

## **Title: Oral Tumour Cell Migration and the Effect of the Local Soluble Factors from the Microenvironment on Signalling Pathways. Is It All about the Akt?**

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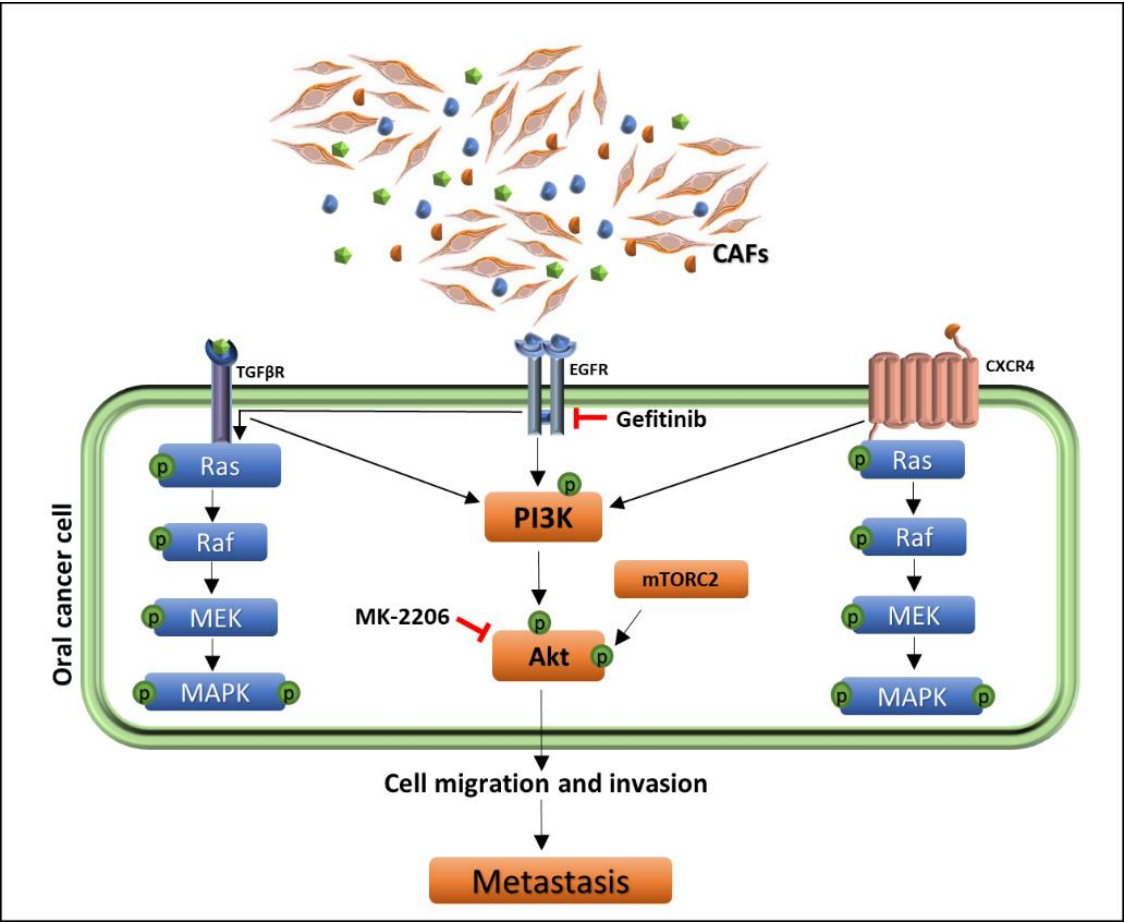
### **Abstract**

Oral cancer cells (TYS) and the signalling pathways involved in metastasis, in response to cancer-associated fibroblasts (CAFs, COM) and normal oral mucosal fibroblasts (MM1) was studied. Metastatic cell behaviour was observed by cell-scatter, 3D-collagen gel migration and 3D-spheroid invasion assays. Akt, MAPK, EGFR, TGF $\beta$ Rii and CXCR4 inhibitors were used to identify the signalling pathways involved. Signalling pathway protein expression and activation were assessed by SDS-PAGE and Western Blotting. COM-CM (conditioned medium) and MM1-CM stimulated cancer cell scattering, which was blocked only by the Akt inhibitor. COM-CM induced scattered cancer cells showed higher levels of Akt phosphorylation than the negative control and MM1-CM. Migration and invasion of YYS cells into the collagen gels from the spheroids was stimulated by CM from both sources, compared to the negative control. COM cells stimulated YYS, cancer cell invasion into the collagen more than MM1 and the control. Akt and EGFR inhibitors effectively blocked CM and COM cell-induced invasion. Akt-silenced cancer cells were not stimulated to migrate and invade by fibroblast-CM and did not survive addition of the EGFR inhibitor. This suggests that CAFs stimulate oral cancer cell migration and invasion in an Akt- dependent manner. EGFR and Akt are potential therapy targets in metastatic oral cancer.

### **Key words**

oral cancer; cancer-associated fibroblasts; metastasis; cell migration; cell invasion; Akt; EGFR

Graphical abstract:



## 1. Introduction

Oral cancer is the sixth most common cancer worldwide with more than 330,000 deaths every year [1]. Despite advances in diagnosis and treatment for OSCC, the poor survival of patients has not been fundamentally changed, mainly because of its high recurrence rate and strong inclination to metastasis [2]. Emerging evidence has found that the context in which malignant cells reside, which is now known as the tumour microenvironment (TME), has been implicated in orchestrating cancer survival and progression through the continuous cross-interaction between cancer cells and the stroma [3-5]. Thus, the concept of a tumour being only a cancer-cell-centred disease has been shifted into complex multicellular interactions between cancer and stromal cells.

In normal tissue, fibroblasts are usually found in a quiescent state and become 'transiently' activated in certain physiological conditions, such as wound healing. However, in the tumour microenvironment fibroblasts, which are the predominantly stromal cells, are perpetually activated without reverting to their normal state or undergoing apoptosis, and they have been termed cancer-associated fibroblasts (CAFs) [6,7]. Several studies have recognised the role of CAFs in the progression of various tumour types, including oral cancer [7,8]. CAFs were found to provide cancer cells with several growth factors and chemokines that encourage tumour growth and invasion, as well as metastasis through remodelling the extracellular matrix [9-11].

In oral cancer, studies showed that CAFs induce epithelial to mesenchymal transition, and thus, invasiveness of cancer cells [12,13]. Another research study revealed that CAFs participated in recruiting and inducing tumour-associated macrophages in the stroma of oral squamous cell carcinoma, thus increasing the immunosuppressive environment [14]. A CAF-rich TME has been found to be associated with increased mortality, in a study conducted on 77 patients with tongue cancer [15]. Among the various growth factors and cytokines that are produced by CAFs, TGF- $\beta$ , EGF and chemokines were found to play a major role in inducing proliferation and invasion of cancer cells [16,17].

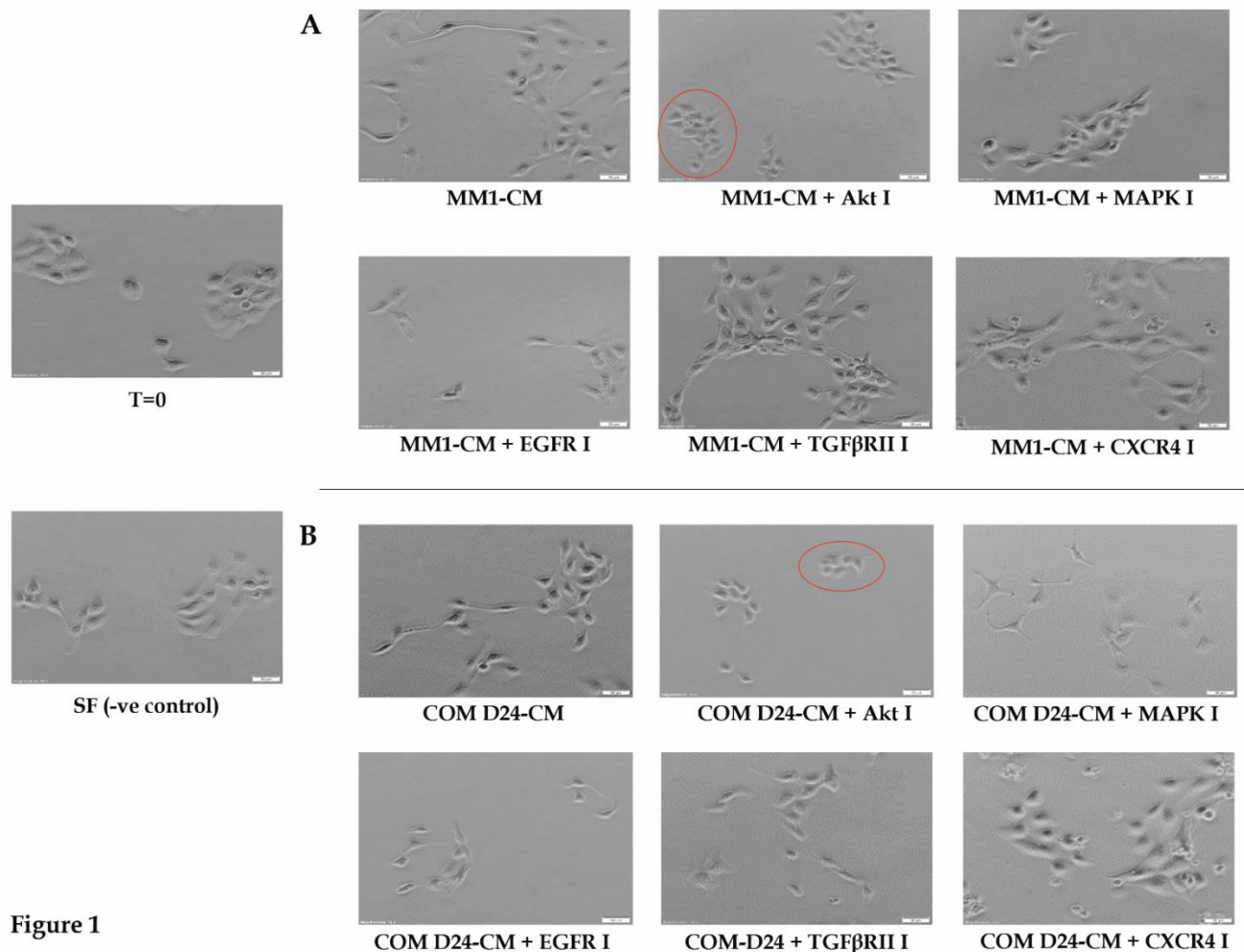
Multiple intracellular pathways were found to be involved in progression and invasion of oral cancer [9,18]. The PI3K/Akt pathway is the most commonly activated pathway in HNSCC [19]. The serine/threonine kinase, Akt, is the downstream target of PI3K which is responsible for cell proliferation, differentiation as well as cell motility and migration [20,21]. Full activation of Akt is achieved once it is phosphorylated at Thr 308 and Ser 473 residues [22].

In this study, we aimed to investigate the role of oral cancer-associated fibroblasts in stimulation of oral cancer cell migration and invasion by developing a novel 3D *in vitro* TME model and identifying the key signalling pathways that might be involved in fibroblast-induced oral cancer metastasis.

## 2. Results

### 2.1 Conditioned medium collected from fibroblasts induced cancer cell scattering in an Akt-dependent manner

Conditioned medium collected from both MM1 and COM D24 cells induced oral cancer cell scattering after 48 hours. Cancer cells scattered out from the compact colonies and their cuboidal shape changed to spindle, mesenchymal type cells in response to conditioned medium from both cell lines. Cells treated with serum-free medium (negative control) did not scatter (Figure 1). MK2206, an Akt inhibitor completely blocked the scattering of cancer cells induced by conditioned medium from both fibroblast cell lines. However, the inhibitors for MAPK, EGFR, TGFBR and the chemokine receptor did not inhibit conditioned medium induced oral cancer cell scattering (Figure 1).



**Figure 1**

Figure 1. Scattering of TYS cells in response to fibroblast CM with or without inhibitors. A) TYS cells treated with MM1 conditioned medium  $\pm$  inhibitors. Cancer cells scattered out from the colonies in response to MM1 conditioned medium and only the Akt inhibitor blocked the scattering of TYS cells (red circle). B) TYS cells treated with COM D24 conditioned medium  $\pm$  inhibitors. Cancer cells scattered out from the colonies in response to COM conditioned medium and only the Akt inhibitor blocked the scattering of TYS cells (red circle). Scattering of the cancer cells was observed for 48 hours and images of the cells captured using an inverted microscope with 10x objective lens. T=0 represents the baseline

cells before adding the test conditions and cells treated with SF-MEM were regarded as a negative control.

2.2 Conditioned medium from oral cancer associated fibroblasts (COM D24) activated Akt more than the negative control in scattered cancer cells

Conditioned medium collected from cultures of MM1 and COMD24 cells activated Akt as indicated by phosphorylation of S473 in the scattered TYS cells 1.6 and 3 times more than that of serum-free medium from negative control cells, respectively. However, MM1-CM did not activate Akt by phosphorylation of the T308 residue, but COM D24-CM activated Akt at T308 in scattered cancer cells 5.5 times more than that negative control cells. MAPK, on the other hand, was not activated by any of the conditioned medium samples (compared to the negative control). The Akt inhibitor completely blocked the activation of Akt in cancer cells treated with conditioned medium. However, inhibitors for TGFβR and the chemokine receptor (CXCR4), did not block the activation of Akt at either residue in the COMD24-CM induced scattered TYS cells (Figure 2A). Full blot images were included as supplementary figure S1 and S2.

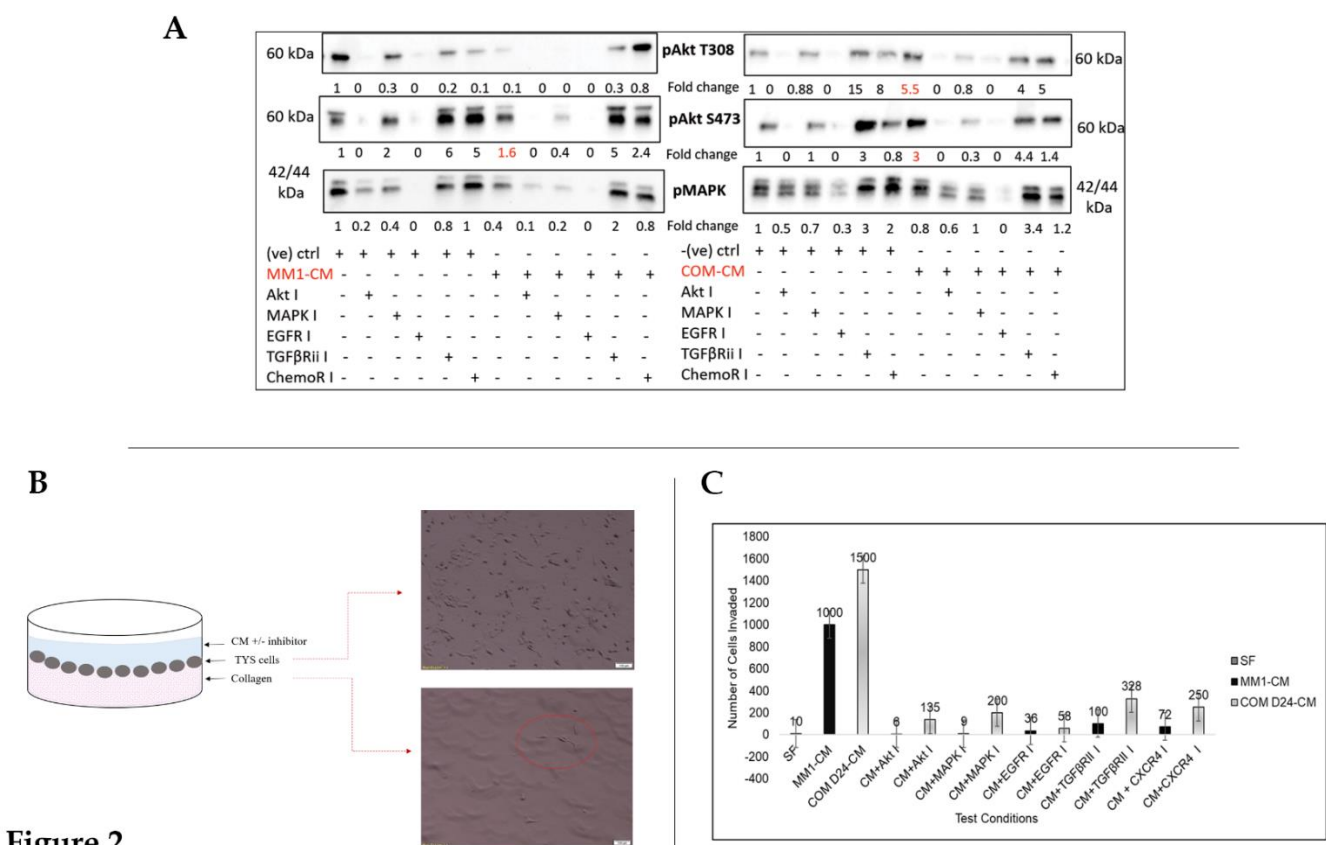


Figure 2

Figure 2. Activation of Akt and MAPK in scattered TYS cells and migration of TYS into the matrix. A) pAkt at T308 and S473 and p42/44-MAPK in TYS cells after 48 hours of scatter assay. COM-CM activated Akt in scattered TYS cells (red font) more than the negative

control and MM1-CM. Fibroblasts CM did not activate MAPK in TYS cells after 48 hours. Blots were normalised against total protein, quantified and data expressed as fold change compared to the negative control. SF-MEM treated TYS cells in scatter assay after 48 hours were regarded as the negative control. B) Graphical representation of the collagen gel migration assay in which TYS cells were plated on top of collagen gels followed by treatment with CM  $\pm$  inhibitors. Migrated cells (red circle) were counted manually by selecting 5 random areas within the gel after 48 hours. C) Bar diagram represents the number of migrated TYS cells into the matrix in response to fibroblasts CM with or without the inhibitors. COM-CM stimulated TYS cells migration into the matrix more than the negative control (SF-MEM treated cells) and that of MM1-CM. The Akt and EGFR inhibitors were the most effective inhibitors in blocking CM-induced TYS migration.

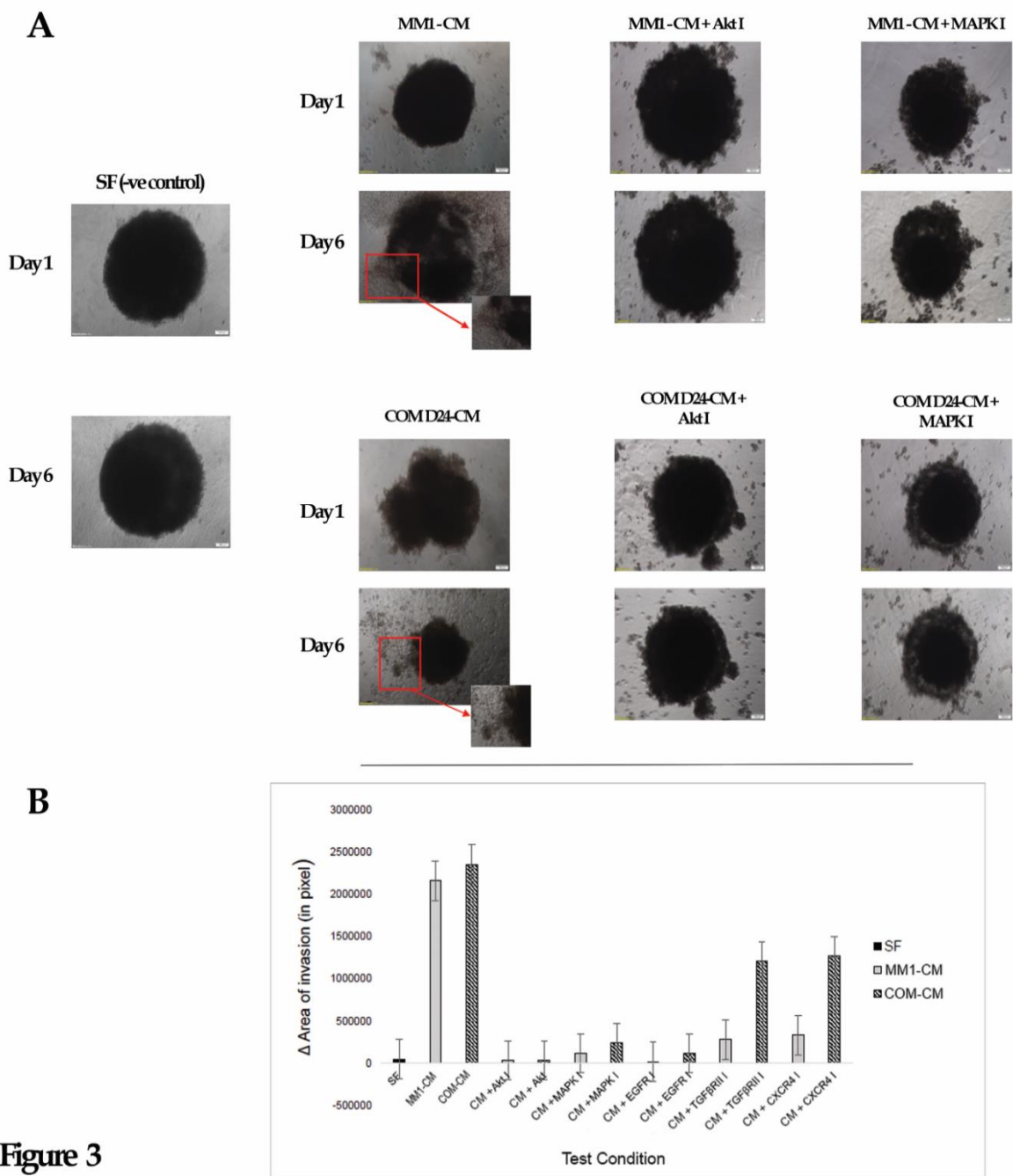
### **2.3 COM D24-CM and MM1-CM stimulated cancer cell invasion into the collagen matrix**

After observing that conditioned medium collected from fibroblasts could induce TYS cell scattering/migration, we aimed to study whether the conditioned medium could induce TYS cell invasion into a collagen matrix treated with /without the inhibitors (Figure 2B). Both MM1-CM and COM D24-CM stimulated TYS cells to invade into the matrix 100 times and 150 times more than the negative control (SF), respectively. COM D24-CM induced TYS cell invasion 1.5 times more than that of MM1-CM. The Akt inhibitor blocked MM1-CM induced cancer cell invasion (by 99%) effectively and COM-CM induced invasion (by 91%) partially as number of cells invaded were more than the control. The MAPK inhibitor blocked MM1-CM induced cancer cell invasion effectively but blocking of COM-CM induced cancer cell invasion was partial, as 20 times more cells than the control still invaded. However, the EGFR inhibitor blocked MM1-CM and COM-CM induced cancer cell invasion effectively. Blocking of MM1-CM and COM-CM induced cancer cell invasion by both TGF $\beta$  receptor inhibitor and CXCR4 inhibitor were also not effective (Figure 2C).

### **2.4 Fibroblast conditioned medium stimulated cancer cell evasion from the spheroids into the collagen matrix more than the control**

Cell's invasion from spheroid into the surrounding matrix appeared as spindle-like projections; whereas, there were no such projections observed where invasion had not been stimulated. There was no change in the area of invasion of TYS spheroids observed in serum-free medium and was used as negative control (Figure 3A). TYS cells from their spheroids invaded into the collagen matrix after day 6, in response to both MM1 and COM D24 conditioned medium. The inhibitors for Akt and MAPK completely blocked the cancer cell invasion from the spheroids induced by both MM1-CM and COM-CM (Figure 3). EGFR inhibitor also completely blocked CM-induced invasion of cancer cells from the spheroids into the matrix (Figure 3B and S3). However, inhibitors of TGF $\beta$ R and CXCR4, did not block COM-CM induced invasion of cancer cells from the spheroids (Figure 3B and S3).





**Figure 3**

Figure 3. TYS cells invasion from their spheroids in response to fibroblasts CM ± the inhibitors. A) TYS spheroids embedded into a collagen matrix followed by the treatment with fibroblast CM with or without the inhibitors. Images of the invasive TYS cells from the spheroids were captured after day 6 using an inverted microscope with a 4X objective lens. Images of the TYS spheroids treated with EGFR I, TGFBR I and CXCR4 I are presented as a supplementary figure. SF-MEM treated TYS spheroids were regarded as negative control B) Graphical representation of TYS invasion into the collagen matrix from the spheroids in response to fibroblast CM ± the inhibitors after day 6. COM-CM stimulated TYS invasion from the spheroids into the matrix, more than the negative control (SF-MEM treated cells) and f MM1-CM. The Akt and EGFR inhibitors were the most effective inhibitors in blocking

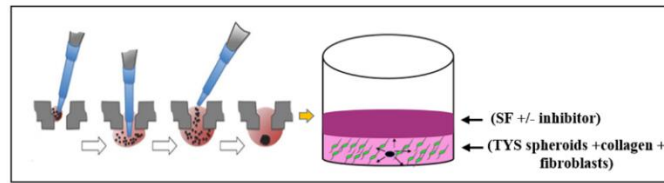
CM-induced TYS invasion. The area of invasion (out from the edge of the spheroid) was calculated using ImageJ software.

## **2.5 COM D24 cells stimulated cancer cells to invade from the spheroids into the collagen matrix and the Akt inhibitor completely blocked this invasion**

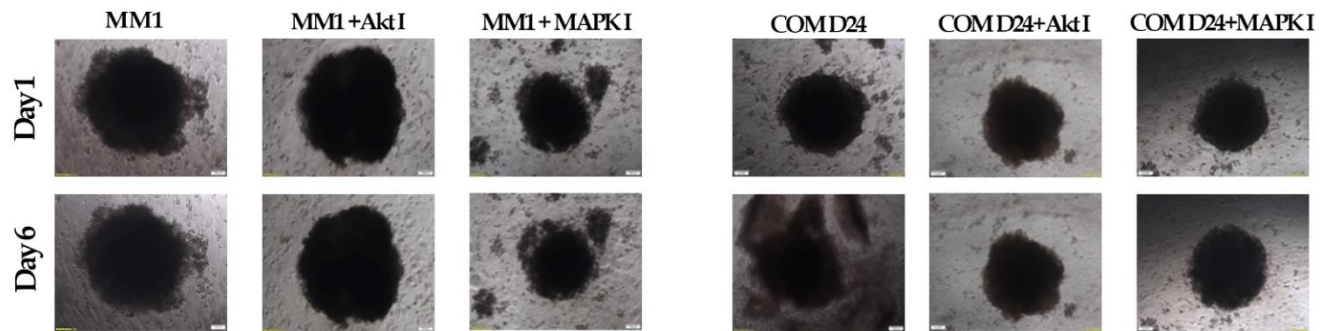
COM D24-cells stimulated TYS cells invasion from the spheroids into the collagen (Figure 4B) 19-fold more than the negative control (SF-MEM treated spheroids) (Figure 4C). Inhibition of the TGF $\beta$  receptor and chemokine receptor by their respective inhibitors resulted in stimulation of the invasion of TYS cells from the spheroids by about 23-fold compared to that of negative control (Figure S4 and 4C). The Akt inhibitor completely blocked COM D24-induced invasion of TYS cells from the spheroids into the matrix (Figure 4B & 4C). The MAPK inhibitor and EGFR inhibitor also blocked COM D24 induced cancer spheroid cell invasion, but not as efficiently as the Akt inhibitor (Figure S4, 4B & 4C). MM1 cells did not stimulate oral cancer spheroid cell migration into the collagen, in comparison to the negative control. COM D24 cells stimulated invasion 17-fold more in comparison to MM1 cells (Figure 4B and 4C).



A



B



C

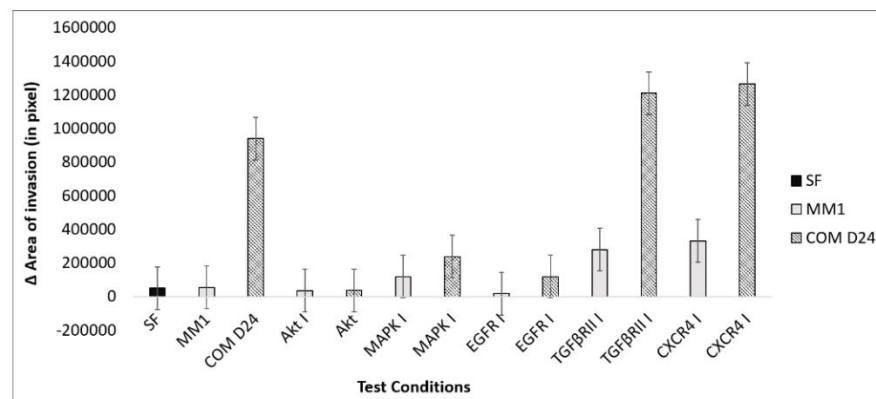


Figure 4

Figure 4. TYS cells invasion from their spheroids in response to fibroblasts ± the inhibitors. A) A 3D TME model that illustrates the development of 3D TYS spheroids by the hanging drop method, adding fibroblasts and embedding the spheroids into collagen. B) TYS invasion from the spheroids in response to fibroblasts with or without exogenous inhibitors. Images of the invasive TYS cells from the spheroids were captured after day 6 using an inverted microscope with 4X objective lens. Images of the TYS spheroids treated with EGFR1, TGFβRI and CXCR4I are presented as a supplementary figure. SF-MEM treated TYS spheroids without the fibroblasts were regarded as the negative control the same as Fig 3A. TYS cells invaded into the matrix from the spheroids in response to COMD24 cells. C) Graphical representation of TYS cells invasion into the matrix from the spheroids in response to fibroblasts ± inhibitors after day 6. COM D24 cells stimulated TYS cells invasion from their spheroids more than the negative control (without fibroblasts) and that of MM1 cells. The Akt and EGFR inhibitors were the most effective inhibitors in blocking COM D24 cells induced TYS cells invasion. However, TGFβR and CXCR4 inhibitors did not block TYS invasion.

The area of invasion (out from the edge of the spheroid) was calculated using ImageJ software.

2.6 EGFR inhibitor was toxic to the Akt silenced oral cancer cells

TYS cells were transfected with shRNA Akt at different MOI. The cell lysates from the cells transfected at MOI 1, 2, 5, 10 and 15 were blotted against pAkt and Pan Akt antibodies and compared with the negative control. MOI 2 has been chosen for transduction as the phosphorylation of Akt T308 and total Akt was observed to be considerably less than that of negative control. Akt silenced TY5 cells were then regarded as shRNA Akt TY5 cells (Figure 5A). Akt-silenced TY5 cells were not stimulated to scatter in response to conditioned medium whereas non-transduced TY5 cells were. Cell death was noted when Akt –silenced TY5 cells were treated with the EGFR inhibitor (Figure 5B). After 6 days of the 3D spheroid assay, it was observed that the Akt-silenced TY5 did not invade into the collagen from their spheroids when treated with either MM1-CM or COM D24-CM, as compared to the non-transfected TY5 cells (Figure 5C). Akt-silenced TY5 cells lost the ability to invade into the collagen matrix completely in comparison to that of non-transfected cells treated with MM1-CM and COM-CM (Figure 5D).

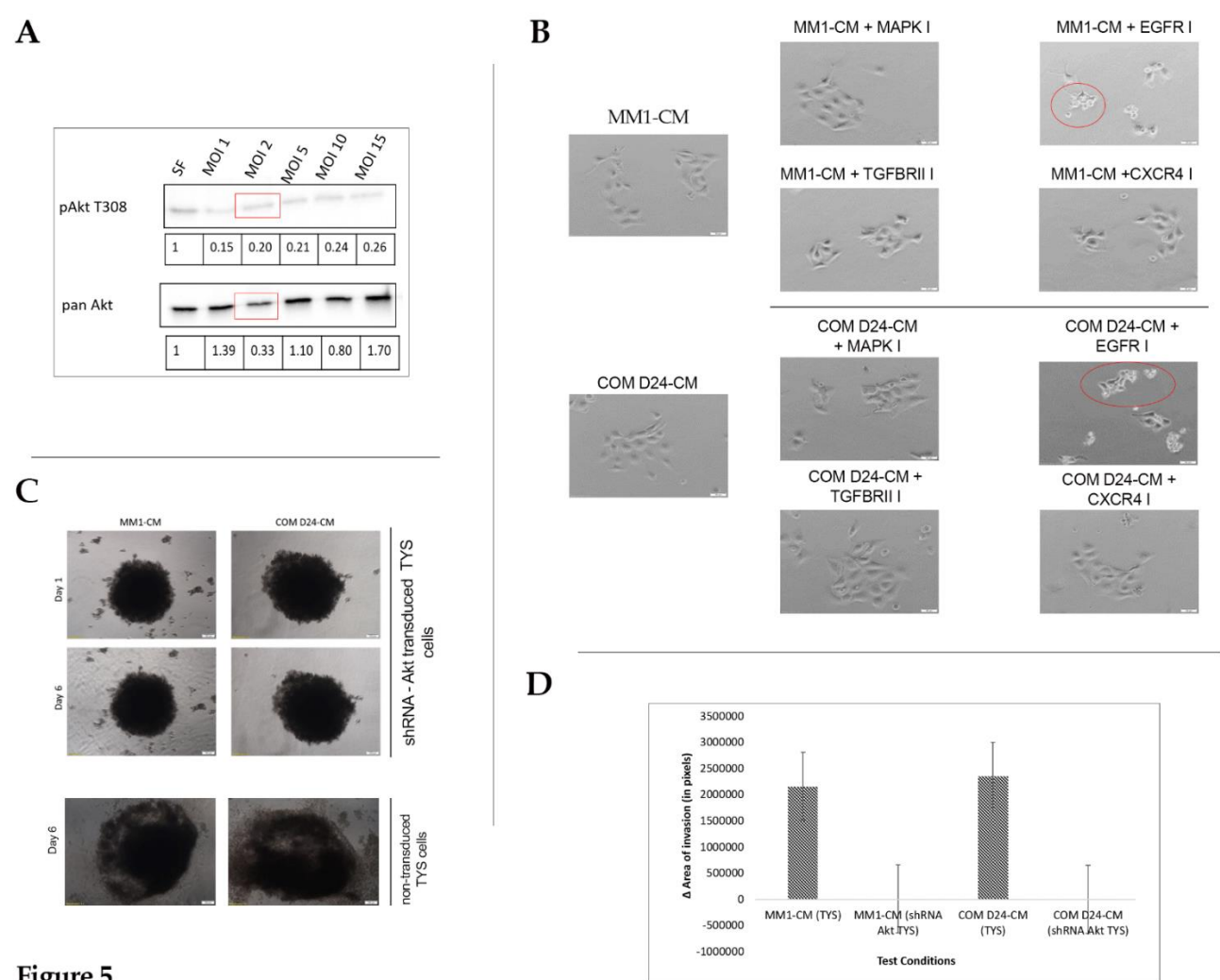


Figure 5

Figure 5. Scattering and invasion of Akt silenced TYS cells. A) Akt expression and activation after silencing the Akt gene using different MOI of shRNA lentivirus. Red rectangles related to MOI 2 were chosen for transduction. B) Scattering of shRNA Akt TYS cells in response to fibroblasts conditioned medium. shRNA Akt TYS cells were not stimulated to scatter in response to fibroblast CM and did not survive when treated with EGFRi (red circle) after 48 hours. C) Invasion of shRNA Akt TYS cells from the spheroids into the collagen matrix in response to fibroblast CM. D) Graphical presentation of Akt-silenced Akt TYS invasion and shRNA Akt TYS cells did not stimulate to invade in response to fibroblasts CM.

### 3. Discussion

CAFs are considered to play a crucial role in tumour progression. A recent study has revealed that CAFs trigger tumour progression in OSCC by secreting various growth factors to the nearby cells [23], as observed in this study. The normal fibroblast (NF) cells that are found in the tumour microenvironment can interact with cancer cells and become 'activated' leading to CAF formation. Thus, both NF and CAF in interaction with cancer cells may promote tumour progression by activating migration and invasion [24]. Both the NF and the CAFs stimulated migratory behaviour of the oral cancer cells in the 2D scatter assay. The results in this assay did not show any clear difference in the migratory behaviour of the cancer cells in response to NF (MM1) and CAF (COM-D24), further invasion experiments were conducted. Invasion assays concluded that CAF-CM induced oral cancer cell invasion more than NF-CM. The 3D spheroid invasion assay confirmed upregulation of CAF-CM or CAF mediated oral cancer cell invasion in comparison to that of normal fibroblasts. Thus, to conclude, CAFs have a pivotal role in migration and invasion in oral cancer. The 3D spheroid invasion assay containing fibroblasts can be a useful method to study the relationships between the tumour microenvironment and metastasis. Akt phosphorylation both at Thr 308 and Ser 473 was upregulated in CAF-CM treated cancer cells, whereas only Ser 473 was upregulated in NF-CM treated cancer cells, compared to the negative control. This may explain why the CAF-CM treated cancer cell invasion was higher than that of the normal fibroblasts. This also proves the long-standing theory that Akt needs to be phosphorylated at both residues to be fully active, stable and to induce certain bioactivities. Through multivariate studies it has been reported that Akt activation is a significant, independent prognostic indicator for OSCC [25]. It is evident from this study that the Akt inhibitor is responsible for blocking both CAF and NF- induced oral cancer cell migration and invasion. Activation of MAPK transforms normal cells to the tumour cells causing a high risk of developing a second primary tumour in oral cancer, distant metastasis is also observed in advanced stages [26]. A higher rate of MAPK phosphorylation was observed in CAF treated oral cancer cells, in comparison to the normal fibroblasts, supporting the theory that MAPK might also be responsible for CAF mediated metastasis in oral cancer. Though MAPK inhibitor and EGFR inhibitor blocked the invasion of oral cancer cells in response to NF and CAF, these inhibitors did not block scattering in 2D cell culture. The Akt inhibitor, however, blocked the migration and invasion of cancer cells effectively in both the 2D and 3D assays.

This data indicates that Akt is a vital molecule to study in order to ascertain the underlying signalling mechanisms involved in fibroblast-induced oral cancer metastasis. TGF $\beta$  has a dual nature as it exhibits both pro- and anti- tumour effects and helps in the acquisition of the CAF phenotype. Many TGF $\beta$  antibodies, kinase inhibitors and antisense oligonucleotides are being assessed for efficacy in phase III trials [8]. TGF $\beta$  has been shown to activate Akt, leading to cell migration in prostate cancer [27]. TGF $\beta$ 1 stimulates the phosphorylation of SMAD, MAPK and Akt in oral cancer, but stimulated the migration of oral cancer cells in an Akt-dependent manner [28]. In prostate cancer, CXCR4 is overexpressed and loss of the tumour suppressor PTEN was reported to activate Akt and regulate the CXCL12/CXCR4 signalling pathway in metastasis [29]. CXCR4 expression was also found to be upregulated in human oesophageal squamous cell carcinoma and modulated cell migration and invasion by Rho-A, Rac-1 and Cdc42 through Akt-dependent mechanisms [30]. CAFs are also reported to promote invasion through the CXCR4 pathway in gastric cancer and a CXCR4 antagonist blocked invasiveness of gastric cancer [31]. Thus, the literature suggests that TGF $\beta$  and the chemokine receptor might have a role in cancer metastasis. The data presented here indicate that inhibitors of TGF $\beta$ R and CXCR4 do not block CAF and NF induced cancer cell migration and invasion since, they did not block Akt phosphorylation, but rather increased it. This unexpected result and the mechanism of action of these two inhibitors needs to be explored further.

Furthermore, silencing the Akt gene in oral cancer cells blocked their scattering and invasion, even when treated with CAF and NF conditioned medium. Akt silenced oral cancer cells did not survive when treated with EGFR inhibitor, in this project. EGFR is over-expressed in most oral cancers and is considered to be a potential therapeutic target for controlling cell proliferation and metastasis in OSCC by inhibiting the PI3K/Akt pathway. Recently, the FDI has approved a drug, called Cetuximab, to be used in metastatic HNSCC which blocks EGFR. However, certain tumours in the head and neck region are resistant to the EGFR inhibitor due to a mutation in the EGFR tyrosine kinase [32]. From our data we have seen that if both Akt and the EGFR are blocked, it leads to cell death, which can be studied further to check its specificity to cancer cells.

## 4. Materials and Methods

### 4.1 Antibodies and Inhibitors

Table 1: Details of antibodies and inhibitors used in this study

Name	Catalogue /ref no.	Company and address	Dilution/ conc. used
Primary antibodies			
Phospho-Akt (Thr308) (C31E5E) Rabbit mAb	2965	Cell Signaling Tech., Denver, MI, USA	1:1000

Phospho-Akt (Ser473) (D9E) XP Rabbit mAb	4060	Cell Signaling Tech., Denver, MI, USA	1:2000
Akt (pan) (C67E7) Rabbit mAb	4691	Cell Signaling Tech., Denver, MI, USA	1:1000
Phospho- MAPK 42/44 Rabbit mAb	9101	Cell Signaling Tech., Denver, MI, USA	1:2000
Secondary antibodies			
Goat anti-rabbit IgG, HRP-linked	7074	Cell Signaling Tech., Denver, MI, USA	1:2000
Chemical inhibitors			
MK2206 (Akt inhibitor)	S1078	Selleckchem, Houston, TX, USA	5 $\mu$ M
Gefitinib (EGFR inhibitor)	4765S	Cell Signaling Tech., Denver, MI, USA	10 $\mu$ M
PD98059 (MAPK inhibitor)	9900L	Cell Signaling Tech., Denver, MI, USA	50 $\mu$ M
TGF $\beta$ RII Kinase Inhibitor VII	616458	Calbiochem, San Diego, CA, USA	5 $\mu$ M
AMD3100 Octahydrochloride hydrate (CXCR4 Inhibitor)	A5602	Cell Signaling Tech., Denver, MI, USA	1 $\mu$ M

## 4.2 Cell culture

The adenoid squamous carcinoma cell line (TYS), derived from a minor salivary gland was a kind gift from Dr. Koji Harada, University of Tokushima, Japan. Normal oral mucosal fibroblasts (MM1) and oral cancer-associated fibroblasts (COM D24) were isolated in-house from explant cultures of biopsies from the Oral Surgery Clinic, Ninewells Hospital, Dundee. Cells were cultured and maintained in minimum essential medium (MEM) supplemented with 10% (v/v) Foetal Calf Serum and 200mM glutamine and incubated at 37°C in a humidified incubator with 5% CO<sub>2</sub>. Prior to growth of MM1 and COM D24 cell lines, culture dishes were coated with collagen 1 (# C-3867, Sigma, St. Louis, MO, USA).

## 4.3 Conditioned medium preparation

MM1 and COM D24 cells were cultured in 90mm dishes until 70-80% confluent. The cells were then washed three times with 4ml of PBS, followed by a final wash with 4ml of serum free-MEM medium (SF-MEM). The cells were then maintained in 5ml of SF-MEM and incubated for two days. The medium was then collected, centrifuged for 5 minutes at 900 RPM, filtered using a 0.2  $\mu$ m filter and stored at -20°C.

#### 4.4 Cell Scatter Assay

The cell-scatter assay was used to investigate the scattering (motility) of TYS cells out from compact colonies, in response to MM1 and COM D24 conditioned medium. 40% COM D24-CM and 25% MM1-CM were found to be the best dilutions, after optimisation. TYS cells were plated in 60mm dishes at a density of  $1 \times 10^4$  cells/ml. Once the cells had formed small colonies (10-15 cells/colony) after 24 or 48 hours, conditioned medium with or without the inhibitors was added and the colonies observed for 48 hours. Assays were repeated at least three times.

#### 4.5 Cell Lysis, SDS-PAGE and Western blot

Scattered TYS cells were washed twice with ice-cold PBS, then ice-cold RIPA buffer containing both phosphatase inhibitors (#88666, Thermo Scientific, MA, USA) and protease inhibitors (# 04906837001, Roche, Bavaria, Germany) was added. Cells were incubated on ice for 10 minutes and the cell lysates were then collected.

For SDS-PAGE and Western blot, lysates were mixed with an equal volume of Laemmli loading buffer (Bio-Rad, Hercules, CA, USA) containing 5% (v/v) 2-Mercaptoethanol and then heated at 95°C for 5 minutes, followed by centrifugation. 20  $\mu$ l of each sample was loaded into 10-well 10% SDS PAGE BioRad pre-cast gels. After SDS-PAGE, protein bands were transferred from the gel onto PVDF membrane, blocked with blocking buffer (1% w/v dried milk in 1x Tris buffered solution with Tween-20), and then incubated with primary antibody overnight at room temperature, followed by incubation with secondary antibody. Finally, blots were developed with BioRad Clarity Western ECL Substrate, chemiluminescence was detected using a GelDoc system (BioRad). Bands on the blots were then normalised against total protein and quantified using Image lab software (BioRad).

#### 4.6 3D-collagen gel assay

Collagen gels (2 mg/ml) were made by mixing collagen 1 (#C-4243, Sigma, USA) with 10X SF-MEM medium and 7.5% (w/v) sodium bicarbonate and incubated for 1 hr to allow complete polymerisation. Then, TYS cells were plated on the top of the gels at a density of  $2 \times 10^4$  cells/well in a 48-well plate, the plate was then incubated for 4 hours to allow cell attachment. After that, the medium was discarded and conditioned medium with or without the inhibitors was added in the wells and incubated for 48-hours. SF-MEM was added to some wells and regarded as negative control. Five areas were chosen randomly inside the gel in each well and pictures were taken of the migrated cells. The mean number of migrated cells per well was calculated and the results were compared to the negative control. The experiments were carried out three times.



#### 4.7 3D Spheroid invasion assay

A 96-well hanging drop plate (#HDP 1096, Perfecta3D® hanging drop plate, 3D Biomatrix Inc, MD, USA) was used to form spheroids from TYS cells. TYS cells grown in 2D dishes were trypsinised, collected by centrifugation and then re-suspended in 1% (v/v) FCS-MEM. Cells were then plated at a density of  $1 \times 10^6$ /ml in the hanging drop plate (as instructed by the manufacturer's protocol), and the medium was changed every day. After 72 hours, cell aggregation and spheroid formation were observed and confirmed under the microscope.

Then, the spheroids were transferred into the collagen solution prepared as described in 2.6 (before polymerisation) by pipetting the hanging droplets with 50  $\mu$ l of serum-free medium, followed by incubation for 1 hour. Test conditions (conditioned medium  $\pm$  inhibitors) were then added onto the top of the gel and incubated for 6 days. The test conditions were replaced every 2 days by adding 50  $\mu$ l of each condition to the well. SF-MEM medium was also added and regarded as a negative control.

MM1 or COM D24 cells was added to the collagen matrix solution at a concentration of  $2.5 \times 10^5$  cells/ml. Then, the spheroids were transferred into the collagen /fibroblast mix and incubated for 1 hour for polymerisation. Test conditions (SF-MEM medium  $\pm$  inhibitors) were then added onto the top of the gel and incubated for 6 days. The test conditions were replaced every 2 days by adding 50  $\mu$ l of each condition to the well.

Images of the spheroids and invasive cells were taken using an inverted microscope (IX70, Olympus, Japan) and processed by CellSense software (Olympus). The maximum invasive areas were measured using ImageJ software (NIH) and compared to the spheroid area (the values quoted are in pixel measurements).

#### 4.8 Gene silencing using Akt 1 shRNA (human) lentivirus

shRNA lentivirus transfection was carried out according to the manufacturer's instructions (Santa Cruz Biotechnology, TX, USA). All the reagents related to the transfection were purchased from Santa Cruz Biotechnology. TYS cells were plated in a 6-well plate at a concentration  $1 \times 10^5$  per well and incubated overnight. On the next day, a mixture of 10% (v/v) FCS-MEM growth medium with polybrene (#sc-134220) at a final concentration 5  $\mu$ g/ml was prepared. The growth medium on the cells was then replaced with 1ml of polybrene-medium mixture per well. The TYS cells were then transfected by adding the required volume of shRNA Akt (#sc-29195-v) or shRNA control lentiviral particles (#sc-108080) to the medium after determining the Multiplicity of Infection (MOI). On the third day, the polybrene-culture medium mix was replaced with 1 ml of 10% (v/v) FCS-MEM growth medium and incubated overnight. On the fourth day, the cells were split into 60 mm dishes at a 1:3 ratio and incubated for 24 hours. On the fifth day, the stable clones were selected by addition of 300 ng/ml puromycin dihydrochloride (#sc-108071). Fresh puromycin-containing medium was added every 3-4 days until antibiotic resistant colonies were identified. Silencing of Akt expression was confirmed by Western blot assay using both pAkt and Pan Akt antibodies. Transfected cells were denoted as shRNA Akt TYS and used in

scatter and 3D spheroid invasion assay to investigate the effect of the fibroblast conditioned medium and inhibitors on the migration and invasion of Akt silenced cancer cells.

## **5. Conclusion**

The 3D model of the tumour microenvironment developed in this project, could be used as a valuable tool to study the association between TME and metastasis. Data from this project suggests that cancer associated fibroblasts stimulate oral cancer cell migration and invasion in a PI3K/Akt signalling pathway dependent manner. This study also suggests that by combining an EGFR inhibitor and an Akt inhibitor might have potential in clinical practice for the treatment of metastatic HNSCC.

Supplementary materials

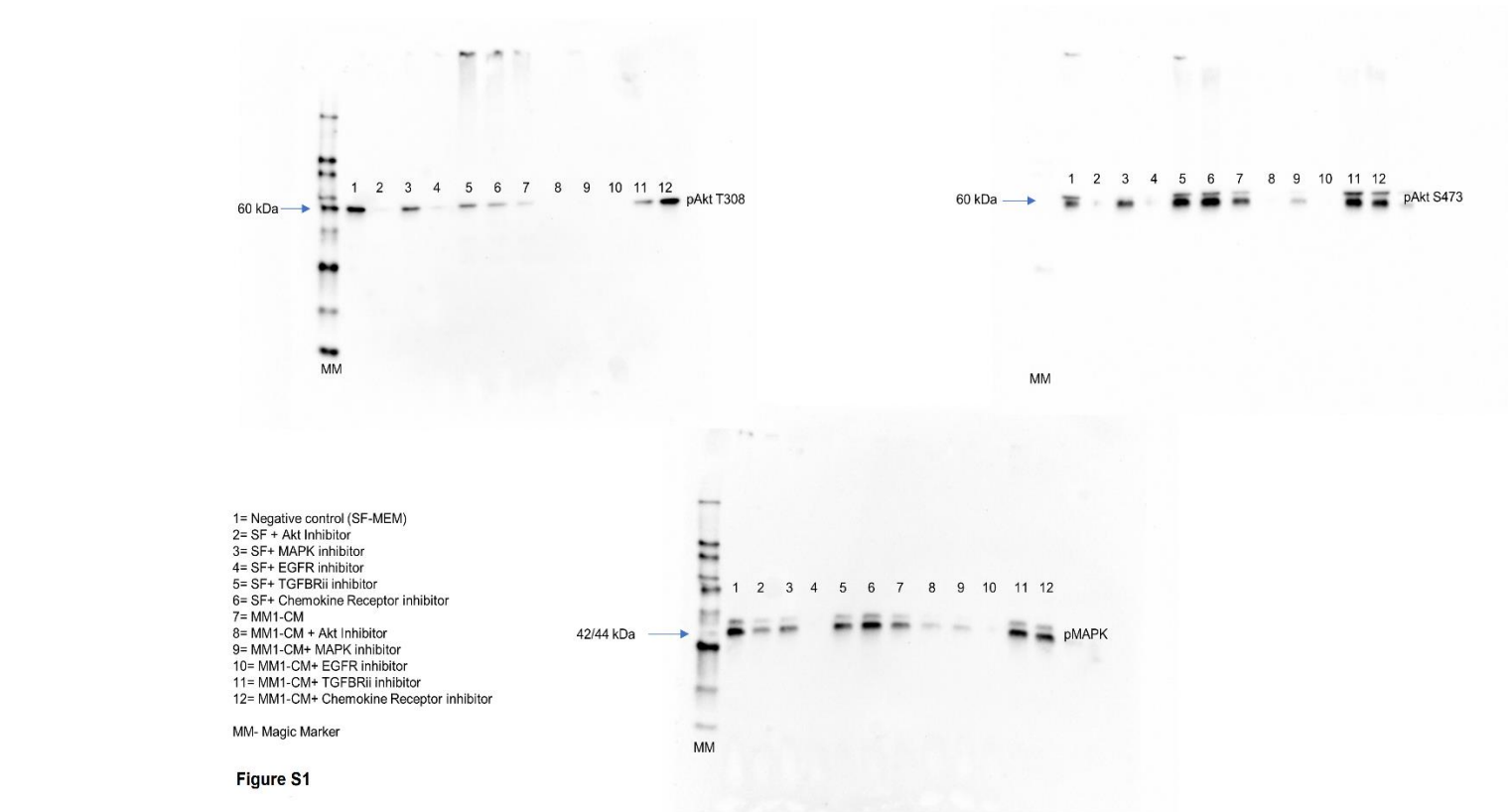


Figure S1: Full blot images of the pAkt T308, pAkt S473 and pMAPK treated with MM1-CM. TYS cells were treated with MM1 conditioned medium with or without the inhibitors for 48 hours. SF-MEM treated cells were regarded as negative control.

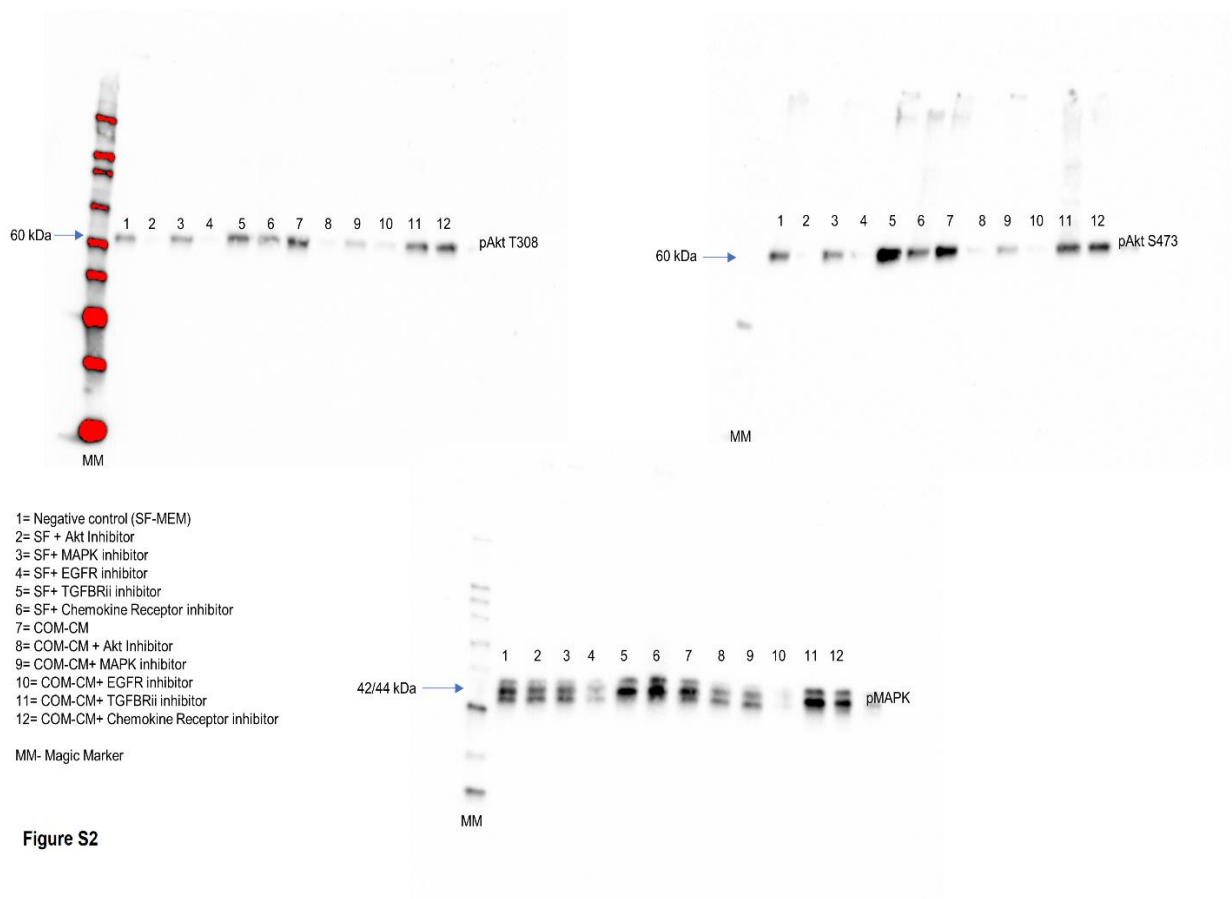
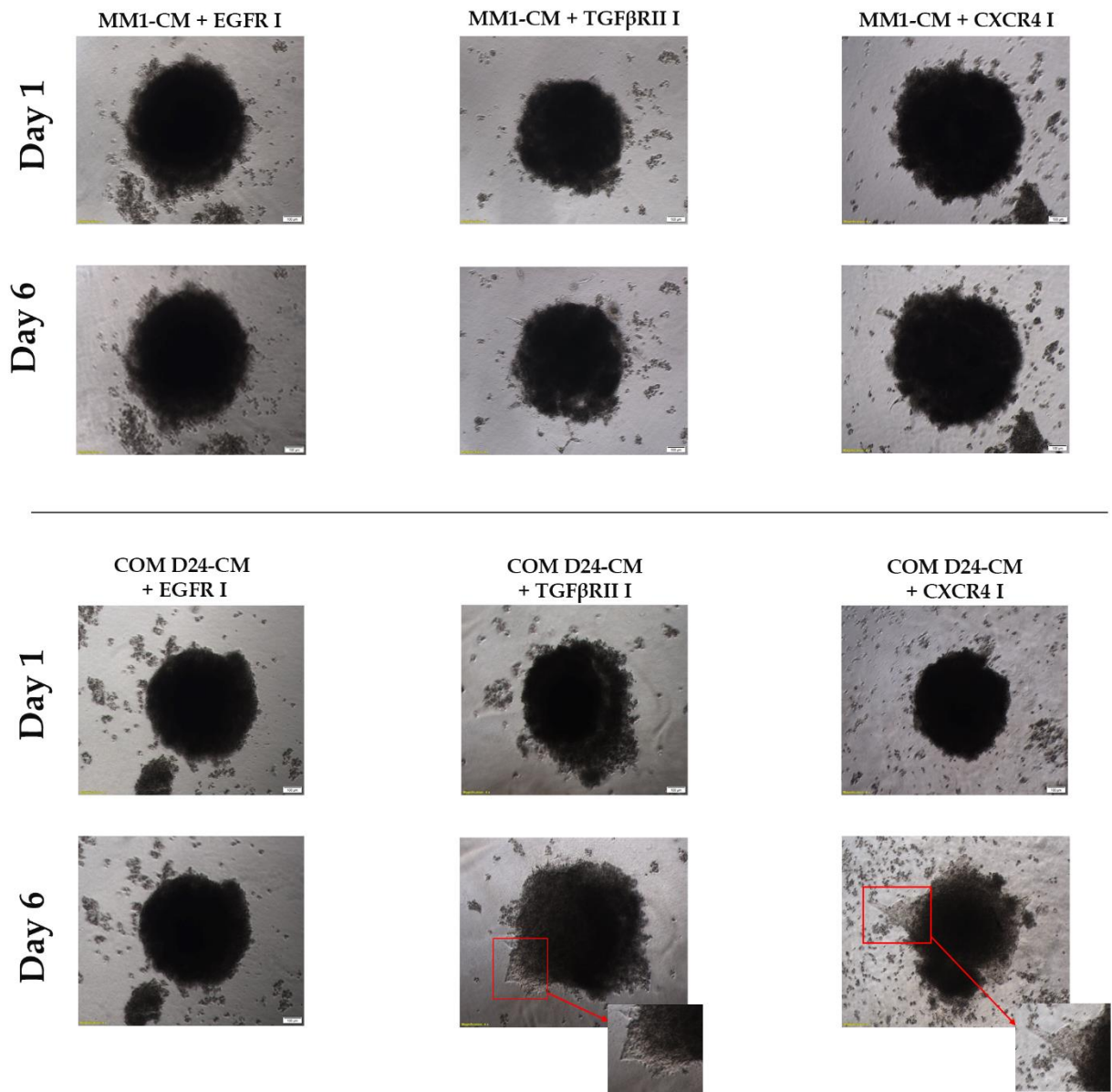
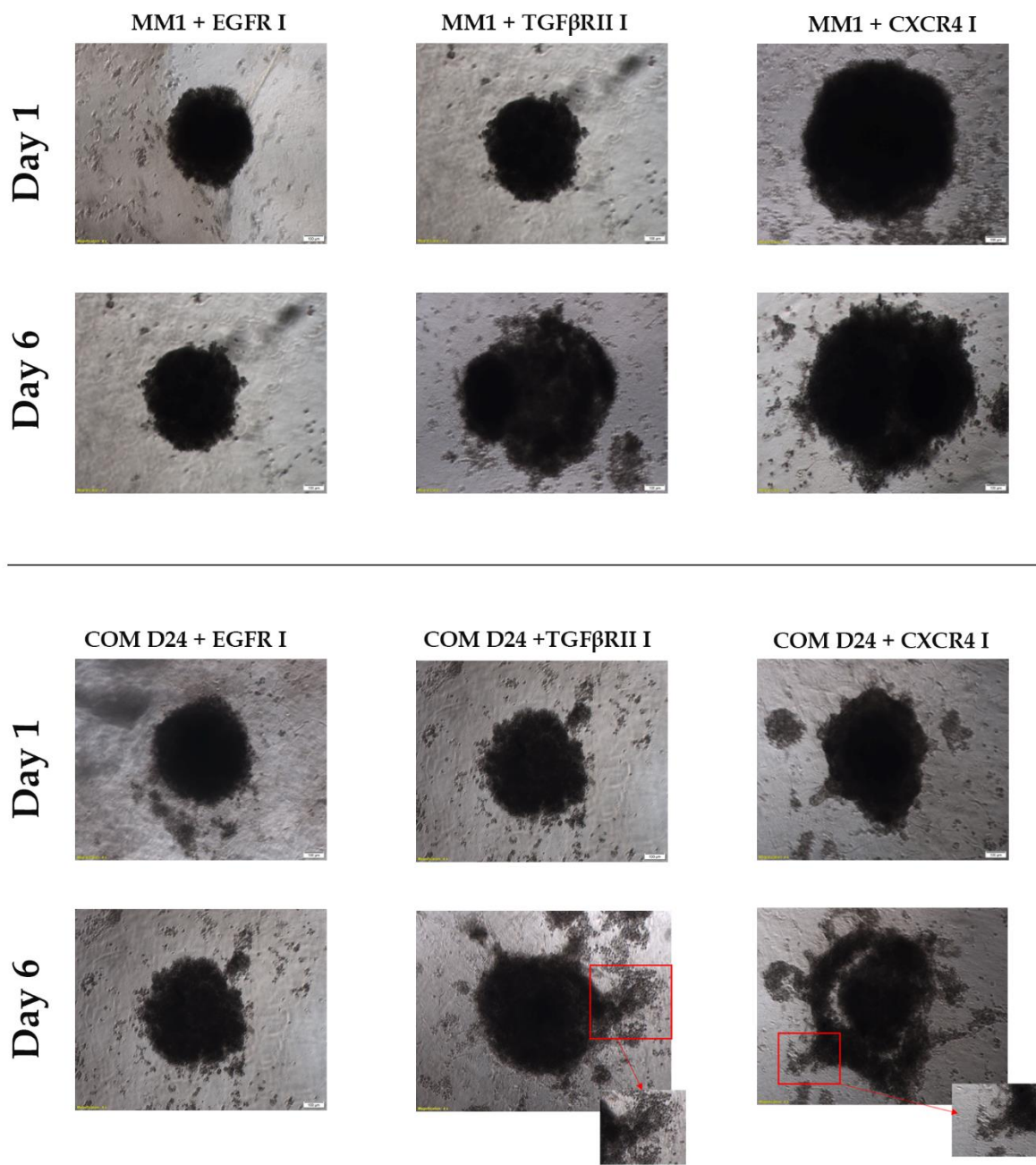


Figure S2: Full blot images of the pAkt T308, pAkt S473 and pMAPK treated with COM-CM. TYS cells were treated with COM conditioned medium with or without the inhibitors for 48 hours. SF-MEM treated cells were regarded as negative control.



**Figure S3**

**Figure S3.** TYS cells invasion from their spheroids in response to fibroblasts CM with the inhibitors. The EGFR inhibitor completely blocked TYS cells invasion into the matrix whereas TGFβR and CXCR4 inhibitors did not block COM-CM induced TYS cells invasion.



**Figure S4**

**Figure 4.** TYS cells invasion from their spheroids in response to fibroblasts with the inhibitors. The EGFR inhibitor completely blocked TYS cells invasion into the matrix whereas TGFβR and CXCR4 inhibitors did not block COM D24-induced TYS cells invasion.



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## Author Contributions

Study concepts and design- MI, IE, SJ; Data acquisition- HA, AG; Quality control of data, data analysis and interpretation- MI, HA AG; Manuscript preparation- HA; Manuscript editing and review- MI, SJ, IE.

## Ethical Approval

This article does not require any human/animal subjects to acquire any ethical approval.

## Conflicting Interests

The authors declare no conflicts of interest.

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