In the following experiments the cells were plated onto dishes at $1x10^5$ cells per dish and allowed to attach. The medium was changed to SF-MEM and the cells left overnight. The following day the medium was changed into test conditions and photomicrographs taken at various time-points (not all data shown). The test conditions are described in the Figure legend and it should be noted that the photomicrographs of the serum free, EGF treated and TGFα treated cells in all parts of Figure 5.6 are the same- they are shown like this for comparison to the inhibitors.

Figure 5.6a shows the effect of the EGFR inhibitor Gefitinib (5μM) on EGF and TGFα stimulated cells. The photographs indicate that both EGF and TGFα induced reduced cell-cell contact and a spindle shaped morphology in the cells Figure 5.6a.iii and v (Coloured arrows- yellow arrows indicate normal epithelial cell morphology and red arrows indicate mesenchymal morphology). Treatment with the EGFR inhibitor Gefitinib inhibits these changes (Figure 5.6a.iv and vi). Note that the cell-cell contacts are intact and the cells retain their tightly formed colony status.

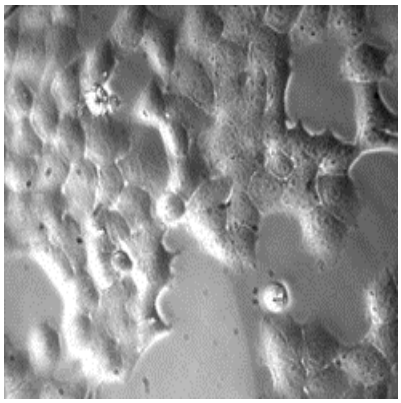
Figure 5.6b shows the effect of the EGFR inhibitor Erlotinib (5μM) on EGF and TGFα stimulated cells. The photographs indicate that both EGF and TGFα induced reduced cell-cell contact and a rounded morphology in the cells Figure 5.6b.iii and v (Coloured arrows- yellow arrows indicate normal epithelial cell morphology and red arrows indicate mesenchymal morphology). It is interesting to note that these changes are different between the two growth factors. Treatment with the EGFR inhibitor Erlotinib

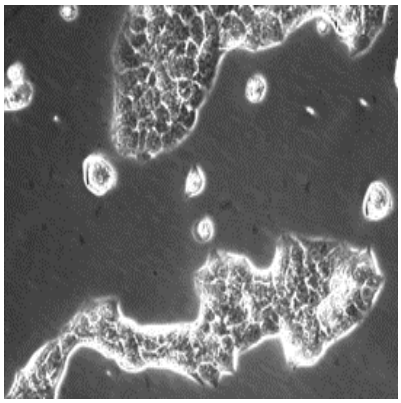
inhibits these changes (Figure 5.6b.iv and vi). Note that the cell-cell contacts are intact and the cells retain their tightly formed colony status. However, the inhibitor treated cells do appear different in morphology from the serum free cells plus inhibitor suggesting that some aspects are inhibited but others are not.

Figure 5.6c shows the effect of the MAPKinase inhibitor PD98059 (25μM) on EGF and TGFα stimulated cells. The photographs indicate that both EGF and TGFα induced reduced cell-cell contact and a spindle shaped morphology in the cells Figure 5.6c.iii and v (Coloured arrows- yellow arrows indicate normal epithelial cell morphology and red arrows indicate mesenchymal morphology). Treatment with the MAP-kinase inhibitor inhibits these changes (Figure 5.6c.iv and vi). Note that the cell-cell contacts are intact and the cells retain their tightly formed colony status.

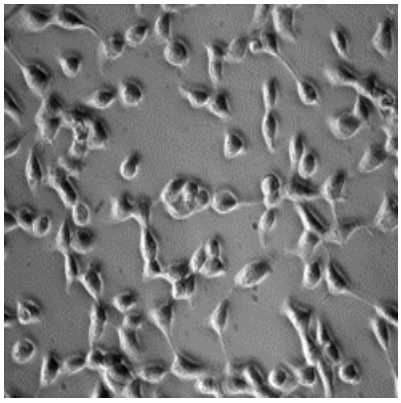
Figure 5.6a: HSG cell line in response to growth factors and Gefitinib:

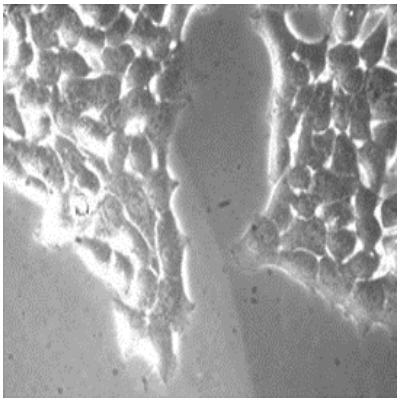
i) Serum free **ii) Gefitinib5µM**



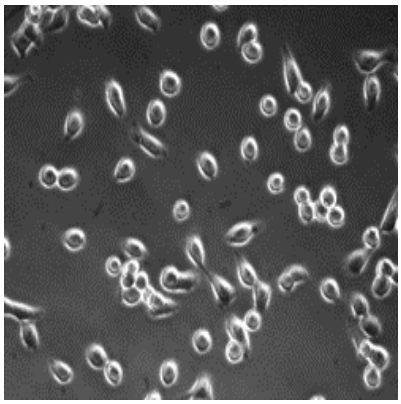


iii) EGF 50ng/ml **iv) Gefitinib 5µM + EGF50ng/ml**





v) TGFα50ng/ml **vi) Gefitinib5µM +TGFα50ng/ml**



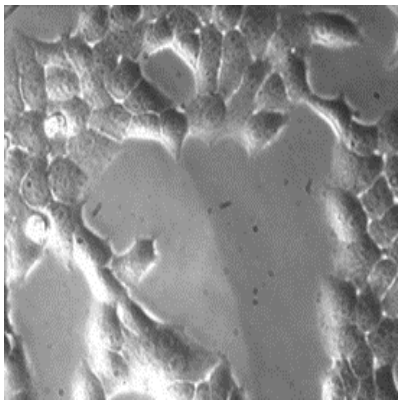
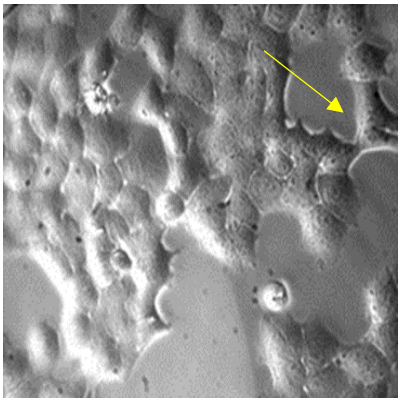
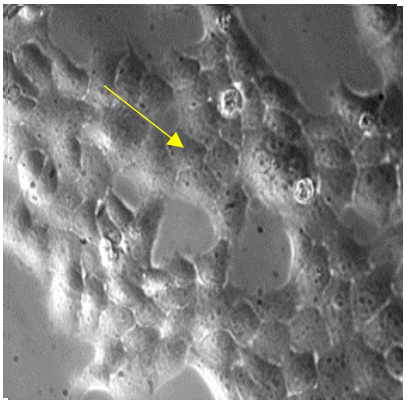


Figure 5.6a: HSG cell line was plated at 1x 10^5 per dish (30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Gefitinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 40x magnification. Treatments: i) SF-MEM, ii) 5µM Gefitinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Gefitnib, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Gefitnib. (yellow arrow indicate epithelial phenotype and red arrow indicate mesenchymal phenotype)

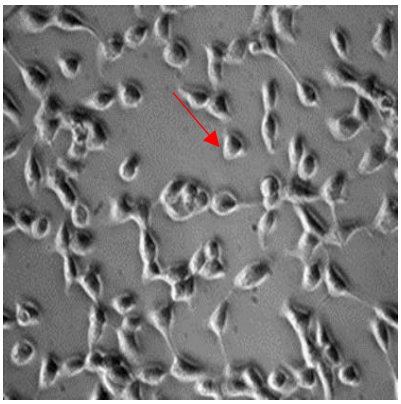
Figure 5.6b: HSG cell line in response to growth factors and Erlotinib:

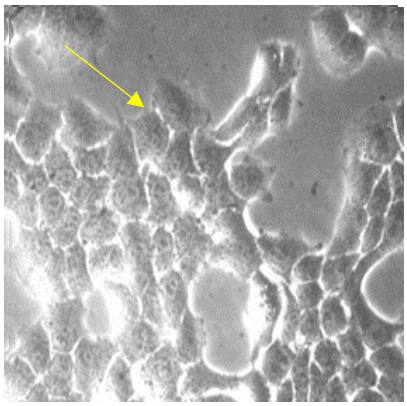
i) Serum free **ii) Erlotinib5µM**



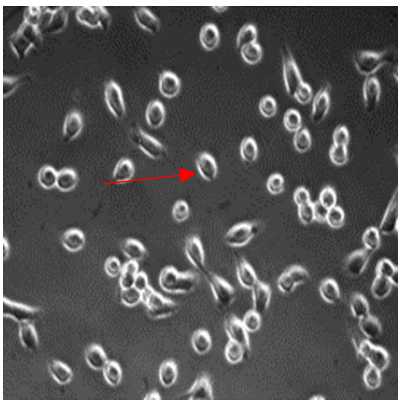


iii) EGF 50ng/ml **iv) Erlotinib 5µM + EGF50ng/ml**





v) TGFα50ng/ml **vi) Erlotinib 5µM +TGFα50ng/ml**



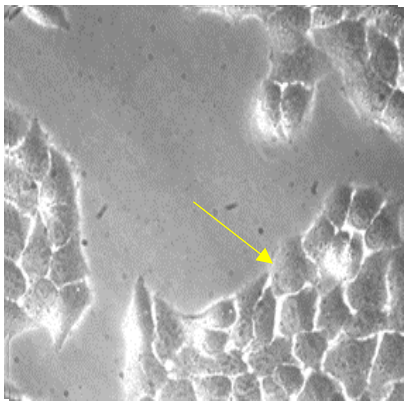
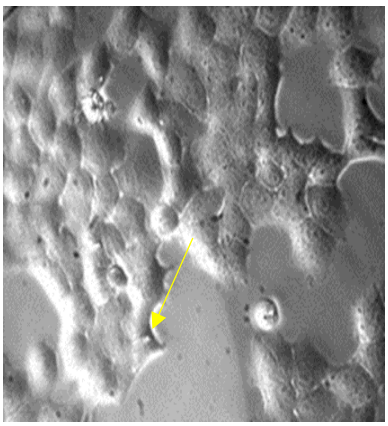


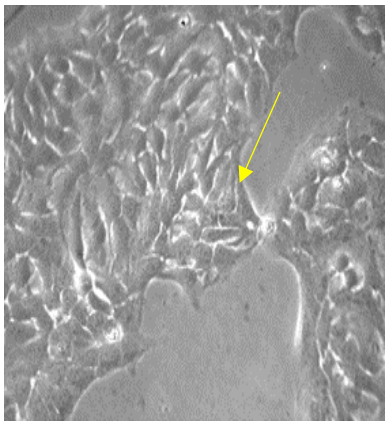
Figure 5.6b: HSG ***cell line was plated at*** **1x 10^5 per dish** ***(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Erlotinib. The photomicrograph was taken after 48 hrs of addition of the test conditions.All micrographs were taken at 100x magnification. Treatments: i) SF-MEM, ii) 5µM Erlotinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Erlotinib,v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Erlotinib. (yellow arrow indicate epithelial phenotype and red arrow indicate mesenchymal phenotype)***

Figure 5.6c: HSG cell line in response to growth factors and

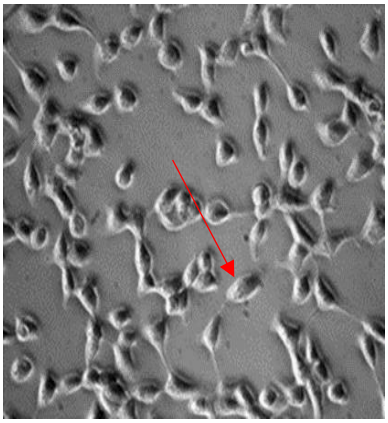
i) Serum free



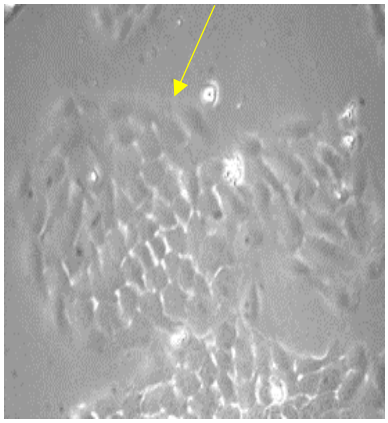
ii) PD98059 25µM



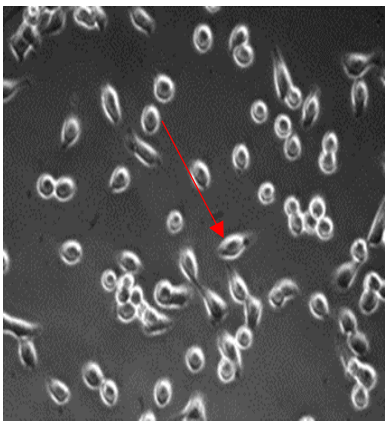
iii) EGF 50ng/ml



iv) PD98059 25µM +



v) TGFα50ng/ml



vi) PD98059 25µM

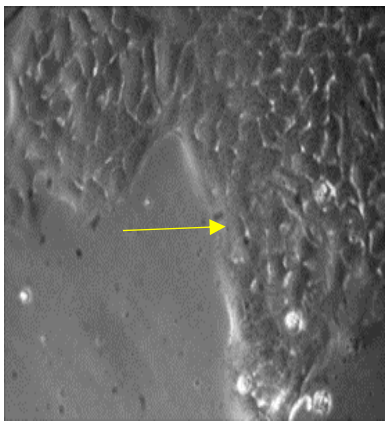


Figure 5.5c: HSG cell line was plated at 1x 10^5 per dish (30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 25µM PD98059. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 40x magnification. Treatments: i) SF-MEM, ii) 25µM PD98059, iii) 50ng/ml EGF, iv) 50ng/ml EGF/25µM PD98059, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/25µM PD98059. (yellow arrow indicate epithelial phenotype and red arrow indicate mesenchymal phenotype)

5.4.6 Results of Cell migration assay

The cells lines that were described in the previous chapter were studied here in this chapter. The idea behind this was to investigate a more deliberate migration of the cells. The Scratch assay has a much more obvious migration point and is measured by gap closure. Cells were grown to confluence and then scratched and the dishes photographed. The cells were then placed in the test medium and photomicrographs taken every 2 hours for 8 hours and then again when the gap closed.

5.4.7 Pilot experiment to investigate the best time point in the scratch assay

The HSG cell line was chosen to investigate the best time point for investigating cell migration by gap closure or scratch assay. An example of preliminary experiment is shown in figure 5.7 in the following experiment the cells were plated onto dishes at $3x10^5$ cells per dish and grown until confluent changing medium 10% FCS every 48 hours. Once confluent the medium was changed to SF-MEM overnight. A scratch was made from one side of the dish to the other. The medium was changed into test conditions (1ng/ml and 10ng/ml of EGF). Photographs were taken at 2 hours, 4 hours, 6 hours and 24 hours. Data in Figure 5.7 indicates that 24 hours was the best time point to take photographs.

5.4.8 Experimental data for TYS cell line in response to growth factors and inhibitors using the gap closure/scratch assay

In the following experiments TYS were plated onto dishes at $3x10^5$ cells per dish and grown until confluent changing medium 10% FCS every 48 hours. Once confluent the medium was changed to SF-MEM overnight. A scratch was made from one side of the dish to the other. The medium was changed into test condition. They were then incubated and the photomicrographs were taken after 24 hours. The test conditions are described in the Figure legend.

Figure 5.8a, b andc shows the effect of Gefitinib (5µM), Erlotinib (5µM) and PD98059 (25µM) on EGF and TGFα stimulated cells. The photographs indicate that both EGF and TGFα induced migration in the cells of TYS cells (Figure 5.9 iii and 5.9v). One hour pre-treatment with Gefitinib, Erlotinib and PD98059 inhibits these migration (figure 5.9 iv,vi,viii,ix,xi,xii).

Figure 5.8 The *TYS cell line plated in presence of the inhibitor Gefitinib and growth factors in Scratch assay.*

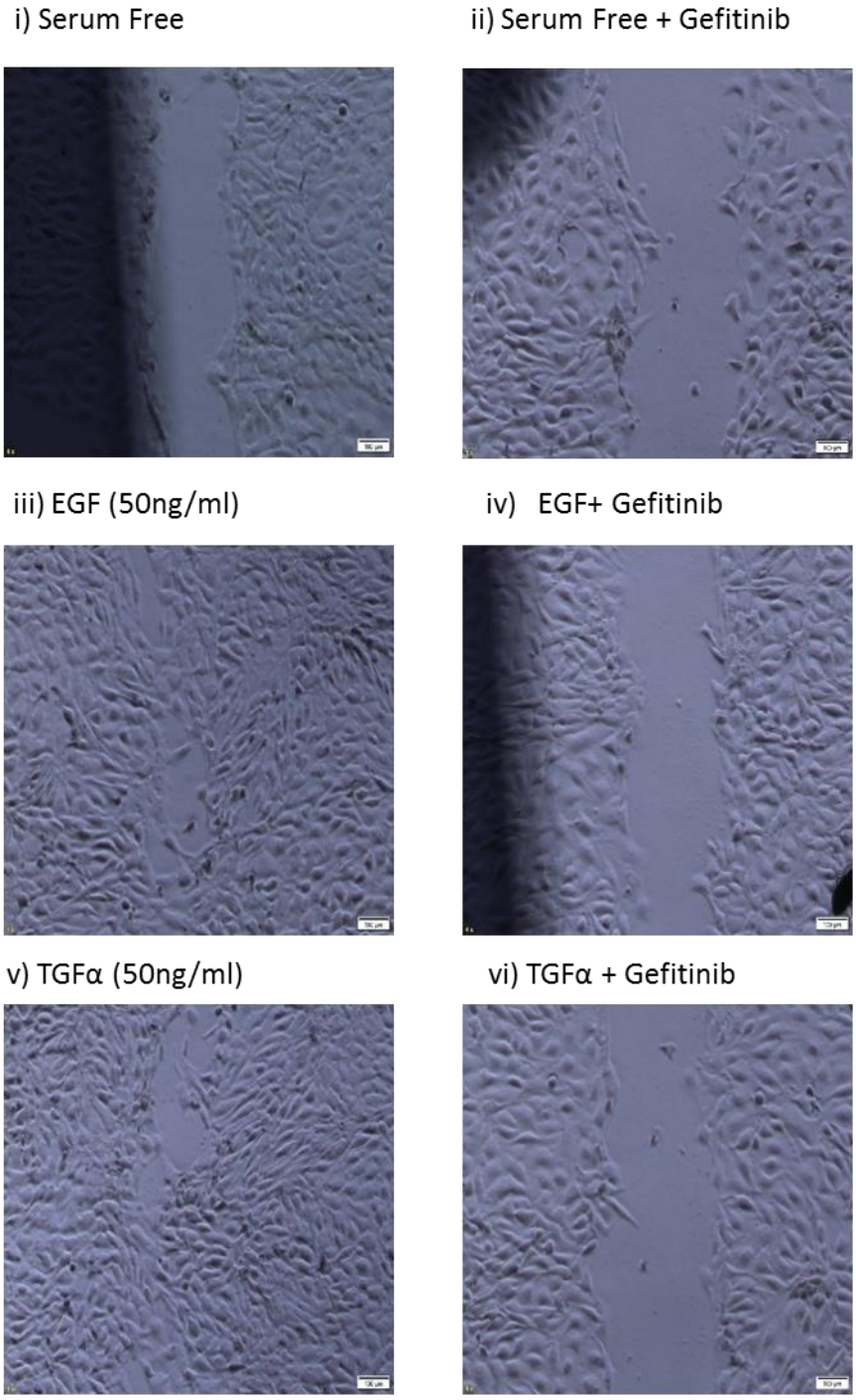


Figure 5.8 The *TYS cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Gefitinib. The photomicrograph was taken after 24 hrs of addition of the test conditions. All micrographs were taken at 10x magnification. Treatments: i) SF-MEM, ii) 5µM Gefitinib iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Gefitinib v) 50ng/ml TGFα vi) 50ng/ml TGFα/5µM Gefitinib.*

Figure 5.8b The *TYS cell line plated in presence of the inhibitor Erlotinib and growth factors in Scratch assay.*

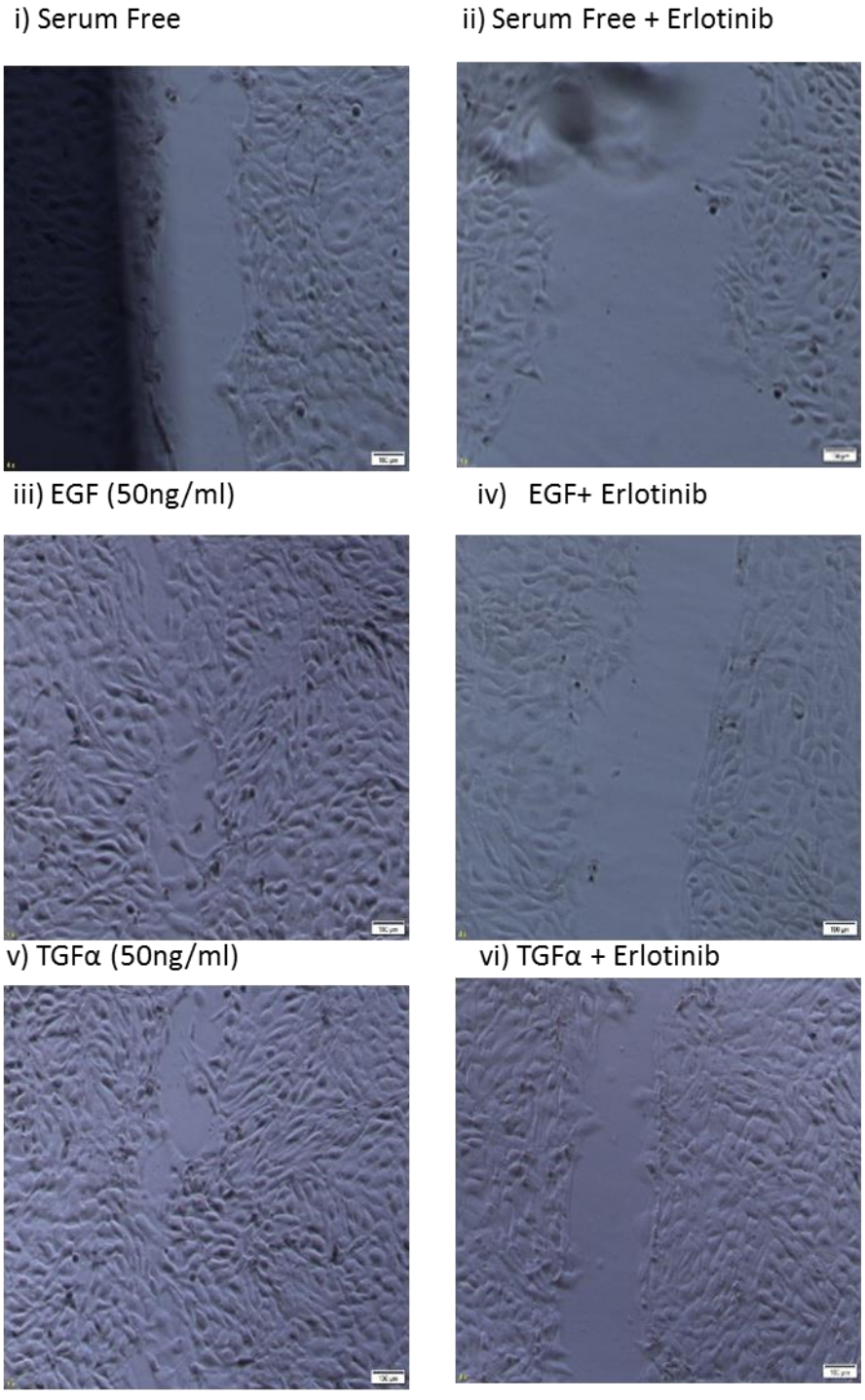


Figure 5.8 The *TYS cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Erlotinib. The photomicrograph was taken after 24 hrs of addition of the test conditions. All micrographs were taken at 10x magnification. Treatments: i) SF-MEM, ii) 5µM Erlotinib iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Erlotinib v) 50ng/ml TGFα vi) 50ng/ml TGFα/5µM Erlotinib.*

Figure 5.8c The *TYS cell line plated in presence of the inhibitor* PD98059 *and growth factors in Scratch assay.*

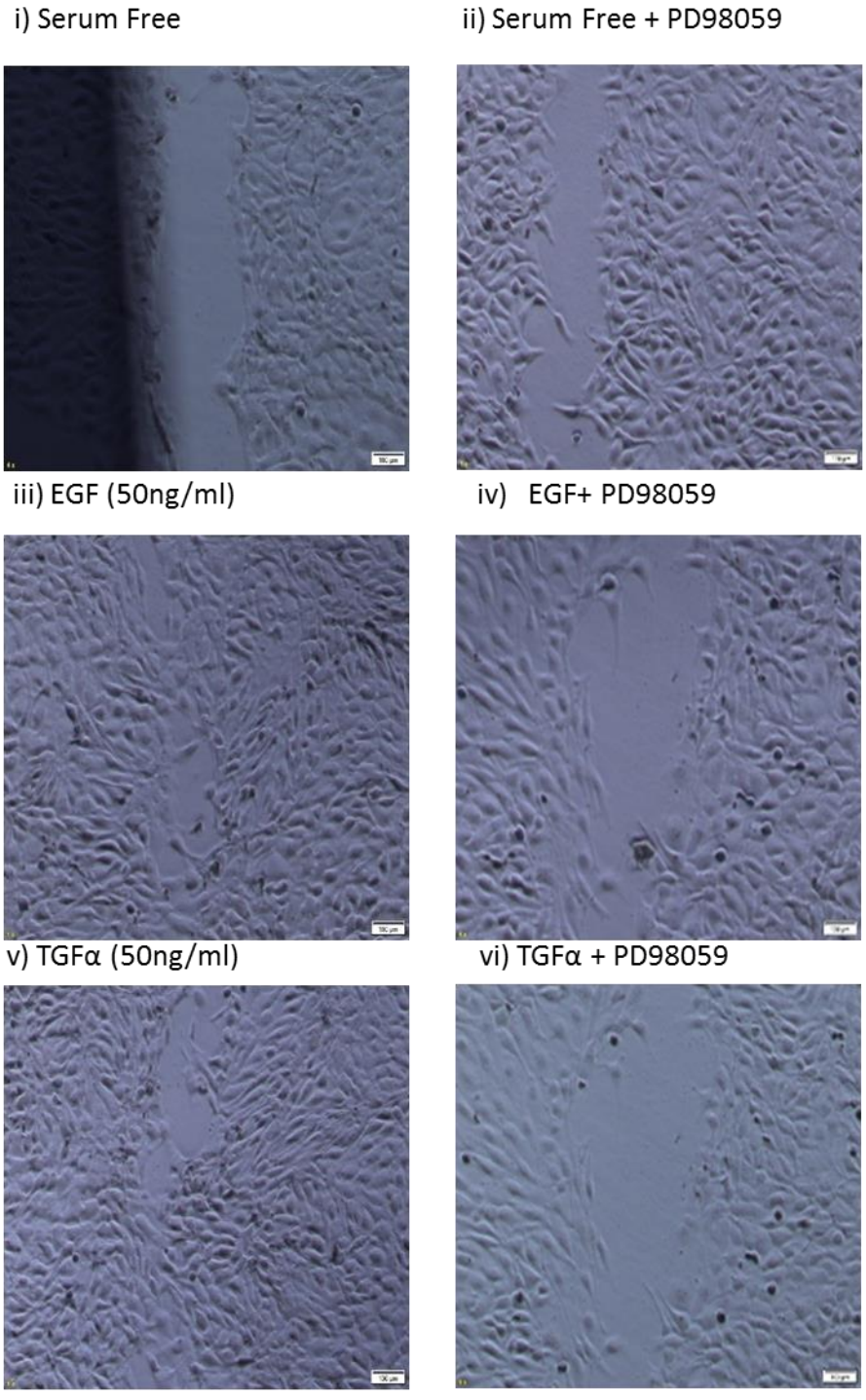


Figure 5.8c The *TYS cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5μM PD98059. The photomicrograph was taken after 24 hrs of addition of the test conditions. All micrographs were taken at 10x magnification. Treatments: i) SF-MEM, ii) 5μM PD98059 iii) 50ng/ml EGF, iv) 50ng/ml EGF/5μM PD98059 v) 50ng/ml TGFα vi) 50ng/ml TGFα/5μM PD98059.*

5.4.9 Experimental data for HaCaT cell line in response to growth factors and inhibitors

In the following experiments HaCaT were plated onto dishes at $3x10^5$ cells per dish and grown until confluent changing medium 10% FCS every 48 hours. Once confluent the medium was changed to SF-MEM overnight. A scratch was made from one side of the dish to the other. The medium was changed into test condition. They were then incubated and the photomicrographs were taken after 24 hours. The test conditions are described in the Figure legend.

Figure 5.9a, b and c shows the effect of Gefitinib (5μM), Erlotinib (5μM) and PD98059 (25μM) on EGF and TGFα stimulated cells. The photographs indicate that both EGF and TGFα induced migration in the cells of HaCaT cells (Figure 5.9 a, b and c). One hour pre-treatment with Gefitinib, Erlotinib and PD98059 inhibits this migration (figure 5.9 a, b and c).

Figure 5.9 The *HaCaT cell line plated in presence of the inhibitor Gefitinib and growth factors in Scratch assay.*

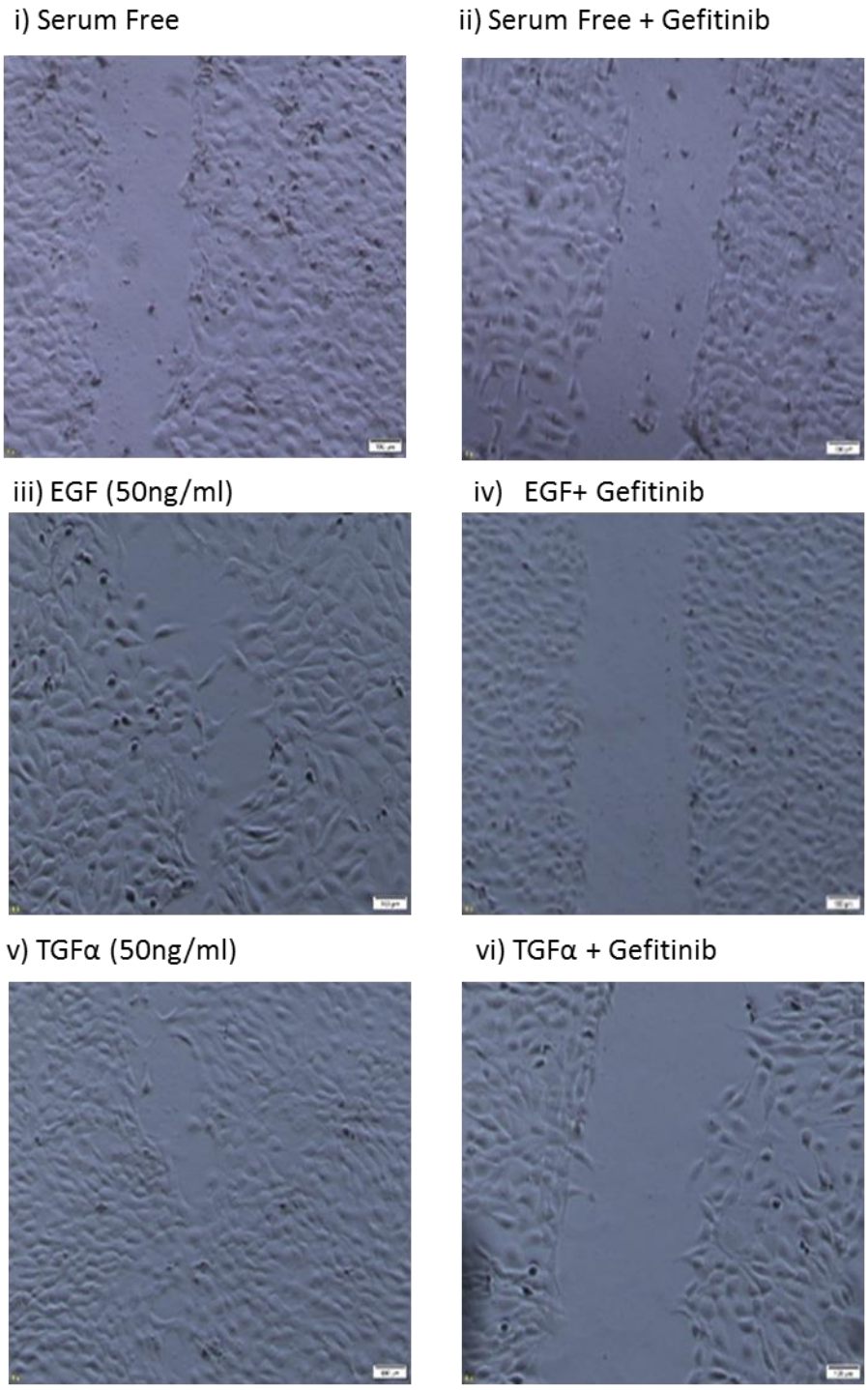


Figure 5.9a The *HacaT cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Gefitinib. The photomicrograph was taken after 24 hrs of addition of the test conditions. All micrographs were taken at 10x magnification. Treatments: i) SF-MEM, ii) 5µM Gefitinib iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Gefitinib v) 50ng/ml TGFα vi) 50ng/ml TGFα/5µM Gefitinib.*

Figure 5.9b The *HaCaT cell line plated in presence of the inhibitor Erlotinib and growth factors in Scratch assay.*

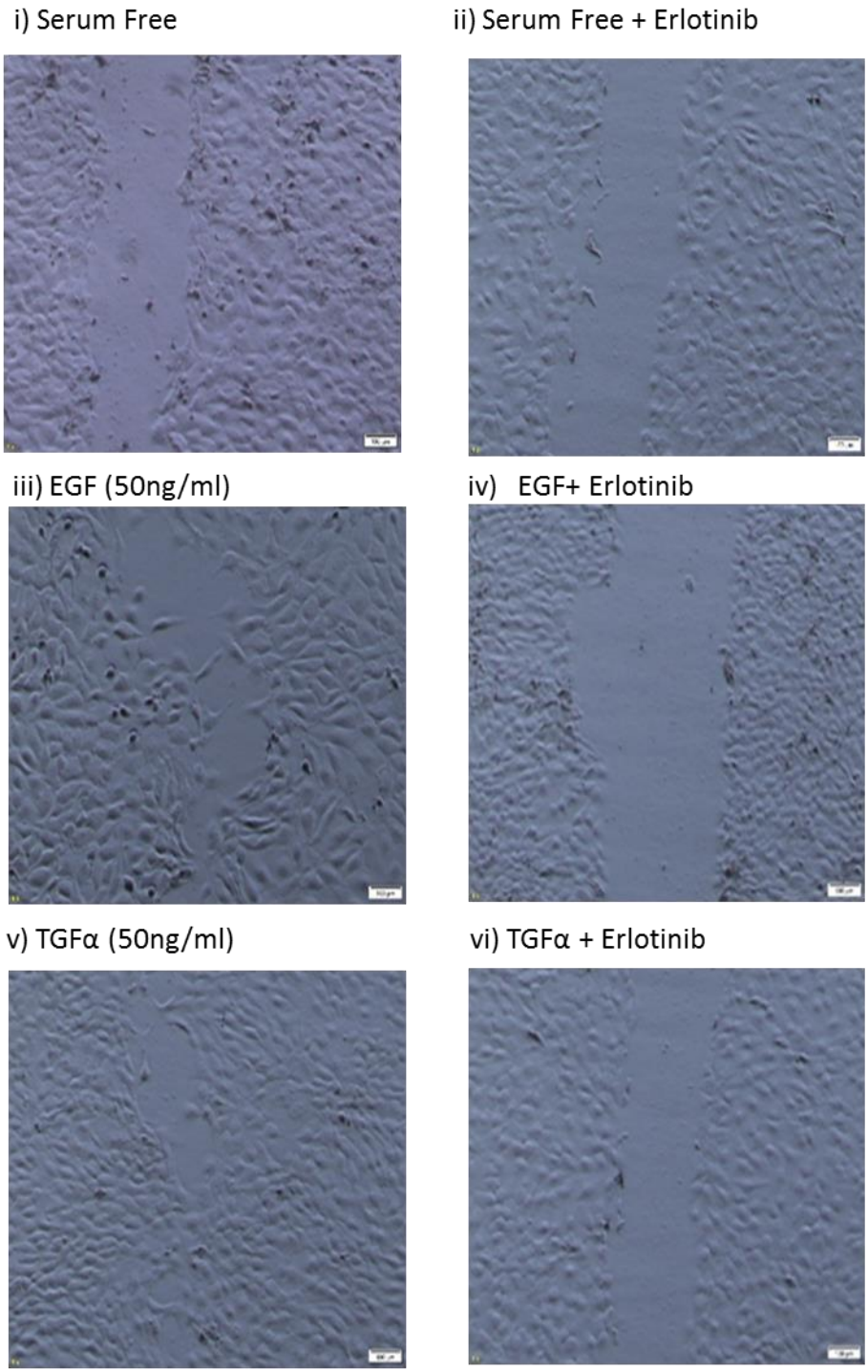


Figure 5.9b The *HacaT cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Erlotinib. The photomicrograph was taken after 24 hrs of addition of the test conditions. All micrographs were taken at 10x magnification. Treatments: i) SF-MEM, ii) 5µM erlotinib iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Erlotinib v) 50ng/ml TGFα vi) 50ng/ml TGFα/5µM Erlotinib.*

Figure 5.9c The *HaCaT cell line plated in presence of the inhibitor PD98059 and growth factors in Scratch assay.*

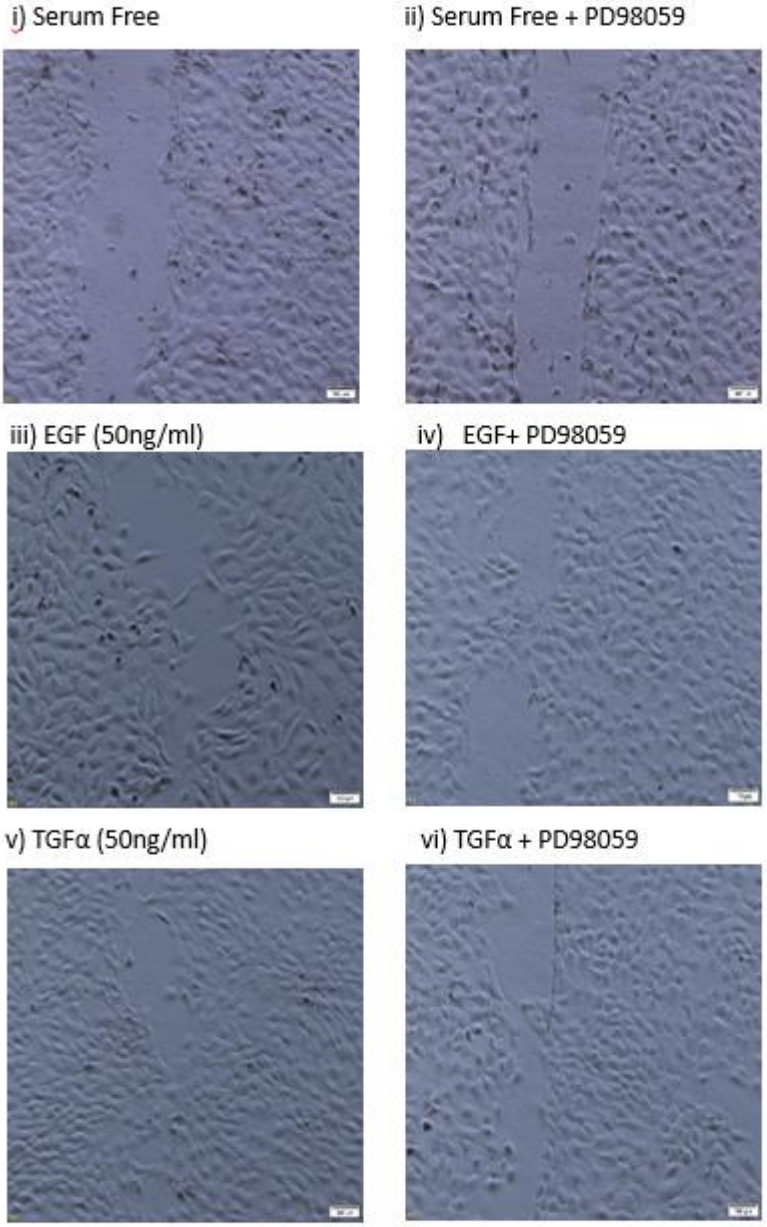


Figure 5.9b The *HacaT cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml) were added with 25μM PD98059. The photomicrograph was taken after 24 hrs of addition of the test conditions. All micrographs were taken at 10x magnification. Treatments: i) SF-MEM, ii) 25μM PD98059 iii) 50ng/ml EGF, iv) 50ng/ml EGF/25μM PD98059 v) 50ng/ml TGFα vi) 50ng/ml TGFα/25μM PD98059.*

5.4.10 Experimental data for HSG cell line in response to growth factors and Inhibitors

In the following experiments HSG were plated onto dishes at $3x10^5$ cells per dish and grown until confluent changing medium 10% FCS every 48 hours. Once confluent the medium was changed to SF-MEM overnight. A scratch was made from one side of the dish to the other. The medium was changed into test condition. They were then incubated and the photomicrographs were taken after 24 hours. The test conditions are described in the Figure legend.

Figure 5.10a, b and c shows the effect of Gefitinib (5µM), Erlotinib (5µM) and PD98059 (25µM) on EGF and TGFα stimulated cells. The photographs indicate that both EGF and TGFα induced migration in the cells of HSG cells (Figure 5.9 a, b and c). One hour pre-treatment with Gefitinib, Erlotinib and PD98059 inhibits these migration (Figure 5.9 a, b and c). It should be noted that single cell migration is visible in the gaps.

Figure 5.10 The *HSG cell line plated in presence of the inhibitor Gefitinib and growth factors in Scratch assay.*

i) Serum Free

ii) Serum Free + Gefitinib

iii) EGF (50ng/ml)

iv) EGF+ Gefitinib

v) TGFα (50ng/ml)

vi) TGFα + Gefitinib

Figure 5.10a The *HSG cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Gefitinib. The photomicrograph was taken after 24 hrs of addition of the test conditions. All micrographs were taken at 10x magnification. Treatments: i) SF-MEM, ii) 5µM Gefitinib iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Gefitinib v) 50ng/ml TGFα vi) 50ng/ml TGFα/5µM Gefitinib.*

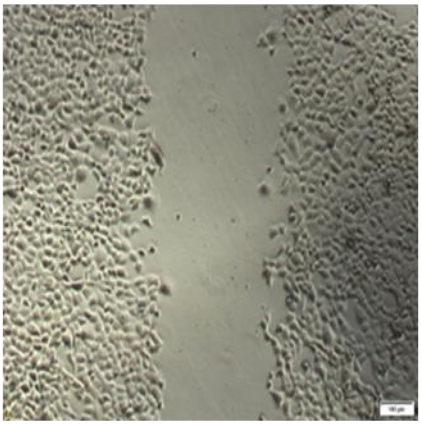
Figure 5.10b The *HSG cell line plated in presence of the inhibitor Erlotinib and growth factors in Scratch assay.*

i) Serum Free

ii) Serum Free + Erlotinib

iii) EGF (50ng/ml)

iv) EGF+ Erlotinib



v) TGFα (50ng/ml)

vi) TGFα + Erlotinib

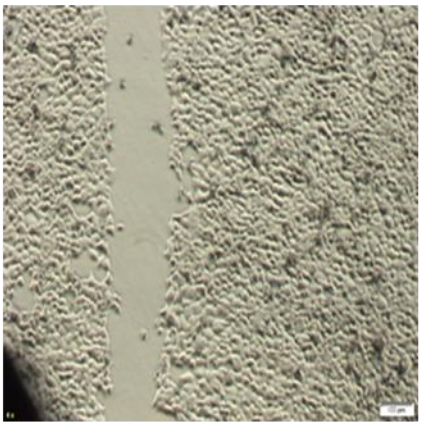


Figure 5.10b The *HSG cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Erlotinib. The photomicrograph was taken after 24 hrs of addition of the test conditions. All micrographs were taken at 10x magnification. Treatments: i) SF-MEM, ii) 5µM Erlotinib iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Erlotinib v) 50ng/ml TGFα vi) 50ng/ml TGFα/5µM Erlotinib.*

Figure 5.10c The *HSG cell line plated in presence of the inhibitor PD98059 and growth factors in Scratch assay.*

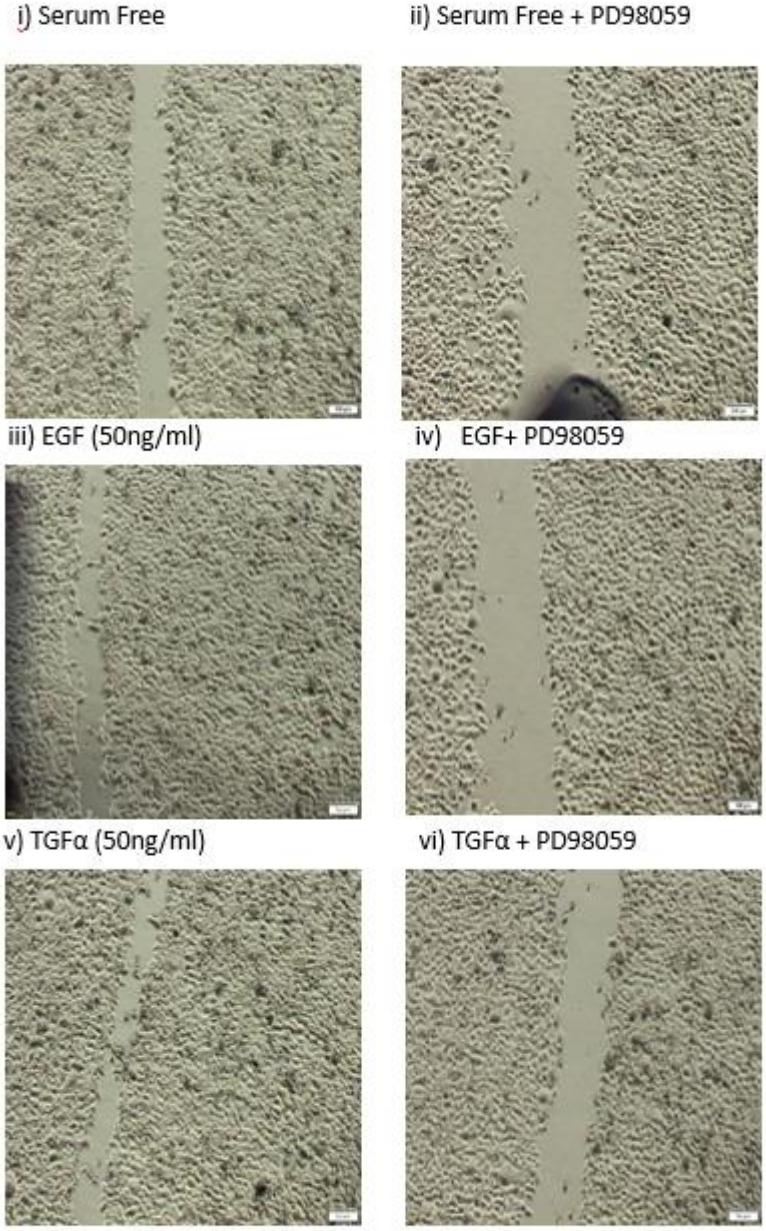


Figure 5.10c The *HSG cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml) were added with 25μM PD98059. The photomicrograph was taken after 24 hrs of addition of the test conditions. All micrographs were taken at 10x magnification. Treatments: i) SF-MEM, ii) 25μM PD98059 iii) 50ng/ml EGF, iv) 50ng/ml EGF/25μM PD98059 v) 50ng/ml TGFα vi) 50ng/ml TGFα/25μM PD98059.*

5.4.11 Experimental data for AZA1 cell line in response to growth factors and Inhibitors

In the following experiments HSG were plated onto dishes at $3x10^5$ cells per dish and grown until confluent changing medium 10% FCS every 48 hours. Once confluent the medium was changed to SF-MEM overnight. A scratch was made from one side of the dish to the other. The medium was changed into test condition. They were then incubated and the photomicrographs were taken after 24 hours.The test conditions are described in the Figure legend.

Figure 5.11 a, b and c shows the effect of Gefitinib (5μM), Erlotinib (5μM) and PD98059 (25μM) on EGF and TGFα stimulated cells. The photographs indicate that both EGF and TGFα induced migration in the cells of HSG cells (Figure 5.11 a, b and c). One hour pre-treatment with Gefitinib, Erlotinib and PD98059 inhibits these migration (Figure 5.11 a, b and c).

Figure 5.11a The *AZA1 cell line plated in presence of the inhibitor Gefitinib and growth factors in Scratch assay.*

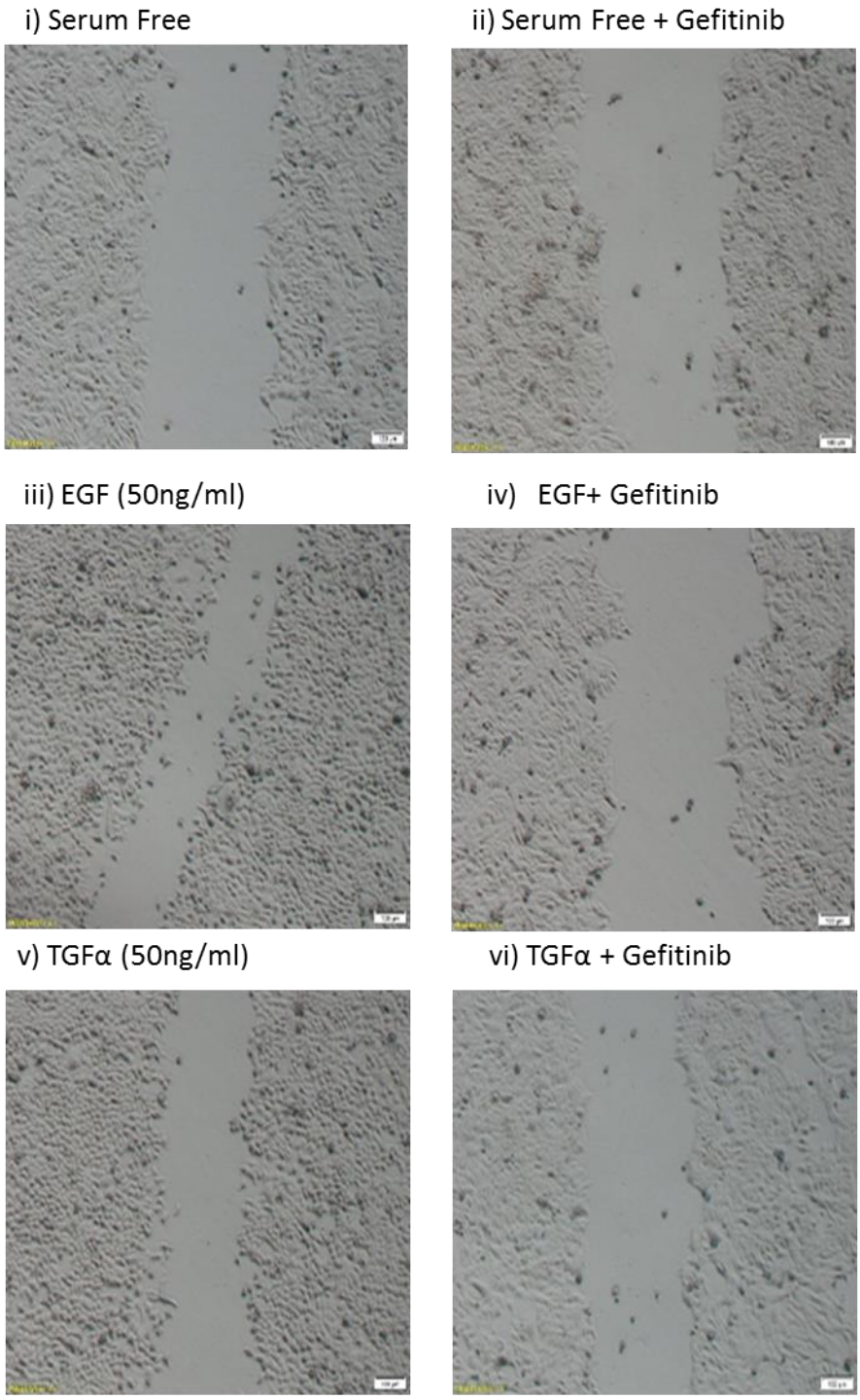
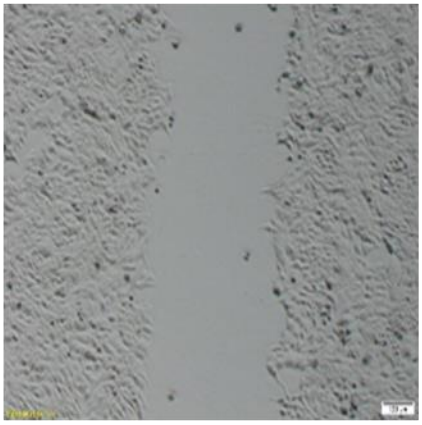


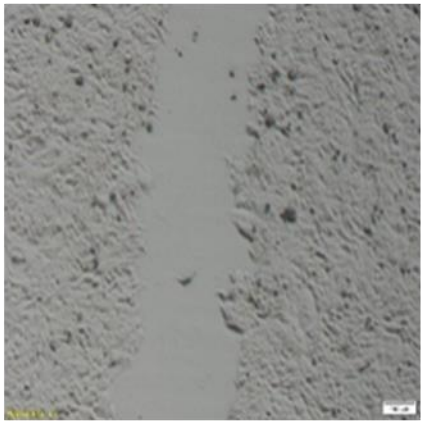
Figure 5.11a The *AZA1 cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5μM Gefitinib. The photomicrograph was taken after 24 hrs of addition of the test conditions. All micrographs were taken at 10x magnification. Treatments: i) SF-MEM, ii) 5μM Gefitinib iii) 50ng/ml EGF, iv) 50ng/ml EGF/5μM Gefitinib v) 50ng/ml TGFα vi) 50ng/ml TGFα/5μM Gefitinib.*

Figure 5.11b The *AZA1 cell line plated in presence of the inhibitor Erlotinib and growth factors in Scratch assay.*

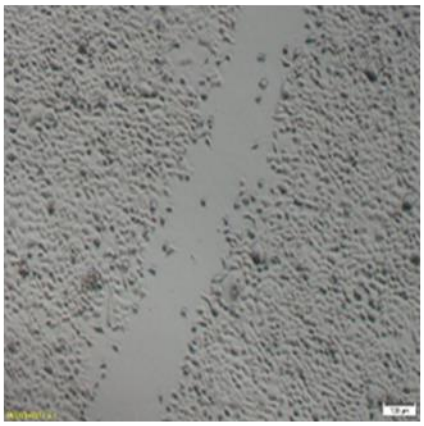
i) Serum Free



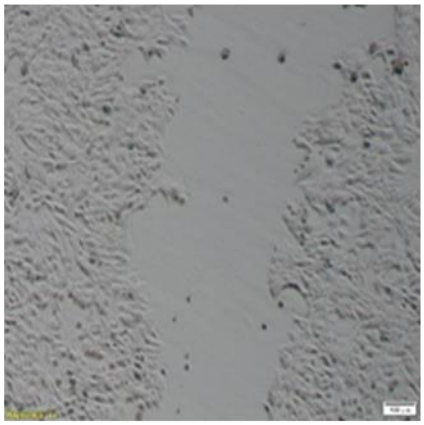
ii) Serum Free + Erlotinib



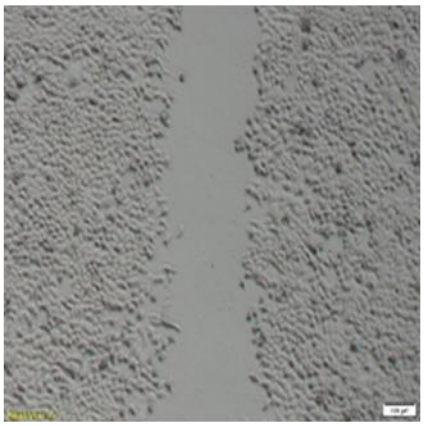
iii) EGF (50ng/ml)



iv) EGF+ Erlotinib



v) TGFα (50ng/ml)



vi) TGFα + Erlotinib

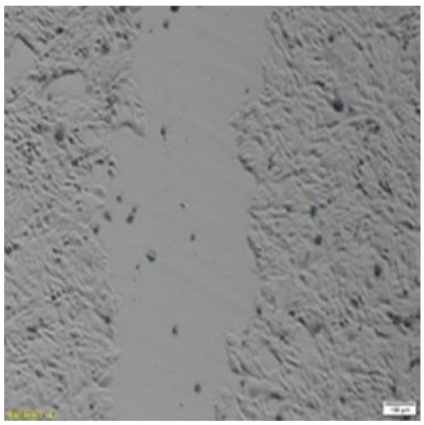


Figure 5.11b The *AZA1 cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Erlotinib. The photomicrograph was taken after 24 hrs of addition of the test conditions. All micrographs were taken at 10x magnification. Treatments: i) SF-MEM, ii) 5µM Erlotinib iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Erlotinib v) 50ng/ml TGFα vi) 50ng/ml TGFα/5µM Erlotinib.*

Figure 5.11c The *AZA1 cell line plated in presence of the inhibitor PD98059 and growth factors in Scratch assay.*

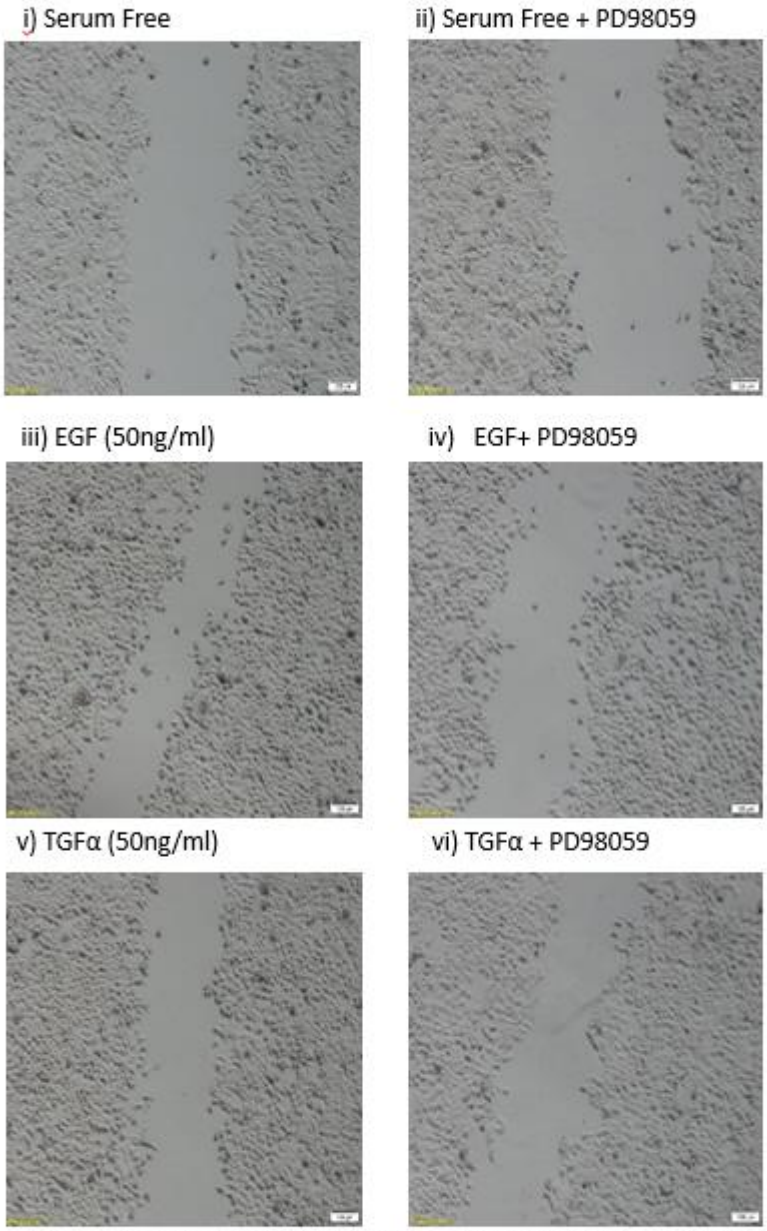


Figure 5.11c The *AZA1 cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml) were added with 25μM PD98059. The photomicrograph was taken after 24 hrs of addition of the test conditions. All micrographs were taken at 10x magnification. Treatments: i) SF-MEM, ii) 25μM PD98059 iii) 50ng/ml EGF, iv) 50ng/ml EGF/25μM PD98059 v) 50ng/ml TGFα vi) 50ng/ml TGFα/5μM PD98059.*

5.4.12 Changes in protein expression by cancer cell lines in response to growth factors and inhibitors

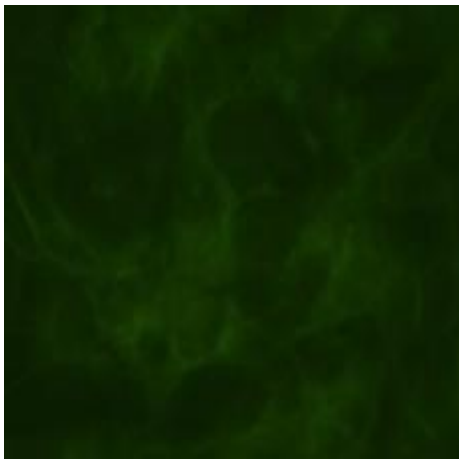
The the previous chapter the four cell lines were investigated in respect of the expression of being epithelium-like (E-Cadherin) or mesenchyme-like (vimentin) and how the growth factors EGF and TGFα may change this in terms of EMT. However, how the growth ractors worked in terms of binding to cell surface receptors and signalling pathways was not inviestigated. However, initial data indicated that the inhibitors worked by blocking EMT. It was decided to invetigate the repcetor expression and signalling pathways involved in this. The experiments undertaken to investigate this were immunoflourescence or SDS-PAGE/Western blotting. In the following experiments only changes in expression in response to the growth factors were investigated.

5.4.13 Localization of EGFR and phosphorylation of EGFR (Tyr1068) residue

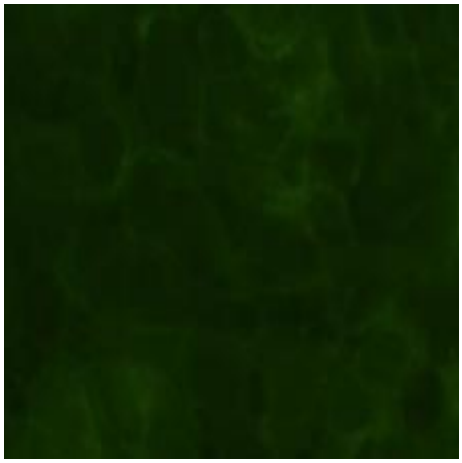
In order to determine the expression of total EGFR on the cell membrane localisation of the receptor was determined by immunofluorescence. The photomicrographs are shown in Figure 5.12, 5.13, 5.14 and 5.15. EGFR expression was found in serum free and growth factors treated dishes of all cell lines. EGFR internalization was captured in some of the dishes. In AZA1 cell line (Figure 5.14), receptor internalization was seen in both the EGF and TGFα treated dishes after 48 hours. The HSG cell lines also showed receptor internalization in both serum free and growth factors treated dishes (Figure 5.15). There is no clear information about receptor internalization in TYS and HaCaT cell lines due to low level of staining (Figure 5.12 and 5.13).

Figure 5.12 The TYS cell line expression of total EGFR

i) Serum Free



ii) EGF (50ng/ml)



iii) TGFα (50ng/ml)

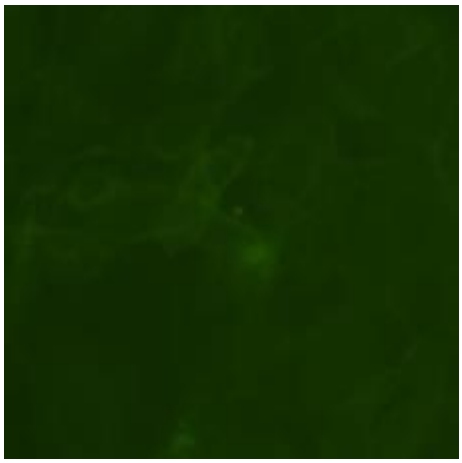
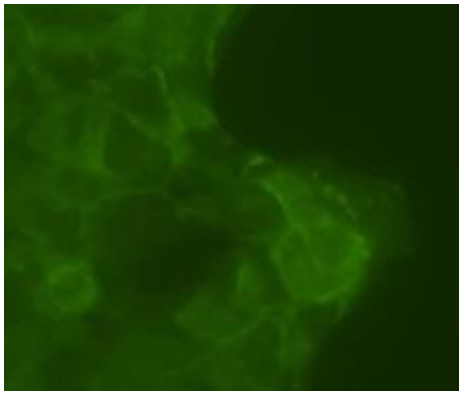


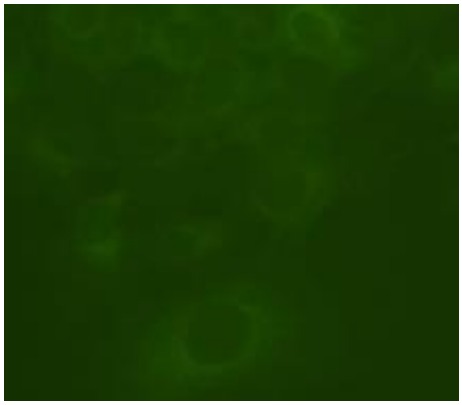
Figure 5.12 The *TYS cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml). Staining for total EGFR. All micrographs were taken at 40x magnification. Treatments: i) SF-MEM, ii) 50ng/ml EGF, iii) 50ng/ml TGFα.*

Figure 5.13 The HaCaT cell line expression of total EGFR

i) Serum Free



ii) EGF (50ng/ml)



iii) TGFα (50ng/ml)

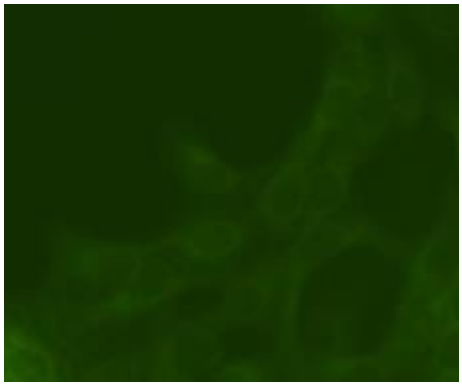
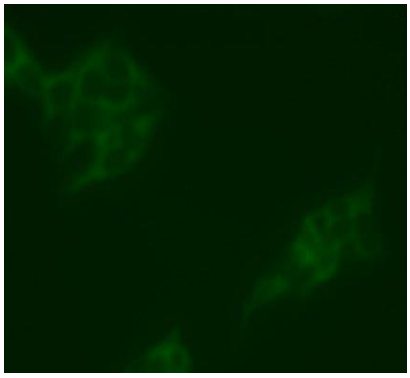


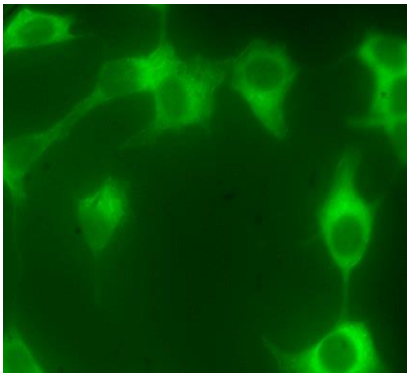
Figure 5.13 The *HaCaT cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml). Staining for total EGFR. All micrographs were taken at 40x magnification. Treatments: i) SF-MEM, ii) 50ng/ml EGF, iii) 50ng/ml TGFα.*

Figure 5.14 The AZA1 cell line expression of total EGFR

i) Serum free



ii) EGF 50ng/ml



iii) TGFα 50ng/ml

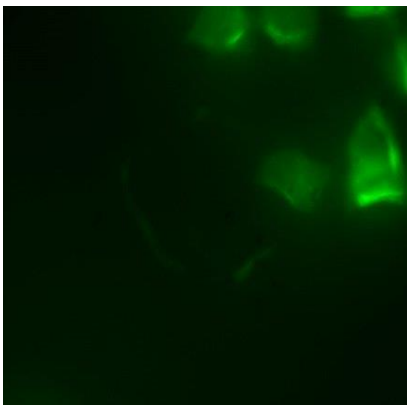
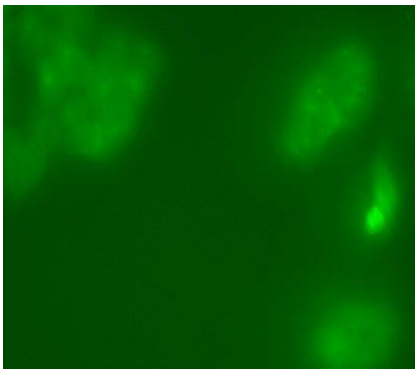


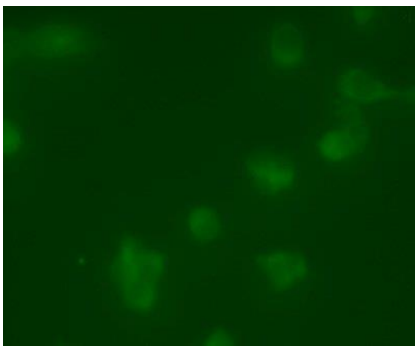
Figure 5.14 The *AZA1 cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml). Staining for total EGFR. All micrographs were taken at 40x magnification. Treatments: i) SF-MEM, ii) 50ng/ml EGF, iii) 50ng/ml TGFα*

Figure 5.15 The HSG cell line expression of total EGFR

i) serum free



ii) EGF 50ng/ml



iii) TGFα 50ng/ml

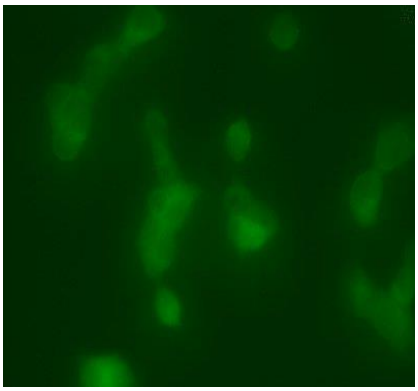


Figure 5.15 The *HSG cell line was plated at* 3x 10^5 per dish *and EGF (50ng/ml) and TGFα (50ng/ml). Staining for total EGFR. All micrographs were taken at 40x magnification. Treatments: i) SF-MEM, ii) 50ng/ml EGF, iii) 50ng/ml TGFα*

5.4.14 Phosphorylation of EGFR (Tyr1068) residue

In the previous section it was noted that the expression of phosphorylated EGFR (Tyr1068) could not be determined in two of the cells lines by localisation studies. It was decided to investigate whether the residue was phosphorylated in the HaCaT and also the Tys cell line. Using Western blotting techniques we could determine the total level of phosphorylation in the cell lines but not where the expression occurred.

Figure 5.16 indicates that EGFR tyrosine residue 1068 was phosphorylated in all conditions even in tyrosine kinase inhibitor treated dishes. Therefore it is safe to assume that the lack of reduction on this residue means that this pathway is not the most important in treatment with the inhibitors.

Figure 5.16: Western blot experiments for EGFR phosphorylation at Tyr 1068 with the 4 cell lines.

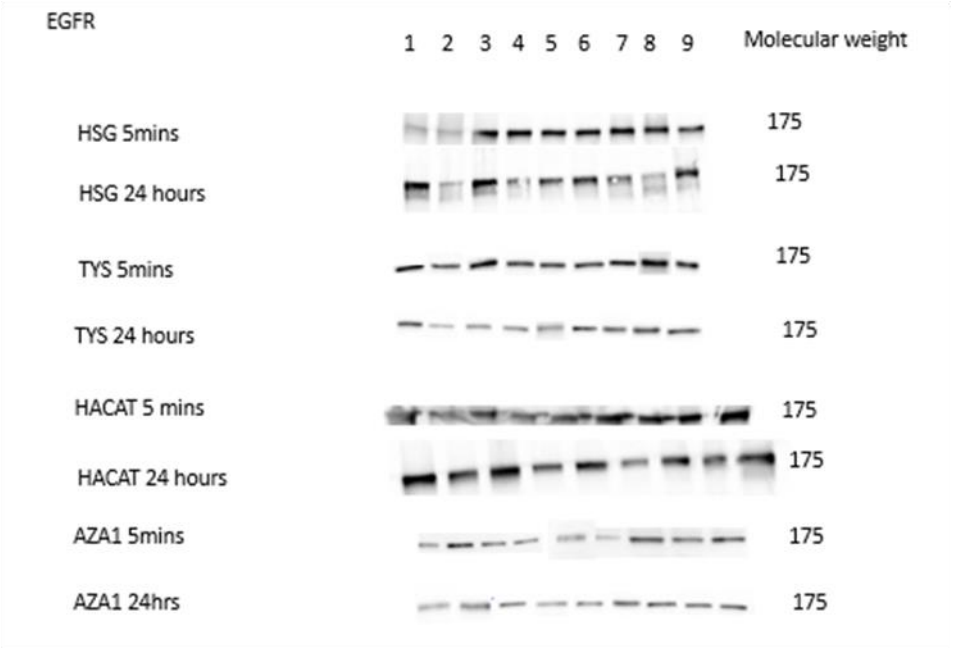


Figure 5.16: EGFR phosphorylation in the 4 cell lines was determined at the concentrations below of growth factor and inhibitor.
The timings are detailed on the blot.
The blots are cropped from the original images.

Lane 1	Serum free
Lane 2	EGF 50ng/ml
Lane 3	TGFα 50ng/ml
Lane 4	5μM Gefitinib+EGF 50ng/ml
Lane 5	5μM Gefitinib+TGFα 50ng/ml
Lane 6	5μM Erlotinib+EGF 50ng/ml
Lane 7	5μM Erlotinib+TGFα 50ng/ml
Lane 8	25μM PD98059+EGF 50ng/ml
Lane 9	25μM PD98059+TGFα 50ng/ml

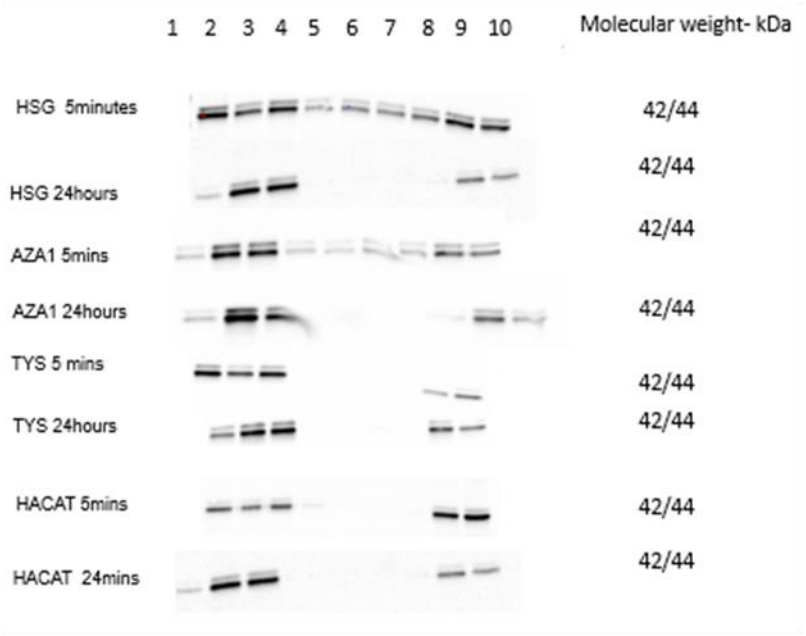
5.4.15 Localization and phosphorylation of MAPK (Thr202/Tyr204) residue

In all four cell lines, MAPK202/204 was expressed in EGF and TGF treated dishes in both 5 mins and 24 hours' time point. Tyrosine kinase inhibitors (Gefitinib and Erlotinib) fully blocked MAPK202/204 expression in TYS and HaCaT cell line while in HSG and AZA1 cell lines, MAPK 202/204 expression was reduced from 5 mins to 24hours time point. In contrast, ERK1/2 inhibitorPD98059 was partially expressed in all the cell lines. There might be a reason why MAPK202/204 was phosphorylated in PD98059 treated dishes. Dose of PD98059 might not be high enough to fully inhibit the phosphorylation. According to manufacturer data sheet, 20µM did not fully inhibit phosphorylation while 50µM completely inhibit the phosphorylation. This could be overcome by increasing concentration.

In immunofluorescence, SF treated dishes have very light expression of MAPK202/204. Addition of 50ng/ml EGF and TGFα50ng/ml for 48 hours caused increase expression of MAPK202/204. In contrast, dishes with (one hour pre-treated with Gefitinib, Erlotinib and PD98059 before addition of EGF and TGFα) blocked MAPK202/204 expression fully.

In immunofluorescence, addition of 50ng/ml EGF and TGFα50ng/ml for 48 hours caused increase expression of phospho MAPK202/204 in 3 cell lines(HSG,AZA1,TYS). In contrast, tyrosine kinase inhibitors blocked its expression fully. ERK1/2 inhibitors(PD98059) inhibit its expression in HSG and AZA1 cell line, not in TYS cell lines. HACAT cell line do not express phospho MAPK202/204 in all conditions.

Figure 5.17: Western blot experiments for MAPK phosphorylation with the 4 cell lines.

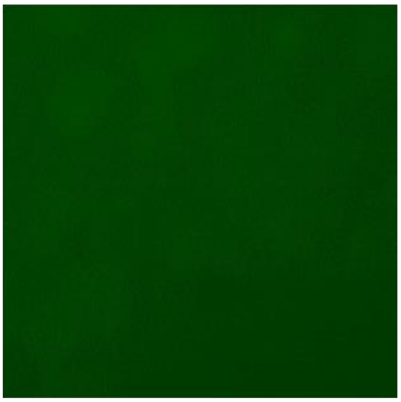


Expression of phosphor-p44/p42 MAPK(Erk1/2) Thr202/Tyr204

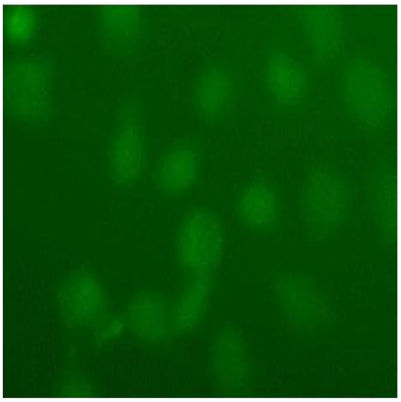
Figure 5.17: *the cells lines were plated at 3x10^5 per dish and each of the dishes were added with EGF (50ng/ml), TGFα (50ng/ml) ,1hr pre-treated with 5μM Gefitinib+EGF50ng/ml, 1 hr pre-treated with5μM Gefitinib+TGFα50ng/ml, 1 hr pre-treated with5μM Erlotinib+EGF50ng/ml, 1 hr pre-treated with5μM Erlotinib+TGFα 50ng/ml, 1 hr pre-treated with25μM PD98059+EGF50ng/ml, 1 hr pre-treated with 25μM PD98059+TGFα50ng/ml for 2 time point(5 minutes and 24 hrs) and then lysis was done and continue SDS page. Cell lysis with serum was put into lane 1, EGF50ng/ml into lane2. TGFα 50ng/ml into lane3, 5μM Gefitinib+EGF 50ng/ml was into lane 4, 5μM Gefitinib+TGFα 50ng/ml into lane 5, 5μM Erlotinib+ EGF 50ng/ml into lane 6, 5μM Erlotinib+ TGFα 50ng/ml into lane 7, 25μM PD98059+EGF 50ng/ml into lane 8, 25μM PD98059+TGFα 50ng/ml respectively and MAPK antibody was used as primary antibody.*

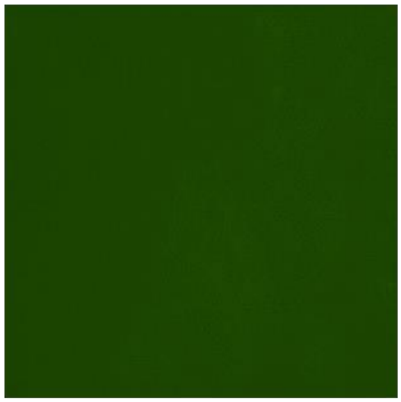
Figure 5.18a: HSG cell line in response to growth factors and Gefitiinib:

i) Serum free **ii) Gefitinib5µM**

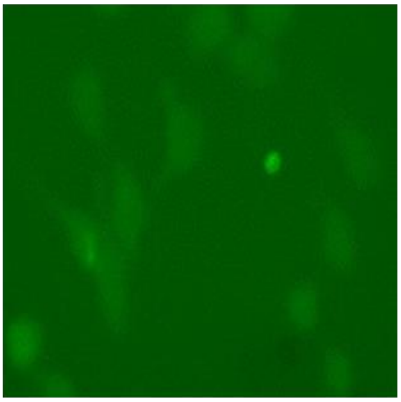


iii) EGF 50ng/ml **iv) Gefitinib 5µM + EGF50ng/ml**





v) TGFα50ng/ml **vi) Gefitinib 5µM +TGFα50ng/ml**



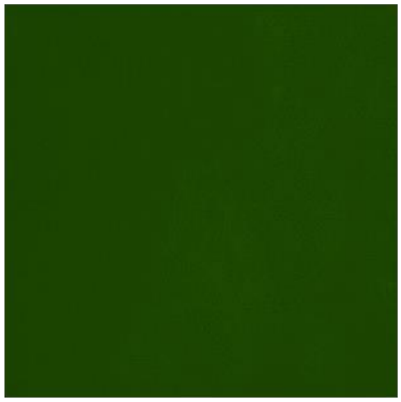
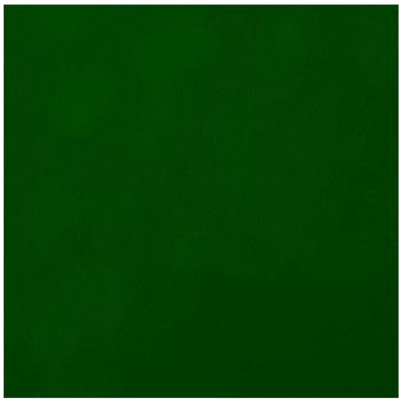


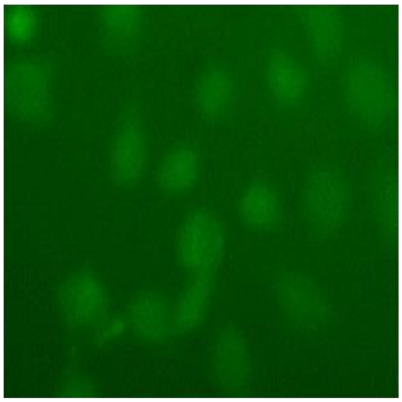
Figure 5.18a: The *HSG cell line was plated at* 1x 10^5 per dish *(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Gefitinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 100x magnification. Treatments: i) SF-MEM, ii) 5µM Erlotinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Erlotinib, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Erlotinib.* *Staining with phospho MAPK*

Figure 5.18b: HSG cell line in response to growth factors and Erlotinib:

i) Serum free **ii) Erlotinib5µM**



iii) EGF 50ng/ml **iv) Erlotinib 5µM + EGF50ng/ml**



v) TGFα50ng/ml **vi) Erlotinib 5µM +TGFα50ng/ml**

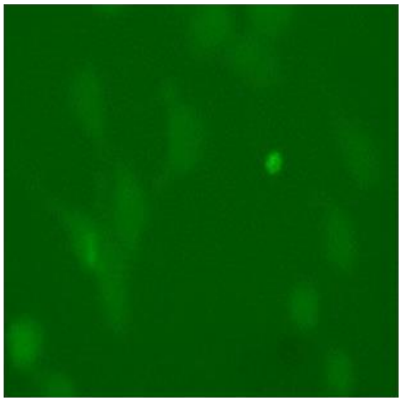


Figure 5.18b: The *HSG cell line was plated at* 1x 10^5 per dish *(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Erlotinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 100x magnification. Treatments: i) SF-MEM, ii) 5µM Erlotinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Erlotinib, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Erlotinib. Staining with phospho MAPK*

Figure 5.18c HSG cell line in response to growth factors and PD98059

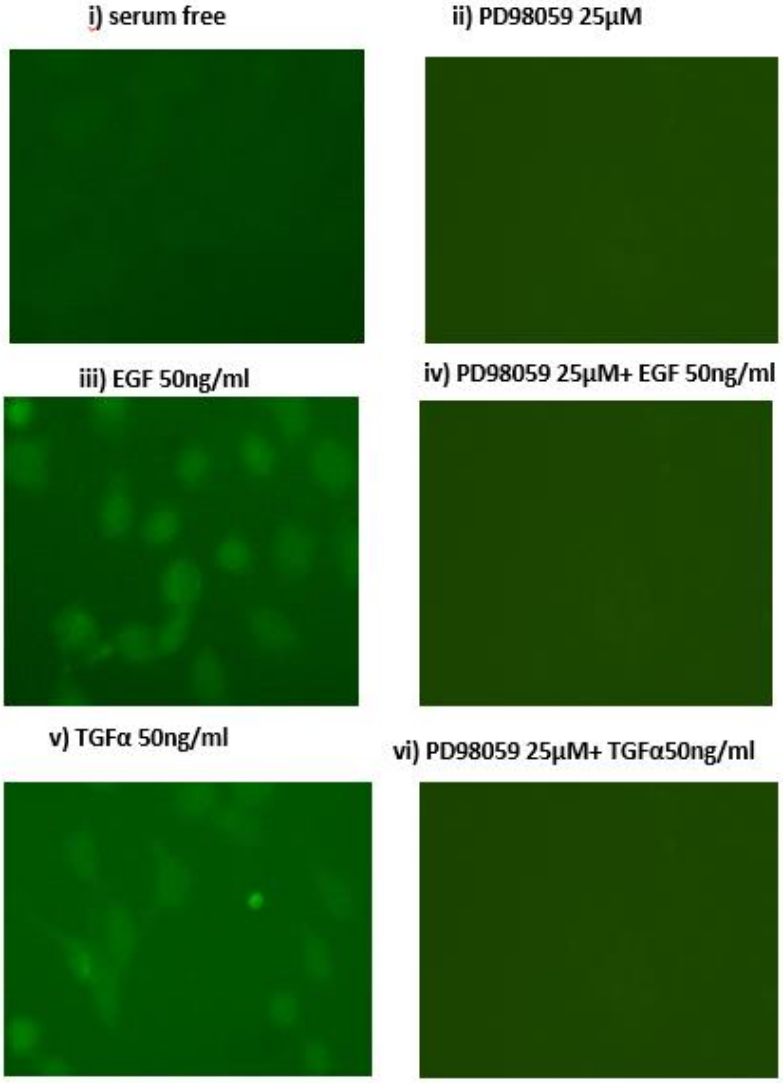
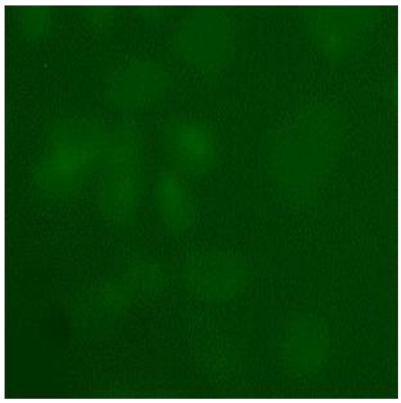


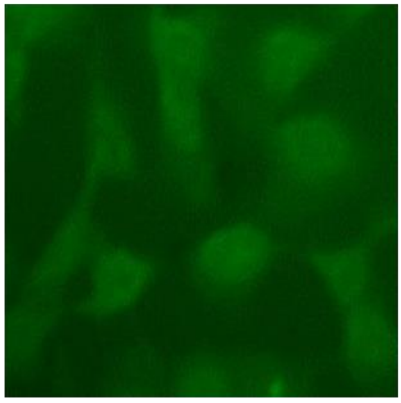
Figure 1.8c HSG cells line was plated at $1x10^5$ per dish(30% confluency) and EGF(50ng/ml) and TGFα(50ng/ml)were added with 25µMPD98059. The photomicrographs were taken after 48 hour addition of the tested conditions. All micrographs were taken at 100xmagnification. Treatment:1)SF-MEM2,2) ii)PD98059 25µM, iii)EGF 50ng/ml, iv) 50ng/ml EGF/ 25µMPD98059, v)TGFα 50ng/ml,vi) 50ng/ml TGFα/ 25µMPD98059 *Staining with phospho MAPK*

Figure 5.19a: AZA1 cell line in response to growth factors and Gefitiinib:

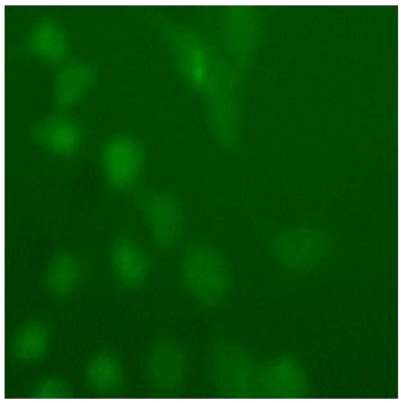
i) Serum free **ii) Gefitinib5µM**



iii) EGF 50ng/ml **iv) Gefitinib 5µM + EGF50ng/ml**



v) TGFα50ng/ml **vi) Gefitinib 5µM +TGFα50ng/ml**



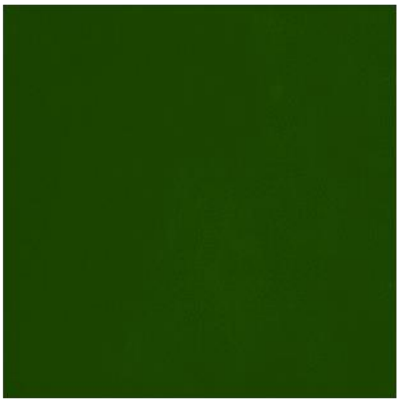
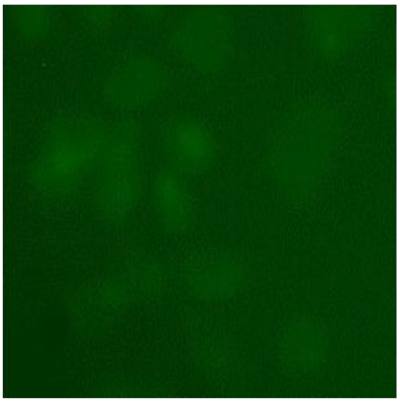


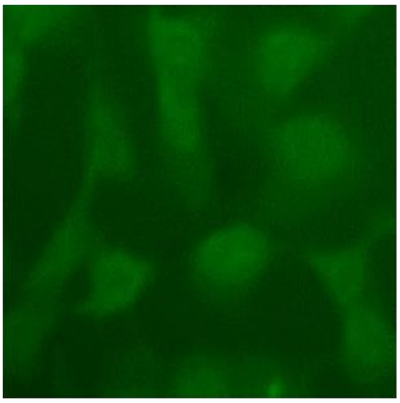
Figure 5.19a: The *AZA1 cell line was plated at* 1x 10^5 per dish *(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Gefitinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 100x magnification. Treatments: i) SF-MEM, ii) 5µM Gefitinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Gefitinib, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Gefitinib.* *Staining with phospho MAPK*

Figure 5.19b: AZA1cell line in response to growth factors and Erlotinib:

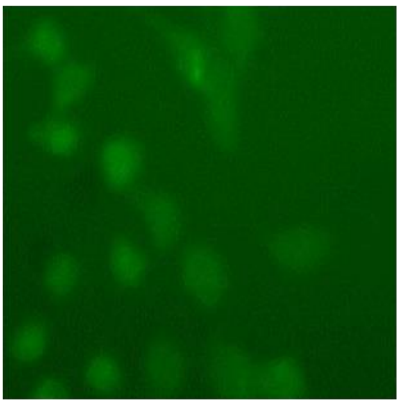
i) Serum free **ii) Erlotinib5µM**



iii) EGF 50ng/ml **iv) Erlotinib 5µM + EGF50ng/ml**



v) TGFα50ng/ml **vi) Erlotinib 5µM +TGFα50ng/ml**



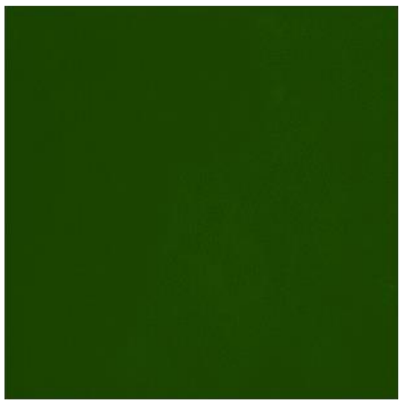


Figure 5.19b: The *AZA1 cell line was plated at* 1x 10^5 per dish *(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Erlotinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 100x magnification. Treatments: i) SF-MEM, ii) 5µM Erlotinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Erlotinib, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Erlotinib.* *Staining with phospho MAPK*

Figure 5.19c AZA1 cell line in response to growth factors and PD98059

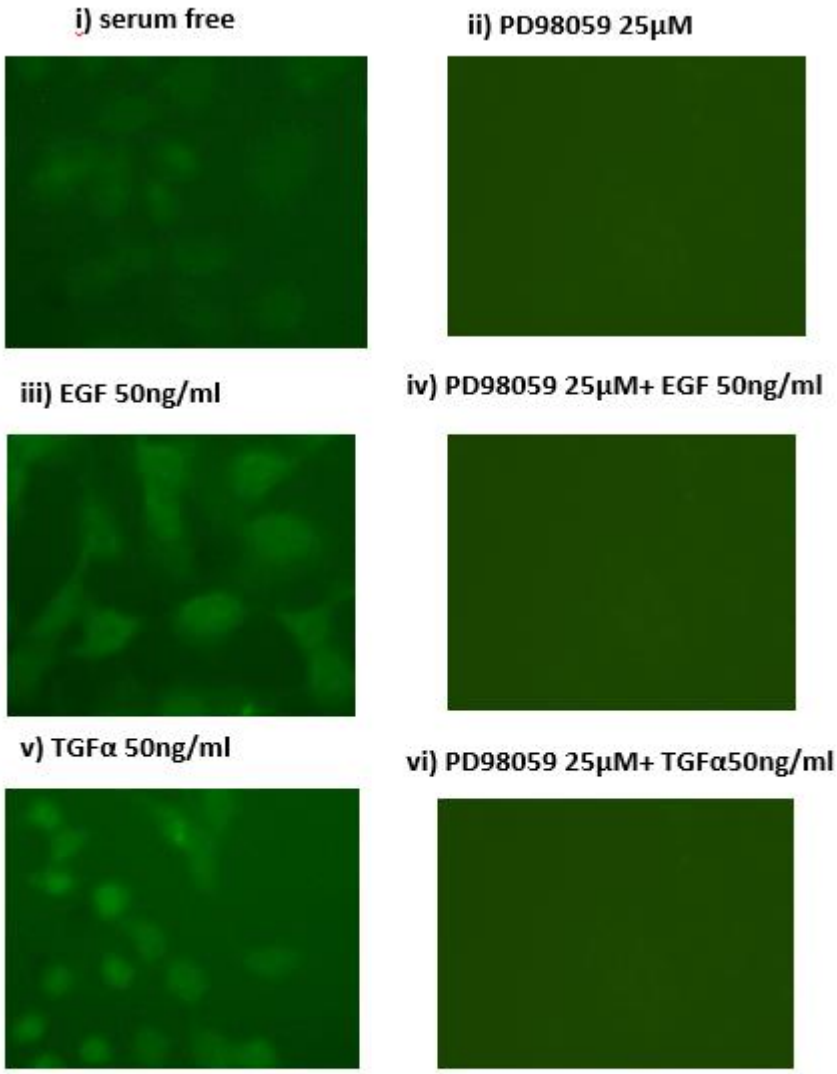
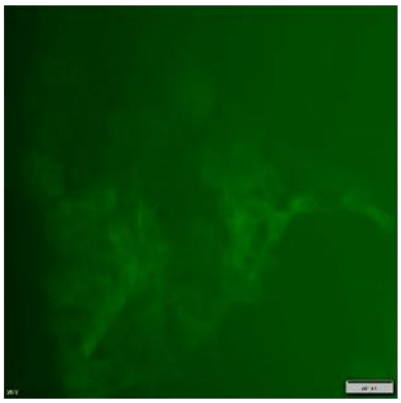


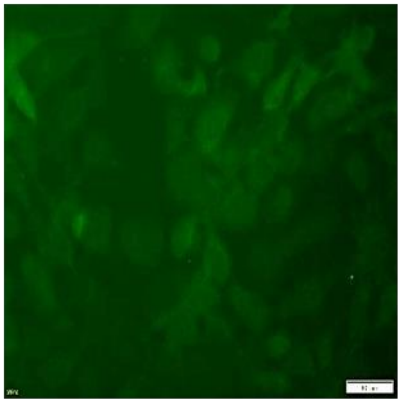
Figure 5.19c AZA1 cells line was plated at 1x10^5 per dish(30% confluency) and EGF(50ng/ml) and TGFα(50ng/ml) were added with 25µMPD98059. The photomicrographs were taken after 48 hour addition of the tested conditions. All micrographs were taken at 100xmagnification. Treatment:1)SF-MEM2,2) ii)PD98059 25µM, iii)EGF 50ng/ml, iv) 50ng/ml EGF/ 25µMPD98059, v)TGFα 50ng/ml,vi) 50ng/ml TGFα/ 25µMPD98059
Staining with phospho MAPK

Figure 5.20a: TYS cell line in response to growth factors and Gefitiinib:

i) Serum free **ii) Gefitinib5µM**



iii) EGF 50ng/ml **iv) Gefitinib 5µM + EGF50ng/ml**



v) TGFα50ng/ml **vi) Gefitinib 5µM +TGFα50ng/ml**

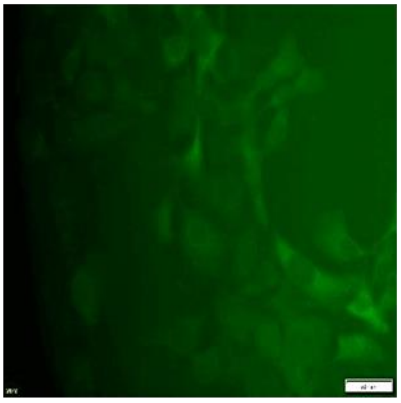
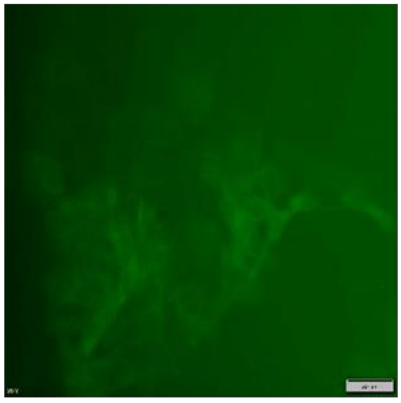
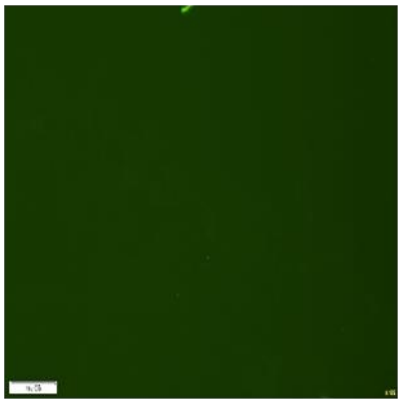


Figure 5.20a: The *TYS cell line was plated at* 1x 10^5 per dish *(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Gefitinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 100x magnification. Treatments: i) SF-MEM, ii) 5µM Gefitinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Gefitinib, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Gefitinib.* *Staining with phospho MAPK*

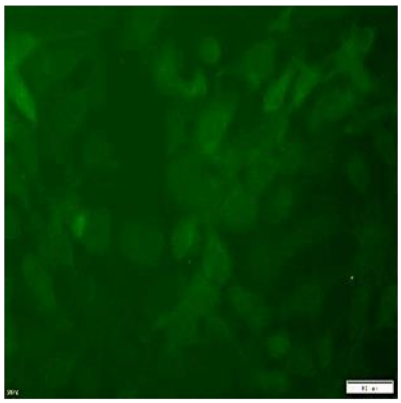
Figure 5.20b: TYS cell line in response to growth factors and Erlotinib:

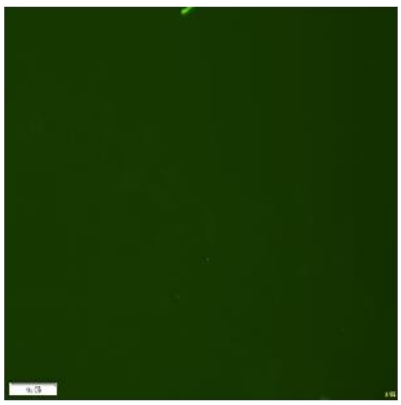
i) Serum free **ii) Erlotinib5µM**



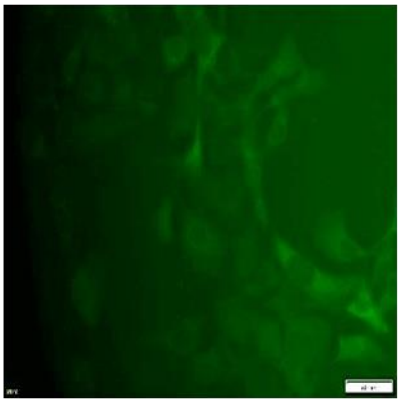


iii) EGF 50ng/ml **iv) Erlotinib 5µM + EGF50ng/ml**





v) TGFα50ng/ml **vi) Erlotinib 5µM +TGFα50ng/ml**



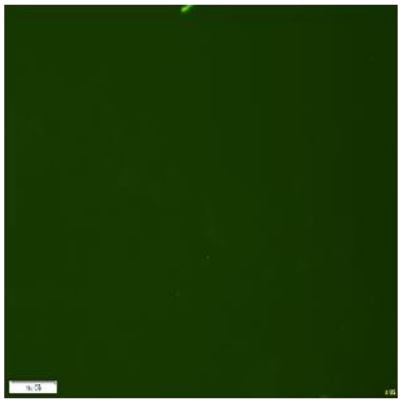


Figure 5.20b: The *TYS cell line was plated at* 1x 10^5 per dish *(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Erlotinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 100x magnification. Treatments: i) SF-MEM, ii) 5µM Erlotinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Erlotinib, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Erlotinib.* *Staining with phospho MAPK*

Figure 5.20c TYS cell line in response to growth factors and PD98059

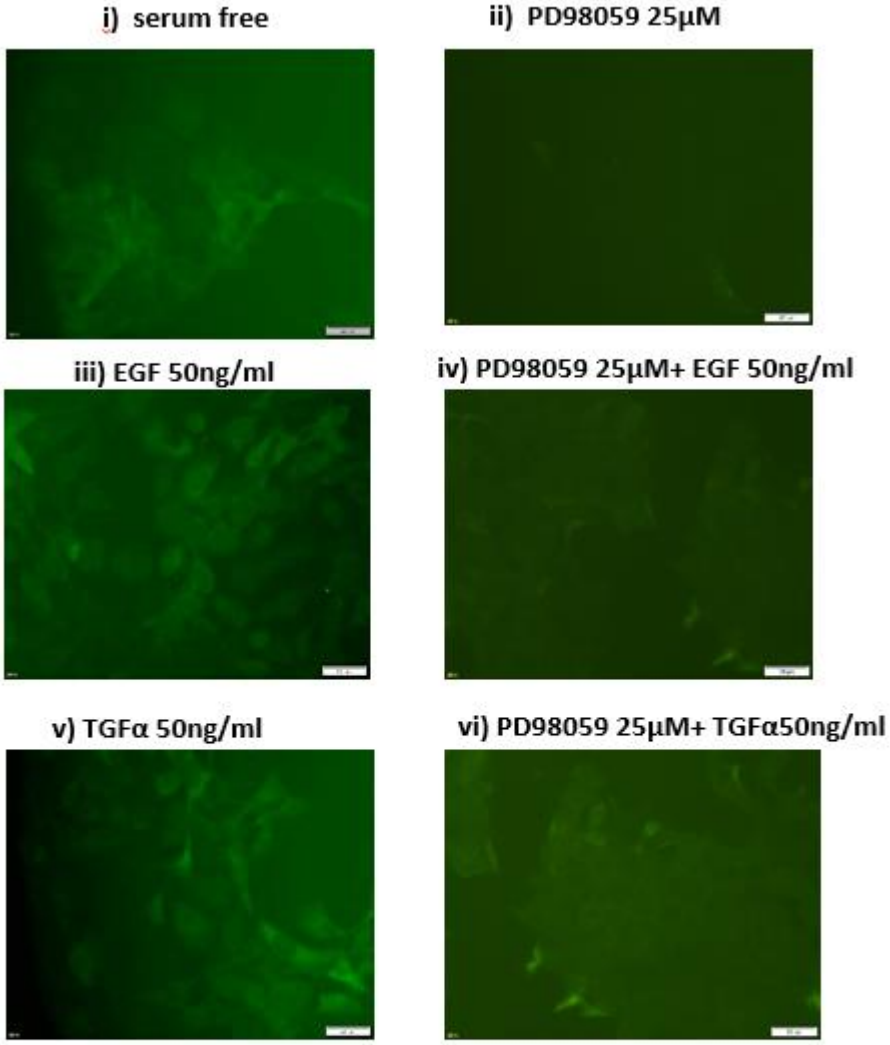
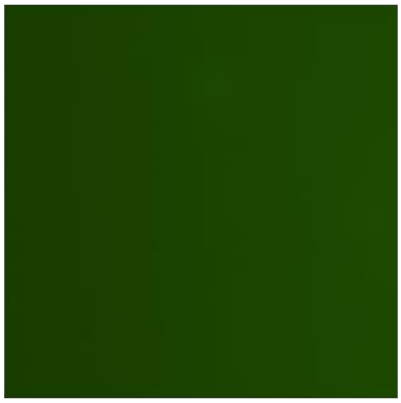
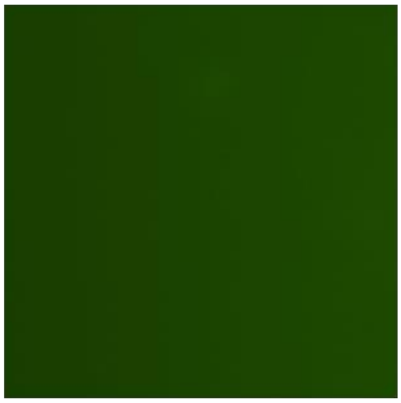


Figure 5.20c TYS cells line was plated at 1x10^5 per dish(30% confluency) and EGF(50ng/ml) and TGFα(50ng/ml) were added with 25µMPD98059. The photomicrographs were taken after 48 hour addition of the tested conditions. All micrographs were taken at 100xmagnification. Treatment:1)SF-MEM2,2) ii)PD98059 25µM, iii)EGF 50ng/ml, iv) 50ng/ml EGF/ 25µMPD98059, v)TGFα 50ng/ml,vi) 50ng/ml TGFα/ 25µMPD98059
Staining with phospho MAPK

Figure 5.21a: HaCaT cell line in response to growth factors and Gefitiinib:

i) Serum free **ii) Gefitinib5µM**





iii) EGF 50ng/ml **iv) Gefitinib 5µM + EGF50ng/ml**

v) TGFα50ng/ml **vi) Gefitinib 5µM +TGFα50ng/ml**

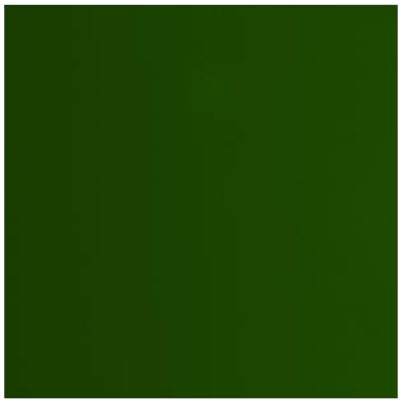
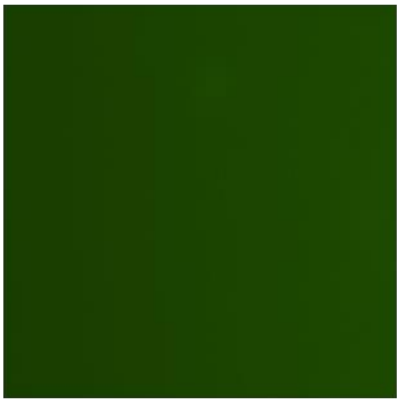


Figure 5.21a: The *HaCaT cell line was plated at* 1x 10^5 per dish *(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Gefitinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 100x magnification. Treatments: i) SF-MEM, ii) 5µM Gefitinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Gefitinib, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Gefitinib.* *Staining with phospho MAPK*

Figure 5.21b: HaCaT cell line in response to growth factors and Erlotinib:

i) Serum free **ii) Erlotinib5µM**



iii) EGF 50ng/ml **iv) Erlotinib 5µM + EGF50ng/ml**

v) TGFα50ng/ml **vi) Erlotinib 5µM +TGFα50ng/ml**

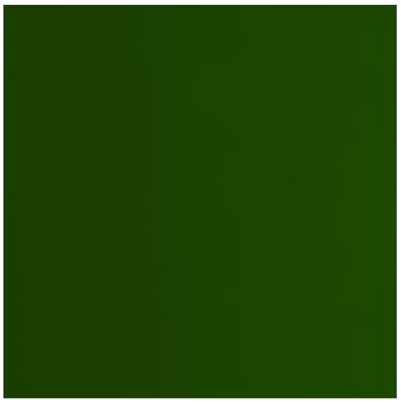


Figure 5.21b: The *HaCaT cell line was plated at* 1x 10^5 per dish *(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Erlotinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 100x magnification. Treatments: i) SF-MEM, ii) 5µM Erlotinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Erlotinib, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Erlotinib.* *Staining with phospho MAPK*

Figure 5.21c HaCaT cell line in response to growth factors and PD98059

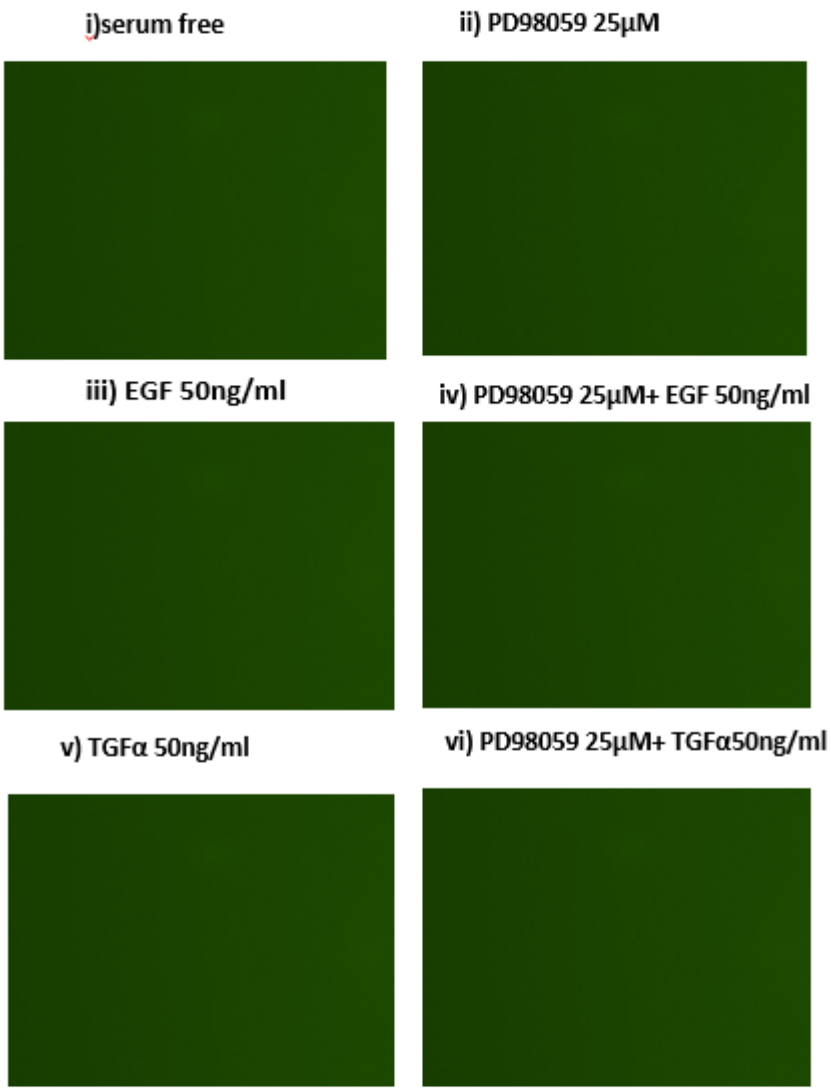


Figure 5.21c HaCaT cells line was plated at 1x10^5 per dish(30% confluency) and EGF(50ng/ml) and TGFα(50ng/ml) were added with 25µMPD98059. The photomicrographs were taken after 48 hour addition of the tested conditions. All micrographs were taken at 100xmagnification. Treatment:1)SF-MEM2,2) ii)PD98059 25µM, iii)EGF 50ng/ml, iv) 50ng/ml EGF/ 25µMPD98059, v)TGFα 50ng/ml,vi) 50ng/ml TGFα/ 25µMPD98059
Staining with phospho MAPK

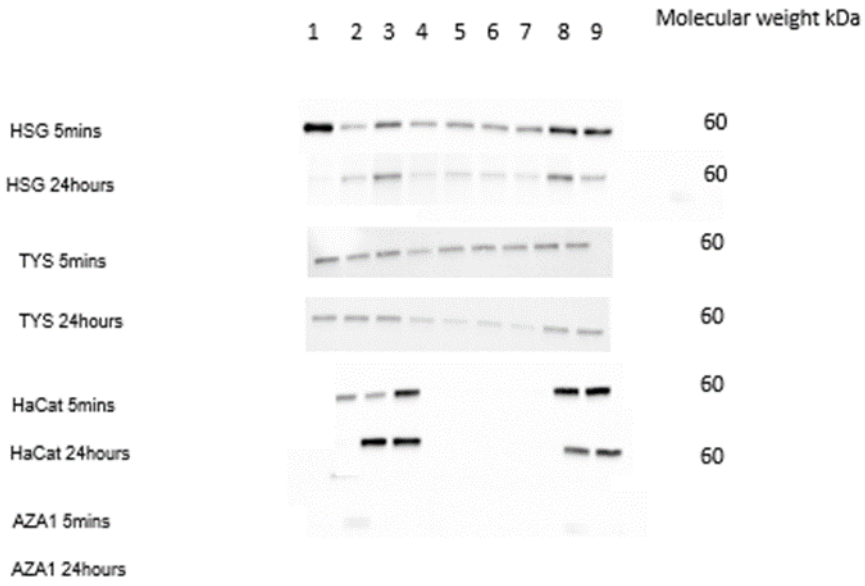
5.4.16 Localization and phosphorylation of Akt (Ser 473) residue

The Akt pathway is the one most studied in our laboratory. The response to both growth factors and all three inhibitors was studied in all 4 cell lines. Studies of immune localization of the phosphorylated protein were inconclusive in 3 of the cell lines (data not shown).

SDS-Page/Western blotting experiments were also interesting in terms of the phosphorylated Akt. Data presented in Figure 22, AZA1 cell line has no AKT phosphorylation in all the test conditions. The HaCaT cell line (normal) has AKT phosphorylation in EGF, TGFα and PD98059 treated dishes while tyrosine kinase inhibitors fully block AKT 473 phosphorylation. TYS and HSG cell lines, AKT was expressed in 5 mins time point in all conditions. However, expression was reduced in tyrosine kinase inhibitor treated dishes at the 24 hours' time point.

Data presented in Figure 23a, b and c shows that the localisation of p473Akt is low in the TYS cell type (and not really seen in the other cell lines). The expression of pAKT-473 was faintly distributed through in EGF and TGFα treated dishes while did not express in serum free and inhibitors treated dishes.

Figure 5.22: Western blot experiments for Akt phosphorylation with the 4 cell lines.



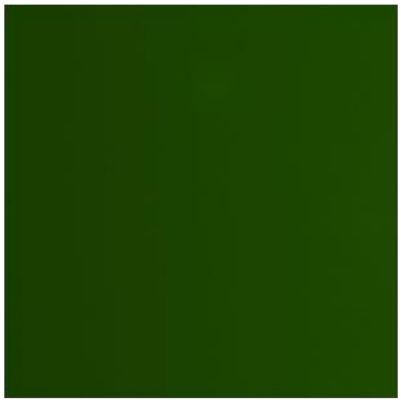
Expression of Akt (Ser 473) Thr202/Tyr204

Figure 5.22: *the cells lines were plated at 3x10^5 per dish and each of the dishes were added with EGF (50ng/ml), TGFα (50ng/ml) ,1hr pre-treated with 5μM Gefitinib+EGF50ng/ml, 1 hr pre-treated with5μM Gefitinib+TGFα50ng/ml, 1 hr pre-treated with5μM Erlotinib+EGF50ng/ml, 1 hr pre-treated with5μM Erlotinib+TGFα 50ng/ml, 1 hr pre-treated with25μM PD98059+EGF50ng/ml, 1 hr pre-treated with 25μM PD98059+TGFα50ng/ml for 2 time point(5 minutes and 24 hrs) and then lysis was done and continue SDS page. Cell lysis with serum was put into lane 1, EGF50ng/ml into lane2. TGFα 50ng/ml into lane3, 5μM Gefitinib+EGF 50ng/ml was into lane 4, 5μM Gefitinib+TGFα 50ng/ml into lane 5, 5μM Erlotinib+ EGF 50ng/ml into lane 6, 5μM Erlotinib+ TGFα 50ng/ml into lane 7, 25μM PD98059+EGF 50ng/ml into lane 8, 25μM PD98059+TGFα 50ng/ml respectively and p473 Akt antibody was used as primary antibody.*

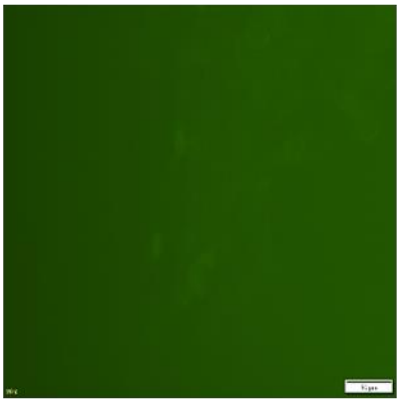
Figure 5.23a: TYS cell line in response to growth factors and Gefitiinib:

i) Serum free

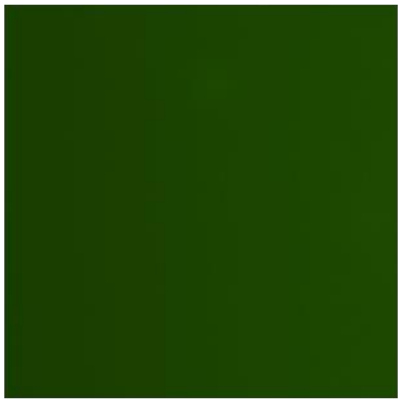
ii) Gefitinib5µM



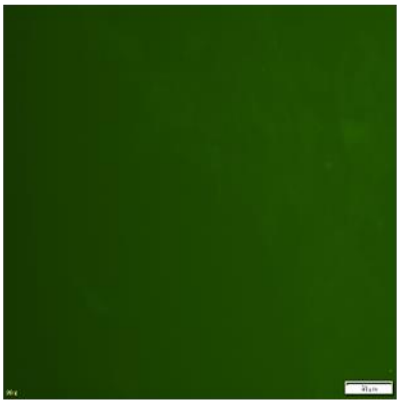
iii) EGF 50ng/ml



iv) Gefitinib 5µM + EGF50ng/ml



v) TGFα50ng/ml

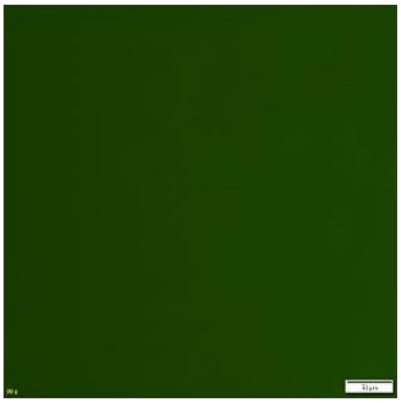


vi) Gefitinib 5µM +TGFα50ng/ml

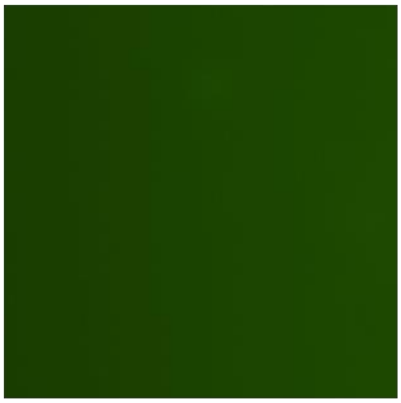
Figure 5.23a: The *TYS cell line was plated at* 1x 10^5 per dish *(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Gefitinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 100x magnification. Treatments: i) SF-MEM, ii) 5µM Gefitinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Gefitinib, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Gefitinib.* *Staining with phospho Akt*

Figure 5.23b: TYS cell line in response to growth factors and Erlotinib:

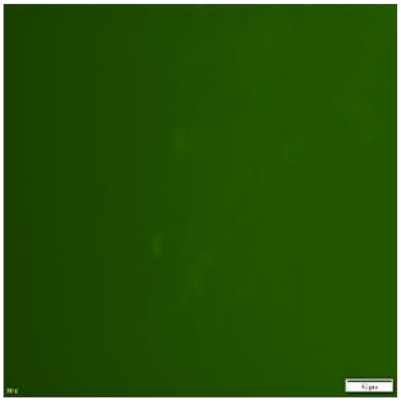
i) Serum free



ii) Erlotinib5µM

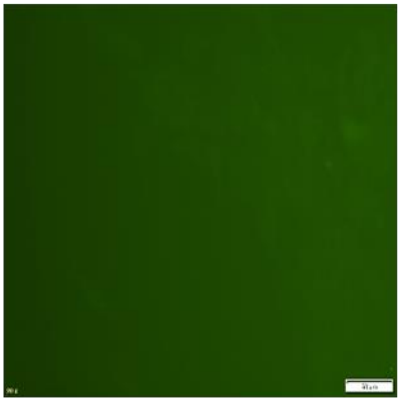


iii) EGF 50ng/ml



iv) Erlotinib 5µM + EGF50ng/ml

v) TGFα50ng/ml



vi) Erlotinib 5µM +TGFα50ng/ml

Figure 5.23b: The *TYS cell line was plated at* 1x 10^5 per dish *(30% confluence) and EGF (50ng/ml) and TGFα (50ng/ml) were added with 5µM Erlotinib. The photomicrograph was taken after 48 hrs of addition of the test conditions. All micrographs were taken at 100x magnification. Treatments: i) SF-MEM, ii) 5µM Erlotinib, iii) 50ng/ml EGF, iv) 50ng/ml EGF/5µM Erlotinib, v) 50ng/ml TGFα, vi) 50ng/ml TGFα/5µM Erlotinib. Staining with phospho Akt*

Figure 5.23c TYS cell line in response to growth factors and PD98059

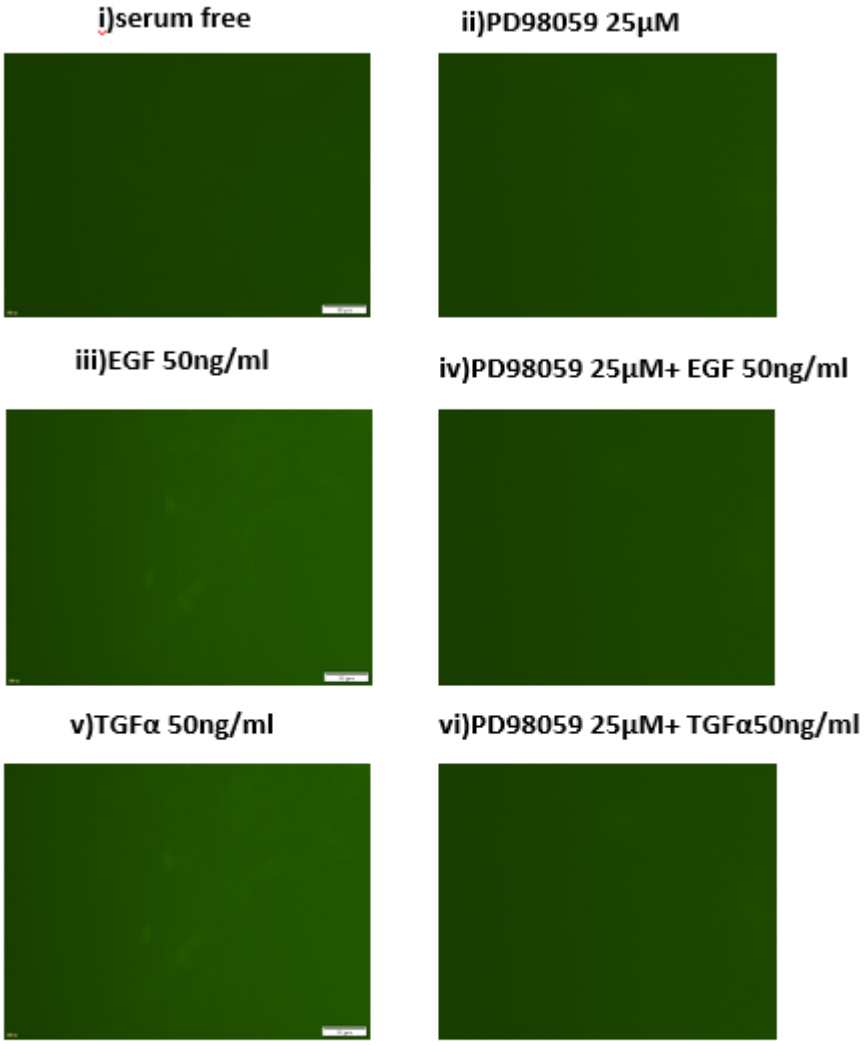


Figure 5.23c TYS cells line was plated at 1x10^5 per dish(30% confluency) and EGF(50ng/ml) and TGFα(50ng/ml) were added with 25µM PD98059. The photomicrographs were taken after 48 hour of addition of the tested conditions. All micrographs were taken at 100xmagnification. Treatment:1)SF-MEM2,2) ii)PD98059 25µM, iii)EGF 50ng/ml, iv) 50ng/ml EGF/ 25µMPD98059, v)TGFα 50ng/ml,vi) 50ng/ml TGFα/ 25µMPD98059 *Staining with phospho AKT*

5.5 Result Summary

The following tables summarise the results for this Chapter and will lead into the Discussion 5.6.

5.5.1 EMT or single cell migration

Table 5.2a EMT or single cell migration

Cell line	Serum free	EGF	TGFα	Gefitinib	Gefitinib+ EGF	Gefitinib+TGFα
HaCaT	Tightly packed colonies, Epithelial characteristic	loosely connected cells with dendritic or spiky projection in some area	loosely connected cells with dendritic or spiky projection in some area	Same as serum free	Same as serum free	Same as serum free
TYS	Partially Mesenchymal Shape cells	More mesenchymal With dendritic projection	More mesenchymal With dendritic projection	Epithelial characteristic	Epithelial characteristic	Epithelial characteristic
AZA1	Epithelial shape colonies	cell scattering, mixture of rounded cells and elongated cell, dendritic or spiky projection in some cells	cell scattering, mixture of rounded cells and elongated cell, dendritic or spiky projection in some cells	Same as serum free	Same as serum free	Same as serum free
HSG	Epithelial shape colonies	cell scattering, mixture of rounded cells and elongated cell, dendritic or spiky	cell scattering, mixture of rounded cells and elongated cell, dendritic or spiky	Same as serum free	Same as serum free	Same as serum free

		projection in some cells	projection in some cells			

Table 5.2b EMT or single cell migration

Cell line	Erlotinib	Erlotinib+ EGF	Erlotinib+TGFα	PD98059	PD98059+ EGF	PD98059+ TGFα
HaCaT	Same as serum free	Same as serum free	Same as serum free	Same as serum free	Same as serum free	Same as serum free
TYS	Epithelial charact-ristic	Epithelial character-istic	Epithelial characteris-tic	Epithelial character-istic	Epithelial characteri-stic	Epithelial character-istic
AZA1	Same as serum free	Same as serum free	Same as serum free	Same as serum free	Same as serum free	Same as serum free
HSG	Same as serum free	Same as serum free	Same as serum free	Same as serum free	Same as serum free	Same as serum free

5.5.2 Scratch assay or collective migration

Table Summary 5.3a Scratch assay or collective migration

Cell lines	Serum free	EGF50ng/ml	TGFα50ng/ml	Gefitinib5µl+ EGF50ng/ml	Gefitinib5µl + TGFα50ng/ml	Erlotinib5µl +EGF50ng/ml
TYS	Visible scratch line	full migration, Gap almost closure	Fully migration, Gap almost closure	same as Serum free	same as Serum free	Same as Serum free
HaCaT	Visible scratch line	fully migration	fully migration	same as Serum free	same as Serum free	same as Serum free
HSG	Visible scratch line	Moderate migration	Moderate migration	same as Serum free	same as Serum free	same as Serum free
AZA1	Visible scratch line	VeryLittle migration	Very Little migration	same as Serum free	same as Serum free	same as Serum free

Table 5.3b Scratch assay or collective migration

Cell lines	Erlotinib5µl +EGF50ng/ml	Erlotinib5µl + TGFα50ng/ml	PD25µl +EGF50ng/ml	PD25µl +TGFα50ng/ml
TYS	Same as Serum free	same as Serum free	Little migration, still intact scratch line	Little migration with intact scratch line
HaCaT	same as Serum free	same as Serum free	Partially migration	Partially migration
HSG	same as Serum free	same as Serum free	same as Serum free	same as Serum free
AZA1	same as Serum free	same as Serum free	same as Serum free	same as Serum free

5.5.3 Expression and Localisation of EGFR and its phosphorylated sub-type (Tyr1068)

Table 5.4 Expression and Localisation of total EGFR

Total EGFR	HSG	AZA1	TYS	HACAT
Serum free	membrane	membrane	Membrane	Membrane
EGF	Nucleus ,diffuse cytoplasm	Diffuse cytoplasm	Diffuse Cytoplasm	Diffuse cytoplasm
TGFα	Nucleus ,diffuse cytoplasm	Diffuse cytoplasm	Diffuse Cytoplasm	Diffuse cytoplasm

Table 5.5 Expression of phosphorylated EGFR sub-type (Tyr1068)

Phospho EGFR (1068)	HSG	AZA1	TYS	HACAT
Serum free	+	+	+	+
EGF	+	+	+	+
TGFα	+	+	+	+
Gefitinib+EGF	+	+	+	+
Gefitinib+TGFα	+	+	+	+
Erlotinib+EGF	+	+	+	+
Erlotinib+TGFα	+	+	+	+
PD98059+EGF	+	+	+	+
PD98059+TGFα	+	+	+	+

5.5.4 Expression and localisation of phospho-p44/p42 MAPK(Erk1/2) Thr202/Tyr204

Table 5.6.: Expression and localisation of phospho-p44/p42 MAPK(Erk1/2) Thr202/Tyr204

MAPK202/204	HSG		AZA1		TYS		HACAT	
	WB (24hr)	IF	WB (24hr)	IF	WB (24hr)	IF	WB (24hr)	IF
Serum free	+	+	+	++	+	+++	+	-
EGF	+++	+++	+++	+++	+++	+++	+ ++	-
TGFα	+ ++	+++	+++	+++	+++	+++	+++	-
Gefitinib only		-		-		-		-
Gefitinib+EGF	-	-	-	-	-	-	-	-
Gefitinib+TGFα	-	-	-	-	-	-	-	-
Erlotinib only		-		-		-		-
Erlotinib+EGF	-	-	-	-	-	-	-	-
Erlotinib+TGFα	-	-	-	-	-	-	-	-
PD98059 only		-		-		+		-
PD98059+EGF	+	-	+	-	+	++	+	-
PD98059+TGFα	+	-	+	-	+	++	+	-

5.5.5 Expression and localisation of phospho-Akt 473

Table 5.7: Expression and localisation of phospho-Akt 473

Akt473	HSG		AZA1		TYS		HACAT	
	WB (24hr)	IF	WB (24hr)	IF	WB (24hr)	IF	WB (24hr)	IF
Serum free		-	-	-	+	-	-	-
EGF	+	-	-	-	+	-	+++	-
TGFα	+	-	-	-	+	-	+++	-
Gefitinib only		-		-		-	-	-

Gefitinib+EGF	+	-	-	-	+	-	-	-
Gefitinib+TGFα	+	-	-	-	+	-	-	-
Erlotinib only		-		-		-	-	-
Erlotinib+EGF	+	-	-	-	+	-	-	-
Erlotinib+TGFα	+	-	-	-	+	-	-	-
PD98059 only		-		-		-	-	-
PD98059+EGF	+	-	-	-	+	-	+++	-
PD98059+TGFα	+	-	-	-	+	-	+++	-

5.6 Discussion

Experimental data supported the hypothesis of the study. Results in this Chapter have focussed on the effect of the inhibitors on migration, EMT and which signalling pathways are likely to be involved. The results are summarised in Table format above (Table 5.6.1, 5.6.2, 5.6.3, 5.6.4 and 5.6.5). The discussion will focus on relating the results with previous studies and clinical situations which these inhibitors may one day be used in.

5.6.1 EMT or Single cell migration

In epithelial characteristic cell lines such as HaCaT, HSG and AZA1, gefitinib, erlotinib and PD98059 fully inhibit the cell morphology and migration induced by EGF and TGFα treatment. In transformed cell line TYS, these inhibitors not only inhibit the EMT and single cell migration but also induce epithelial characteristic or MET transformation. Results from this experiments proved that Inhibiting MAPK pathway alone is enough for EMT or single cell migration. Only MAPK pathway is responsible for single cell migration in these cell lines (Table 5.6.1).

5.6.2 Collective cell migration or scratch assay

Result from scratch assay suggested that intensity of cell migration between each cell line was different. AZA1 is the least migrate cell line while HSG had moderate cell migration. TYS and HaCaT are migrate full intensity in response to EGF and TGFα. (Table 5.6.2)

We also found out tyrosine kinase inhibitors (Gefitinib and Erlotinib) inhibit the collective cell migration in all the cell line. Data from this study support the hypothesis of the study. Since all the cell line response to tyrosine kinase inhibitors, we can expect our cell lines did not harbour Ras mutation.

In HSG and AZA1 cell lines, PD98059 (ERK1/2 inhibitor) treated dishes did not show any sign of migration which lead to question that only be responsible for cell migration.

In contrast, TYS and HaCaT cells were shows sign of migration in PD98059 treated cell line showing that ERK1/2 might not be only signalling pathway which control the collective cell migration in these cell lines. There might be additional pathway involving the cell migration. Effect of PD98059 in collective cell mgration might be cell line dependent.

By comparing the two techniques, we found out single cell migration and collective cell migration use different signalling mechanism. Similar idea has found in an article (Lintz M, 2017).

5.6.3 EMT and drug resistant to Tyrosine kinase inhibitor

Established literatures stated that EMT believed to be related with drug resistance to tyrosine kinase inhibitor(Weng *et al.,*2019)(Maseki *et al.,*2012)(Frederick *et al.,*2007). One study

found that vimentin was expressed in non-small cell lung cancer cell lines insensitive to erlotinib while little or no vimentin was found in erlotinib sensitive lung cancer cell lines In contrast, E-cadherin was lost in majority of Erlotinib insensitive NSCLC cell lines while it is expressed in erlotinib sensitive cell lines (Thomson *et al.,*2005).

However, cells line used in this project were well response to TKIs. TYS which is partially mesenchymal shape cells were well response to TKIs. Some TYS cells were even transformed into MET. This leads to question that EMT is not the cause of drug resistance but the signs of drug resistance. Theoretically, TKIs could not be able to block down stream mutation such as Ras or PI3K. Without testing those mutation in those research paper, it is illogical to say EMT are related with drug resistant to TKIs.

5.6.4 Phosphorylation of EGFR (Tyr-1068)

In western blot, EGFR tyrosine residue 1068 was phosphorylated in all condition even in tyrosine kinase inhibitor treated dishes. So, it is best to see whether other tyrosine residues are phosphorylated. In human epidermoid cancer cell lines with high EGFR overexpression, tyrosine kinase inhibitors blocked all ligand phosphorylation sites except Y992 and Y1068 (Guo *et al.,* 2003)

Phosphorylation of tyrosine residue Y1068 has been suggested to be a predictive biomarker for tyrosine kinase inhibitors treatment for patient with wildtype EGFR in lung cancer patients (Wang *et al.,* 2012, Sette *et al.,* 2015) .Phosphorylation of tyrosine residue 1068 might be act as predictive biomarker for tyrosine kinase inhibitors in this experiments.

5.6.5 EGFR localization

In serum free condition, total EGFR was found in cell membrane. EGFR internalization was found after growth factor treatment. Diffuse cytoplasmic staining and membranous were found in TYS, AZA1 and HaCaT cell lines.

Report from other study found diffuse cytoplasmic staining of EGFRvIII in addition to strong membranous staining (Sok *et al.,* 2006). Another study stated that majority of EGFR vIII was expressed in cytoplasm while wild type EGFR was expressed in both membrane and cytoplasm (Chang *et al.,* 2013)

In HSG cell line, EGFR was stained in nucleus in addition to diffuse cytoplasmic and membranous staining.

It should be noted that EGFR has the potential to be translocated to the nucleus. Nuclear internalization of EGFR has been reported to acts as transcription factor (Lin *et al.,* 2001).

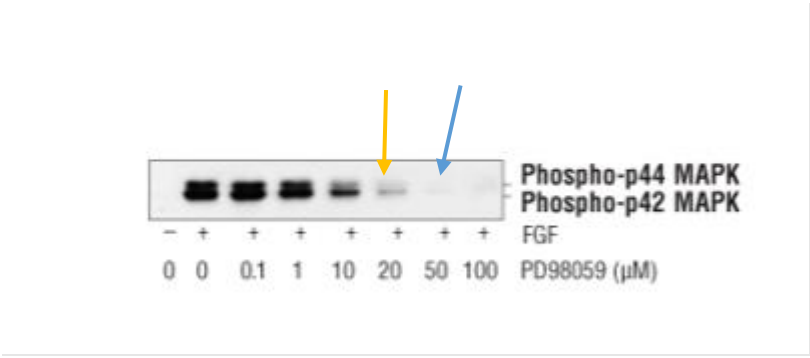
It might be possible, EGFR might acts as transcription factor in HSG cell lines. It lead to question why EGFR was internalized differently followed by growth factor treatment. In the future, it would be the best to look at the EGFR to get more detail information since current data are not enough to make a conclusion.

5.6.6 Phosphorylation of phosphor-p44/p42 MAPK (Erk1/2) Thr202/Tyr204

In all the cell line, MAPK 202/204 was expressed in EGF and TGFα treated cells. An hour pre-treated with Tyrosine kinase inhibitor inhibit its expression while PD98059 (selective ERK1/2 inhibitor) could not inhibit the expression of phosphorylated MAPK202/206 was still expressed in serum free condtion which was likely to be the same level of expression as

PD98059 treated dishes. The cells might need to express a certain intensity of phospho MAPK202/204 to see cell changes such as EMT and cell migration.

Another possible reason why MAPK202/204 was expressed in experiment is the amount used in experiment (25µM PD98059) is not enough for fully block it phosphorylation despite it fully blocked cell morphology change. Following picture from data sheet showed that MAPK 202/204 was expressed in 20µM PD98059 treated lane (yellow arrow) and its expression was completely blocked in 50µM PD98059 (blue arrow). It might be good idea to increase the concentration of PD98059 in future experiments.



5.6.7 Phosphorylation of Akt 473

Western blot results showed that. AKT 473 was not phosphorylated in AZA1 cell line. AKT 473 expression was found in HSG, TYS and HaCaT cell line. In HaCaT, tyrosine kinase inhibitor were able to block AKT473 expression completely. PD98059 did not block AKT473 expression since PD98059 only target to MAPK pathway. There was certain degree of AKT473 expression in tyrosine kinase inhibitors dishes of TYS and HSG cell line. However, morphological changes and migration of these cells were blocked with tyrosine kinase inhibitors which might mean

that AKT473 phosphorylation is not related with these changes. This leads to a new hypothesis for future projects.

In immunofluorescence, Akt 473 was faintly expressed in EGF and TGFα treated dishes of all cell lines and can consider negative. There was no expression of AKT473 in in Gefitinib, Erlotinib and PD98059 treated dishes. Data from these experiments are not enough to draw conclusion about involvement of AKT473 in EGFR signalling pathway. In the future it might be of interested to widen the concentration range of antibodies to overcome the poor image problems.

Chapter 6: General Discussion

This Chapter will bring together all my results for a final overarching discussion. Chapter 4 (4.5), and Chapter 5 (5.5) give a full summary of the results while Chapter 4 (4.6) and Chapter 5 (5.6) give a detailed discussion. The aim of this Chapter is to discuss this work in the context of past and current developments in the field, elaborate on the advances that this work brings to the field and give a focus for future work following on from my studies.

The main highlights of my results and discussion are (the facts are shown in normal font and the interpretation is in italics):

- EGF and TGFα does not have an effect on cell proliferation.
- EGF and TGFα induce EMT-like morphological change and single cell migration.
- The plating density of the cells play an important role in morphological change and single cell migration.
- Four cell lines (one control and three cancer cell lines) have own unique characteristics in both morphological change and migration
- Gefitinib, Erlotinib and PD98059 inhibit both morphological change and single cell migration.
- *MAPK pathway might be only pathway responsible for morphological change and single cell migration in cell lines used in this project*.
- EGF and TGFα induce collective cell migration
- Gefitinib, Erlotinib inhibit collective cell migration.
- PD98059 partially inhibit collective cell migration in TYS and HaCaT cell line while it fully inhibit collective cell migration in AZA1 and HaCaT.
- *Role of the MAPK signalling pathway in collective cell migration is cell line dependent.*
- *MAPK alone might not responsible for collective cell migration.*

- *Single cell migration and collective might not be use the same signalling pathway in cell migration*
- *These result match with my view and one that is widely held that the idea of personalized therapy might be the best to cure cancer.*
- Addition of EGF and TGFα to the cell culture conditions takes time for changes in expression to occur (Both growth factors added to the cells for 48 hours did not change intensity of E-cadherin and Vimentin expression).
- *It might be better to look at EMT markers after 72 hours growth factor treatment.*
- HSG and AZA1 cell lines are morphologically similar to epithelial cells while expressing mesenchymal marker.
- *For the future, it might be better to passage the cells onto Matrigel or 3D collagen to have a more in vivo like culture conditions.*
- All the cell lines used in this project were responsive to TKIs including the TYS cells which is a cell line that morphologically undergoes EMT.
- This is at odds with others who have reported that EMT is believed to be related with resistance to TKIs.
- *This leads to question: is it that EMT is not the cause of drug resistance but the signs of drug resistance?*
- *Theoretically, TKIs could not be able to block downstream pathways due to a mutation in proteins such as Ras or PI3K.*
- In the Western blot, EGF and TGFα were found to induce phosphorylation of MAPK202/204 in all the cell lines and Tyrosine kinase inhibitors were able to block it. The tested concentration of PD98059 was not able to block phosphorylation. It might be good idea to increase the concentration.

Limitations of research

One of the Limitations of research include inaccessibility of pure squamous cell line because the cell lines used in this project were chosen on the basis of ethnicity (Asian) rather than site of cancer.

Lack of ideal positive control in Chapter 5 for Gefitinib, Erlotinib and PD98059 could also be regarded as limitation of the study.

There are also technological limitation based on the methods.

Automated cell counter TC10: it is unable to differentiate between dead cells and live cells. Dead cells therefore may be counted as live cells. It could also recognised a big clump of cells as single cell.

Scatter assay: While taking pictures, shot between time 0 and time 48 hours may not be same area of the dishes.

Scratch assay: Scratch line may not be uniform therefore a machine made scrach system is now available rather than the man made scratch used here. As above scatter assay, while taking picture, the shot between time 0 and time 24 hours may not be same area.

Immunofluorescence: technique does not have a uniform scoring system and interpretation is highly subjective.

Because of these limitations, Data are not strictly quantitive. In the future, it would be the better to use techniques (BrdU , time lapse imaging)which can provide strictly quantitave data.

Recommendation for future research

One of my developing views is that personalised therapy could be a way forward. Some trials or studies already exist where PI3K inhibitor such as Buparlisib (BKM120), Alpelisib (BYL719), PX-866, Copanlisib and mTOR inhibitor such as rapamycin (sirolimus), Temsirolimus (CCI-779), Everolimus (RAD001) and PI3K/mTOR dual inhibitor such as SF1126, Gedatolisib, Dactolisib (BEZ235) are used either alone or in combination with chemotherapy or in HNSCC clinical trials (Cai *et al.,* 2017, Wang *et al.,* 2017, Jung *et al.,* 2018).

It may be that epigenetic and environmental factors are important and that studies of cells that have had phenotypic change are a good future model in developing therapies. AZA1 cells are HSG cells treated with 5-Azacytidine. It is a potent growth inhibitor and cytotoxic agent. It has ability to inhibits DNA methyltransferase causing DNA demethylation and allows transcription factors to bind DNA and reactivate tumour suppressor genes. In the clinic, Azacytine is mainly used to treat myelodysplastic syndrome and some cases of acute myeloid leukemia as a hypomethylation agent. In vitro, it can be used to remove methyl group. In research 5-azacitidine is used to promote the cardiomyocyte differentiation of stem cells. In contrast, it promotes transdifferentiation of cardiac cells into skeletal myocyte. 5-Azacytidine inhibit T cell proliferation, blocks the cell cycle, inhibit the production of proinflammatory cytokines (Sánchez-Abarca *et al.,* 2010).

In South East Asia low dose of arecoline, which is the major alkaloid of areca nut was found to induce cell proliferation, cell cycle progression at G2/M phase and upregulation of c-myc while high dose of arecoline induced a cytotoxic response (Chuerduangphui *et al.,* 2018).

Since starting this study I have become increasing interested in geographic mutations as part of the future of drug design and therapy. EGFR TKD mutation varies with geographic location. The EGFR mutation had not been studied world-wide. Limited data has been produced from Africa, the Middle East, Central Asia, and Australia. Reports suggest that EGFR mutation is uncommon in the West (Loeffler-Ragg *et al.,* 2006) whilst its mutation is found more frequently in South Korea and South East Asia region (Perisanidis, 2017, Vatte *et al.,* 2017).The mechanisms behind the different rates of *EGFR* mutation in different ethnicities remains to be determined. The South East Asia region is a densely populated area of the world with a population of around 600 million and it definitely needs attention in terms of more research. It is a worry since none of TKIs targeting EGFR mutations has got FDA approval for HNSCC.

The mutation of the Ras family is more common in Asia when compared with the West (Saranath *et al.,* 1991, Chang *et al.,* 1991, Xu J *et al.,* 1998, Das *et al.,* 2000). Mutation of Ras is low in the west (Europe and North America). In fact, only 5% of head and neck cancer patients in UK harboured the H-Ras mutation (Chang *et al.,* 1991). Another study conducted in USA also found low occurrence of H-Ras mutation (Xu J *et al.,* 1998). In contrast, 35% of betel quid chewing related oral SCC in western India has H-ras mutation (Saranath, *et al.,* 1991). Further studies conducted in Eastern India, reported that H- and K-ras genes mutations in head and neck cancer patients at a frequency of 28 % and 33%, respectively (Das N *et al.,* 2000). A study conducted in Taiwan (which is another betel quid chewing region) found that 18% tumour specimens contained K-*ras* mutation (Kuo *et al.,* 1994).

This leads to question of whether Ras mutation is caused by specific carcinogen in areca nuts or related with ethnicity. This is an area where funding for these studies in countries such as Myanmar will be the future. Future research coming from my studies will involve the study of EGFR in Asian Head and Neck Cancer patient. As discussed in the above, EGFR

mutation and Ras mutation are more common in Asia than the West. Currently the only FDA approved EGFR target for head and neck cancer is Cetuximab (which only works on extra cellular ligand binding domain of EGFR). Cetuximab will not be effective on downstream EGFR mutated and Ras mutated patients. Further work needs to analyse whether EGFR tyrosine kinase inhibitors and Ras inhibitors would be an appropriate therapy for subsets of populations such as from South East Asia. In order to hopefully save the life of millions of Asian head and neck cancer patients, clinical research need to be done not just on a Western group of people, but on all the ethnic groups. Asian people should receive equal chances to get the benefit from Anti-cancer drugs. The cell lines and tissue samples from SEA patients are needed to allow us to continue to develop/discover new and improved drugs. Funding would be required for this from NGO's, governments and pharmaceutical companies. My work will be to coordinate a team of scientists and clinicians working together for the benefit of people from South East Asia.

Chapter 7: References

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