The role of seminal fluid in cervical squamous carcinoma progression: Impact on cell proliferation, EMT, motility and gene expression.

Nonkululeko Mkwanazi

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Declaration

I, Nonkululeko Mkwanazi, hereby declare that the contents of this thesis is my own unaided work, except where acknowledgements indicate otherwise; and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University. I am now presenting this thesis for academic examination towards the Degree of Doctor of Philosophy in Medical Biochemistry.

Sign: Date: 12 December 2022

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Habakkuk 2:3 “For the revelation awaits an appointed time; it speaks of the end and will not prove false. Though it lingers, wait for it; it will certainly come and will not delay” - Thanks be to God.

Abbreviations

ADC	Adenocarcinoma
AP-1	Activator protein-1
ASC	Adenosquamous carcinoma
BM	Basement membrane
BSA	Bovine serum albumin
CAF	Cancer associated fibroblast
CCL2	C-C motif ligand 2
COX	Cyclooxygenase enzyme
CREB	cAMP response element binding protein
CSF-2	Colony-stimulating factor 2
CXC8	Chemokine 8/ Interleukin-8
CXCR4	Chemokine receptor 4
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EC50	Half maximal effect concentration
EDTA	Ethylenediamine tetra acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EMT-TF	EMT inducing transcription factor
ERK	Extracellular signal-regulating kinase
EP	E-prostanoid
F-actin	Filamentous actin
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FMRT	Female reproductive tract
GM-CSF	Granulocyte-macrophage colony stimulating factor
Gro-α	Growth regulated oncogene alpha
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia inducible factor 1 alpha
HPV	Human Papilloma virus
Hr-HPV	High risk HPV
HSIL	High-grade intraepithelial lesions
HSV	*Herpes simplex virus*
INI-43	Inhibitor of nuclear import 43
JNK	cJun N-terminal kinase
LGL	Lethal giant larvae
LMICs	Low-to-middle income countries
LPS	Lipopolysaccharide
LSIL	Low-grade intraepithelial lesions
luc	Luciferase reporter gene
MAPK	Mitogen-activated protein kinase

MDCK	Modin-durby canine kidney
MEK	Mitogen-activated protein kinase
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteases
NES	Nuclear export signal
NFAT	Nuclear factor of activated T-cells
NF-kB	Nuclear factor kappa B
NLS	Nuclear localization signal
NPC	Nuclear pore complex
NSAIDs	Non-steroidal anti-inflammatory drugs
PATJ	PALS1-associated tight junction
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin
PGE2	Prostaglandin E2
PI3K	Phosphoinositide-3 kinase
PKA	cAMP dependent protein kinase
PMA	Phorbol-12-Myristate-13-acetate
PPAR	Peroxisome proliferator-activated receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Quantitative real time PCR
SCC	Squamous cell carcinoma
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SF	Seminal fluid
SSA	Sub-Saharan Africa
STAT	Signaling transducer activator of transcription
STI	Sexually transmitted infection
TBST	Tris buffered saline
TGF-B	Transforming growth factor beta
TME	Tissue microenvironment
TNF-α	Tumour necrosis factor alpha
TZ	Transformation zone
VEGF	Vascular endothelial growth factor

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Abstract

Cervical cancer is the leading cause of cancer related deaths and the second most common cancer amongst South African women. The key cause for cervical cancer development is sexual transmission and persistent infection with high-risk Human Papillomavirus (HPV). However, it takes several years from infection to cervical cancer development, suggesting that other factors contribute to the disease. Exposure of neoplastic epithelial cells to Seminal Fluid (SF) has been shown to promote cell proliferation in culture and growth of explants in mice injected with HeLa cervical adenocarcinoma cells. Since the majority of cervical cancer cases are squamous cell carcinoma, in this study, we examined the effect of SF on cancer cell proliferation, EMT, motility and gene expression using two squamous cell carcinoma cell line model systems, SiHa and Me180. This study shows that SF significantly enhanced cell proliferation in both cell lines. Using confocal microscopy and phalloidin staining, it was further shown that SF caused morphological changes and induced stress fibre formation. SF upregulated the expression of EMT transcription factors Snail, Twist and ZEB1. EMT induction was confirmed by the increase of N-cadherin and a decrease in E-cadherin protein expression. Additionally, results showed that the induction of EMT transcription factors Snail, Twist and ZEB1 by SF occurs via EP4 receptor, ERK1/2 and COX signaling pathways. To investigate the effect of SF on migration and invasion, transwell migration assays were used. SF significantly enhanced directional cell migration and invasion of SiHa and Me180 cells. Cell invasion was associated with an increase in MMP-2 and MMP-9. SF also induced proinflammatory and angiogenic gene expression in cervical squamous carcinoma cells. SF mediated induction of inflammatory and angiogenic genes was shown to be associated with AP-1 and NF-kB transcription factors. A small molecule inhibitor of nuclear import, INI-43 inhibited the nuclear localization and activity of SF activated NF-kB as well as the expression of SF induced

inflammatory and angiogenic genes. Employing ectocervical tissue biopsies, SF caused the upregulation of EMT transcription factors, MMPs, inflammatory and angiogenic genes. Taken together, these results suggest that SF may play a role in promoting EMT and enhances the migratory and invasive potential of cervical squamous cell carcinoma. These findings together implicate SF as a possible factor that may promote cervical cancer progression.

CHAPTER 1 : LITERATURE REVIEW

1.1 Cervical cancer

Cervical cancer is the fourth most common cancer in women world-wide. According to the Globacon estimates, a total of 604 000 new cervical cancer cases and 342 000 deaths were recorded in 2020[1]. The highest regional incidence and mortalities reported are seen in Africa, where rates are estimated to be 7-10 times higher than observed in the western world[2]. Cervical cancer is a preventable disease and curable if detected and treated early[3]. However, cervical cancer is unfortunately a disease that highlights global inequalities. According to the World Health Organization, cervical cancer affects low- and middle- income countries (LMICs) more, which is evident in the incidences of death being three times higher in LMICs as compared to high income countries[4]. Of the new cases recorded, the majority of the cases (85%) and deaths (90%) occurred in LMICs, highlighting cervical cancer as a major public health problem in LMICs[5].

In South Africa, cervical cancer is the second most common cancer amongst women after breast cancer. Cervical cancer also shows the highest age incidence amongst women aged between 15-44 and is the leading cause of death in South Africa[6]. Despite cervical cancer being preventable many women in sub-Saharan Africa present with the disease late making it more challenging to treat[7]. The main aetiology of cervical cancer is persistent infection with High-Risk Human Papilloma virus (hr-HPV)[8]. Other cofactors of cervical cancer include low socioeconomic class, HIV infection, multiple sexual partners, and smoking. These cofactors either influence the risk of HPV infection or the progression to cervical cancer[9].

1.2 The Anatomy of the cervix

The cervix is the lowermost part of the uterus. It consists of two parts namely the ectocervix and the endocervix. The ectocervix, which is the outermost part of the cervix, is lined by multiple layers of squamous epithelial cells which protects the cervix against infection[10] and the endocervix is lined by columnar epithelial cells (Figure 1.1)[5]. The region where the endo- and ectocervix meet is known as the transformation zone (TZ) and is the area where more than 90% of cervical cancers develop[11]. It has been shown that infection with Human Papillomavirus (HPV) in the cervix is greatest at the TZ. Though it is still unclear why the TZ is more prone to malignant transformation, it has been hypothesized that the basal cells at the transformation zone are more susceptible and accessible to HPV infection[12].

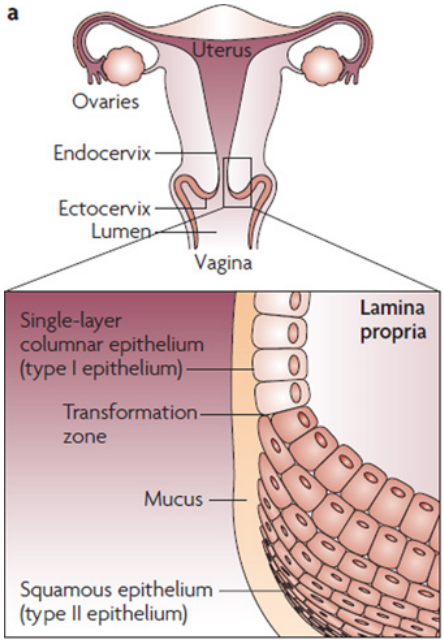


Figure 1.1: Anatomy of the cervix showing the endocervix, ectocervix and transformation zone. The endocervix is lined with columnar epithelium and the ectocervix with squamous epithelium. The transformation zone is the junction where the endo- and ectocervical cells meet[13].

1.3 Human Papilloma Viruses (HPV) and cervical cancer

HPVs are small non-enveloped viruses with a double stranded DNA genome of approximately 8KB[14]. There are multiple HPV types of which some are classified as low-risk and others high-risk. High-risk HPVs (hr-HPV), also known as oncogenic HPVs, have been established as carcinogens in the cervix and are the key aetiological agent of cervical cancer. World-wide, 99% of cervical cancers contain HPV[15]. Most HPV infections can be cleared naturally within 1-2 years however, a subset of women experience persistent infection[16]. Persistent infection with hr-HPV is the main factor driving cervical dysplasia and increases the risk of cervical cancer development[8]. Currently there are more than 200 HPV types that have been identified[17]. The HPV types within the beta-species are usually benign and infect the skin[18]. These infections cause cutaneous warts that usually clear over time. The alpha-species consists of approximately 40 HPV types that are sexually transmitted and develop in the genital mucosa[19]. Of the 40 HPV types, 12 are classified as hr-HPV types which are HPV- 16, 18, 31, 33, 35, 39, 45, 51, 52, 56 and 59[20]. HPV-16 is the most common hr-HPV found in cervical cancers, accounting for 70% of cases while HPV-18 accounts for approximately 20%[19]. HPV infection occurs at a site of epithelial abrasions (micro wounds) that are caused by friction during coitus[21]. The transformation zone is especially more susceptible to transformation by hr-HPV[22]. The virus infects the basal keratinocytes and spreads to other cells through the HPV life cycle[23].

HPV genome integration into the hosts genome has been associated with persistent HPV infection which has been proven to play a role in cervical carcinogenesis[24,25]. Though persistent infection with hr-HPV is necessary in cervical malignancy it is not sufficient for the progression to cancer. Mutations and genomic instabilities caused by chromosomal

rearrangement are important contributors in malignant transformation[26]. There is evidence that viral integration results in genetic alterations that initiate tumorigenesis, proposing genome integration as a driver in cervical carcinogenesis[27]. The consequences of HPV genome integration includes loss of function of tumour suppressor genes, increase in oncogene expression and inter and intra-chromosomal rearrangement[28]. Integration of HPV into tumour suppressor genes has been shown to inactivate host tumour suppressor genes resulting in uncontrolled cell growth[29] and deregulation of key oncogenes[25]. Integration of HPV genome into the host's genome has been accepted as a key event in cervical malignant transformation[25]. In addition, the role of oncogenic HPV proteins E5, E6 and E7 has been identified as crucial in cervical malignant transformation[30].

HPV E6 and E7 are primary oncoproteins that cooperatively mediate progression to malignancy through various mechanisms including interrupting the function of retinoblastoma (Rb) and p53, therefore driving aberrant cell proliferation[26]; inducing immortalization through the activation of telomerase[30] and inducing DNA damage and genomic instability[31]. The role of E7 in malignant transformation has been shown to occur through its ability to inhibit Rb. Inhibition of Rb results in the release of E2F, an important transcription factor that regulates DNA synthesis, Cyclin function and promotes progression into S-phase of the cell cycle. The release of E2F results in the activation of Cyclin A which drives cell turnover. Consequence to the inhibition of pRb by the E7 oncoprotein is an increase in the tumour suppressor p53[32]. The p53 protein is a master tumour suppressor that induces G1-S cell cycle arrest, induces DNA repair and activates apoptosis as mechanisms to inhibit tumour progression. However, in the case of HPV, oncoprotein E6 plays a role in blocking p53 activity by degrading p53. Absence of p53 activity results in unrestricted cell cycle

progression, inhibition of apoptosis and the accumulation of chromosomal mutations due to a lack of DNA repair[33]. In addition, the E6 protein plays a role in cell immortalization and invasive cancer development through its telomerase activity. The E6 oncoprotein activates the transcription of human telomerase reverse transcriptase (TERT), an enzyme that plays an essential role in replicating DNA sequences (telomerase) that are required for immortalization[26]. Although E6 and E7 provide the major transforming activities of hr-HPV viruses, the E5 oncoprotein has been shown to augment their function therefore enhancing their transforming ability[34]. Together, these HPV oncoproteins disrupt signaling pathways in the host cells to maintain continuous proliferation which allows for viral replication but also causes an accumulation in mutations and genetic instability[26].

Currently there are three HPV vaccines available namely; the bivalent vaccine (Cervarix),the quadrivalent HPV vaccine (Gardasil) and its later version, 9-valent vaccine (Gardasil-9) [35,36]. All of these vaccines protect against HPV 16 and 18 which are linked to majority of cervical cancer cases world- wide[19]. These HPV vaccines are readily available and are effective at preventing certain HPV infections however, they may not provide protection against hr-HPV infection in sexually active women already exposed to hr-HPV. Therefore, cervical cancer prevention strategies for sexually active women are dependent on effective screening methods and early detection.

1.4 Cervical Cancer Diagnosis and Histology Subtypes

Cervical cancer is one of the few gynaecological cancers that can be diagnosed through screening methods. Since persistent infection with hr-HPV has been identified as the main risk factor for cervical cancer development , it has been accepted that HPV status is important for cervical cancer screening[37]. The two types of diagnostic tests used for cervical cancer screening are the Papanikolaou test (Pap Smear) and the HPV diagnostic test. The pap smear is a well-established screening test for identification of precancerous lesions[38] and allows for analysis of cellular abnormalities using cytological methods[39].

Cervical cancer presents as three primary histological subtypes; Squamous cell carcinoma (SCC), Adenocarcinoma (ADC) and Adenosquamous carcinoma (ASC)[40]. Differences in biological behaviour, tumour growth, immune escape, sensitivity to chemotherapy and metastasis among SCC, ADC and ASC have been shown[41-42]. Other uncommon types of cervical cancer include small cell, adenoid cystic and lymphoma, to name a few, which also affect the cervix[43]. However, these cervical cancer types are rare. SCC is a cervical carcinoma that develops in the flat cells of the ectocervix and compromises the majority of cervical cancers accounting for more than 70% of cervical malignancies [44]. Adenocarcinoma (ADC), develops in the glandular cells found in the endocervix and is the second most common cervical cancer type, accounting for about 15% of cases. Unlike SCC, ADC is more difficult to diagnose by cervicovaginal cytology because this screening method is highly sensitive and specific for squamous lesions but poor sensitivity has been reported for ADC[45]. Adenosquamous carcinomas (ASCs) are tumours that contain a mixture of squamous and glandular malignant cells[42]. Similar to ADC, increasing incidences of ASC specifically in young women have been observed[46].

Though cervical cancer screening methods have been proven to be beneficial in managing the burden of cervical cancer, LMICs still have the highest burden of cervical cancer due to limited access to public health facilities and poor implementation of screening and treatment methods. Therefore, the majority of women present with the disease in the late stages[4]. As with other carcinomas, cervical cancer presents with all the classic hallmarks of cancer as described by Hanahan and Weinberg (2011)[47].

1.5 The Hallmarks of cancer

In the early 2000s, Hanahan and Weinberg proposed the notion that normal cells evolve progressively into neoplastic cells through the acquisition of key capabilities termed the hallmarks of cancer (Figure 1.2). The Hallmarks of cancer rationalize the need of cancer cells to acquire these capabilities to undergo the complex multistep process of human tumorigenesis. Initially, six hallmarks which include sustained proliferative signaling, evading growth suppressors, enabling replicative immortality, resisting cell death, inducing angiogenesis and activation of invasion and metastasis; were described[48]. In 2011, after a decade of novel research, the hallmarks of cancer were revised where two additional hallmarks were added namely: Deregulating cellular energetics and avoiding immune distraction. In addition, two enabling characteristics were added which include; genetic instability & mutation and tumour promoting inflammation[47].

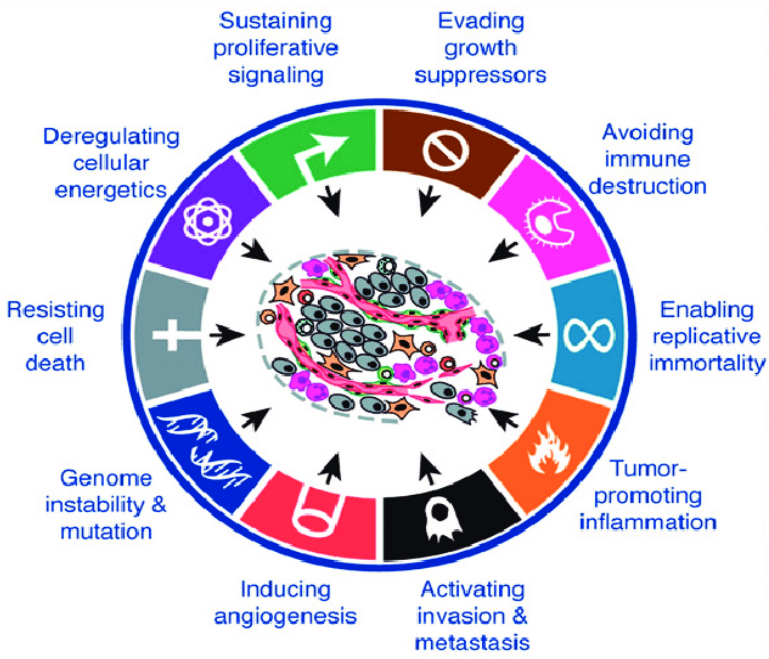


Figure 1.2 The Hallmarks of cancer as described by Hanahan and Weinberg[47].

Three of the hallmarks of cancer; sustained proliferative signaling, activation of invasion & metastasis and tumour promoting inflammation will be discussed further as it relates to the research in this thesis.

i. Sustained proliferative signaling as a requirement for cancer progression

One of the main characteristic of neoplastic cells is their ability to proliferate uncontrollably. In normal cells, cell proliferation is controlled by growth signals that instruct cells to enter and progress through cell growth and cell division[49]. However, in cancer cells this control is deregulated resulting in sustained proliferation. Cancer cells use various mechanisms to sustain their proliferative signaling. One mechanism occurs via the release of their own growth factors to stimulate their own cell growth via autocrine signaling[48,50]. Cancer cells can also release bioactive molecules that instruct surrounding cells to release growth factors that signal proliferation in cancer cells through paracrine signaling[51]. Cancer cells also achieve

sustained cell proliferation by deregulation of the expression of growth receptors. In many cancers, growth factor receptors are often overexpressed leading to the hyperresponsiveness of cancer cells to ambient levels of growth factors[47,52]. As an example, overexpression of the epidermal growth factor receptor (EGFR) has been observed in several carcinomas such as colon, breast, lung, ovarian, cervical and pancreatic carcinomas[53].

Self-sufficient cell proliferation is also driven by oncogenic growth signaling pathways such as the phosphoinositol-3 kinase (PI3K), mitogen-activated protein kinase (MAPK)/ERK and mTOR signaling transduction pathways[54,55]. One of the major genes shown to be mutated in many cancer types is the Ras gene which is upstream of the (MAPK)/ERK signaling pathway. Due to signaling cross talk many signaling pathways in cancer are interconnected[48]. For example, research has shown that Ras interacts directly with the survival promoting PI3 Kinase causing growth factors to not only evoke growth signals but also concurrently evoke survival signals[56]. These mechanisms and mutations highlight the ability of cancer cells to sustain their proliferative trait.

ii. Tumour promoting inflammation

The role of inflammation as cancer promoting has been established[57]. Inflammation was identified as an enabling characteristic because in addition to being a hallmark of cancer, inflammation can contribute to other hallmarks of cancer by supplying factors to the tumour microenvironment[47]. These bioactive molecules include growth factors that sustain proliferation, survival factors that inhibit cell death, proangiogenic factors that induce angiogenesis and extracellular matrix-modelling enzymes that play a role in angiogenesis, invasion and metastasis[58–60].

In the tumour microenvironment, cancer cells release signals that operate to hijack the host immune cells to drive tumour survival. Major inflammatory mechanisms shown to be corrupted by tumour cells include the NF-kB signaling pathway, immune checkpoints and proinflammatory signaling in the form of cytokine and chemokine release[47].

iii. Tumour invasion and metastasis

Metastasis is a biological process which involves the dissociation of tumour cells from the primary tumour and migrating to nearby or distant organs to subsequently colonize the site to form a secondary tumour[61]. Metastasis has been shown to be the primary cause of cancer deaths in more than 90% of carcinomas[62]. The metastatic process is a well-organized process known as the invasion-metastatic cascade. This process requires extensive changes in cell-cell interactions and cell-matrix interactions to allow cancer cells to migrate and invade[47]. The well documented epithelial-mesenchymal transition (EMT) program has been implicated in tumour migration and invasion. EMT has been described as a program whereby epithelial cells can acquire abilities to facilitate their migration and invasion[63]. In addition, tumour cells have the ability to induce the rearrangement and degradation of the extracellular matrix to pave a path for their invasion[48,47].

1.6 The process of Epithelial-mesenchymal transition (EMT)

EMT has increasingly been recognized as an important process during cancer progression and metastasis[64]. EMT is a highly regulated process that occurs during embryogenesis, chronic inflammation and fibrosis[65]. However, when this process becomes deregulated as seen in cancer, EMT can drive tumorigenesis. EMT is a reversible biochemical process which is characterized by the loss of epithelial markers and the subsequent gain of mesenchymal gene expression[65]. This transition leads to a loss of adherent junctions therefore freeing

mesenchymal cells to migrate and invade. EMT has been shown to be induced by numerous factors including integrins, epidermal growth factor (EGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), cytokines, hepatocyte growth factor (HGF) and transforming growth factor (TGF)-β[64]. These factors then activate downstream signaling pathways such as RhoA and Rac1, Ras, phosphoinositol-3 kinase (PI3K), MAPK and Notch all of which are well known EMT inducing pathways[66]. A major characteristic of the EMT process is the loss or downregulation of E-cadherin and subsequent upregulation of N-cadherin[67]. A set of transcription factors including Snail, Twist, Slug and ZEB1/2 have been shown to orchestrate the EMT process by inhibiting E-cadherin[64].

1.6.1 EMT inducing transcription factors

The EMT programme is activated by various EMT inducing transcription factors (EMT-TFs). These pleiotropic transcription factors interact with regulators that control proteins involved in cell-cell contact, cell polarity, cytoskeleton structure and extracellular matrix degradation[68]. The major EMT-TFs that play a role in orchestrating EMT are the Snail, Twist1/2 and ZEB1/2 transcription factors. These transcription factors activate EMT by repressing E-cadherin and activating the transcription of genes associated with the mesenchymal state (e.g., N-cadherin)[69,70]. Snail represses E-cadherin by binding the E-boxes in the E-cadherin gene promoter[71]. Similarly, ZEB1 represses E-cadherin directly and induces gene expression of N-cadherin and vimentin which is another EMT marker[71]. Twist on the other hand, represses E-cadherin transcription indirectly[64]. In addition to controlling genes that activate EMT, EMT-TFs also control genes that are involved in cell polarity. Snail and ZEB1 have been reported to

repress genes such as crumbs, PALS1-associated tight junction protein (PATJ) and lethal giant larvae (LGL) (a cytoskeleton protein) all of which play a role in controlling cell polarity[72].

EMT-TFs have also been implicated in cell invasion. Snail and ZEB2 have been shown to activate the expression of MMPs which facilitate basement membrane degradation[73]. In addition, Twist expression has been associated with increased migration and invasion in hepatocellular carcinoma[74]. EMT-TFs have also been implicated in cancer stemness. Various studies have shown that the induction of EMT also enhances self-renewal and stemness in neoplastic cells[75–77]. As major inducers of EMT, EMT-TFs progressively drive the conversion of epithelial cells to motile mesenchymal cells. The morphological changes that occur during EMT also associate with the rearrangement of the cell cytoskeleton.

1.6.2 Cytoskeleton rearrangement during EMT signaling

A key feature of EMT is the reorganization of the cytoskeleton. The cytoskeleton plays an important role in cell shape, migration and intracellular cargo transport[78]. During EMT induction a dramatic rearrangement of the cytoskeleton occurs where cortical actin[79], found in epithelial cells, is reorganized into actin stress fibers[80] in the mesenchymal state (Figure 1.3). The formation of stress fibers has been shown to play a role in elasticity and migration of mesenchymal cells[81]. Actin cytoskeleton rearrangement is mainly regulated by Rho family GTPases which consist of RhoA, Rac and Cdc42. RhoA GTPase regulates stress fiber formation while Rac and Cdc42 mediate lamellipodia and filipodia formation[66]. All these elements play an essential role in cell motility and invasion that is observed in tumour cells in the mesenchymal state.

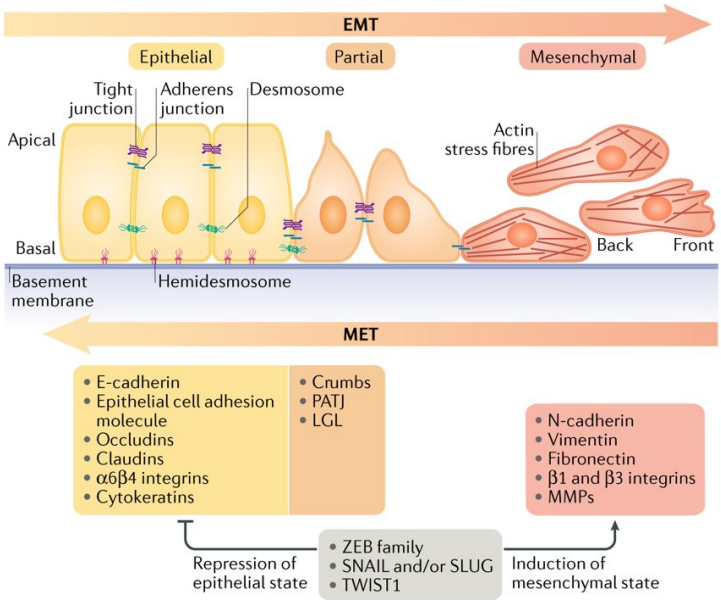


Figure 1.3. EMT program. Epithelial cells are tightly held together by tight junctions and characterized by the expression of epithelial markers. When EMT is activated by EMT-TFs, mesenchymal markers are upregulated and cytoskeleton rearrangement is induced resulting in stress fiber formation[69].

1.7 Migration and invasion in cancer

Metastasis is a biological process which involves the dissociation of tumour cells from the primary tumour and migrating to a nearby or distant organ to subsequently colonize the site to form a secondary tumour[61]. Metastasis has been shown to be the primary cause of cancer deaths in more than 90% of carcinomas[62]. The biggest danger of metastasis is the dissemination of tumour cells from the primary site. The process of dissemination can occur through two processes namely localized invasion of tumour cells into surrounding tissue or metastatic dissemination where tumour cells migrate to a secondary distant site[82]. In order for tumour cells to invade, tumour cells need to undergo various changes and alteration such as i) enabling properties that allow them to detach from the primary site, ii) reorganization of the extracellular matrix (ECM) matrices to allow for invasion and iii) activation of migration for movement through the matrices[83]. During the process of metastasis, tumour cells disseminate

and migrate to a secondary site through a complex multistep process known as the metastatic cascade[84].

Interestingly, both processes of invasion and metastasis utilize two common strategies to achieve cell migration. The first process is the EMT program, where EMT plays an important role in mesenchymal cell migration as it loosens cancer cells from the primary tumour and infers motile capabilities in these newly transformed mesenchymal cells[85]. The second strategy is the activation of cell migration through stimuli. Research has shown that cancer cells can move under two stimuli namely, basal motility from signaling via adhesion receptors and a faster migration known as growth factor receptor- mediated migration[82]. Solid tumours are able to produce autocrine and paracrine factors that activate signaling networks that actuate motility[86]. In growth factor mediated motility, several studies have shown classic growth factors such as EGF and HGF playing a role in inducing cell migration of carcinoma cells[87–90]. Interestingly, a study using breast cancer cells showed that EGF secreted by macrophages acted as a chemoattractant for tumour cells to migrate to the blood vessel[91], highlighting the role of EGF in cell migration. Another class of soluble molecules shown to play a role in driving cell motility in cancer are chemokines. It has been shown that chemokines that signal via the chemokine receptor 4 (CXCR4) can drive metastasis by increasing cell motility via autocrine and paracrine signaling[92–94]. Another receptor, the CXCR3 receptor, known to act as a chemoattractant for immune cells has similarly been shown to enhance motility *in vitro*[95,96] and metastasis *in vivo*[97,98] through chemokine receptor signaling. Together these observations show that factors such as growth factors and chemokines can enhance cell migration in cancer.

Growth factors have also been shown to play a role in the process of invasion. The invasion of tumour cells into the surrounding tissue requires tumour cells to breach the extracellular matrix (ECM) of the surrounding tissue. The basement membrane (BM), is a specialized ECM which organizes the epithelial cells in the surrounding tissue. The BM also contains various growth factors embedded in it[99]. The architecture of the BM acts as an intrinsic barrier for invading tumour cells which needs to be overcome for invasion to continue[61]. In order to overcome the physical barrier of the BM, tumour cells secrete matrix metalloproteases (MMPs). MMPs have been shown to be upregulated in carcinomas[100]. The MMPs degrade the BM and other ECM which opens up the path for invading tumour cells. Degradation of the BM also leads to the liberation of pro-motility signals (growth factors) of which tumour cells use to facilitate their migration[101]. Many pro-motility growth factors are either produced as pro-factors (e.g., EGF) that require proteolytic action for processing or are activated after liberation from the ECM (e.g., HGF). These proteases liberate growth factors to act in paracrine signaling to drive cell migration[82]. Though further research is required to understand the exact mechanisms behind invasion and metastasis, it is evident that tumour cells utilize various mechanisms and signaling pathways to overcome the challenges of invasion and metastasis.

1.8 Signal transduction pathways implicated in tumorigenesis

Cancer is a disease driven by genetic and epigenetic alterations that result in abnormal cell proliferation and hallmarks of cancer[102,103]. These oncogenic mutations also result in the dysregulation of various signal transduction pathways that control cell fate, cell growth and cell motility. Mutations in cancer can activate oncogenes that cause the hyperactivation of signaling pathways or the inactivation of tumour suppressor genes[103]. While signaling pathways in normal cells are carefully regulated, in tumour cells many signaling pathways are dysregulated and therefore drive tumorigenesis. Some of the most common oncogenic signaling pathways shown to be dysregulated in cancer include the EGFR, MAPK/ERK and COX/PG signaling pathways.

1.8.1 EGFR signaling in cancer progression

EGFR is a member of the ErbB family tyrosine kinase receptors that modulate growth factor-induced signaling. The EGFR signaling pathway is often dysregulated in cancer due to EGFR overexpression, mutation or amplification[104]. Overexpression of EGFR has been reported in various cancers and this overexpression has been correlated with poor patient prognosis in patients with head and neck cancer[105], oesophageal, bladder, cervical and ovarian cancer[106].

EGFR signaling has been shown to play a role in cell proliferation, cell growth, migration and cell differentiation[107]. EGFR is activated by growth factor ligand binding which then leads to the activation of intracellular signaling pathways which regulate cell cycle progression, cell proliferation, survival and migration[108–110]. The two main intracellular pathways activated by EGFR are the MAPK/ERK and PI3K/Akt pathways[110]. The MAPK/ERK signaling pathway is a

well-known signaling pathway that controls cell cycle progression and cell proliferation, while the PI3K/Akt pathway plays an important role in cell survival and inducing antiapoptotic signals in cancer[111]. Therefore, EGFR can be seen as a master regulator of a network of signaling pathways that may drive multiple hallmarks of cancer. EGFR signaling has also been shown to play a role in EMT.

It has been shown that EGF stimulation or EGFR overexpression results in the downregulation of E-cadherin[112]. Down regulation of E-cadherin was shown to be initiated by the EMT transcription factor, Snail, following chronic exposure (days) to EGF, implicating EGFR in EMT induction[113]. The EGF/EGFR axis was also shown to induce EMT by activating Zeb and Slug EMT transcription factors, in cholangiocarcinoma cells[114]. In a study using human oral squamous carcinoma cells, EGF was shown to induce EMT and cancer stem cell like properties through EGFR/PI3K/Akt signaling[115]. Since EMT has been shown to enhance cell migration and invasion, the role of EGFR in invasion has also been investigated.

In order for tumour cells to invade the surrounding tissue, the ECM is degraded through a process known as proteolysis[116]. This process is heavily controlled by MMPs. Interestingly, EGFR activation has been shown to upregulate many MMPs including MMP-1, MMP-7, MMP-9, MMP-10, MMP-13 and MMP-14 in various tumours types such as glioma, pancreatic and bladder cancer[117–121]. The inhibition of EGFR and the subsequent inhibition of MMPs has been shown to reduce invasion *in vitro*[117,118]. In addition, inhibition of MMPs has been shown to inhibit EGF-mediated invasion in carcinoma cells[122]. These findings demonstrate that EGFR signalling can drive ECM degradation through MMP upregulation. In addition to driving invasion, EGF has been shown to increase motility of tumour cells. In a breast cancer model,

metastatic cells showed directional migration towards endogenous and exogenous sources of EGF[91,123]. As a master regulator of various intracellular signaling pathways, EGFR activation also promotes cell migration and invasion via downstream activation of PI3K, MAPK/ERK and Rho GTPase [116]. Due to the fact that EGFR is overexpressed in various cancers and that EGFR ligand binding stimulates a network of intracellular signaling pathways , targeting EGFR has been described as an anticancer therapy[124].

1.8.2 The role of MAPK/ERK in cancer progression

The MAPK kinase pathway is one of the most well studied signaling pathways that is associated with tumorigenesis. It is a conserved pathway known to play a role in cell proliferation, survival and differentiation[125]. The MAPK signaling pathway is activated by binding of a ligand/growth factors to membrane receptors which leads to the activation of Ras which activates MAPKs. The MAPK cascade consists of a group of signaling pathways that include the extracellular signal-regulated kinase (ERK)1/2, JNK1/2/3 and p38 MAPK pathwys[126,127]. The ERK, JNK and p38 differ in their structure, stimuli and function. The ERK pathway is activated by growth factors, hormones or proinflammatory stimuli while the JNK1/2/3 and p38 pathways are activated by stress and proinflammatory stimuli[128,129]. The Ras-dependent ERK1/2 pathway is the most well characterized MAPKs pathway. Dysregulation of the ERK1/2 pathway has been shown to drive cell proliferation and tumour cell survival[125]. Accumulating evidence has also implicated the ERK1/2 pathway in cell motility and invasion.

1.8.2.1 Role of ERK1/2 signaling in sustained cell proliferation

The ERK1/2 signaling pathway is a well-known pathway that controls cell proliferation and cell growth. This signaling pathway is carefully regulated in normal cells however, in tumour cells the ERK1/2 pathway is often dysregulated[130]. During resting state, ERK1/2 is associated with the MEK protein kinase and localized in the cytoplasm in its inactive state[131]. Upon activation, ERK1/2 becomes phosphorylated which results in the dissociation of ERK1/2 from MEK. Within 15 minutes of activation, ERK1/2 is rapidly translocated into the nucleus where it activates cell cycle regulatory transcription factors that activate the cell cycle and drives cell proliferation[132]. Research has shown that the nuclear localization of ERK1/2 is mainly required for the induction of cell proliferation, while negative feedback loops can be activated by cytoplasmic ERK1/2[133]. Therefore, studies have targeted ERK1/2 as a strategy to inhibit cancer cell proliferation.

A study by Plotnikov et al. (2015), showed that blocking ERK1/2 nuclear entry significantly inhibited cell proliferation in a range of cancer cell lines[134]. Though ERK1/2 mutations are rare in cancers[135], hyperphosphorylation of ERK1/2 is observed in over 85% of cancers which plays a role in the enhanced uncontrolled proliferation of tumour cells[133]. ERK1/2 hyperphosphorylation is often induced by upstream ontogenetic mutations (e.g., Ras gene mutation)[136]. Therefore, unlike normal cells, tumour cells have the ability to activate and sustain their own uncontrolled cell proliferation by dysregulating the ERK1/2 signaling pathway.

1.8.2.2 The role of ERK1/2 signaling in EMT

ERK1/2 signaling has been reported to induce EMT[137,138]. It has been shown that hyperactivation of ERK1/2 signaling cooperates with TGF-β signaling to induce EMT in carcinoma cells. Further evidence demonstrated that carcinoma cells treated with an ERK1/2 inhibitor (PD98059) blocked EMT induction and reversed EMT[139]. ERK1/2 signaling has also been shown to induce the expression of Snail family transcriptional repressors. In MDCK cells, treatment with PD98059, significantly reduced Snail promoter activity and blocked Snail induced EMT induction in response to TGF-β stimulation[140]. In a colon cancer model, ERK1/2 activation was shown to activate Slug and subsequently repress E-cadherin expression[140]. In a study using lung carcinoma cells, EMT was induced by the ERK-ZEB1 signaling pathway which resulted in enhanced migration and invasion[141]. A study using a 3D melanoma model showed the induction of EMT which was mediated via the ERK-Twist1-MMP1 signaling pathway and was associated with enhanced invasion.[142] Together this evidence highlights the role of ERK1/2 signaling in EMT induction.

1.8.2.3 Role of ERK1/2 signaling in invasion and migration.

Malignant cells are mostly characterized through their ability to invade surrounding tissue which has been shown during the process of metastasis[140]. As previously mentioned, in order for tumour cells to invade they firstly degrade the ECM[99]. Biochemical studies as well as transcriptional profiling has shown that ERK1/2 regulates the expression of various MMPs such as MMP-1,-2,-3,-7,-9 and 14[120,143,144] that play a role in ECM degradation during invasion. In addition ERK1/2 signaling has been implicated in invasion. Sung et al (2017), showed a reduction in the migration and invasion of ovarian cancer cells after treatment with a ERK1/2

inhibitor[145]. The natural product, Sulforaphane, was shown to block invasion via ERK1/2 signaling in glioblastoma cells[146].

For malignant cells to successfully invade they also have to migrate. Studies have implicated ERK1/2 in mesenchymal motility of epithelial cells[147]. The mechanism of ERK1/2 associated enhanced motility occurs via the Rho GTPase signaling which regulates the formation of protrusions (filipodia and lamellipodia) required for cell migration[125]. In colon cancer cells activation of ERK1/2 signaling was shown to induce Rho and Rac expression leading to protrusion formation [148]. Another target of ERK1/2 is the myosin light chain kinase (MLCK). MLCK together with ROCK regulate migration through actin cytoskeleton rearrangement. Research has shown that ERK1/2 governs cell migration through the phosphorylation of MLCK which activates membrane protrusions that drive cell motility[149].

1.8.3 Cyclooxygenase (COX)/PGE signaling and cancer

Cyclooxygenases (COX) are enzymes that convert arachidonic acid to prostaglandins(PG). There are two isoforms of COX enzymes namely COX-1 and COX-2[150]. COX-1, is described as constitutively expressed in most tissues and is known to be responsible for homeostatic PG synthesis[151]. COX-2 is known to be induced by inflammatory stimuli[152]. The role of COX-2 and PGs in cancer related inflammation has been investigated[153–157]. In contrast, COX-1 is not well described as yet, however, both isoforms have been shown to be overexpressed in several cancers[158]. The COX pathway is a well-known inflammatory pathway that has been described in tumorigenesis[159]. One of the prostaglandins produced in the COX pathway is prostaglandin E2 (PGE2). Secreted PGE2 acts in an autocrine or paracrine manner by binding PGE2 receptors

1,2,3 and 4 (EP1-4)[160]. EP receptors then activate a range of downstream signaling pathways that mediate cell proliferation, EMT, stem cell phenotypes and angiogenesis (Figure 1.4)[160].

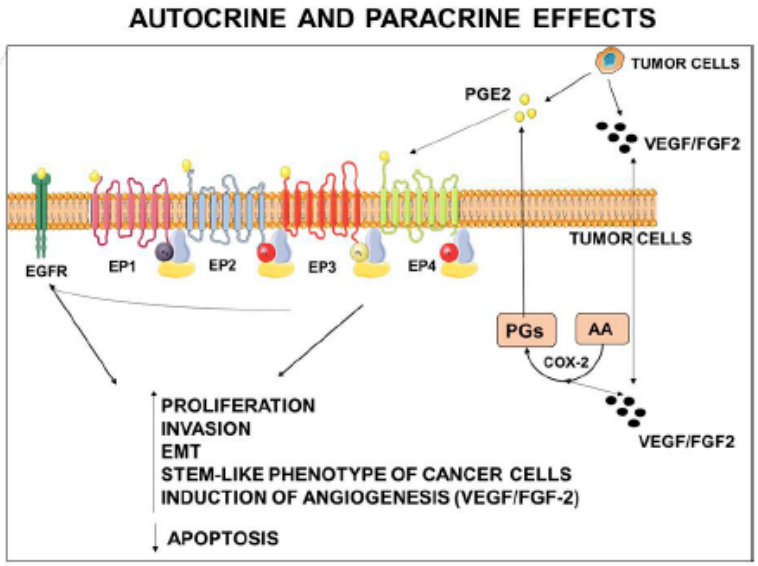


Figure 1.4: Autocrine and paracrine effects of PGE2. Secreted PGE2 from tumours or produced by COX-1/2 binds the various EP receptors to activate signalling pathways that drive proliferation, invasion, EMT, stemness and angiogenesis[160].

The link between COX enzymes and tumorigenesis was discovered in the early 90s. In a study by Thun et al. (1991), evidence from an epidemiological study revealed that regular intake of aspirin reduced the risk of colorectal cancer, suggesting a link between COX signaling and tumorigenesis[161]. Since then, elevated expression of COX enzymes have been reported in various cancers[162,163]. Additionally, COX signaling has been implicated in various hallmarks of cancer.

As previously described, the two major pathways shown to play a role in cell proliferation and survival are the Ras-MAPK-ERK and PI3K/Akt pathways. These pathways are activated by various stimuli. Interestingly, PGE2 has been reported to activate pro-survival and proliferative

pathways such as PI3K/Akt, ERK and EGFR in cancer[164]. It has also been shown that PGE2 promotes survival in murine intestinal adenomas by transactivating the nuclear peroxisome proliferator-activated receptor (PPAR)-delta via the PI3K/Akt signaling pathway[165]. In another study, PGE2 was shown to activate the MAPK-ERK signaling pathway and enhance cell proliferation in non-small cell lung cancer cells[166]. Therefore, highlighting the role of the COX/PGE2 signaling pathway in proliferation and survival. In addition to proliferation and survival, PGE2 has also been implicated in invasion and metastasis.

The first evidence of the involvement of PGE2 in metastasis was in human oesophageal cancer cells where invasive and metastatic potential in nude mice was found to be linked to elevated PGE2 expression[167]. Among the EP receptors, EP2 and EP4 have been shown to play a role in breast cancer metastasis[168,169]. PGE2 signaling via the EP2 receptor was shown to increase tumour proliferation and angiogenesis[170]. Another study showed that silencing of EP2 resulted in reduced cell invasion which was caused by a decrease in MMP-2 and MMP-9 expression where overexpression of EP2 resulted in increased tumour volume and metastasis[171]. Research has further shown that signaling via EP4 results in enhanced cell proliferation, migration and invasion in breast cancer cells[172,173].

The role of COX enzymes in metastasis has been previously reported. Overexpression of COX-2 was shown in colorectal cancer cells and was correlated to metastatic potential via MMP-1 and MMP-2 expression[174]. In non-small lung cancer cell lines COX-2 was shown to increase invasion by upregulating CD44[175]. In a endometrial cancer study showing the invasive properties of cancer cells after oxytocin stimulation, results showed that cell invasion was activated via the PI3K/AKT pathway which in turn activated COX-1 and COX-2 and upregulated

PGE_2 production. Both enzymes were shown to be necessary as COX-1 upregulated MMP-14 expression while COX-2 upregulated MMP-2[176]. What should be noted is signaling in the tumour microenvironment (TME) does not occur in isolation. Many of these oncogenic signaling pathways are pleiotropic, therefore play a role in other hallmarks of cancer that drive tumorigenesis. One hallmark that has been identified as an enabler and shown to influence core hallmarks of cancer is tumour promoting inflammation.

1.9 The role of inflammation in tumorigenesis

In the 19th century Rudolf Virchow discovered the link between inflammation and cancer based on observations of most cancers developing in a site of chronic inflammation and the presence of immune cells in tumour biopsies[177]. It is now accepted that cancer related inflammation is an important characteristic of cancer[178], with tumour promoting inflammation being identified as one of the hall marks of cancer[179]. In fact, it has been shown that chronic inflammation or unresolved inflammation is associated with increased risk of cancer development and progression in most cancer types[177,180,181].

In normal physiology, upon injury or infection, the immune system is activated as a protective response required to eliminate harmful stimuli such as irritants, pathogens and sterile lesions[180]. This immune response is characterized by the activation of innate immunity and the adaptive immunity. This immune response is a fast self-contained response (acute) where immune activation and signaling is terminated after healing[182]. Unlike the acute inflammatory response which stops after healing, the inflammatory response in cancer has been described as persistent and non-resolving[183]. As described by Dvork, inflammation during cancer development can be seen as a "wound that never heals"[184], therefore better known as chronic

or persistent infection. Persistent exposure to carcinogens, viral or bacterial infection have been identified as risk factors of cancer development[185]. Some cancer risk factors include *Helicobacter pylori* infection in gastric cancer, Hepatitis B or C in Hepatocellular carcinoma and HPV in cervical cancer[186–188]. Chronic inflammation and persistent tissue injury lead to transformation in the epithelial architecture and surrounding stroma, which enhance abnormal genetic mutation and epigenetic changes in epithelial cells[182]. Chronic inflammation is also known to drive immunosuppression therefore creating an ideal environment for tumour development[189].

1.9.1 Inflammatory cytokines and chemokines associated with cancer

Cytokines and chemokines are signaling molecules secreted by inflammatory cells and tumour cells. Cytokines play an important role in autocrine and paracrine signaling in the tumour microenvironment (TME) which drives chronic inflammation, promotes transformation of malignant epithelial cells, blocks tumour immune surveillance and drives metastasis[190]. Many different proinflammatory cytokines are associated with cancer. Some examples of important inflammatory cytokines include tumour necrosis factor-alpha (TNF-α), IL-1 and IL-6[57].

TNF-α

One of the main cytokines produced by macrophages is TNF-α. TNF-α is known as the main mediator of cancer related inflammation and a key factor in chronic inflammation[191]. TNF-α has been shown to exert its effects by activating proinflammatory signaling pathways such as NF-kB and c-Jun N-terminal kinase (JNK). Interestingly, TNF-α is described as a double edged sword due to its ability to be both anti- and pro-tumorigenic. As a tumour suppressor TNF-α

can initiate cancer cell death[192]. In contrast, as a tumour promoter TNF-α drives cell proliferation, invasion and metastasis as well as angiogenesis[193].

IL-1

IL-1 is a key inflammatory cytokine that exists as two isoforms namely IL-1α and IL-1β. Both isoforms have been shown to be equally potent in activating the inflammatory process[194]. IL-1α and IL-1β are secreted by macrophages and induce inflammation by activating a cascade of pro-inflammatory cytokines[195]. IL-1 has been shown to be upregulated in several carcinomas including breast, colon, lung, pancreatic and cervical carcinomas. As an inflammatory cytokine IL-1α has been implicated in tumorigenesis. IL-1α has been shown to be a driver of colon cancer which is associated with chronic inflammation. Tumour cells, tumour-infiltrating immune cells and stromal cells have been shown to secrete IL-1α in the tumour microenvironment (TME). IL-1 signaling has been shown to activate COX-2 and IL-6 which in turn drives tumour survival[196]. Furthermore, the IL-1α promotor contains binding sites for AP-1 and NF-kB transcription factors, both of which upregulate the expression of IL-1α during inflammation[196–198]. In addition to cancer related inflammation, IL-1α has been shown to be associated with metastasis and poor survival in patients with gastric and head and neck carcinomas[199,200].

Similarly, the IL-1β cytokine has also been implicated in various tumour progressive cell processes including cell proliferation, differentiation and apoptosis[201]. IL-1β also interacts with cells in the TME to drive metastasis. IL-1β can promote stromal cells in the TME to produce angiogenic factors that drive tumour angiogenesis, endothelial activation and immunosuppression[202]. IL-1β is also an important activator of various proinflammatory signaling pathways e.g. NF-kB[190]. In a study using cervical cancer adenocarcinoma cells, IL-1β

was shown to enhance cell proliferation, migration and invasion through the induction of C-C motif ligand 2 (CCL2) and the activation of NF-kB signaling[203].

IL-6

Similar to other proinflammatory cytokines, IL-6 plays an important role in immune response and inflammation. IL-6 is a multifunctional cytokine that has been shown to be associated with signaling transducer activator of transcription-3 (STAT3) signaling. The IL-6/JAK transduction and STAT3 signaling have been shown to play an important role in various cancers such as lung, breast, colon, ovarian and prostate cancer[204,205]. In a study using patient derived cancer associated fibroblasts (CAFs) from lung cancer, IL-6 secreted by CAFs activated JAK2 and STAT3 thereby driving tumour metastasis[206]. Interestingly, IL-6 has also been implicated in cervical cancer growth. A study by Wei et al. (2003), showed that IL-6 promoted *in vivo* tumour growth of C33A human cervical cancer cells and this was associated with upregulation of VEGF via the STAT3 signaling pathway[207].

1.9.2 The role of chemokines in cancer progression

Chemokines are also key players in cancer related inflammation[208]. Many chemokines have been detected in neoplastic tissues as products of stromal cells and tumour cells. Chemokines are molecules that play a role of regulating directional movement of immune or other cells to the tumour which plays an important role in tumorigenesis[209]. Chemokines are mainly known for leukocyte recruitment in tumours[57].

Chemokines can be divided into two groups CXC or CC chemokines. CXC chemokines are known to activate neutrophils and lymphocytes while CC chemokines act on monocytes, dendritic cells, natural killer cells and eosinophils[210]. In addition to leukocyte recruitment, chemokines have been shown to play a role in neo-angiogenesis, tumour cell proliferation, invasion and metastasis[209]. A common chemokine secreted by human tumours is CXC8 (IL-8). IL-8 and Growth-related oncogene (Gro)-α (CXCL1) chemokines have been shown to drive proliferation, angiogenesis and migration of melanoma cells[57]. Previous research has shown that solid tumours such as prostate, breast and ovarian cancers constitutively express IL-8[211,212]. A study by Jia et al. (2018) showed that IL-8 expression in cervical cancer tissue was significantly higher in comparison to normal cervical tissue[213]. It was further shown that exogenous IL-8 upregulated the expression of IL-8 receptors (IL-8A and IL-8B) and ERKs which lead to enhanced cell proliferation and migration in HeLa cells[213]. This highlights that chemokines are pleiotropic as they play multiple roles in tumorigenesis.

1.9.3 Key orchestrators of cancer related inflammation

Key players of cancer related inflammation also include inflammatory transcription factors. Though there are various transcription factors implicated in cancer related inflammation, in this review we will focus on NF-kB, STAT3 and Activator protein-1 (AP-1). These transcription factors are activated in the TME and are involved in the process of cancer related inflammation[214–216].

1.9.3.1 NF-kB as a mediator of inflammation

NF-kB is an important orchestrator of innate immunity and inflammation[217]. NF-kB has been identified as a molecular bridge between tumour cells, TNF-α and inflammatory cells[218]. NF-kB transcription can be activated by various stimuli, such as cytokines (TNF-α and IL-1β), growth factors (EGF), bacterial and viral products (lipopolysaccharides), reactive oxygen species (ROS) and angiogenic stress factors inside the cell[219]. Activation of the NF-kB signaling pathway results in the expression of key target genes including proinflammatory cytokines, adhesion molecules, enzymes in prostaglandin synthesis (COX-2) and angiogenic factors[220], all of which play a role in driving cancer related inflammation. NF-kB activity promotes cell proliferation, inhibits apoptosis, drives angiogenesis and induces EMT[219].

1.9.3.2 STAT3 signaling in cancer related inflammation

STAT3 is another important mediator of cancer related immunity. Along with NF-kB, STAT3 is a point of convergence for multiple oncogenic signaling pathways[215]. Persistent activation of STAT3 promotes cell proliferation, survival and invasion while blocking anti-tumour immunity in tumour cells[220]. Interestingly, STAT3 plays a dual role in tumour inflammation by either promoting proinflammatory signaling pathways such as NF-kB and IL-6/JAK pathways or by opposing NF-kB mediated T-helper 1 anti-tumour immunity[221]. A cross- talk between STAT3 and NF-kB has been identified in tumours. In tumours, STAT3 directly interacts with NF-kB member RELA, trapping it in the nucleus therefore contributing to constitutive NF-kB activation[222]. Target genes of NF-kB (e.g. IL-6) have also been shown to activate STAT3 transcription[223]. Together these transcription factors have been shown to be crucial for cancer related inflammation and pro-inflammatory cytokine production. It should be noted,

that transcription of NF-kB and STAT3 can only occur within the nucleus where transcription leads to the production of key proinflammatory cytokines and mediators.

1.9.3.3 Activator protein-1 (AP-1) signaling in cancer related inflammation

AP-1 is a heterodimeric transcription factor consisting of subunits from the Jun and Fos subfamilies[224]. AP-1 has been identified as a critical transcription factor involved in various cellular processes such as proliferation, apoptosis, survival and cell migration[225]. In particular, AP-1 has been implicated as a major inflammatory transcription factor in cancer. Evidence supports the role of AP-1 in the initiation and development of inflammatory diseases such as cancer. AP-1 activation is regulated by the MAPK/ERK pathway. Similar to NF-kB, AP-1 transcription can be activated by various stimuli. Upon activation, AP-1 activates the expression of various cytokines such as TNF-α, IL-1, IL-6, IL-2, IFNγ and GM-CSF[225]. In addition, AP-1 also regulates many genes that are regulated by another inflammatory transcription factor known as the nuclear factor of activated T cells (NFAT)[226]. Research has shown that AP-1 activation drives TNF-α mediated triple negative breast cancer progression[216]. In another study, the joint action of AP-1, STAT3 and NF-kB transcription was reported to enhance expression of cytokine and chemokines in breast cancer cells[227]. As a mediator of proinflammatory cytokines and chemokines, the AP-1 transcription factor has been implicated in cancer related inflammation.

The link between cancer and inflammation has been established. The inflammatory response is characterised by the recruitment of inflammatory cells and the secretion of inflammatory cytokines and chemokines that play a role in driving inflammation[228]. Over the years, genetic research has revealed a new understanding of inflammation by identifying the cell's nucleus

as central to the inflammatory response. The transcription of inflammatory genes is dependent on transcription factors, that activate a range of inflammatory genes through regulatory networks[229]. This knowledge lead to the identification of the transcriptional paradigm of inflammation. The transcriptional paradigm of inflammation was first described by Hawiger et al (2019), where they hypothesized that denying transcription factors entry into the nucleus could be an approach to control the inflammatory response[229]. Initial evidence showed that IL-2 expression in T-cells could be controlled by inhibiting three transcription factors' (NFAT, NF-kB and AP-1) nuclear entry through the use of cell permeable peptides that disrupt nuclear import[230]. Another study supported this notion, showing that the inhibition of TNF-α expression in a murine mice model challenged with LPS through peptide-directed inhibition of nuclear import of NF-kB, NFAT and Jun[231]. It is now understood that a host of inflammatory transcription factors (NF-kB, STAT, AP-1, NFAT) assemble in the nucleus in order to activate the expression of inflammation-associated genes. The nuclear import of these transcription factors is thus hypothesized to be critical for their role in cancer associated inflammation and biology[229].

1.9.4 Nuclear import of transcription factors associated with inflammation

The nuclear entry of most transcription factors is controlled by the nuclear import pathway. Specialized transport proteins known as Karyopherins play a role in shuttling transcription factors into the nucleus[232]. Karyopherin Beta 1 (KpnB1) is a transport protein that is part of the Karyopherin B superfamily. The Karyopherin B family consists of various Karyopherin B proteins that play a role in nuclear-cytoplasmic shuttling[233]. KpnB1, also known as Importin B, is a nuclear import protein that imports proteins or transcription factors into the nucleus

via the nuclear pore complex[234]. KpnB1 can import proteins directly (non-classical) or by the assistance of an adaptor protein, known as Karyopherin alpha (Kpnα), in the classical pathway[235,236]. Cargoes are recognized by KpnB1 through signals known as nuclear localization signals (NLS). After recognition, cargoes are bound and transported into the nucleus[237]. Therefore, transcription factors with specific NLSs are dependent on KpnB1 for nuclear import. Interestingly, KpnB1 has been implicated in cancer related inflammation[238].

Research has shown that the inhibition of KpnB1 affects various cellular pathways. This is because a host of proteins or transcriptions factors are dependent on KpnB1 shuttling for correct cellular localization for gene transcription[234]. This is indeed the case for certain proinflammatory transcription factors. KpnB1 has been reported to have a role in the nuclear import of NF-kB[239], STAT3[240] and AP-1[241]. A study done in prostate cancer cells showed that the inhibition of KpnB1 by siRNA or using an inhibitor of KpnB1 (Importazole) blocked nuclear entry of p50 NF-kB[242]. Another study demonstrated that the inhibition of KpnB1 using siRNA or a small molecular inhibitor of KpnB1 (INI-43) blocked the nuclear entry of NF-kB and AP-1 which in turn inhibited the expression of their target genes in cervical cancer cell lines[238]. This evidence demonstrates the role of nuclear import in regulating inflammation in cancer.

1.10 Cervical cancer and inflammation

For many years the relationship between chronic inflammation, High-grade intraepithelial lesions (HSIL) and invasive cervical cancer has been described[243,244]. Persistent infection with hr-HPV has been identified as the main causative factor for cervical cancer[8]. Through assessment of cervical tumour tissue compared to healthy tissue, it was shown that increased

macrophage count in cervical epithelium may aggravate HPV induced wounds and infection[245]. Therefore, highlighting the role of inflammation in HPV- associated cancers.

Proinflammatory signaling pathways are responsible for the induction of inflammation[246]. These signaling pathways could also be drivers of HPV-induced chronic inflammation and cytokine production in cervical cancer. A major pathway that has been implicated in cervical cancer is the COX-PG inflammation pathway[247]. It has been shown that HPV oncoproteins (E5, E6 and E7) activate the COX-PG pathway by driving COX-2 and PGE upregulation[248,249]. HPV E6 and E7 have also been shown to activate the EGFR-Ras-MAPK pathway to drive COX-2 expression therefore inducing inflammation[250]. HPV E5 has also been shown to upregulate COX-2 expression where it acts via NF-kB and AP-1 signaling[251]. In addition to COX-2 overexpression, PGE2 has also been shown to be elevated in cervical carcinoma. PGE2 and its receptors have been implicated in cervical cancer[252]. Previous research has shown the upregulation of COX-1 in cervical adenocarcinoma cell lines [253]. A study by Sales et al. (2001) showed the upregulation of COX-2 and increased PGE2 production occurring via the EP2/EP4 receptors in cervical adenocarcinoma cells[248]. Together, this evidence shows the crucial role played by the COX-PG inflammatory pathway in driving inflammation in cervical cancer.

Signaling pathways implicated in chronic inflammation also produce inflammatory cytokines. Following HPV integration in the host genome, it has been shown that NF-kB is activated. Activation of NF-kB by E6 and E7 has been reported to play a role in cellular transformation in cervical cancer[254]. As a potent producer of proinflammatory cytokines, it is no surprise that NF-kB activation and elevated inflammatory cytokines are observed in cervical cancer. A study showed the upregulation of proinflammatory cytokines TGF-β, IL-10, IL-6, IL-23 and IL-17 with a downregulation IFN-γ in patients with invasive cervical cancer[247]. Proinflammatory cytokines

and chemokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-8, IL-6, IL-α and IL-1β have been shown to be significantly elevated in the plasma of patients with invasive cervical cancer[255]. Though persistent HPV infection and chronic inflammation have been identified as major mediators of cervical cancer, other factors may play a role in the transformation from HPV infection to invasive cervical cancer. Interestingly, one such factor, that has been implicated in cervical cancer progression is seminal fluid.

1.11 Seminal fluid and the female reproduction

Seminal fluid (SF) is a complex biological fluid which has, for many years, been assumed to have a single function of sperm delivery[256]. However, it has now been shown that SF plays a crucial role in transmitting signaling agents that interact with the female reproductive tract to facilitate conception and embryo development[257]. SF contains a range of bioactive signaling molecules including cytokines, growth factors, prostaglandins, glycans, nucleic acids and other small molecules[258,259]. Research has shown that when SF enters the female reproductive tract (FMRT) an active female response is elicited. SF agents interact directly with epithelial and immune cells in the mucous lining of the reproductive tissue. The binding of bioactive molecules present in SF to target receptors on target cells, activates signal transduction pathways that exert direct effects on cell function or induce gene expression programs[256]. SF interaction with the epithelial lining of the female reproductive tract stimulates the synthesis of proinflammatory cytokines such as IL-1, IL-8, IL-6, Colony-stimulating factor 2 (CSF-2), GM-CSF and other cytokines, leading to recruitment of dendritic cells, monocytes and lymphocytes to the cervix[260,261]. During this phase, T-cell generation also occurs and together this response allows maternal tolerance for embryo implantation[262]. This inflammatory-like response drives

various reproductive processes which together ensure successful conception and immune modulation in the female reproductive tract.

In humans, the inflammatory-like response characterized by the upregulation of cytokines and leukocyte recruitment is not just seen in the lumen but extends through the epithelium and into the stromal layer of the FMRT[261]. Studies using donor semen showed that immediately after administration of seminal fluid, an influx of neutrophils into the cervix was observed[263]. In a study using human cervical biopsies before and 12hrs after unprotected vaginal sex, induction of various cytokines including CSF2, IL-6, IL-8 and IL-1α was observed[261]. It should be noted that direct contact of SF with the cervix is required to elicit this inflammatory response as this effect was not seen in biopsies of women who had coitus using a condom[261]. In addition to epithelial cells, bioactive molecules present in SF can interact directly with immune cells in the reproductive tract to promote immune regulation and tolerance[256].

Various agents in SF are responsible for the active response seen in the female reproductive tract. SF contains a wide range of cytokines including CSF1, IL-6, IL-8, IL-1 and TNF-α[258,264,265]. Other potent mediators found in abundance in SF include TGF-β and PGE_2. There are three TGF-β isoforms; TGF-β1, TGF-β2 and TGF-β3 that exist at varying concentrations in SF[266]. TGF-β present in SF is synthesized in the male seminal vesicle and prostate in its latent form. After coitus, activation of TGF-β is facilitated by the acidic pH of the vaginal environment, proteases (Plasmin) and MMP produced in the female reproductive tract[267]. Once activated TGF-β interacts with cervical epithelial cells via its receptor to elicit the cervical immune response. Evidence of TGF-β activity has been shown in an *in vivo* mouse model[268]. Research showed that administration of TGF-β elicited the expression of cytokines such as IL-1, CSF2 and TNF-

α in the murine uterus. Following administration, leukocyte influx was observed similar to the inflammatory response caused by SF[269]. In SF, TGF-β has been identified as a key mediator that plays a role in establishing a favourable immune environment to support implantation and foetal development[256].

Compared to other body fluids, human SF contains high concentrations of prostaglandins, especially PGE. The highest reported prostaglandins in SF include PGE_2, 19-hydroxyprostaglandin E1 and 19-hydrocyprostaglandin E2 (19-OH-PGE)[270]. PGE_2 is an important mediator of immune function. Addition of 19-OH-PGE to human cervical explants was shown to induce IL-8 and COX-2 expression[271,272]. Consistent with these observations, Sharkey et al showed that SF also induced the expression of COX-2 in human vaginal and cervical epithelial cells[260]. SF incubated with human cervical adenocarcinoma cells was shown to upregulate fibroblast growth factor 2, COX-2, VEGF, EP2 and EP4 as well as induce EGFR and ERK1/2 signaling[273,274]. In normal conditions the inflammatory response elicited by SF in the female reproductive tract is crucial for successful reproduction however, in pathological conditions, such as cancer, this inflammatory response may be exaggerated or dysregulated leading to exacerbated effects.

1.12 The role of SF in cervical cancer

The link between inflammation and cervical tumorigenesis has been accepted and shown to promote the process of carcinogenesis[178]. In HPV-associated malignancies such as cervical cancer, the COX-PGE2-PTGERs inflammatory signaling pathway together with the cAMP/CREB cascade play a role in the carcinogenic nature of HPV. This highlights inflammation as a co-factor for HPV-dependent carcinogenesis. Therefore, chronic inflammation initiated by HPV oncoproteins E5, E6 and E7 together with additional inflammatory signaling characterized by

an upregulation of PGE2 and Prostagladin receptors can drive cervical tumorigenesis[275]. Interestingly, emerging research has implicated SF in cervical cancer progression. With SF containing a significant amount of bioactive molecules that have been shown to interact directly with the epithelial cells in the cervix[256], it was hypothesized that SF may have an effect on cervical cancer cells. Since cervical cancer is exacerbated by chronic inflammation, factors that activate inflammation may worsen the disease. In the context of women with neoplastic lesions, repeated exposure of cervical epithelial cells to pro-inflammatory mediators present in SF could initiate an exacerbated inflammatory response and drive pathways which regulate tumorigenesis.

Indeed, it has been shown that SF has multiple effects on cervical cancer cells. SF has been shown to upregulate proinflammatory enzymes COX-1 and COX-2 with a subsequent upregulation of PGE2 in cervical adenocarcinoma cells[276]. Research by Muller (2006) and Sutherland (2012) confirmed that SF upregulates COX-1 and COX-2 as well as VEGF, IL-6 and IL-11 in cervical adenocarcinoma cell lines[273,277]. Studies by Sharkey et al.(2012) showed that SF upregulates CSF-2, IL-1α, IL-6 and IL-8 in cervical explants exposed to SF[261]. Research has further shown that SF drives tumour growth and blood vessel size in xenograft mouse models[277]. In addition, SF has been shown to upregulate angiogenic chemokines and regulate vasculature through the activation of COX and NF-kB signaling pathways in HeLa cells[278]. A study by Adefuye et al (2016) showed that SF upregulated IL-1α in cervical cancer cells and tissue[279]. Together this evidence highlights that bioactive molecules present in SF elicit a proinflammatory response in cervical adenocarcinoma cells. It is therefore likely that SF can regulate other tumorigenic pathways in the cervix to further drive hallmarks of cancer. The majority of the research to date has primarily focused on SF mediated inflammation and

cervical cancer. Since many tumorigenic pathways are pleiotropic and overlap in cervical cancer progression we hypothesize that in sexually active women with neoplastic lesions, repeated exposure of cervical epithelial cells to factors in SF may enhance inflammation and other hallmarks such as EMT, migration and invasion of which to our knowledge have not been investigated as yet. Since some oncogenic pathways that have been implicated in cancer related inflammation have also been shown to play a role in migration and metastasis, it is likely that SF mediated inflammation may enhance other cervical cancer phenotypes. Therefore, understanding the mechanism of SF mediated signaling in cervical cancer progression is important for identifying therapeutic targets and providing information for sexually active women at risk of cervical cancer.

1.13 Significance

In South Africa, cervical cancer is the second most common cancer amongst women after breast cancer[6]. Despite cervical cancer being a preventable disease, this disease affects LMICs more than HICs with many women presenting with the disease at the late stages[7]. Persistent infection with hr-HPV has been established as the main aetiology of cervical cancer[9]. As previously mentioned, HPV infection and chronic inflammation have been shown to drive cervical cancer progression[243]. With SF containing a range of bioactive molecules that have been shown to activate tumorigenic pathways in cervical cancer, it is likely that SF may drive other tumorigenic hallmarks. Previous evidence has shown that SF activates oncogenic pathways such as PI3K/Akt[279], EGFR/MAPK/ERK[278,280] and COX-PG[276] in cervical adenocarcinoma cells, all of which have been implicated in various hallmarks of cancer. In addition, SF has been shown to enhance cell proliferation in cervical adenocarcinoma cells.

Therefore, this study aims to further investigate the effect of SF on cervical cancer phenotypes such as EMT, migration and invasion using cervical squamous carcinoma cells because squamous carcinomas make up majority of cervical cancer cases. This will provide a better understanding of the role and mechanism of SF in cervical squamous carcinoma progression.

SF has been shown to upregulate proinflammatory cytokines and activate proinflammatory signalling in cervical epithelial cells[249]. Previous work in our laboratory has shown that SF exacerbates cervical cancer by driving inflammation. Hence, elucidating mechanisms for inhibiting SF mediated inflammation in cervical cancer may be a favourable approach to prevent cervical cancer related inflammation. A target that has been shown to have anti-inflammatory effects in cervical cancer is the nuclear import pathway via KpnB1[238,281]. Here, we investigate whether inhibition of nuclear import via KpnB1 can abrogate SF mediated proinflammatory and angiogenic gene expression. This study will provide deeper understanding of the role and mechanism by which SF regulates proliferation, EMT, migration and the expression of inflammatory and angiogenic genes to augment cervical cancer progression in sexually active women and will serve as a lead in developing novel treatments for cervical cancer.

1.14 Project Aim

The aim of this study is to investigate the effects of SF on cervical cancer proliferation, EMT, migration and invasion as well as elucidate the signaling pathways involved. The study will also investigate whether inhibition of nuclear import pathways can abrogate SF induced inflammation and angiogenesis in cervical squamous carcinoma cells. As a proof of concept approach the effects of SF on EMT, MMPs and inflammation was also investigated using cervical tissue biopsies.

Objectives:

i) To investigating the effects of seminal fluid on proliferation, EMT, migration & invasion in squamous carcinoma cell lines

ii) To investigate the signaling pathways required in SF mediated proliferation, EMT, migration and invasion in cervical squamous carcinoma cells.

iii) To investigate the effect of nuclear import inhibition via Karyopherin Beta 1 on SF regulated proinflammatory and angiogenic genes in cervical squamous carcinoma cell lines.

iv) To investigate the effect of SF on EMT and inflammation in cervical tissue explants.

Chapter 2:

Materials and Methods

2.1 Human Ethics statement

Ethics approval for the collection of seminal fluid and cervical tissue biopsies was obtained from the University of Cape Town Ethics Committee (HREC/REF: 567/2018). The study was explained to all participants and written consent was obtained from all subjects before sample collection.

2.2 Inhibitors and ligands

The chemical inhibitors: SC560, Cyclooxygenase-1 (COX-1) inhibitor; NS398, Cyclooxygenase-2(COX-2) inhibitor; AG1478, epidermal growth factor receptor (EGFR) kinase inhibitor and PD98059 extracellular signal-regulated kinases ½ kinase (ERK1/2) inhibitor were purchased from Calbiochem (Merk, Darmstadt, Germany). Inhibitor of nuclear import 43 (INI-43) chemically known as *3-(1H-benzimidazol-2-yl)-1-(3-dimethylaminopropyl) pyrrolo[5,4-b]quinoxaline-2-amine))* was purchased from Molport Chemicals (MolPort-000-492-602, Riga, Latvia). Prostaglandin E2, EP2 receptor antagonist (AH9809), and EP4 receptor antagonist (L-161,982) were purchased from Sigma Chemical Company (Cape Town, South Africa). Inhibitors and ligands were prepared and stored according to the manufacturer's instructions.

2.3 Cell lines

Human squamous cervical carcinoma cell lines, SiHa and Me180 were obtained from the American Type Culture Collection (Rockville, MD, USA).

2.4 Plasmids

The NF-kB/p65 reporter construct in a pGL4 vector which contains five p65 binding sites that drives transcription of luciferase reported gene (luc2P) was purchased from Promega (Madison, WI, USA). The 4X-AP-1 promoter-reporter construct, which contains four AP-1 Luc binding sites upstream of a minimal promoter sequence from the albumin gene and firefly luciferase[282] was used for the Dual-Luciferase assay, was gifted by Professor M. Birrer (Harvard Medical School, MA, USA).

2.5 Antibodies

The following antibodies were used for immunofluorescence or western blotting: anti-NFkB/ p65 (ser536), sc-101752; anti-Ki67 (M-19), sc7846; E-cadherin (H-108), sc-7870 and anti-c-Jun(H79), sc1694 were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). The Phospho-c-Jun (Ser63) #9261 antibody was purchased from Cell Signaling Technology. N-cadherin (CH-19) antibody was purchased from Invitrogen and anti-KpnB1 (ab45938) antibody was purchased from Abcam. Anti-GAPDH (G9545) rabbit antibody was purchased from Sigma-Aldrich.

2.6 METHODS

2.6.1 Cell culture

SiHa and Me180 cells were grown and maintained in Dulbecco's Modified Eagles Medium (DMEM, Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% Fetal Calf Serum (FCS, Gibco, Life Technologies, CA, USA), 100U/mL penicillin and 100μg/mL streptomycin. For serum-free medium, Dulbecco's Modified Eagles Medium supplemented with 100U/mL

penicillin and 100μg/mL streptomycin was used. Cells were cultured at 37 °C in a humidified incubator with 5% CO_2.

2.6.1.1 Cell passaging

When cells reached 80% confluency they were passaged. To passage, cells were removed from the incubator and media was aspirated. Cells were washed with 1XPBS and dissociated from the plate by trypsinisation using 4mL trypsin-EDTA at 37°C. Cell culture plates were incubated at 37°C for 5 minutes to aid cell detachment. Once cells were detached, the trypsin -EDTA cell suspension was neutralized using 4mL fresh culture media. The cell suspension was centrifuged at 500x g for 3 minutes to pellet cells. The supernatant was aspirated off and cell pellet was resuspended in fresh culture media before seeding cells in a new 100mm cell culture plate at a ratio of 1 in 10.

2.6.1.2 Cryopreservation

For long time storage of cells, cells were grown to 80-90% confluency before trypsinisation and centrifugation as described in section 2.6.1.1. After centrifuge, the supernatant was aspirated off and the cell pellet was resuspended in cold freezing media at a concentration of 10 x 10^5 cells/mL. The cell suspension was then transferred to cryovials and stored at -80°C for 2 days before transferring cells to liquid nitrogen. To reconstitute cells from liquid nitrogen, a single cryovial containing cell suspension was rapidly thawed in the water bath (37°C) before being added to 9mL fresh culture media in a 100mm culture dish[i]

[i] Solutions and Buffers found in Appendix I

2.6.1.2 Mycoplasma Testing

Cells were checked regularly for mycoplasma infection. Cells were cultured in penicillin-streptomycin free medium for 3 days after which they were harvested and plated on coverslips. Cells were then fixed with cold methanol and stained with Hoechst nuclear fluorescent stain. Coverslips were mounted on microscope slides using mowiol. Cells were visualized using a fluorescent microscope (Zeiss Axiovert, Carl Zeiss, Jena, Germany).

2.6.2 Cervical Tissue Explants

Normal cervical tissues were collected from women undergoing vaginal or total abdominal hysterectomies for non-malignant conditions at the Urogynaecology unit at Groote Schuur Hospital, Cape Town, South Africa. Cervical biopsies were immediately transported in 1XPBS on ice to the laboratory where they were cut into pieces and divided into experiment and control before being serum-starved overnight prior to SF stimulation.

2.6.3 Semen collection and preparation

Semen samples were collected from healthy male volunteers attending the Andrology laboratory of the Reproductive Medicine unit at Groote Schuur hospital, Cape Town. Sexual abstinence of at least 72 hours was maintained by all donors prior to ejaculation. Ejaculates were collected in sterile specimen jars following voluntary self-masturbation. Samples collected for this study complied with the 2010 WHO reference parameters for human semen characteristics[283]. Samples with parameters outside of the range were excluded from the study. The individual ejaculates were collected and transported to the laboratory where they were processed. Seminal fluid (SF; sperm-free supernatant of the ejaculate) was separated from the ejaculate by centrifuge at 15 000x g at 4°C for 20 minutes. Seminal fluid from 10

healthy individual volunteers was pooled and aliquoted (500μL) and stored at -80°C. Prior to experimental use, seminal fluid was thawed and diluted in serum-free medium to a final dilution of 1:50. Seminal fluid has been shown to exert no toxic effect on cell viability at this dilution[284,279].

2.6.4 Seminal fluid treatment

SiHa and Me180 cells were seeded in complete DMEM media and allowed to attach overnight. The following day, the complete DMEM was removed, and cells were serum-starved by adding serum-free DMEM media for 24hrs. Cells were then treated with SF diluted in serum free media at a final dilution of 1:50 or serum-free media with Phosphate buffered saline (PBS) (control) for 4, 8, 16 and 24hrs.

2.6.5 Treatment with chemical inhibitors/antagonists

SiHa and Me180 cells were seeded at a density of 4 x 10^5 cells in 60mm tissue culture dishes and allowed to attach overnight in a humidified incubator (37°C and 5% CO_2 (v/v)). The following day, the complete DMEM media was removed, and cells were serum starved as described in section 2.6.4. After 24hrs, serum-free media was removed, and cells were treated with the chemical inhibitors (Table 1) in the absence or presence of SF (1:50 dilution) or vehicle (DMSO) in serum-free media.

Table 1. Chemical inhibitors and concentrations used [278,285–288]

Compound	Target	Final Concentration
SC-560	COX-1	10μM
NS398	COX-2	8-10μM
AG1478	EGFR	100-200nM
PD98059	ERK1/2	50μM
AH6809 (Antagonist)	EP2 receptor	20μM
L-161,982 (Antagonist) [289]	EP4 receptor	20μM

2.6.6 RNA extraction

RNA was extracted from cells using TRIzol (Invitrogen Life Technologies) reagent following the manufacturers protocol. Briefly, 4 x 10^5 cells /60mm plate were seeded and allowed to adhere overnight. Cells were then serum-starved for 24hrs and treated with SF or chemical inhibitors following methods in section 2.6.4 and 2.6.5. After treatment, cells were lysed with 600μL Trizol/ 60mm tissue culture dish and cell lysate collected in Eppendorf test tubes. 120μL Chloroform was added to each sample and shaken vigorously for 10 seconds. Samples were allowed to stand for 5 minutes after which phase separation was achieved by centrifugation of samples at 12,000x g for 15 minutes at 4°C. The aqueous layer of each sample was transferred to a fresh tube and 300 μL Isopropanol was added to each sample. Samples were incubated at room temperature for 5min and centrifuged at 12 000x g for 10 minutes at 4°C to pellet the RNA. After centrifugation, the supernatant was removed and the RNA pellet was washed by adding 600μL 75% Ethanol, vortexing and then centrifuge at 12 000x g for 5 minutes at 4°C. Supernatant was removed, and RNA pellet was air dried for 10 minutes before

resuspending pellet in 30μL nuclease free water. RNA concentration and purity was determined (A260/A280>1.80) using the Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.6.6.1 Cervical tissue RNA extraction

Collected cervical tissue samples were cut into pieces and treated with SF (1:50) in serum-free DMEM for 24hrs followed by RNA extraction the following day. Cervical tissue (50-100mg) was homogenized in 1mL TRIzol using a bead homogenizer for 5 minutes per sample. Samples were allowed to stand on ice for a further 5 minutes to ensure complete dissociation of nucleoprotein complexes. 200μL of chloroform per 1mL of TRIzol was added to each sample, and tubes were inverted 10 times to ensure mixing. Samples were centrifuged at 12 000x g for 15 minutes at 4°C. Aqueous layer was transferred to fresh tubes on ice and 500μL of ice -cold isopropanol per mL TRIzol was added to each sample. Sample were gently inverted and allowed to stand on ice for 5 minutes followed by centrifugated at 12 000x g for 10 minutes at 4°C. RNA pellet was washed with ice cold 75% ethanol and centrifuged at 12 000x g for 5 minutes at 4°C. RNA pellet was air dried, and steps followed as described in section 2.6.6.

2.6.7 Reverse transcription (cDNA synthesis)

Diluted RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), following the manufacturers instruction. A 2x Reverse transcription mix was prepared as seen in Table 2. For each reaction, 1μg RNA was made up to a total volume of 10μL using nuclease free water into individual polymerase chain reaction (PCR) tubes. 10μL of the 2x reverse transcription mix was added to RNA prepared reaction on ice. PCR tubes

were sealed and briefly centrifuged in a desktop centrifuge. Reverse transcription was performed using the Applied Biosystems 2700 Gene Amp PCR system thermal cycler set at 25°C for 10 minutes (annealing), 37°C for 120 minutes (elongation) and 85°C for 5 minutes (Denaturation) for a total volume of 20μL. For large experiments the reverse transcription was scaled up according to manufacturer's instructions. cDNA samples were stored at -20°C until further use.

Table 2. 2x Reverse transcription mix (20μL reaction)

Components	**Volume**
10X RT Buffer	2.0μL
25X dNTP Mix (100mM)	0.8μL
10X Random Primers	2.0μL
MultiScribe™ Reverse Transcriptase	1.0μL
RNase Inhibitor	1.0μL
Nuclease-free water	3.2μL

2.6.8 Quantitative real-time PCR (RT-qPCR)

Quantitative real-time PCR was performed using the StepOne Applied Biosystems Real-time PCR system (Applied Biosystems, USA). Gene expression was determined using 2uL cDNA, primers (Table 3) and the Luna® Universal qPCR Master Mix (NEB) according to the manufacturers protocol. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. All primers were synthesized by Inqaba Biotechnical Industries (Pretoria, South Africa). A melt curve was performed for each RT-qPCR reaction, and purity of product was determined by a single peak. Analysis and fold change was calculated using the comparative threshold cycle (C_T) method.

Table 3. Primers used for RT-qPCR

Target gene	Sequence	Product size	Tm (°C)	Cycle no.
Snail1 For [290]	5' CTTCCAGCAGCCCTACGAC 3'	71 bps	55°C	40
Snail1 Rev	5' CGGTGGGGTTGAGGATCT 3'			
Twist1 For [290]	5' AGCAAGATTCAGACCCTCAAGCT 3'	55 bps	55°C	40
Twist Rev	5' CCTGGTAGAGGAAGTCGATGTACCT 3'			
ZEB1 For [291]	5' TCCCCATCACCTCTAAACCTT 3'	122 bps	60 °C	40
ZEB1 Rev	5' CCCTGTTGCTTTGGTAGTGAA 3'			
COX-1 For [277]	5' TGTTCGGTGTCCAGTTCCAATA 3'	93 bps	55°C	40
COX-1 Rev	5' ACCTTGAAGGAGTCAGGCATGAG 3'			
COX-2 For [277]	5' CCTTCCTCCTGTGCCTGATG 3'	80 bps	57 °C	40
COX-2 Rev	5' ACAATCTCATTTGAATCAGGAAGCT 3'			
IL-6 For [277]	5' GCCGCCCCACACAGACA 3'	70 bps	57 °C	40
IL-6 Rev	5' CCGTCGAGGATGTACCGAAT 3'			
IL-11 For [277]	5' CCCAGTTACCCAAGCATCCA 3'	89 bps	58 °C	40
IL-11 Rev	5' AGACAGAGAACAGGGAATTAAATGTGT 3'			
VEGF-A For[277]	5' TACCTCCACCATGCCAAGTG 3'	103 bps	57 °C	40
VEGF-A Rev	5' TAGCTGCGCTGATAGACATCCA 3'			
Gro-α For [278]	5' GTTTTCAAATGTTCTCCAGTCATTATG 3'	80 bps	60 °C	45
Gro-α Rev	5' CCGCCAGCCTCTATCACAGT 3'			
IL-1α For [279]	5' TGTATGTGACTGCCCAAGATGAA 3'	79 bps	57 °C	40
IL-1α Rev	5' CTACCTGTGATGGTTTTGGGTATC 3'			
IL-8 For	5' CTGTGTGAAGGTGCAGTT 3'	112 bps	58 °C	40
IL-8 Rev	5' CACTCTCAATCACTCTCAGTTC 3'			
KPNB1 For [292]	5' CCAGTGCCGAGTGGAATG 3'	191 bps	55°C	40
KPNB1 Rev	5' AAATCCCTGACCCTCCTTC 3'			
MMP-2 For	5' TGGCGATGGATACCCCTTT 3'	117 bps	55°C	40
MMP-2 Rev [238]	5' TTCTCCCAAGGTCCATAGCTCAT 3'			
MMP-9 For	5' CCGGACCAAGGATACAGTTT 3'	108 bps	60 °C	40
MMP-9 Rev	5' GCGGTACATAGGGTACATGAG 3'			
GusB For [292]	5' CTCATTTGGAATTTTGCCGATT 3'	81 bps	55°C	40
GusB Rev	5' CCGAGTGAAGATCCCCTTTTTA 3'			

For= Forward primer; Rev= Reverse Primer

2.6.9 E_{50} Determination

The EC_{50} concentration of INI43 in SiHa and Me180 cells was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Sigma-Aldrich, USA). 5000 cells in 90μL complete DMEM media were seeded in triplicate and allowed to adhere overnight. Cells were treated with varying concentrations of INI43 ranging from 1-50μM. Incubation with INI-43 was done for a period of 48hrs before the addition of 10μL MTT reagent, followed by 100μL solubilization reagent 4hrs later. The following day, absorbance was read at OD595nm using a microplate reader (BioTeK, Winooski,VT, USA). Absorbance readings were used to plot a dose-response curve and EC_{50} values were calculated using GraphPad Prism V5.0 software.

2.6.10 Cell Proliferation

Cell proliferation was investigated using the MTT assay. 1500 cells were plated in a 96-well plate in triplicates and allowed to adhere overnight. The following day, media was aspirated, and cells were serum starved for 24hrs. The following day, serum-free media was removed and replaced with 0.1% FBS containing media in the absence or presence of SF (1:50 dilution). Cell growth was measured over five days using the MTT reagent whereby each day 10μL MTT was added to the appropriate wells followed by 100μL of solubilization reagent 4hrs later. Absorbance was measured the following day at OD595nm.For proliferation assay using chemical inhibitors, cells were plated in a 96-well plate then serum starved before treatment with the chemical inhibitors in the absence or presence of SF (1:50), with vehicle control (DMSO) included in 0.1% DMEM media. MTT reagent was then added after three days and

followed by solubilization solution 4hrs after. Absorbance was measured and chemical inhibitors in the present of SF were normalized to chemical inhibitor only readings.

2.6.11 Cell morphology imaging

For cell morphology analysis, 5 x 10^4 cells were seeded in 6-well tissue culture plates and allowed to adhere overnight. The following day cells were serum starved for 24hrs. The next day, cells were treated with SF (1:50) or vehicle control (PBS). Live cell images were taken at 4, 8, 16 and 24hrs using a phase contrast light microscope.

2.6.12 Actin staining

To visualize F-actin arrangement phalloidin staining was performed. 5 x 10^4 cells were seeded on a coverslip in 6-well plates. The following day cells were serum starved for 24 hours after which cells were treated with SF(1:50) for 4, 8, 16 and 24hrs or vehicle (PBS). After treatment, cells were washed with 1XPBS before fixation with 4% paraformaldehyde. Cells were then washed with 0.04% PBS-Tween and blocked in 1% BSA for 30 minutes. F-actin was labelled with 50µg/ml Phalloidin-Tetramethylrodaine B isothiocyanate (Sigma-Aldrich) in 1% BSA for 30 minutes. Cells were washed twice with 1XPBS, followed by nuclei staining with Hoechst stain diluted to 1:5000 in PBS. Coverslips were mounted on glass slides using mowial and allowed to dry overnight in the dark. Fluorescent images were captured at 63X oil immersion objective using the Zeiss LSM880 Airyscan Confocal microscope with AxioVision Zeiss software. For F-actin visualization in the presence of chemical inhibitors, cells were seeded on coverslips and serum starved. The following day, cells were treated with chemical inhibitors in the absence or presence of SF (1:50) for 24hrs, with vehicle control (DMSO) included. After treatment, cells were fixed, stained and visualized as described above.

2.6.13 Immunofluorescence microscopy

Cells were seeded on coverslips in 6-well plates. Following serum starvation and treatment, the cells were fixed with cold 4% paraformaldehyde for 15 minutes at room temperature. Fixative was removed and cells were washed twice with 1XPBS. Cells were permeabilized by adding 0.25% Triton X-100 in PBS for 10 minutes. Following permeabilization, cells were washed thrice with 1XPBS (5 minutes/wash). Cells were then blocked using 1% BSA in PBST and 0,3M glycine for 30 minutes. Cells were subsequently incubated with primary antibody, either NF-kB p65 primary antibody or anti-Ki67 antibody was used at a dilution of 1:100 in 1% BSA in PBST and incubated overnight at 4 °C. Cells were then washed with 1x PBS thrice at 5 minute intervals. Secondary antibody, GαR-Cy3 for NF-kB p65 and DαG Alexa488 for Ki-67, was diluted at a concentration of 1:300 in 1% BSA in PBST and placed on cells for 1hr in the dark at room temperature. Secondary antibody was removed and cells were washed with PBS before adding nuclei staining Hoechst at a dilution of 1:5000 in PBS for 5 minutes. Hoechst staining was removed and coverslip was mounted on microscope slides using mowial. Immunofluorescent imaging was done at 40X water immersion using the Zeiss LSM880 Airyscan Confocal microscope with AxioVision Zeiss software (Carl Zeiss, Jena, Germany).

2.6.14 Western blot analysis

2.6.14.1 Whole cell lysate collection

After relevant treatment of cells in 60mm tissue culture plates, protein was harvested by lysing cells with complete RIPA buffer containing a cocktail of protease inhibitors (Roche, Basel, Switzerland) and 0.1M Sodium Orthovanadate to inhibit phosphatase activity. Cells were removed using a cell scraper and transferred to a fresh tube on ice. Cell lysates were sonicated for 12 seconds before centrifugation at 10 000x g for 10 minutes at 4 °C to remove cell debris. The protein supernatants were transferred to fresh tubes and stored at -80 °C until further use.

2.6.14.2 BCA Protein quantification

Protein samples were thawed and quantified in 96-well plates using the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Absorbance readings for protein samples and BSA protein standards were read at OD595nm, after which a standard curve was plotted to calculate protein concentration. Using the BSA results, equal amounts of protein were made up with RIPA and 4X Loading dye.

2.7.14.3 SDS-Polyacrylamide gel electrophoresis

Ready prepared protein samples containing 4x loading dye were heated at 90°C for 5 minutes on a heating block to denature the samples. Equal amounts of protein (25μg) in 30μL total volume were loaded into 10% SDS-polyacrylamide gels, with a molecular weight marker included in lane one, and electrophoresis was run at 170V for 1hr. Proteins were then transferred to a Hybond ECL nitrocellulose membrane (Amersham Biosciences, UK) using a wet transfer system for 1hr at 100V.

2.6.14.4 Immunoblotting and chemiluminescent detection

Following transfer, membranes were blocked in 5% non-fat skim milk in TBST (Appendix I) for 1hr on a desktop shaker at room temperature. After blocking, the membrane was incubated with primary antibody (Table 4) overnight at 4°C. The following day, the membrane was washed three times at 5-minute intervals with TBST before adding secondary antibody for 1hr on a desktop shaker at room temperature. Membranes were washed three times for 5 minutes each and protein bands were detected using Lumiglo (KPL, Inc., Gaithersburg, MD, USA) or Lumiglo Reserve chemiluminescent substrate (KPL, Gaithersburg, MD, USA). The protein bands were detected and captured using the chemiluminescent setting on the iBright imaging system (Invitogen™, ThermoFisher).

Table 4: Western blot antibody concentrations and conditions

Primary antibody	**Primary antibody condition**	**Secondary antibody**	**Secondary antibody conditions**	**Chemiluminescent substrate**
E-cadherin	1:1000 5% Milk	GαR	1:5000 5% Milk	Lumiglo Reserve
N-cadherin	1:500 5% Milk	GαM	1:5000 5% Milk	Lumiglo Reserve
Anti-KPNB1	1:1000 TBST	GαR	1:5000 TBST	Lumiglo
Anti-p-c-Jun	1:500 5% BSA	GαR	1:3000 TBST	Lumiglo Reserve
Anti-c-Jun	1:500 5% BSA	GαR	1:5000 5% Milk	Lumiglo Reserve
Anti-GAPDH	1:5000 5% Milk	GαR	1:5000 5% Milk	Lumiglo

2.6.14.4 Stripping and re-probing

In order to re-probe on membranes, membranes were stripped of bound antibody by washing the membrane in stripping buffer (Appendix I) for 7 minutes on each side. The buffer was neutralized by washing the membrane in 1M Tris-EDTA for 3 minutes followed by washing

with TBST for 1 minute. After stripping, membrane was blocked and probed with a new primary antibody.

2.6.15 Migration Assay

In order to investigate the effect of SF on cell motility, Polyethylene Terephthalate hanging cell culture inserts (8μm) for 24-well plates were used for the migration assay (Merk, Millipore, Darmstadt, Germany). This assay is an in vitro technique that investigates the motility of cells through a membrane. 100 x 10^4 cells were plated in a 6-well plate and allowed to adhere for 24hrs. The following day, cells were trypsinized and resuspended in 0,1% FBS containing DMEM. Cells were seeded at equal amounts (3x 10^4 cells/insert) into the upper chamber of the 24-well migration inserts. Migration inserts were placed into lower chambers containing 0,1% FBS DMEM Each condition was plated in triplicate. Cells were allowed to migrate overnight (16hrs). The following day, cells were fixed using 100% Methanol and cells that did not migrate were removed by cotton swob. Inserts were then stained with 0,5% crystal violet solution for 10 minutes after which excess crystal violet was removed by rinsing inserts with water. Migration inserts were allowed to dry overnight. Stained cells were imaged using the Zeiss Primovert inverted phase contrast microscope (Carl Zaiss, Jena, Germany) and migrated cells were counted. Migration was normalized to MTT cell viability absorbance readings to normalize for cell death. For chemical inhibitor treatment, cells were plated and allowed to adhere. The following day cells were treated for 8hrs before trypsinization and plating cells into the top chamber and placing the insert into the bottom chamber containing SF (1:50) in 0,1% FBS DMEM media.

2.6.16. Invasion Assay

Transwell invasion chambers were prepared by coating 24-well migration hanging inserts with 0,2% Gelatin[293]. Briefly, 2% gelatin solution (Type B, Merk) was used to prepare a 0,2% gelatin solution using sterile water. Migration hanging inserts were coated with 0,2% gelatin solution by adding 100μl solution per insert in a 24-well plate. Inserts were incubated at 37C for 15 minutes to allow gelatin solution to polymerize. Excess gelatin was gently aspirated off, and inserts were allowed to dry at room temperature in a tissue culture hood for 2hrs before introducing medium and cells. 8 x 10^5 cells were plated in the top chamber of the invasion insert containing 0,1% FBS in the absence or presence of SF (1:50). The inserts were placed in a lower chamber with 0,1% FBS DMEM media in the absence or presence of SF. Other conditions included SF (1:50) at the top & SF at the top and bottom to investigate the effect of SF. More cells had to be seeded to allow invasive cells to pass through the extracellular matrix (gelatin). Cells were allowed to invade overnight, and the following day cells were fixed and stained following the protocol in section 2.

2.6.17 Dual Luciferase Assay

To assay for NF-kB/p65 and AP-1 transcriptional activity a Dual Luciferase assay (Promega, USA) was used. Cells were seeded at a density of 3 x 10^4 cells/well in a 24-well plate and allowed to adhere. The following day cells were transfected with 100ng NF-kB/p65 luciferase reporter plasmid or 100ng AP-1 luciferase reporter and 10ng pRL-TK Renilla using Genecillin™ transfection reagent (Celtic Molecular Diagnostic, South Africa) overnight. Transfection mix was removed the next day and cells were pre-treated with INI-43 (10μM) for 1hr followed by SF (1:50) stimulation for 4hrs before cells were lysed using 100μL 1x Passive lysis buffer (Promega, USA) for 15 minutes on a desk top shaker, with frequent tapping to aid lysis.

Luciferase firefly readings were taken on the Glomax 96 microplate luminometer (Promega, USA) using the Dual Luciferase kit. Luciferase firefly readings were normalized to Renilla luciferase reporter readings in the same lysate.

2.6.18 Statistical Analysis

Experiments were performed in triplicates or quadruplicates, unless specified otherwise. Results expressed as mean$\pm$SEM (Standard error of mean) and repeated at least 3 independent times. For data comparisons, Student t-test was performed using Graph pad prism V5.0 or V8.0 (GraphPad software, San Diego, USA). A p value < 0,05 was considered statistically significant.

Chapter 3:

Investigating the effects of seminal fluid on proliferation, EMT, migration & invasion in cervical squamous carcinoma cell lines

3.1 INTRODUCTION

Cervical cancer is the fourth most common type of cancer amongst women worldwide[1]. Of the various cervical cancer types, squamous carcinoma accounts for 70% of cervical cancer cases[44]. The global burden of cervical cancer is unevenly distributed with the majority of cervical cancer incidences and mortalities occurring in Sub-Saharan Africa (SSA)[294]. In 2018, 21.7% of all cancer deaths were attributed to cervical cancer, making it the most common cause of cancer mortalities in SSA[295]. The highest age standardized incidence rate (43,1 per 100 000) in the world is in South Africa[295], highlighting cervical cancer as a major public health burden in South Africa. Unlike other cancers, cervical cancer is curable when diagnosed and treated early[3]. However, in LMICs poor access to prevention, screening and treatment interventions contributes to majority of cancer deaths[296]. In Africa, up to 95% of cervical cancer cases are diagnosed when women have late-stage disease[297].

A major cause of cancer-related death is metastasis[62]. As a key hallmark of cancer, metastasis is a multistep process whereby tumor cells migrate and invade a secondary site[62]. A critical process that has been shown to be associated with metastasis is the EMT process. EMT is characterized by the transformation of epithelial cells towards the motile mesenchymal phenotype[298]. This transformation results in a loss of cell-cell adhesion, change in cell polarity, an upregulation of mesenchymal markers and a subsequent downregulation of epithelial markers[299]. EMT occurs by various signaling pathways that lead to the activation of EMT-

inducing transcription factors namely Snail, Twist, Slug and ZEB. These transcription factors drive EMT by inhibiting epithelial markers (e.g., E-cadherin, B-catenin) and upregulating mesenchymal markers (e.g., N-cadherin, Vimentin, MMPs)[300]. EMT has been shown to drive migration and invasion in cancer cells[85]. A prerequisite for the mesenchymal morphology, migration and invasion of cancer cells is the dynamic reorganization of the actin cytoskeleton[301]. During the EMT process, cancer cells undergo a remodeling of the cell cytoskeleton, which includes changes in cell shape, the formation of actin protrusions and stress fibers. The actin cytoskeleton plays a critical role in cell shape and cell motility[302]. Research has shown that the rearrangement of the actin cytoskeleton into F-actin stress fibers during EMT aids the formation of protrusions (lamellipodia) therefore promoting motility[66]. Due to the link between EMT and metastasis, the EMT process has become a key area of research in cancer therapy[303].

Like other cancers, cervical cancer is also prone to metastasis to distant sites such as the lungs[304]. Another key factor that has been shown to promote cancer progression is inflammation. It has been established that hr-HPV plays a role in driving cancer related inflammation in cervical cancer[243,244]. This inflammation is characterized by proinflammatory factors such as cytokines and chemokines that activate various oncogenic pathways and drive cervical cancer progression[208,246,305]. Therefore, factors that initiate inflammation in the cervix may play a role in cervical cancer progression. Interestingly, seminal fluid (SF) has been implicated in the progression of cervical cancer. This is due to the inflammatory response elicited by SF in the female reproductive tract following coitus in the absence of condom barrier[261]. Previous research in our laboratory has shown that SF drives cervical adenocarcinoma cell proliferation *in vitro* and *in vivo*[277]. Additionally, SF has been shown to

upregulate proinflammatory cytokines[261,271,273,278,279] and activates oncogenic signaling pathways such as COX-PG, PI3K, EGFR and ERK1/2 in adenocarcinoma cervical cancer cells[273,276,279,280]. Since SF is a biological fluid rich in a host of bioactive molecules[258], its direct interaction with epithelial cells of the cervix is thought to associate with cervical cancer progression. While there is evidence that SF promotes inflammation and proliferation of adenocarcinoma cervical cancer cells, little is known about the role of SF in the progression of other cancer phenotypes such as EMT and migration in cervical squamous carcinoma cells.

This chapter aims to investigate the role of SF in cervical cancer cell biology using squamous carcinoma cell lines as a model system. Here, we investigated the effects of SF on cervical cancer cell proliferation, morphology, actin cytoskeleton arrangement, EMT, migration and invasion.

3.2 RESULTS

3.2.1 Investigating the effect of SF on the proliferation of cervical squamous carcinoma cell lines

One of the hallmarks of cancer cells is their ability to sustain proliferative signaling. In normal cells, cell proliferation is regulated while in cancer cells this regulation is lost leading to enhanced cell proliferation[306]. In addition to inherent mutations in tumor cells that drive the ability to sustain uncontrolled proliferation, growth factors have also been shown to play a role in tumor cell proliferation[307]. In this study we investigated whether SF, which is rich in a host of growth factors and signaling molecules, has a role in the cell proliferation of cervical squamous carcinoma cell lines.

3.2.1.1 SF enhances the proliferation of cervical squamous carcinoma cell lines

In order to investigate whether SF had an effect on cervical cancer cell proliferation, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay was used. The SF used in all experiments was pooled from 10 individual SF samples to remove patient-patient variation. The concentration of SF used was 1:50 which has been used in previous research in our laboratory and has been shown to exert no toxic effects on cell viability[284,279]. SiHa and Me180 squamous epithelial cells were treated with SF over a period of 5 days and proliferation was monitored using the MTT assay. Our results show a significant increase in cell proliferation within 2 days post SF treatment in both SiHa and Me180 cells indicating that SF stimulates cell proliferation in cervical squamous carcinoma cells (Figure 3.1A, B).

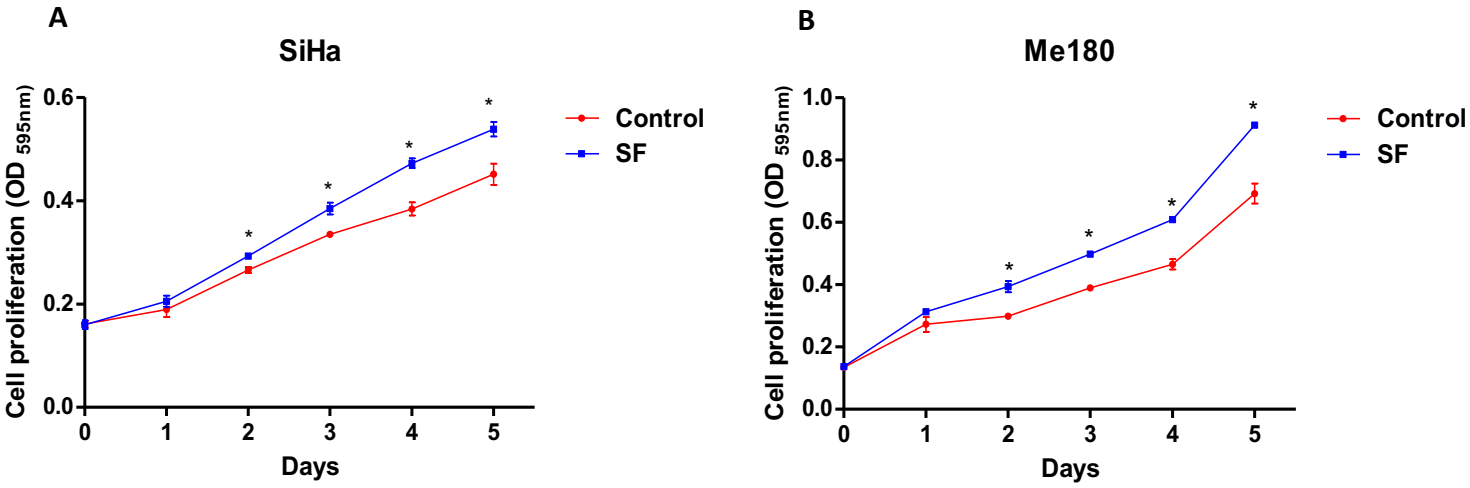


Figure 3.1 SF enhances cell proliferation in cervical squamous carcinoma derived cell lines. A) SiHa and **B)** Me180 cells were incubated with SF at a dilution of 1:50 over 5 days. MTT viability assay was used to measure cell proliferation. Result shown is the mean ± SEM of experiments performed in triplicate and repeated at least three independent times. (*P<0,05).

3.2.1.2 SF enhances Ki-67 positive cells in cervical squamous carcinoma cells

Having observed that SF treatment significantly enhanced the cell proliferation of SiHa and Me180 cells, we investigated the expression of Ki-67, a proliferation marker, using immunocytochemistry. Ki-67 positive cells were quantified following 24, 48 and 72hrs of SF treatment in SiHa and Me180 cells. In SiHa cells, representative immunofluorescent images that show Ki-67 positive cells were captured and quantified (Figure 3.2A). Quantification of Ki-67 showed no significant enhancement of Ki-67 positive cells between control and SF after 24hrs for SiHa cells. However, quantification showed a significant increase in the percentage of Ki-67 positive cells for SF treatment in comparison to control after 48hrs (73,4 vs 62.4 %) and 72hrs (84,2 vs 64,3%) respectively (Figure 3.2B).

In Me180 cells similar results were observed where an increase in Ki-67 positive cells was observed in immunofluorescent images after 48hrs (Figure 3.3A). Quantified results showed no significant difference in the percentage of Ki-67 positive cells at 24hrs. Results showed a significant increase in Ki-67 positive cells in Me180 cells treated with SF in comparison to control at 48hrs (73,5 vs 63,5%) and 72hrs (85,7 vs 67,6 %), respectively (Figure 3.3B). These results show that SF treatment increases the percentage of Ki-67 positive cells in SiHa and Me180 cells. Taken together this data confirms that SF enhances cell proliferation in cervical squamous carcinoma cell lines.

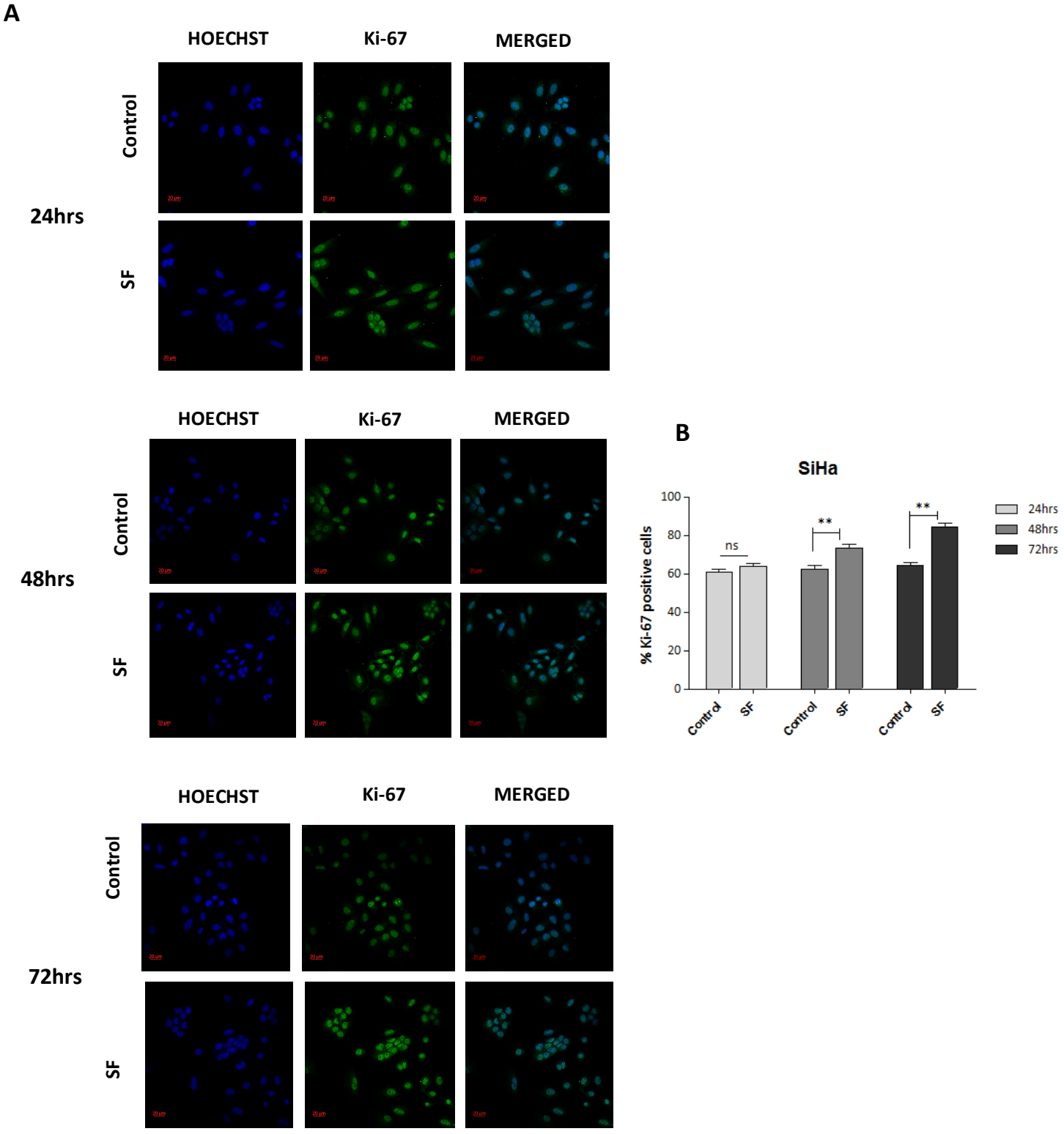


Figure 3.2 SF enhances the percentage of Ki-67 positive SiHa cells A) SiHa cervical cancer cells were plated on glass coverslips and treated with control (PBS) or SF (1:50) for 24, 48 and 72hrs. anti-Ki-67 was Alexa488 labelled (green) while nuclei were stained with Hoechst (blue). **B)** Quantification of Ki-67 positive cells per treatment condition covering 300 cells in difference fields of view. Scale bar=20µm, Magnification x400. Results shown are the mean ± SEM of experiments performed three independent times. (*P<0,05),(**P<0,01).

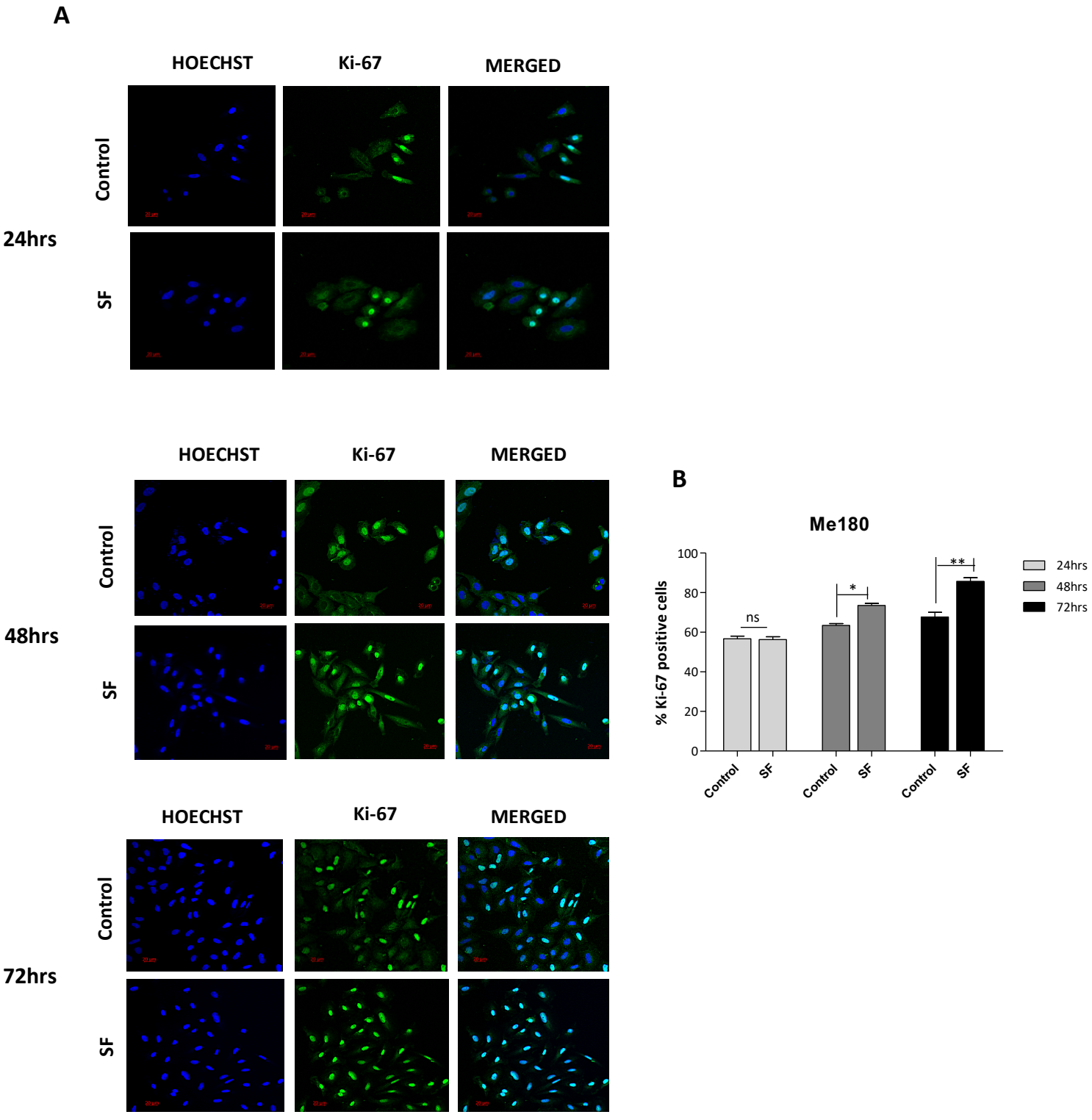


Figure 3.3. SF enhances the percentage of Ki-67 positive Me180 cells A) Me180 cervical cancer cells were plated on glass coverslips and treated with control (PBS) or SF (1:50) for 24, 48 and 72hrs. anti-Ki-67 was Alexa488 labelled (green) while nuclei were stained with Hoechst (blue). **B)** Quantification of Ki-67 positive cells per treatment condition covering 300 cells in difference fields of view. Scale bar=20μm, Magnification x400. Results shown are the mean ± SEM of experiments performed three independent times. (*P<0,05),(**P<0,01).

3.2.2 Investigating the effect of SF on cervical squamous carcinoma cell morphology and cytoskeleton arrangement

While performing cell proliferation assays observations using phase contrast microscopy showed changes in cell morphology in response to SF exposure. In order to confirm these observations, phase contrast images of cells treated with control or SF were taken. Since cell morphology is linked to the cytoskeletal arrangement in a cell, we also investigated the effect of SF on filamentous actin (F-actin) using phalloidin staining.

3.2.2.1 SF induces cell morphological changes in cervical squamous carcinoma cells

In order to investigate the effect of SF on cervical cancer cell morphology, SiHa and Me180 cells were plated and treated with SF. Phase contrast microscopy was used to observe cell morphology. Images of cells treated with SF were taken at 4, 8, 16 and 24hrs. Phase contrast images of SiHa and Me180 cells showed a morphological change from a tightly packed "cobblestone" morphology typical of epithelial cells to a more spindle-like morphology which was prominent at 24hrs of SF treatment (Figure 3.4). Phase contrast images also showed that Me180 cells appeared more sensitive to the morphological change induced by SF treatment.

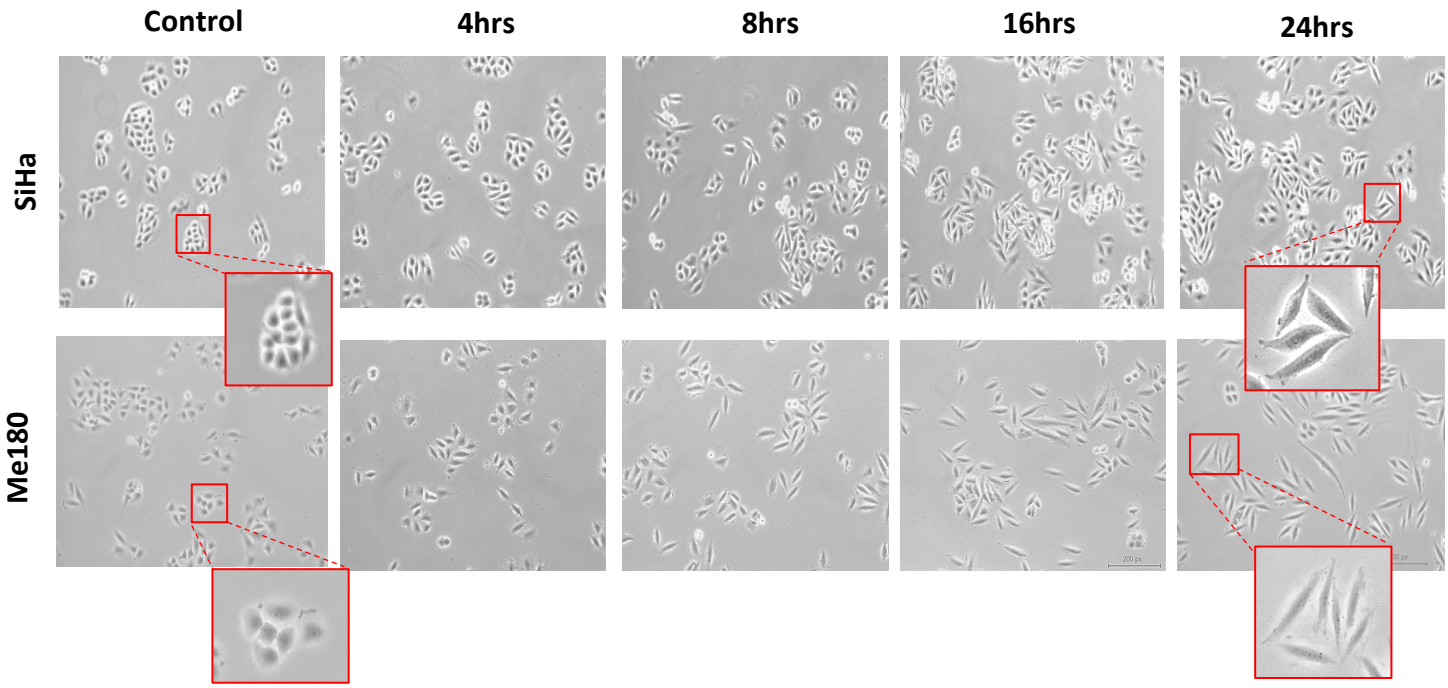


Figure 3.4. **SF causes morphological changes in cervical squamous carcinoma cells.** Visible morphological changes from "cobblestone"- like to "spindle"- like cells were observed in SiHa and Me180 cells after SF (1:50) treatment compared to control using phase contrast microscopy. Representative images captured using magnification 100x are shown.

3.2.2.2 SF induces actin cytoskeleton rearrangement and stress fibers formation in cervical squamous carcinoma derived cell lines

The actin cytoskeleton plays a fundamental role in various cellular processes including migration, morphogenesis and cytokinesis[308]. Having observed a morphological change from a more "cobblestone" to a more spindle-like morphology after SF treatment, we postulated that SF may cause changes to the cytoskeleton of squamous carcinoma cells. In order to investigate the effect of SF on the cytoskeleton, SiHa and Me180 cells were stained with Phalloidin which binds filamentous actin (F-actin) allowing for the visualization of the cytoskeleton using fluorescent microscopy. Fluorescent images confirmed changes in cell morphology in response to SF treatment in SiHa cells (Figure 3.5A). Quantified results further showed a significantly higher percentage of cells containing F-actin stress fibers in SF treated cells at 4, 8, 16 and 24hrs compared to control in SiHa cells (Figure 3.5B). Similarly, immunofluorescent images confirmed morphological changes in cell morphology and the presence of stress fibers in Me180 cells (Figure 3.6A). Me180 cells also showed a higher percentage of cells containing F-actin stress fibers at 4, 8, 16 and 24hrs of SF treatment compared to control cells (Figure 3.6B). Together, these results show that SF induces F-actin cytoskeletal rearrangement and stress fiber formation in cervical squamous carcinoma cells.

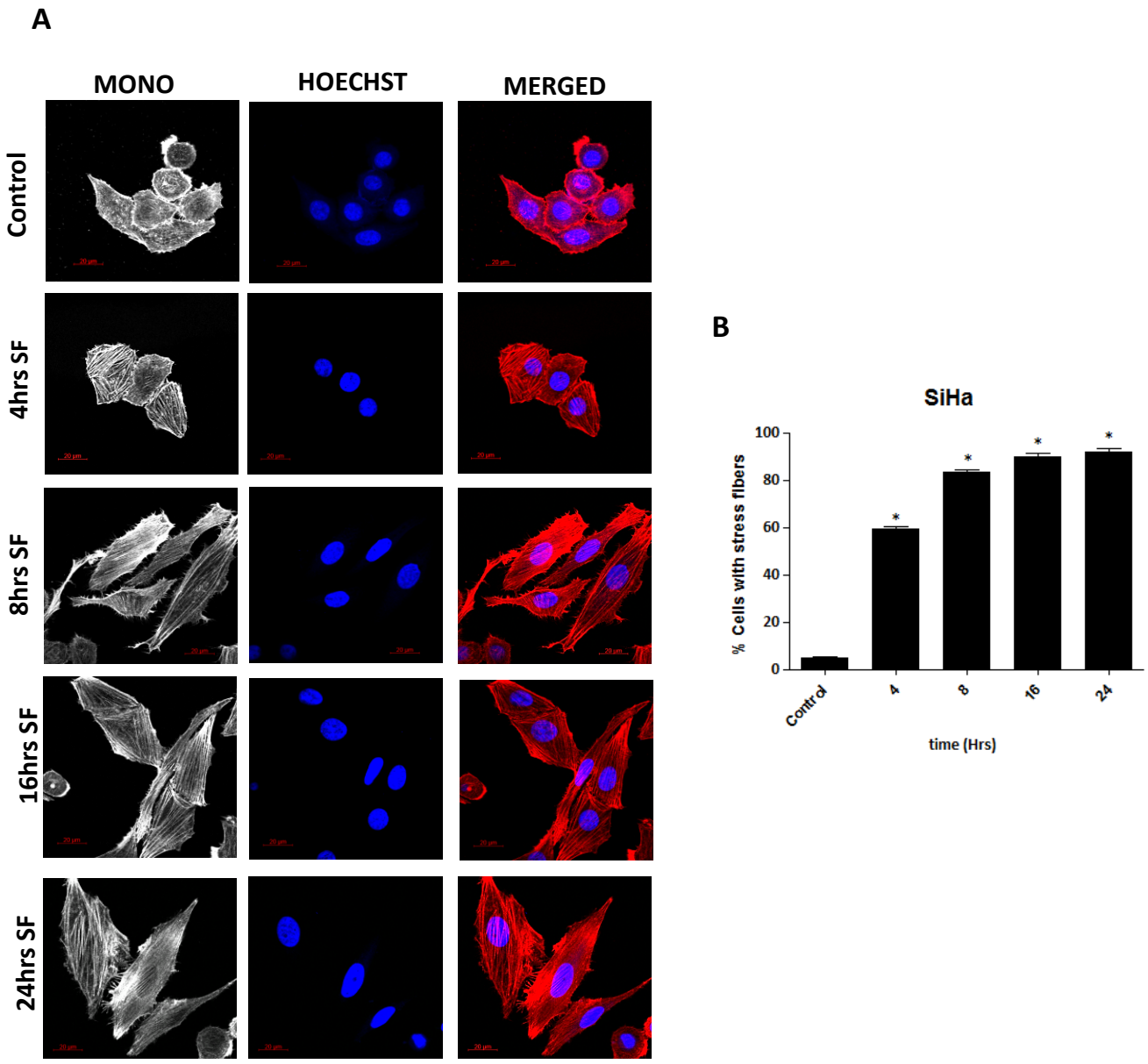


Figure 3.5. Analysis of F-actin rearrangement in SiHa cells following SF treatment using confocal microscopy A) Representative immunofluorescent images of SiHa cells stained with Phalloidin (cy3) for F-actin and Hoechst (blue) for the nuclei following treatment with vehicle (PBS) or SF for 4, 8, 16, 24hrs. Scale bar= 20µm, Magnification= 630X. **B)** Bar graph shows percentage of cells with stress fibers (200 cells were scored per condition in three independent experiments). Results shown are the mean ± SE of experiments performed three independent times. (*P<0,05). MONO=monochromatic

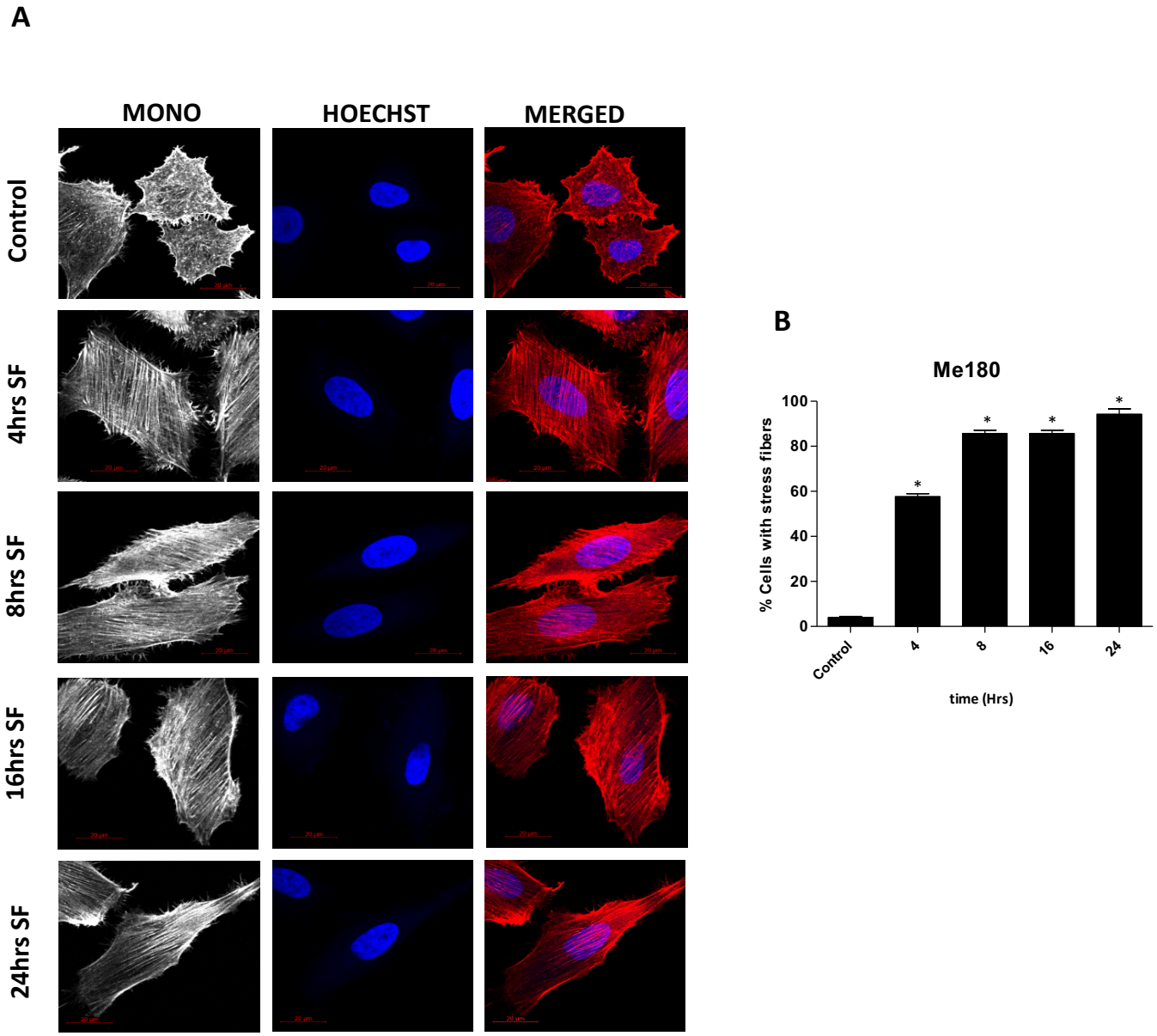


Figure 3.6. Analysis of F-actin rearrangement in Me180 cells following SF treatment using confocal microscopy
A) Representative immunofluorescent images of Me180 cells stained with Phalloidin (cy3) for F-actin and Hoechst (blue) for the nuclei following treatment with vehicle or SF for 4, 8, 16, 24hrs. Scale bar= 20µm, Magnification= 630X. **B)** Bar graph shows percentage of cells with stress fibers in each condition (200 cells were scored per treatment across three independent experiments). Results shown are the mean ± SE of experiments performed three independent times. (*P<0,05). MONO= monochromatic

3.2.3 Investigating the effect of SF on Epithelial mesenchymal transition (EMT) induction in cervical squamous carcinoma cells

Actin stress fibers have been shown to play a role in maintaining cell shape, aiding cell migration and intracellular cargo transport. Interestingly, actin stress fiber formation has been shown to accompany epithelial mesenchymal transition (EMT)[79] where epithelial cells undergo a phenotypic change to a more mesenchymal phenotype with altered elasticity and migration. This altered elasticity is required for movement through tightly constricted spaces[309]. Having observed a change in cell morphology and the induction of stress fiber formation in SiHa and Me180 cells treated with SF, we hypothesized that SF could be inducing EMT. The EMT process is activated by various signaling pathways which activate EMT inducing transcription factors such as Snail, Twist and ZEB1. These transcription factors play a role in inhibiting epithelial markers (e.g., E-cadherin) and upregulating mesenchymal markers (e.g., N-cadherin) which drive cells to acquire a mesenchymal migratory phenotype[300].

3.2.3.1 SF upregulates EMT inducing transcription factors Snail, Twist and ZEB1

To investigate the potential role of SF on EMT induction, SiHa and Me180 cells were treated with vehicle or SF for 4, 8, 16 and 24hrs and mRNA expression of Snail, Twist and ZEB1 was measured by RT-qPCR. In SiHa cells, SF significantly induced Snail, Twist and ZEB1 mRNA expression. For Snail, SF induced expression was observed at 4, 8 and 16hrs with peak induction seen at 8hrs while Twist mRNA upregulation was observed at 8, 16 and 24hrs SF treatment. For ZEB1, SF induced mRNA expression was observed at 4, 8 and 24hrs with peak induction occurring at 4hrs (Figure 3.7A).

RT-qPCR results of Snail, Twist and ZEB1 mRNA expression in Me180 cells also showed the induction of these EMT transcription factors by SF treatment. For Snail, SF induced expression was observed at 4, 8, 16hrs and 24hrs SF treatment, with peak induction occurring at 4hrs while Twist mRNA expression was induced at 8 and 16hrs SF treatment, where peak induction was observed at 16hrs. In addition, ZEB1 mRNA expression was upregulated at 4 and 8hrs SF treatment, with peak induction occurring at 8hrs (Figure 3.7B). Together this data suggests that SF activates the expression of EMT inducing transcription factors Snail, Twist and ZEB1.

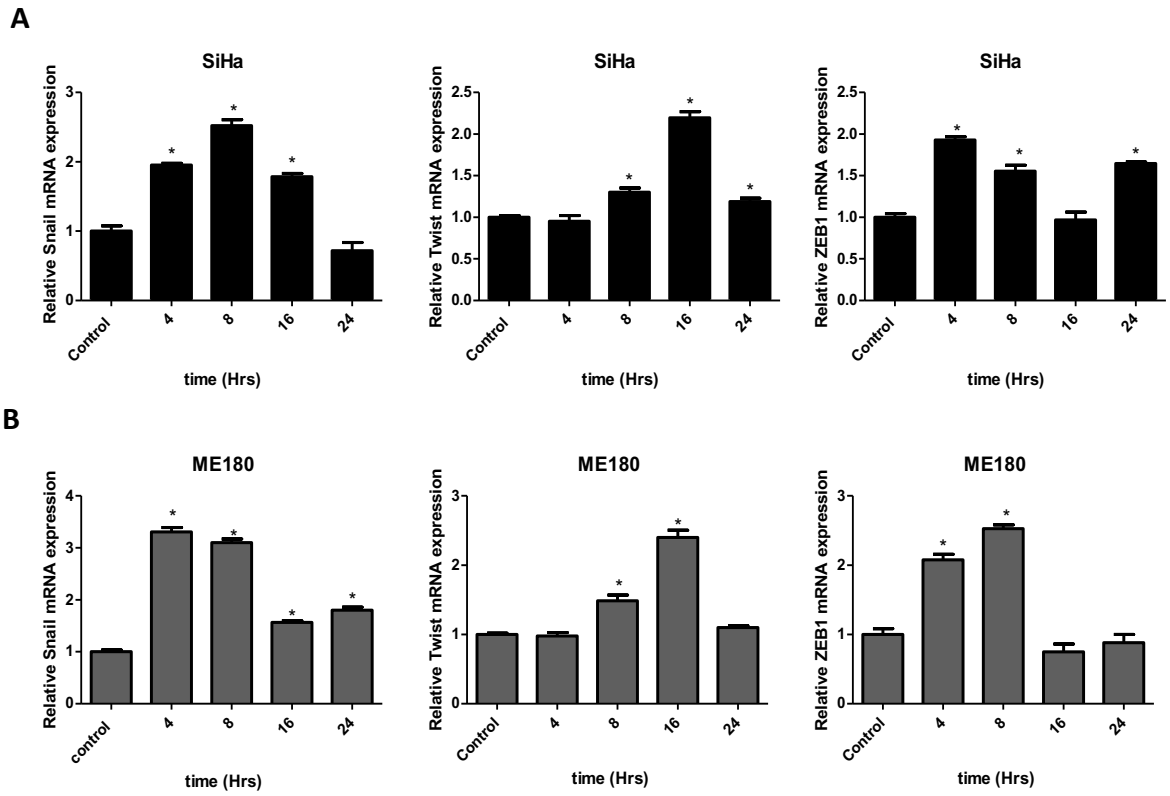


Figure 3.7. SF upregulates the EMT inducing transcription factors Snail, Twist and ZEB1 mRNA expression. Relative Snail, Twist and ZEB1 mRNA relative expression in **A)** SiHa and **B)** Me180 cells treated with SF (1:50) for 4, 8, 16 and 24hrs in comparison to vehicle (PBS) as determined by RT-qPCR. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Results shown as mean $\pm$ SEM of experiments performed in triplicate and repeated three independent times. (*P<0,05).

3.2.3.2 Investigating the effect of SF on E-cadherin and N-cadherin protein expression in squamous carcinoma cells.

Having established that SF stimulates the expression of EMT-inducing transcription factors, we next investigated the effect of SF on the expression of E-cadherin (epithelial marker) and N-cadherin (mesenchymal marker). As the induction of EMT is characterized by the downregulation of epithelial markers and the subsequent upregulation of mesenchymal markers, we investigated whether SF downregulated E-cadherin and upregulated N-cadherin protein expression in cervical squamous carcinoma cell lines. Western blot analysis was used to monitor the expression of E-cadherin and N-cadherin in SiHa and Me180 cells treated for 4, 8, 16 and 24hrs with vehicle or SF. In SiHa cells, the results showed that the expression of E-cadherin was slightly reduced at 4, 8 and 24hrs of SF treatment compared to control cells (Figure 3.8A). For N-cadherin, expression levels were constant with a subtle upregulation observed at 24hrs SF treatment. In Me180 cells, a slight downregulation of E-cadherin expression was observed at 4, 8 and 24hrs whereas a more rapid increase of N-cadherin protein expression, starting at 4hrs SF treatment was observed (Figure 3.8B).

Taken together, these results suggest that in response to SF treatment there is an increase in the expression of the EMT transcription factors (Snail, Twist and ZEB1) which in turn leads to the altered expression of E-cadherin and N-cadherin in SiHa and Me180 cells.

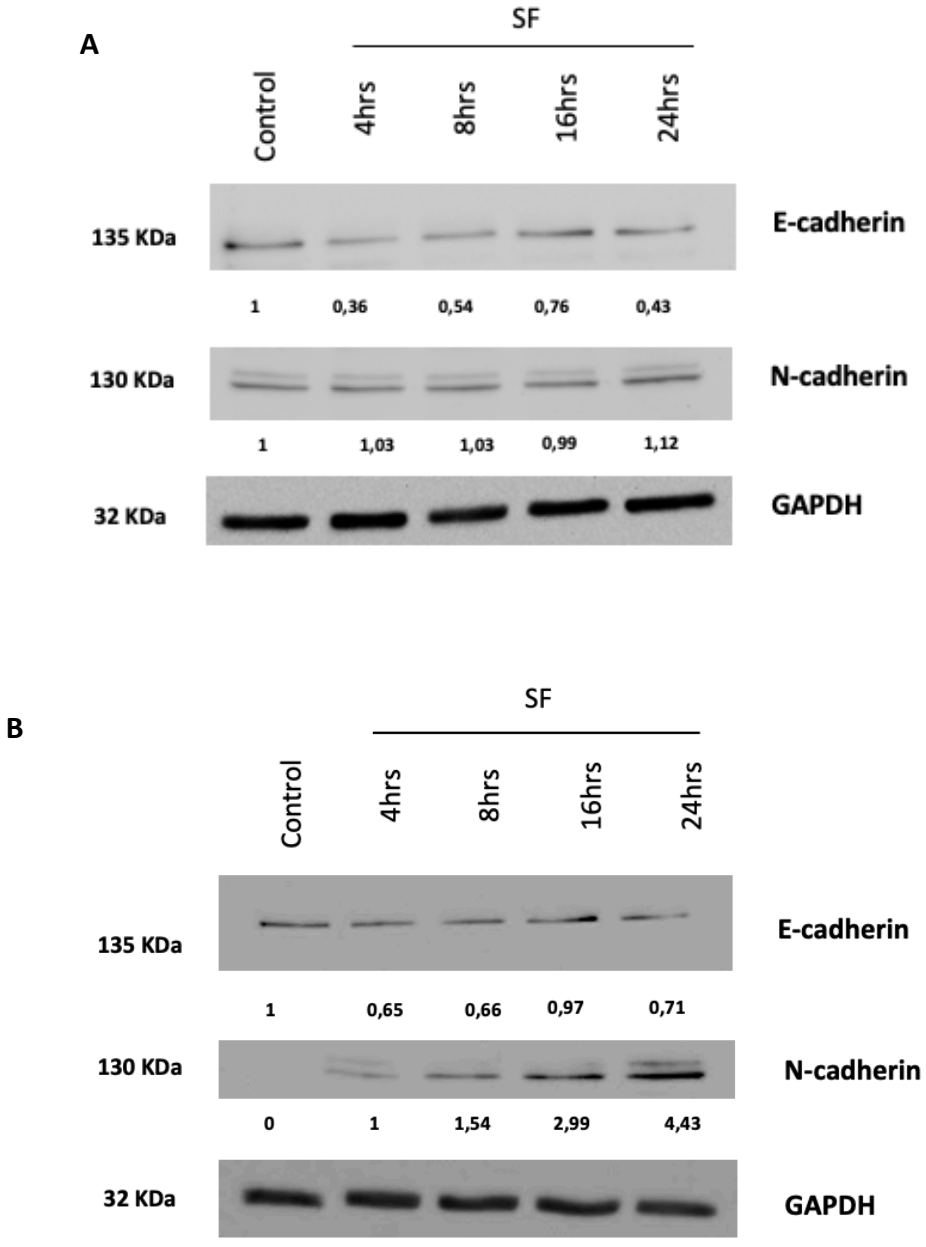


Figure 3.8. Western blot analysis of E-cadherin and N-cadherin expression after SF treatment in squamous carcinoma cells. A) SiHa and **B)** Me180 cells were treated with vehicle or SF (1:50) for 4,8,16 and 24hrs after which western blot analysis was used to monitor expression of E-cadherin and N-cadherin. GAPDH was used as a loading control and densitometry analysis was normalized to GAPDH. Results shown are representative images of experiments repeated at least three independent times.

3.2.4 The effect of SF on cancer cell migration and invasion of cervical squamous carcinoma cell lines.

Having observed the effect of SF on EMT induction, we next investigated the effect of SF on cancer cell migration and invasion in squamous carcinoma derived cell lines. As EMT has been linked to increased migration, we hypothesized that SF could enhance migration and invasion in cervical squamous carcinoma cells.

3.2.4.1 SF induces directional migration in cervical squamous carcinoma cells.

To determine the effect of SF on migration of squamous carcinoma cells a Transwell migration assay was used. This assay allows migratory cells to move from the upper chamber to the lower chamber via a 8μm pore size filter. Cells that have migrated are then stained and quantified. Since the effect of SF on migration was unknown, we placed SF (1:50) in different compartments of the chamber to monitor the effect of SF on cell migration. SiHa and Me180 cells were placed in the top chamber with SF in various compartments (top only, top & bottom and bottom only) and allowed to migrate for 16hrs. Cells were then fixed and stained after which images were taken of cells that migrated through (Figure 3.9A). Migrated cells were quantified. Results were normalized to viable cells as determined by an MTT viability assay. In SiHa cells, results show a 2-fold increase in migration when SF was placed in the top & bottom chamber and a 5-fold increase was observed when SF was placed in the bottom chamber relative to control (Figure 3.9B). Similar results were seen in Me180 cells where a 7-fold increase in migration was observed when SF was placed in the top & bottom and a 10-fold increase in migration was observed when SF was placed in the bottom chamber (Figure

3.9B). These results show that SF significantly enhances cell migration of SiHa and Me180 cells, acting as a chemoattractant towards which the cells moved.

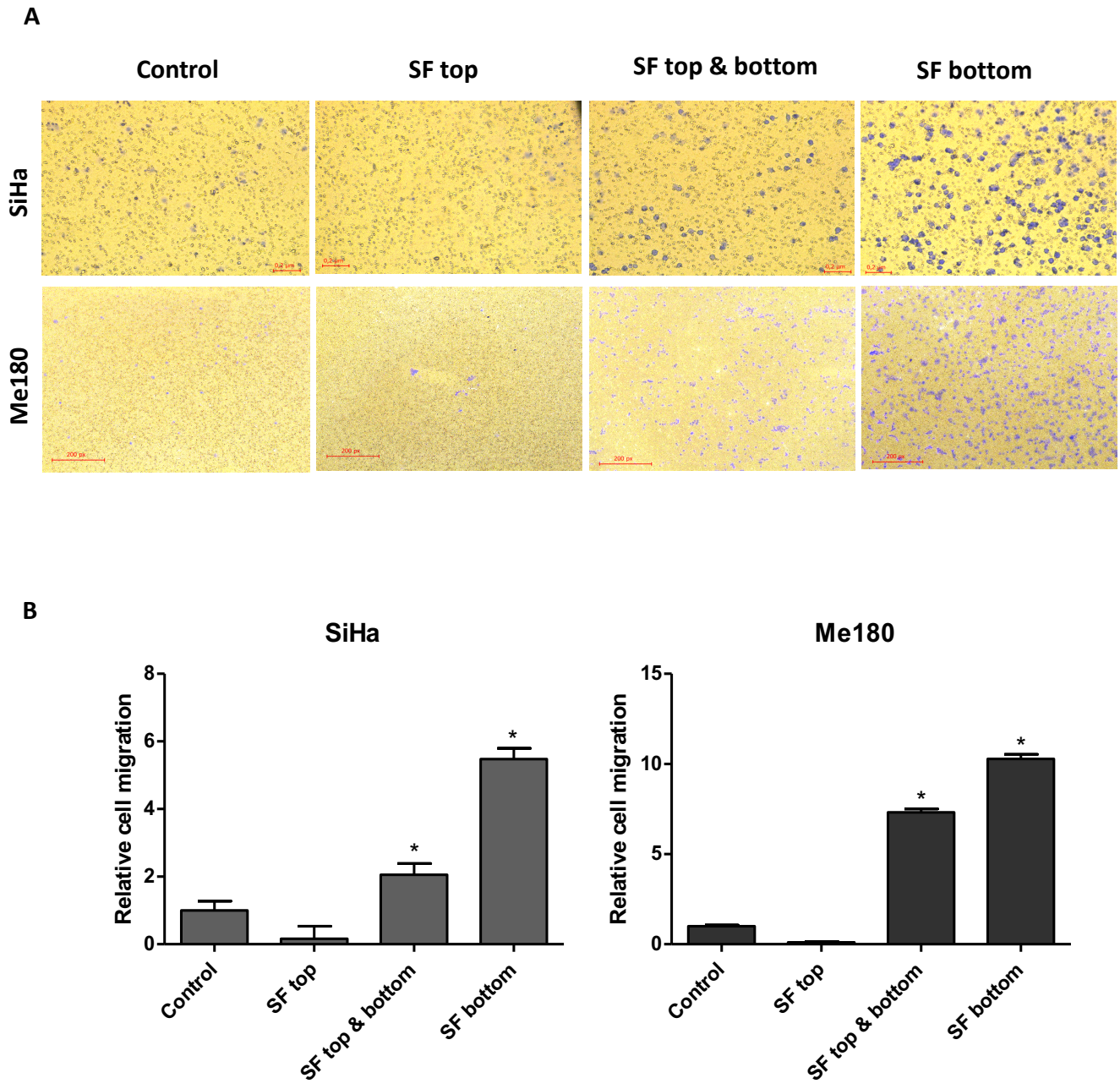


Figure 3.9. SF induces directional cell migration in cervical squamous carcinoma cells. A) Representative images from transwell migration chamber showing migrated SiHa and Me180 cells. Migrated cells shown in purple stain. Scale bar= 0,2μm, Magnification 100X. **B)** Quantification of SiHa and Me180 transwell migration assay relative to control and normalized to a MTT viability assay. Results shown are the mean± SEM of experiments performed in triplicate and repeated three independent times. (P<0.05).

3.2.4.2 SF stimulates directional invasion in cervical squamous carcinoma cells.

In order to investigate the effect of SF on cancer cell invasion, transwell migration inserts (8μm pore) were coated with 0.2% collagen-degraded gelatin[293] which acts as the extracellular basement membrane barrier. SiHa and Me180 cells were plated in the top chamber and SF placed in various compartments of the transwell migration system. Cells were allowed to invade over 16hrs after which cells were fixed, stained, and quantified (Figure 3.10A). Results were normalized to viable cells and quantified relative to control. The results show that SF enhanced cell invasion in SiHa and Me180 cells. In SiHa cells, a 5-fold increase was observed when SF was placed in the top & bottom chamber and a 10-fold increase was observed when SF was placed in the bottom chamber as compared to control (Figure 3.10B). Similarly, Me180 cells showed enhanced invasion of 10-fold (SF top & bottom) and 17-fold (SF bottom) as compared to control (Figure 3.10B). Interestingly, Me180 cells showed higher migration and invasion as compared to SiHa cells. Together these results suggest that SF enhances directional migration and invasion in SiHa and Me180 cells.

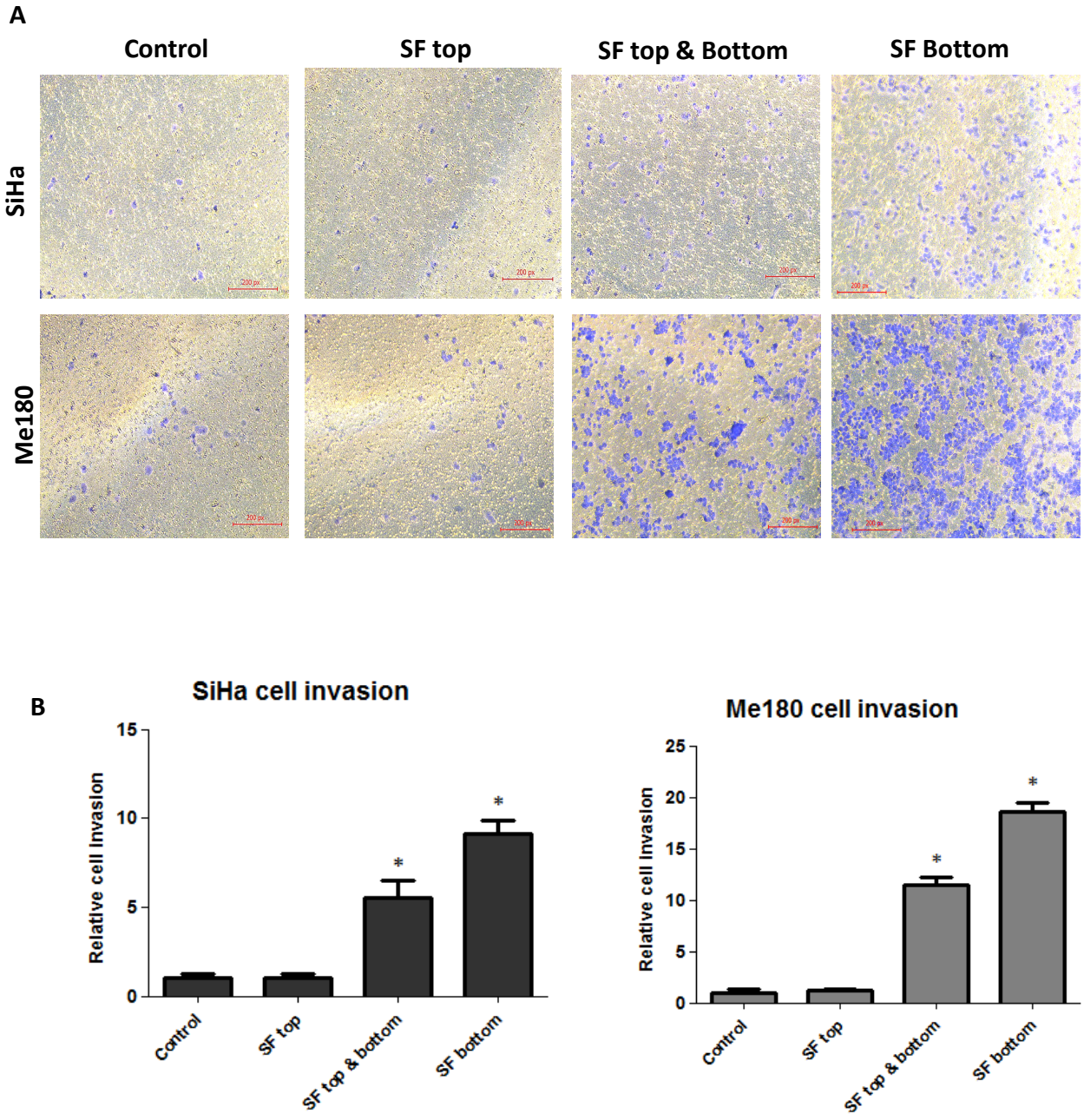


Figure 3.10 SF stimulates directional cell invasion in cervical squamous carcinoma cells. A) Representative images from transwell migration chamber coated with 0.2% gelatin showing invasion of SiHa and Me180 cells. Scale bar= 0,2μm, Magnification 100X. **B)** Quantification of transwell invasion assay relative to control and normalized to a MTT viability assay. Results shown are the mean± SEM of experiments performed in triplicate and repeated three independent times. (*P<0,05).

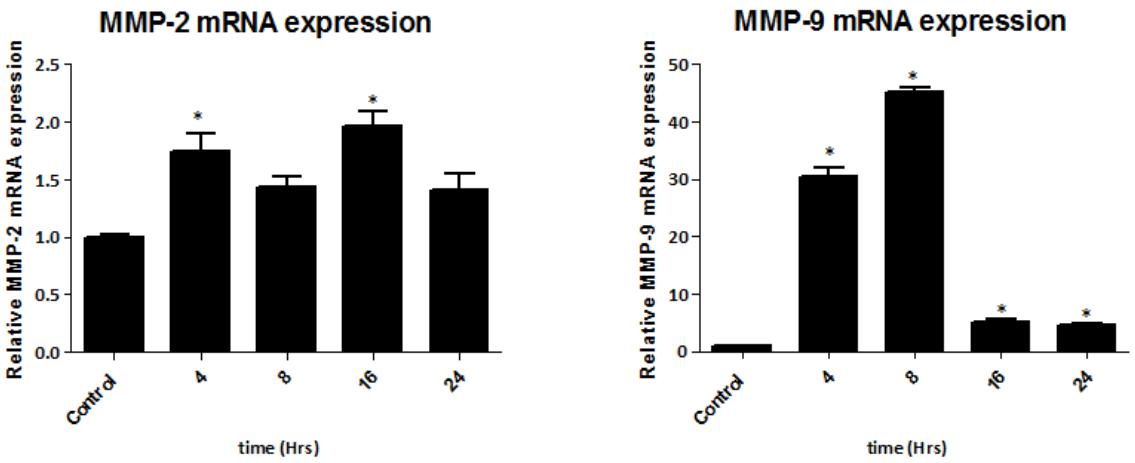
3.2.5 SF upregulates MMP-2 and MMP-9 mRNA expression in cervical squamous carcinoma cells.

MMPs are a family of proteolytic enzymes that play a role in extracellular matrix (ECM) degradation during invasion[310]. In addition to invasion, MMPs have also been implicated in cell proliferation, migration, angiogenesis and EMT[311].

Having observed the induction of invasion by SF, we next investigated the effect of SF on MMP-2 and MMP-9 (Gelatinases) mRNA expression. SiHa and Me180 cells were treated with vehicle or SF for 4, 8, 16 and 24hrs and expression of MMP-2 and MMP-9 was measured. Results showed that SF treatment significantly upregulated MMP-2 and MMP-9 mRNA expression in SiHa and Me180 cells. In SiHa cells, results showed that SF significantly induced MMP-2 mRNA expression at 4, 16 and 24hrs SF treatment in comparison to control, where peak MMP-2 mRNA expression was observed at 16hrs (Figure 3.11A). Similar to SiHa cells, SF treatment induced MMP-2 mRNA expression at 4, 16 and 24hrs SF treatment as compared to control in Me180 cells. Peak induction of MMP-2 mRNA expression was observed at 4hrs in Me180 cells (Figure 3.11B).

In addition to MMP-2 mRNA expression we investigated the mRNA expression of MMP-9. Results revealed a more significant induction of MMP-9 mRNA expression in both SiHa and Me180 cells. In SiHa cells, SF significantly induced MMP-9 mRNA expression at 4, 8, 16 and 24hrs SF treatment relative to control, with peak induction observed at 8hrs. In Me180 cells, SF induced MMP-9 mRNA expression at 4, 8, 16 and 24hrs, with peak induction observed at 8hrs (Figure 3.11B). Together these results show that SF treatment induces the expression of gelatinases MMP-2 and MMP-9 mRNA expression with SF treatment showing a stronger induction of MMP-9 in both SiHa and Me180 cells.

A



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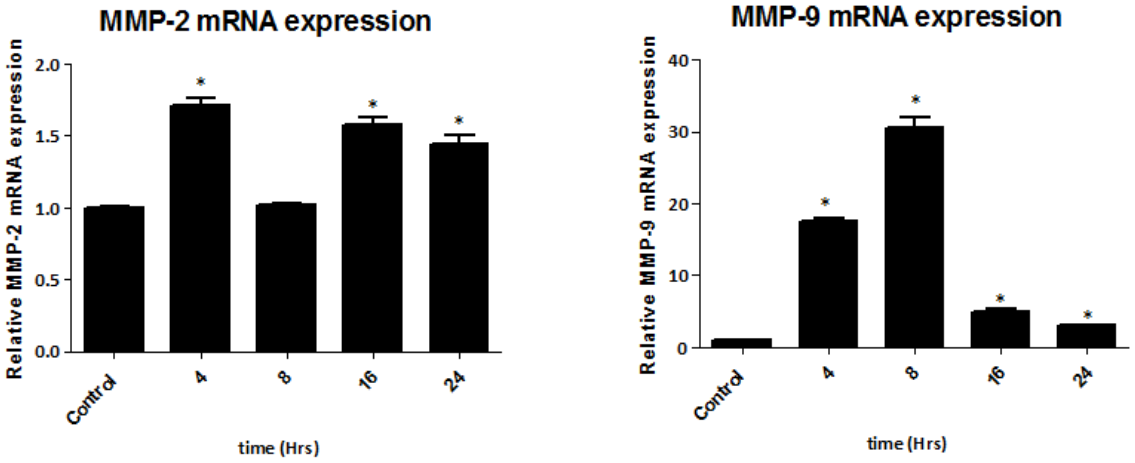


Figure 3.11 SF induces MMP-2 and MMP-9 mRNA expression in squamous carcinoma cell lines. Relative MMP-2 and MMP-9 mRNA expression in **A)** SiHa and **B)** Me180 cells treated with SF (1:50) for 4, 8 ,16 and 24hrs relative to control as determined by RT-qPCR. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Results shown as mean ± SEM of experiments performed in triplicate and repeated three independent times. (*P<0,05).

3.3 DICUSSION

It is now accepted that SF is not simply a medium for sperm transportation but also plays a role as a key regulator of cellular processes in the female reproductive tract (FMRT). This is due to signaling molecules present in SF that have been shown to interact with cells in the female reproductive tract, modulating the female immune response for successful implantation and embryo development[312,268]. Introduction of SF to the FMRT during coitus elicits strong molecular and cellular responses in the female tract resulting in an upregulation of proinflammatory cytokines and leukocyte recruitment[273,261]. These responses have been attributed to factors in SF since these immune responses were not observed in sexually active women using barrier contraceptive[261]. In addition to the immune response elicited by SF, neoplastic cervical cells in sexually active women will potentially be under direct stimulation of the bioactive molecules found in SF which could drive tumorigenic processes.

Indeed, previous research in our laboratory has shown that SF upregulates a host of proinflammatory cytokines and chemokines such as IL-1, IL-6, IL-8 and IL-11 in adenocarcinoma cervical cancer cells[273,278,279]. A study by Sharkey et al (2012), showed an upregulation of cytokines and chemokines in cervical tissue explants after SF stimulation[261]. SF was also shown to upregulate angiogenic chemokines IL-8 and growth regulated oncogene alpha (Gro-α) in HeLa cells and also regulated vasculature[278].This evidence highlights that SF may have a role in the biology of cervical cancer cells.

In this study, the effects of SF on cell proliferation, EMT, migration and invasion were investigated using two cervical squamous carcinoma epithelial cell lines as a model system. Our data showed that SF significantly enhances cervical squamous carcinoma cell proliferation.

This is in agreement with a previous study by Sutherland et al. (2012) where it was reported that SF enhances cervical adenocarcinoma cell proliferation. This study further showed that SF enhances proliferation *in vivo* using a xenograft model[277]. In a study using adenocarcinoma cells, it was reported that SF treatment significantly enhanced cell proliferation *in vitro*[280]. Interestingly, in a study that focused on the effects of SF on endometriosis, it was reported that SF significantly enhanced cell proliferation of tissue derived endometrial cells in women with endometriosis [313]. These findings suggest that bioactive molecules found in SF can enhance cell proliferation. Our data, as well as previous studies, provides further evidence that SF enhances cervical squamous and adenocarcinoma cell proliferation.

As previously discussed, metastasis is responsible for most cancer-related deaths and the acquisition of mesenchymal migratory cell phenotypes in cancer is the driving factor of motility[314]. In carcinomas, the induction of EMT has been linked to cells becoming motile and having increased invasive capability[64]. Our study revealed morphological changes of SiHa and Me180 cells and a significantly higher percentage of cells containing stress fibers in SF treated cells compared to controls. Traditionally, cell morphogenesis and actin remodeling occurs down-stream of the EMT program[315]. The Actin cytoskeleton plays a pivotal role in cell shape and migration and the cells transition from the epithelial phenotype to an elongated mesenchymal phenotype during EMT, requires actin rearrangement[79]. Indeed, this has been shown in a study by He et al. (2015), where it was reported that stimulation of SiHa cells with TGF-β induced morphological changes, F-actin stress fiber formation and upregulation of mesenchymal markers indicative of EMT induction[316]. In another study by Saito et al. (2020), overexpression of Aldolase A lead to EMT like morphological changes in cervical adenocarcinoma cells where cell morphology changes and stress fiber formation was also observed[317].

Therefore, our study shows that molecules in SF induce actin rearrangement which results in the formation of stress fibers and a change in cell morphology typical of the EMT program.

The EMT program is activated by various signaling pathways that converge to activate the transcription of EMT-TFs such as Snail, Twist or ZEB. The Snail and ZEB transcription factors directly inhibit E-cadherin, therefore suppressing the epithelial phenotype, whilst Twist indirectly inhibits E-cadherin and is a potent mesenchymal marker inducer[318]. Activation of any of these transcription factors drives EMT. In our study, data revealed a significant upregulation of transcription factors associated with EMT including Snail, Twist and ZEB1 after SF treatment. A study by Ibrahim et al (2018) showed some similar findings where 10% seminal plasma induced the mRNA expression of Snail which was independent of TGFβ1 in endometrial tissue and endometrial cells, though statistically insignificant. They showed a significant upregulation of ZEB2 after 6hrs of 10% seminal plasma stimulation of endometrial tissue. Interestingly, Twist mRNA expression was persistently down regulated in endometrial tissue and cells treated by 10% seminal plasma, though insignificant[319]. In a study by Ji et al (2020), TGFβ was shown to induce Snail, Twist and Zeb1 mRNA expression after 24hrs in CaSki cells. While our study showed no significant upregulation of Snail, Twist and Zeb1 at 24hrs SF treatment, the induction of Snail and Twist by TGFβ in the study by Ji et al (2020) shows a slightly higher mRNA upregulation while Zeb1 upregulation is in a similar range to our results with SF[320].

In our study, SF treatment slightly reduced E-cadherin protein expression in SiHa and Me180 cells. N-cadherin protein levels were slightly elevated at 24hrs SF treatment for SiHa cells and a marked time dependent increase in protein expression was observed in Me180 cells. In

addition, Me180 untreated cells showed barely any N-cadherin expression as compared to SiHa untreated cells. This observation could be attributed to cell line specific effects. Similar observations of differential N-cadherin protein expression in SiHa and Me180 cells has been shown by Wang et al (2018)[321] and Zhou et al (2020)[322]. In cancers, during the EMT program, N-cadherin upregulation and E-cadherin repression also known as the "cadherin switch" has been associated with enhanced migration and invasion that associates with poor patient survival[323–325]. Our findings demonstrate that SF induces EMT in cervical squamous carcinoma cells which could attribute to the increased migration and invasion observed for both SiHa and Me180 cell lines.

Research has shown that tumor cells can acquire the ability to sense and migrate towards chemokines or growth factors (chemotactic/directional migration)[326]. Our results showed that SF induced directional cell migration and invasion in cervical squamous carcinoma cells. This directional cell migration and invasion has been shown in other models of carcinoma. A study by Biswenger et al (2018) showed that EGF induced directional migration of MDA-MB-231 cells[327]. In a study using colorectal cancer cells, Lysophosphatidylserine was shown to stimulate directional migration via the PI3K/Akt pathway[328]. Therefore, in our study, it is likely that components in SF may cause cervical squamous carcinoma cells to migrate and invade towards SF.

For cancer cells to invade they need to pass through the dense extracellular matrix (ECM). Cancer cells require the proteolytic activity of ECM modifying enzymes to invade surrounding tissue. Our study shows that SF induces MMP-2 and MMP-9 mRNA expression in both SiHa and Me180 cells. In a study using adenocarcinoma cells, SF (1:50) treatment was shown to

inhibit MMP-2 but significantly stimulated MMP-9 mRNA expression[329]. In a study by Guo et al (2019), it was reported that SF significantly induced the protein expression of MMP-9 in HeLa cells[280]. Though differences in MMP-2 induction by SF have been reported, our result as well as others show that SF upregulates MMP-9 in adenocarcinoma and squamous carcinoma cell lines. Our results also showed a rapid induction of MMP-9 at 4-8hrs SF treatment which subsided at 16hrs. This SF-induced rapid induction followed by a dramatic drop is not uncommon for SF and has been previously shown in other genes such as IL-8, VEGF and COX-2 after 8hrs SF treatment in cervical adenocarcinoma cells[273,330].

In summary, this study reports that SF promotes cervical squamous carcinoma cell proliferation, actin organization, EMT, migration and invasion. Our results show that SF plays a role in enhancing the migratory and invasive potential of cervical squamous carcinoma cells and that this associates with the induction of EMT. These findings together implicate SF as a possible factor that may drive migration and invasion in invasive cervical cancer in sexually active women. Given the fact that SF is rich in a host of signaling molecules, elucidating the mechanism of SF induced proliferation, EMT and migration would be beneficial in the understanding of the effects of SF on tumorigenesis in sexually active women.

Chapter 4:

Signaling pathways involved in SF mediated proliferation, EMT and migration in cervical squamous carcinoma cells

4.1 INTRODUCTION

Research in the past few decades identified SF as an important regulatory component needed to promote reproductive success. The cervix is the first site that interacts with SF in the female reproductive tract (FMRT) during coitus[331]. Evidence has shown that the bioactive molecules in SF interact with the epithelial cells and immune cells in the mucosal lining of the FMRT. This occurs when the bioactive molecules of SF bind to receptors of target cells and subsequently initiate signal transduction pathways that activate direct, immediate effects on the cells or induce delayed response through gene expression[256]. Exposure of cervical cells to SF stimulates various gene expression that play a role in cytokine signaling, inflammation, antigen presentation and leukocyte migration[331]. The signaling transduction induced by SF in cervical epithelial cells plays a pivotal role in fertilization however, extend beyond fertility because these agents may also induce signaling pathways in neoplastic cervical cells.

Previous work in our laboratory demonstrated that SF induced the cyclooxygenase (COX)-PG pathway through the upregulation of both COX-1 and COX-2 as well as the upregulation of the E-series prostaglandin receptors (EP1, EP3 and EP4)[276]. The role of cyclooxygenases and prostaglandins in cancer related inflammation has been extensively studied in various cancers including cervical cancer[332]. The cyclooxygenases exist in two main isoforms namely, COX-1 and COX-2. A number of studies have shown the aberrant expression of COX-2 in several

cancers[333]. Unlike, COX-2, the role of COX-1 in cancer is not well described as yet. Known to be constitutively expressed in various cell types, COX-1 was known to play a role in homeostatic cell function[334]. However, increasing evidence has shown that COX-1 expression is elevated in cancers including breast [335] and prostate cancer[336]. A previous study in our laboratory showed that both COX-1 and COX-2 expression was elevated in squamous carcinoma and adenocarcinoma cells of the human cervix[253]. This evidence suggests that both COX isoforms may have a role in cervical tumorigenesis.

In addition to the COX pathway, SF has been shown to activate other signaling pathways that have been implicated in tumorigenesis. In a previous study by Muller et al. (2006), it was shown that SF activates the ERK1/2 pathway via the EGFR and EP4 receptors and that this activation leads to COX-2 and VEGF expression[273].ERK1/2 is a kinase that belongs to the mitogen-activated protein kinases (MAPK) family. In tumorigenesis, the ERK1/2 signaling pathway is known to play a role in survival and proliferation[337]. In the non-oncogenic state MAPK is tightly regulated to ensure activation and de-activation of ERK1/2 to meet requirements of cell growth and morphology[338]. However, in cancer the dysregulation of the MAPK cascade results in a chronically activated ERK1/2 that persistently stimulates aberrant cell growth[103]. The role of ERK1/2 in regulating cell growth and differentiation has been shown; however, a number of studies have also implicated ERK1/2 in cell migration, invasion and metastasis[337,339].

With human SF being a complex biological fluid rich in array of bioactive molecules such as PGE2, EGF, TGF-β and various cytokine and chemokines[331]; it is no surprise that its interaction with receptors on target cells leads to the activation of various signaling pathways in the

FMRT. Extensive research in our laboratory has identified oncogenic signaling pathways activated by SF in cervical adenocarcinoma cells. In this study we aim to investigate whether COX-1, COX-2, EGFR, and ERK1/2 signaling pathways play a role in SF mediated cell proliferation, actin cytoskeletal rearrangement, EMT and migration. This was achieved through the use of small molecule inhibitors of COX-1 (SC560), COX-2 (NS398), EGFR (AG1478) and ERK1/2 (PD98059) as well as, E-series prostaglandin receptor antagonists that were used to monitor SF-induced biological effects.

4.2 RESULTS

4.2.1 SF induced cell proliferation in cervical squamous carcinoma cell lines is mediated via COX-1 and ERK pathways

Having established that SF significantly enhances cell proliferation at 72hrs in squamous carcinoma cell lines, next we investigated the signal transduction pathways required for SF induced proliferation using a panel of small molecule inhibitors. Briefly, cells were plated in a 96-well plate in triplicate, media was removed after 24hrs and cells were treated with SF or vehicle in the absence or presence of chemical inhibitors of COX-1 (SC560), COX-2 (NS398), EGFR (AG1478) and ERK1/2 (PD98059) in 0.1% FBS DMEM media. Cell viability was measured using the MTT Assay. Absorbance readings for SF in the presence of chemical inhibitors were normalized to absorbance readings of the chemical inhibitors only and plotted relative to control.

Our results show that SF significantly induced cell proliferation after 72hrs as compared to control. The COX-1 inhibitor (SC560) and ERK1/2 inhibitor (PD98059) significantly inhibited SF induced cell proliferation in both SiHa and Me180 cell lines. Incubation of SF and selective COX-2 (NS389) and EGFR (AG1478) inhibitors had no inhibitory effect in both SiHa and Me180 cell lines (Figure 4.1.A, B). These results suggest that SF mediated cell proliferation requires the activation of the COX-1 and ERK1/2 pathways in cervical squamous carcinoma cell lines.

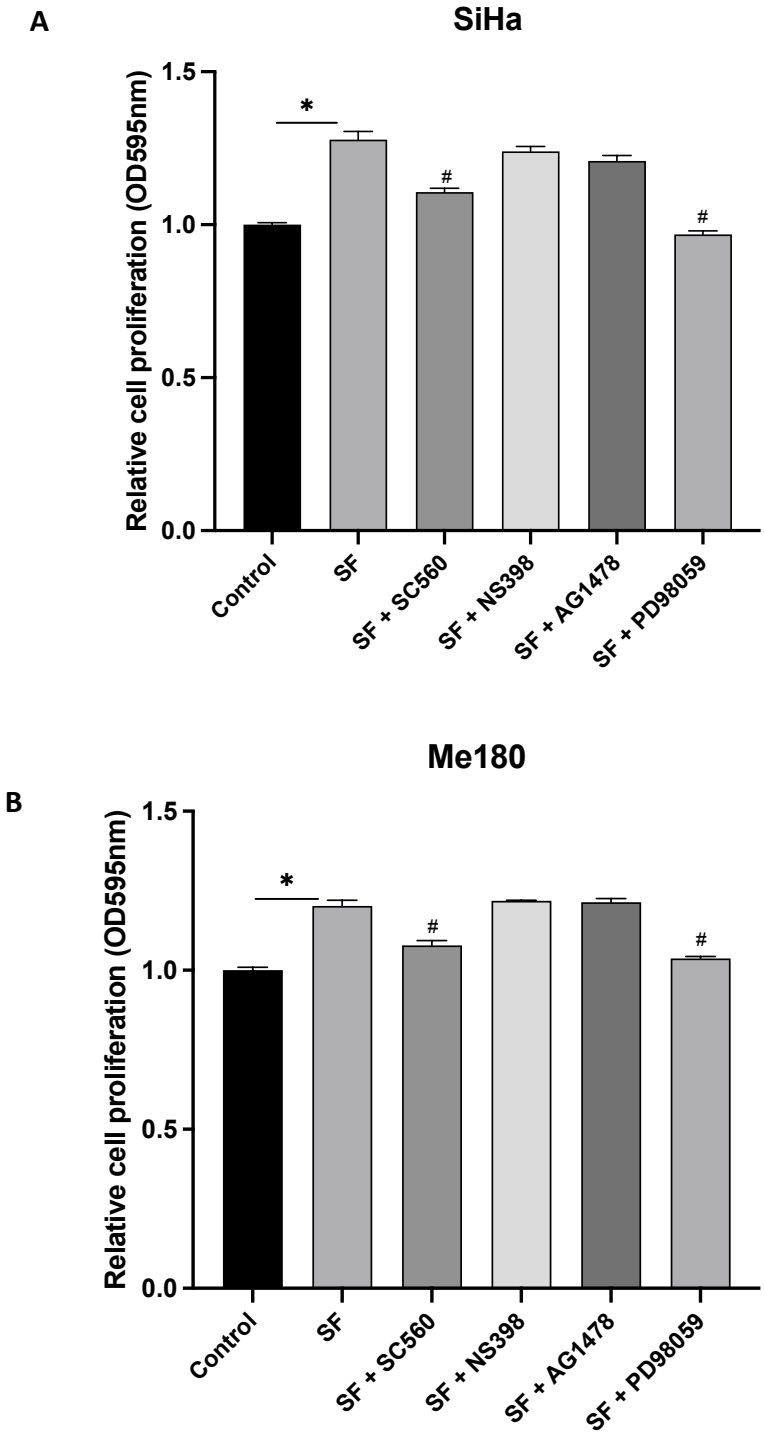


Figure 4.1. SF induced cell proliferation is mediated via the COX-1 and ERK1/2 signaling pathways in cervical squamous carcinoma cells. A) SiHa and **B)** Me180 cells were treated with SF (1:50) or control (DMSO) in the presence/absence of chemical inhibitors to COX-1 [SC560, 5μM], COX-2 [NS398, 5μM], EGFR [AG1478, 100nM] or ERK1/2 [PD98059, 25μM]. Proliferation was monitored after 72hrs. Results shown are the mean ±SEM of experiments performed in triplicate and repeated three independent times. ($^{\#}$or*P<0,05). *=Control vs SF and #= SF compared to SF + inhibitors.

4.2.2 SF mediated actin cytoskeleton rearrangement is mediated via the EGFR, ERK1/2 and COX-1 signaling pathways

The actin cytoskeleton is a dynamic structure that is essential in various cellular functions such as cellular trafficking, EMT, motility and invasion[340]. The rearrangement of the actin cytoskeleton is evident in the EMT process. Research has shown that the cortical organization of actin filaments is characteristic of epithelial cells whereas actin stress fibers are found in more mesenchymal cells[341]. This actin rearrangement in cancer cells can be activated by various signaling pathways.

In this study we investigated the signaling pathways required for the SF mediated mesenchymal morphological change and stress fiber formation. SiHa and Me180 cells were seeded on coverslips and serum starved for 24hrs. The following day, cells were treated with SF or vehicle in the presence of chemical inhibitors to COX-1 (SC560), COX-2 (NS398), EGFR (AG1478) and ERK1/2 (PD98059) for 24hrs after which cells were fixed and stained with Phalloidin to visualize filamentous actin (F-actin) and Hoechst stain to visualize the nucleus. Immunofluorescent images were captured for SiHa (Figure 4.2A) and Me180 (Figure 4.3A) cells and scored for stress fibers.

In SiHa cells, the COX-1 inhibitor (SC560) almost completely inhibited SF-induced morphological changes (Figure 4.2A). Quantification of the percentage of cells with stress fibers showed a marked reduction of cells with stress fibers in cells treated with the COX-1 inhibitor (SC560, 83% reduction) as compared to SF only (Figure 4.2B). In cells treated with the EGFR inhibitor (AG1478) or the ERK1/2 inhibitor (PD98059), immunofluorescent images showed a combination of both cobble- stone and spindle-like (mesenchymal) cells. Quantification of cells with stress fibers showed a reduction of cells containing stress fibers in cells treated with

AG1478 (45% reduction) or PD98059 (50% reduction) as compared to SF only. Addition of the COX-2 inhibitor (NS398) to SF stimulated cells had no effect on SF-induced morphological changes and stress fiber formation (Figure 4.2B). Similar results were observed in Me180 cells, where treatment with the COX-1 inhibitor (SC560) blocked SF induced cell morphology changes (Figure 4.3A) and stress fiber formation (92% reduction) as compared to SF only. A mixed morphology was observed in cells treated with the EGFR inhibitor (AG1478) or the ERK1/2 inhibitor (PD98059) with a subsequent reduction in the percentage of cells with stress fibers in AG1478 (34.7% reduction) and PD98059 (35% reduction) treated cells as compared to SF only. Similarly, treatment with the COX-2 inhibitor (NS398) showed no inhibitory effect on SF mediated cell morphology and stress fiber formation (Figure 4.3B). No alteration in cell morphology and stress fiber formation was observed between control cells and cell treated with the chemical inhibitors in the absence of SF (Appendix II). These results demonstrate that SF mediated actin cytoskeleton rearrangement requires the activation of the EGFR, ERK1/2 and COX-1 signaling pathways but does not require the COX-2 pathway.

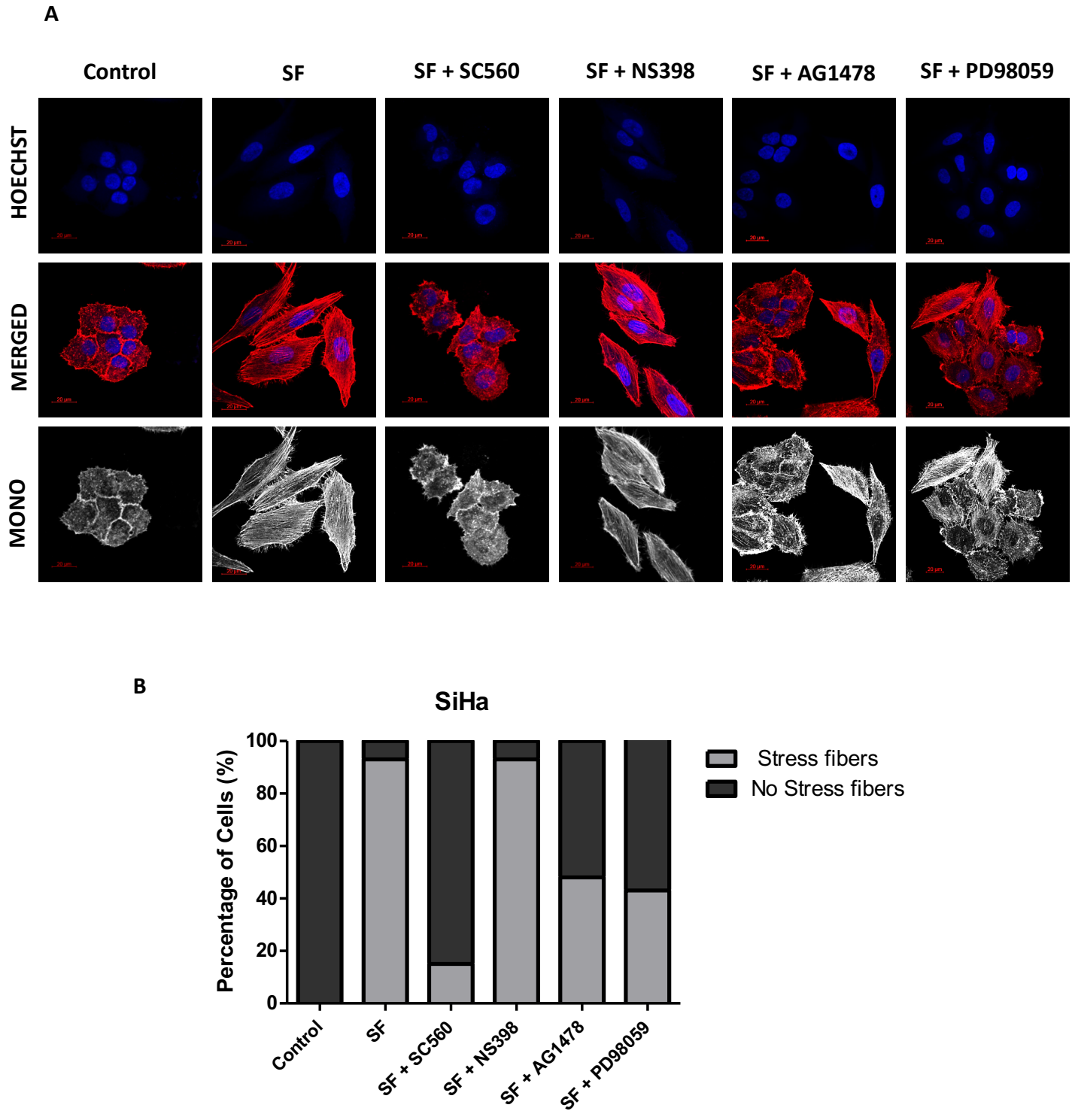
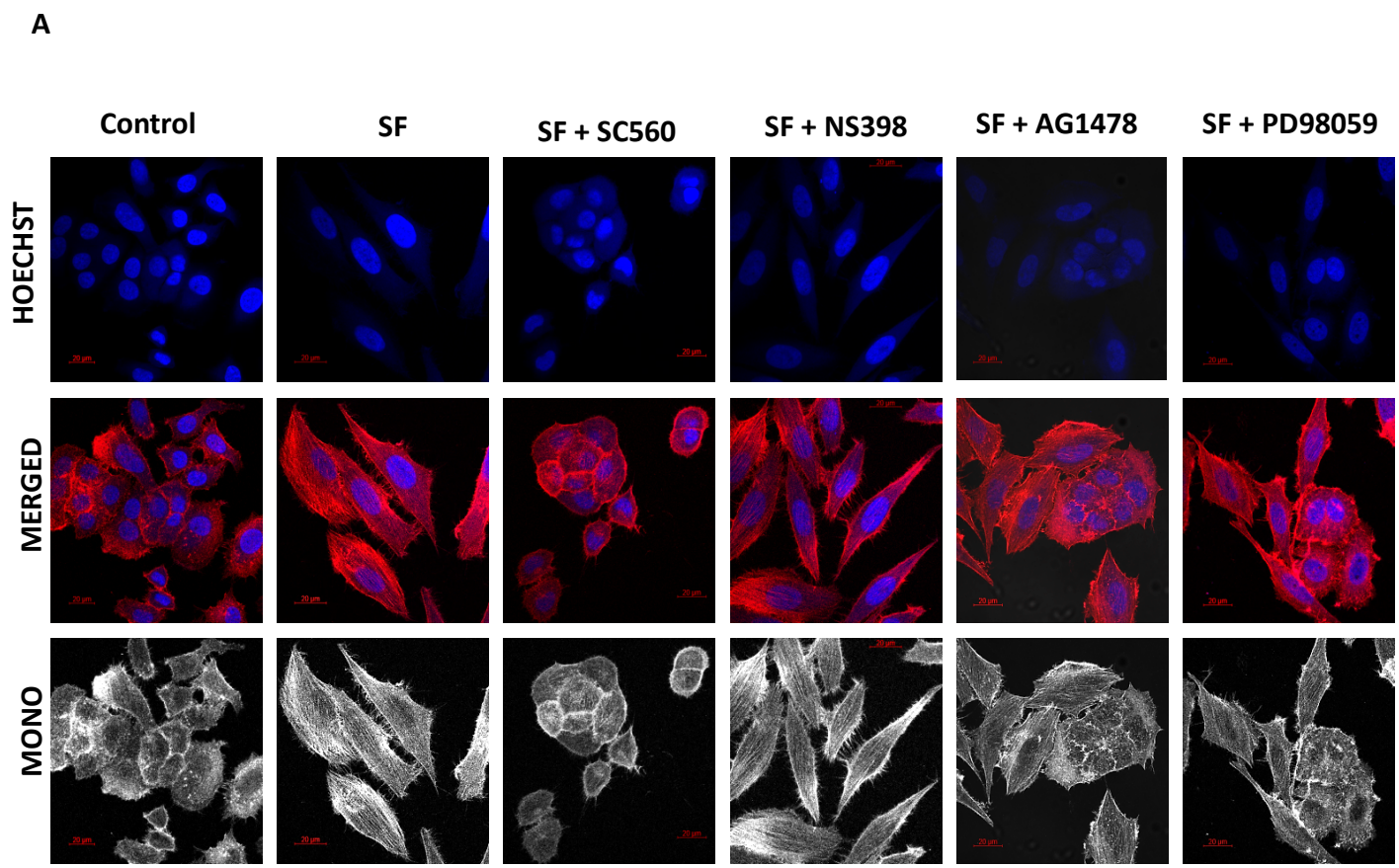


Figure 4.2. SF induced actin cytoskeleton rearrangement is mediated via the EGFR, ERK1/2 and COX-1 signaling pathways in SiHa cells. A) Representative immunofluorescent images of SiHa cells stained with Phalloidin (cy3) for F-actin and Hoechst (blue) for the nuclei following treatment with SF (1:50) or control (DMSO) in the presence of chemical inhibitors to COX-1 [SC560, 10μM]; COX-2 [NS398, 10μM]; EGF receptor [AG1478, 200nM] and ERK1/2 [PD98059,50μM] for 24hrs. Scale bar= 20μm, Magnification= 630X. **B)** Quantification of the percentage of cells with stress fibers per treatment condition (300 cells were scored per condition). Experiments were performed three independent times.



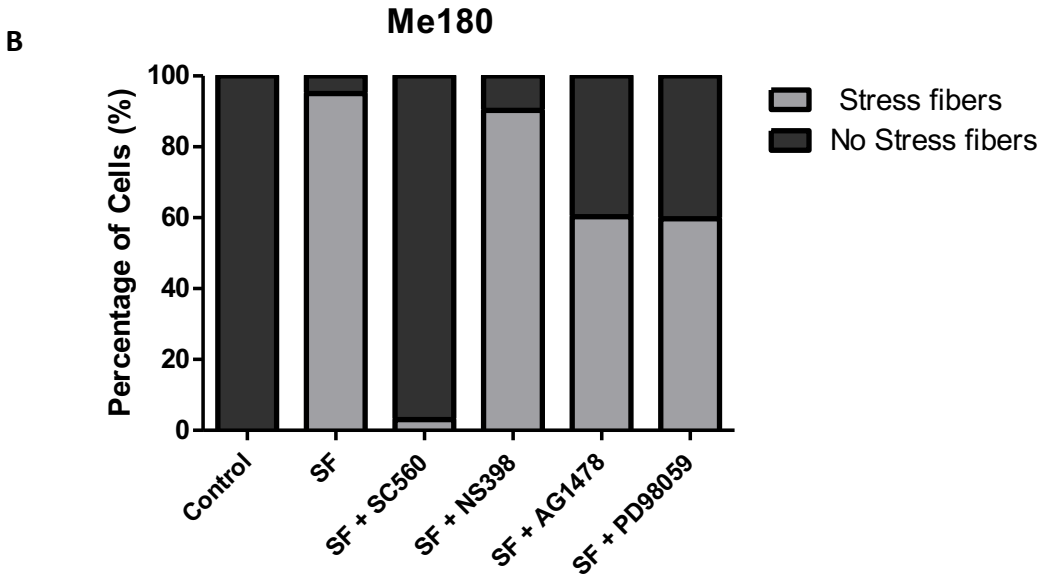


Figure 4.3. SF induced actin cytoskeleton rearrangement is mediated via the EGFR, ERK1/2 and COX-1 signaling pathways in Me180 cells A) Representative immunofluorescent images of Me180 cells stained with Phalloidin (cy3) for F-actin and Hoechst (blue) for the nuclei following treatment with SF (1:50) or control (DMSO) in the presence of chemical inhibitors to COX-1 [SC560, 10μM]; COX-2 [NS398, 10μM]; EGF receptor [AG1478, 200nM] and ERK1/2 [PD98059,50μM] for 24hrs. Scale bar= 20μm, Magnification= 630X. **B)** Quantification of the percentage of cells with stress fibers per treatment condition (300 cells were scored per condition). Experiments were performed three independent times.

4.2.3 Investigating the signal transduction pathways associated with SF induced EMT transcription factors Snail, Twist or ZEB1 in squamous carcinoma cells

The EMT program is orchestrated through the activation of key EMT transcription factors (EMT-TFs) of the Snail, Twist and ZEB families. The Snail and ZEB transcription factors directly inhibit E-cadherin whilst the Twist transcription factor indirectly inhibits E-cadherin but is known as a potent mesenchymal marker inducer[318]. The activation of any one of these transcription factors drives EMT through the repression of epithelial markers and the upregulation of mesenchymal markers[342].Various signaling pathways can activate EMT including TGF-β, EGFR, Notch, MAPK and Wnt signaling, among others[318,343,344]. Earlier results showed that SF induced the mRNA expression of the EMT-TFs Snail and ZEB1 at 4hrs and Twist at 8hrs. Therefore, in this study, we investigate which signaling pathways are involved in SF mediated Snail, Twist and ZEB1 upregulation.

4.2.3.1 SF induced Snail mRNA expression involves the COX-1 and ERK1/2 signaling pathways

In order to investigate the signaling pathways mediating SF induced Snail mRNA expression, SiHa and Me180 cells were pre-treated with inhibitors of COX-1 (SC560), COX-2 (NS398), EGFR (AG1478) or ERK1/2 (PD98059) for 1hr followed by stimulation with SF or vehicle (DMSO) for 4hrs and gene expression analysed by RT-qPCR. In both SiHa and Me180 cells a significant difference was observed for the inhibitor only control as compared to the control (Figure 4.4. A, B). To correct for the response of the chemical inhibitors, percentage inhibition for each chemical inhibitor was calculated by comparing the inhibition by the inhibitor only with the control(un-treated) and the effect of inhibitor in the presence of SF in comparison

to SF stimulation (see Equation 1). In SiHa cells, results showed that SF induced Snail upregulation was inhibited by COX-1 (SC560) and ERK1/2 (PD98059) inhibitors where the percentage inhibition was 27% and 19%, respectively, which was statistically significant (Table 4.1). Treatment with COX-2 (NS398) or EGFR (AG1478) inhibitors showed no inhibition of SF mediated Snail upregulation. In Me180 cells, treatment with SC560 blocked SF mediated Snail upregulation by 79%. In addition, treatment with PD98059 inhibited SF induced Snail upregulation with an inhibition of 14%, which was statistically significant. Similar to SiHa cells, treatment with COX-2 (NS398) and EGFR (AG1478) inhibitors had no effect on SF mediated Snail upregulation in Me180 cells (Table 4.1). In particular, Me180 cells showed a higher sensitivity to the COX-1 inhibitor in comparison to SiHa cells. Taken together these results suggest that SF induced Snail upregulation in SiHa and Me180 cells involves the COX-1 and ERK1/2 signaling pathways and do not involve COX-2 and EGFR pathways.

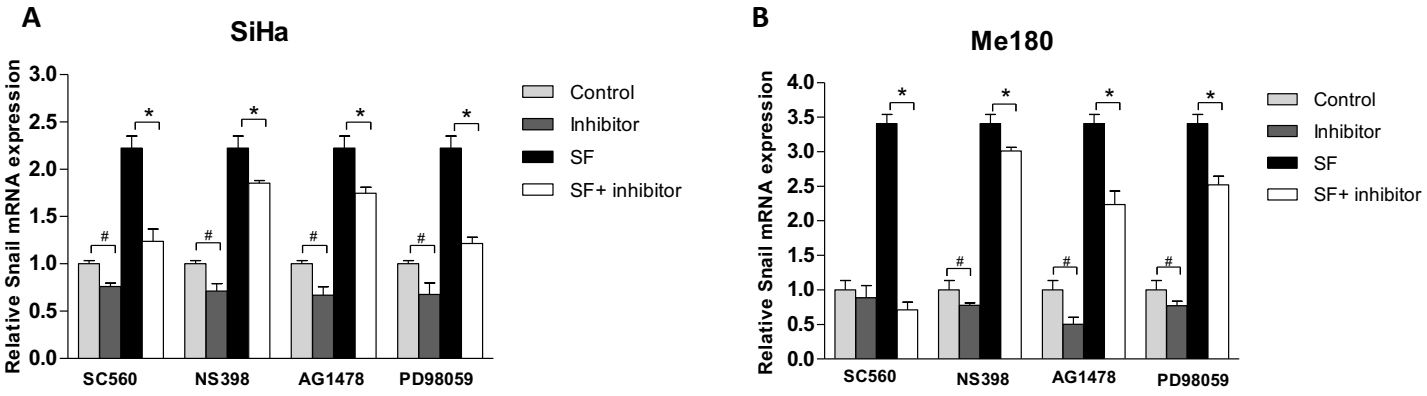


Figure 4.4. SF induced Snail upregulation involves the COX-1 and ERK1/2 signaling pathways. A) SiHa and **B)** Me180 cells were pre-treated with inhibitors SC560 [10µM], NS398 [10µM], AG1478 [200nM] or PD98059 [50µM] for 1hr followed by stimulation with SF (1:50) or control (DMSO) for 4hrs. Chemical inhibitor in the absence of SF were included as controls. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Data are represented as mean ± SEM from three independent experiments. (*P<0,05).

Table 4.1: Percentage inhibition of SF induced Snail mRNA expression by chemical inhibitors (taking into account the chemical inhibitor only effects)

Chemical inhibitors	SiHa Percentage Inhibition	Me180 Percentage inhibition
SC560	27%	79%
NS398	no inhibition	no inhibition
AG1478	no inhibition	no inhibition
PD98059	19%	14%

Equation 1: $$\%\,\boldsymbol{Inhibition} = \left[\left(\mathbf{1} - \left(\frac{\boldsymbol{Inhibitor+SF}}{\boldsymbol{SF}}\right)\right) \times \mathbf{100}\right] - \left[\left(\mathbf{1} - \left(\frac{\boldsymbol{Inhibitor}}{\boldsymbol{Control}}\right)\right) \times \mathbf{100}\right]$$

4.2.3.2 SF induced Twist mRNA expression involves the COX-1 and ERK1/2 signaling pathways

To investigate which signaling pathways are associated with SF induced Twist mRNA expression, SiHa and Me180 cells were pre-treated with the inhibitors of COX-1 (SC560), COX-2 (NS398), EGFR (AG1478) or ERK1/2 (PD98059) for 1hr followed by an 8hr stimulation with SF or vehicle control. Similarly, percentage inhibition was calculated to correct for the inhibition of the chemical inhibitors on their own (Figure 4.5A, B). In SiHa cells, the results showed that SF mediated Twist upregulation was significantly inhibited by SC560 and PD98059 with an inhibition of 40% and 37%, respectively (Table 4.2). Similar to Snail mRNA results, SF mediated Twist upregulation was unaffected by treatment with inhibitors of COX-2 (NS398) or EGFR (AG1478). In Me180 cells, SF mediated Twist upregulation was significantly inhibited by treatment with the COX-1 inhibitor (SC560) with an inhibition of 50%. In addition, SF mediated Twist upregulation was inhibited by treatment with PD98059, showing an inhibition of 15%, which was statistically significant. Treatment with NS398 or AG1478 showed no inhibition of SF mediated Twist induction in Me180 cells (Table 4.2). Taken together, our results show that SF induced Twist upregulation in SiHa and Me180 cells is

mediated via the COX-1 and ERK1/2 signaling pathways and do not involve the COX-2 and EGFR pathways.

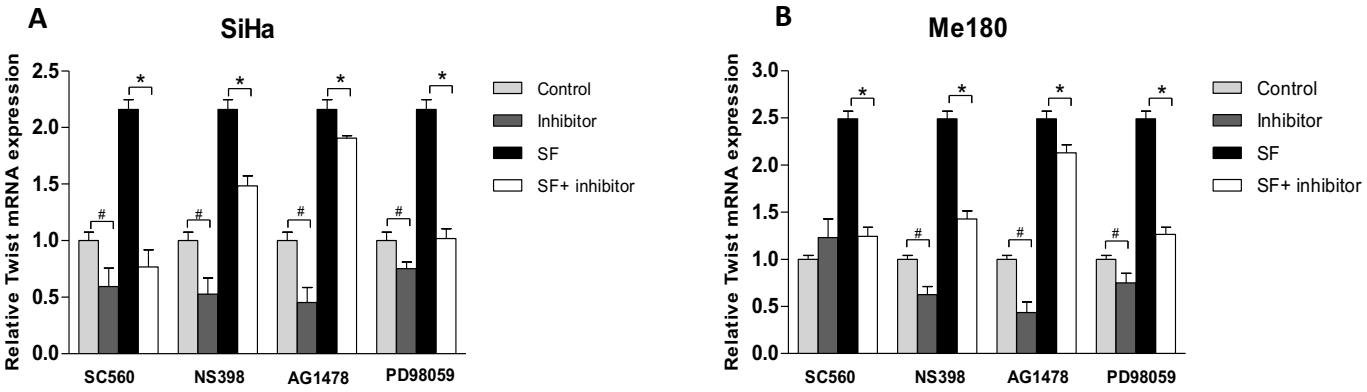


Figure 4.5. SF induced Twist upregulation is mediated via COX-1 and ERK1/2 signaling pathways. A) SiHa and **B)** Me180 cells were pre-treated with inhibitors SC560 [10µM], NS398 [10µM], AG1478 [200nM] or PD98059 [50µM] for 1hr followed by stimulation with SF (1:50) or control (DMSO) for 8hrs. Chemical inhibitor in the absence of SF were included as controls. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Data are represented as mean ± SEM from three independent experiments. (*P<0,05).

Table 4.2: Percentage inhibition of SF induced Twist mRNA expression by chemical inhibitors (taking into account the chemical inhibitor only effects)

Chemical inhibitors	SiHa Percentage Inhibition	Me180 Percentage inhibition
SC560	40%	50%
NS398	no inhibition	no inhibition
AG1478	no inhibition	no inhibition
PD98059	37%	15%

4.2.3.3 SF induced ZEB1 mRNA expression involves the COX-1 or COX-2, EGFR and ERK1/2 signaling pathways

To identify the signal transduction pathways involved in SF mediated ZEB1 upregulation, SiHa and Me180 cells were pre-treated with chemical inhibitors for 1hr followed by stimulation with SF or vehicle for 4hrs and gene expression was analysed by RT-qPCR. Similar to the other EMT transcription factors, our results show that the chemical inhibitors in the absence of SF had an inhibitory effect on ZEB1 mRNA expression, therefore percentage inhibition was calculated to correct for the inhibition of the chemical inhibitors on their own (Figure 4.6.A, B). In SiHa cells, SF meditated ZEB1 upregulation was inhibited by treatment with COX-1 (SC560) and EGFR (AG1478) inhibitors at 69% and 49% respectively. In addition, upregulation of ZEB1 induced by SF was significantly inhibited by inhibitors of COX-2 (NS398) and ERK1/2 (PD98059). Inhibition quantification showed a marked inhibition of 98% for NS398 and an inhibition of 11% for PD98059 which was statistically significant (Table 4.3). In contrast, Me180 cells treated with SC560 showed a minor inhibition of SF mediated ZEB1 upregulation with an inhibition of 5% however, treatment with the COX-2 (NS398) and EGFR (AG1478) inhibited SF mediated ZEB1 upregulation with inhibitions of 74% and 85% respectively. Furthermore, SF induced ZEB1 mRNA expression was inhibited by treatment with PD98059 showing an inhibition of 9%, which was statistically significant (Table 4.3). Taken together our results suggest that SF induced ZEB1 upregulation is mediated via EGFR, COX-1 or COX-2 signaling pathways and may involve ERK1/2 signaling to a less extent. Common amongst all the investigated EMT transcription factors is the involvement of either COX-1 or COX-2 and ERK1/2 signal transduction pathways. Therefore, we further investigated the possible role of these signaling pathways in SF-mediated EMT.

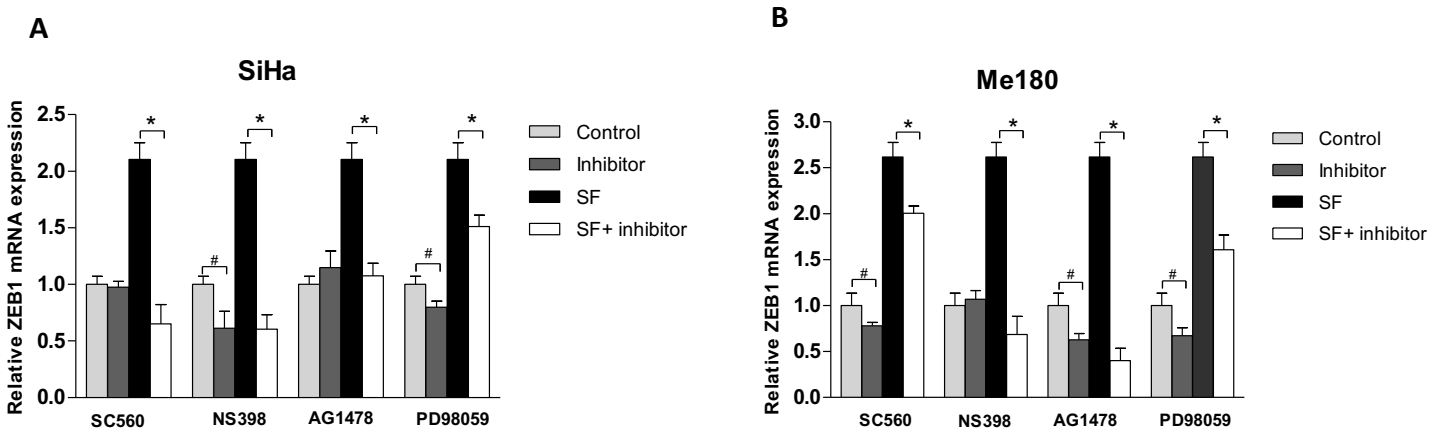


Figure 4.6. SF induced ZEB1 upregulation is mediated via COX-1 and ERK1/2 signaling pathways. A) SiHa and **B)** Me180 cells were pre-treated with inhibitors SC560 [10µM], NS398 [10µM], AG1478 [200nM] or PD98059 [50µM] for 1hr followed by stimulation with SF (1:50) or control (DMSO) for 4hrs. Chemical inhibitor in the absence of SF were included as controls. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Data are represented as mean ± SEM from three independent experiments. (*P<0,05).

Table 4.3: Percentage inhibition of SF induced ZEB1 mRNA expression by chemical inhibitors (taking into account the chemical inhibitor only effects)

Chemical inhibitors	SiHa Percentage Inhibition	Me180 Percentage inhibition
SC560	69%	5%
NS398	98%	74%
AG1478	49%	85%
PD98059	11%	9%

4.2.4 SF induced Snail, Twist and ZEB1 upregulation is mediated via the EP4 receptor

COX enzymes with PG synthase enzymes are known to catalyze the rate limiting step in the conversion of arachidonic acid to prostaglandins such as PGE2[345]. PGE2 then initiates its effects through autocrine/paracrine signaling via the E-series receptors (EP1-EP4)[346]. Interestingly, several studies have shown that EP receptors, particularly EP2 and EP4, are upregulated in cervical and endometrial carcinomas implicating them in carcinogenesis[253,248,347,348]. Previous research from our laboratory elucidated a role for PGE2 signaling via EP2 or EP4 by SF in cervical adenocarcinoma cells[279,273]. Having observed the inhibition of SF mediated EMT-TFs upregulation by either COX-1 or COX-2 inhibitors, it was hypothesized that the activation of Snail, Twist or ZEB1 could be occurring via the EP2 or EP4 receptors.

To determine a role of EP2 or EP4 in SF mediated EMT signaling, SiHa and Me180 cells were pre-treated with EP2 antagonist (AH9809) or EP4 antagonist (L-161.982) for 1hr followed by stimulation with SF or vehicle for 4hrs (Snail and ZEB1) or 8hrs (Twist) after which RT-qPCR was used to investigate Snail, Twist and ZEB1 mRNA expression. In both SiHa and Me180 cells, no inhibitory effect on SF induced Snail, Twist and ZEB1 induction in the presence of the EP2 receptor antagonist (AH9809) was observed (Figure 4.7A, B). However, in both SiHa and Me180 cells SF induced Snail, Twist and ZEB1 mRNA expression was significantly blocked by treatment with the EP4 antagonist (L-161.982) (Figure 4.8A, B). Together these results reveal that SF induced Snail, Twist and ZEB1 mRNA expression in SiHa and Me180 cells requires the activation of the EP4 receptor but not EP2 receptor activation. Furthermore, seminal fluid PGE2 may be one of the possible ligands involved in SF induced Snail, Twist and ZEB1 upregulation.

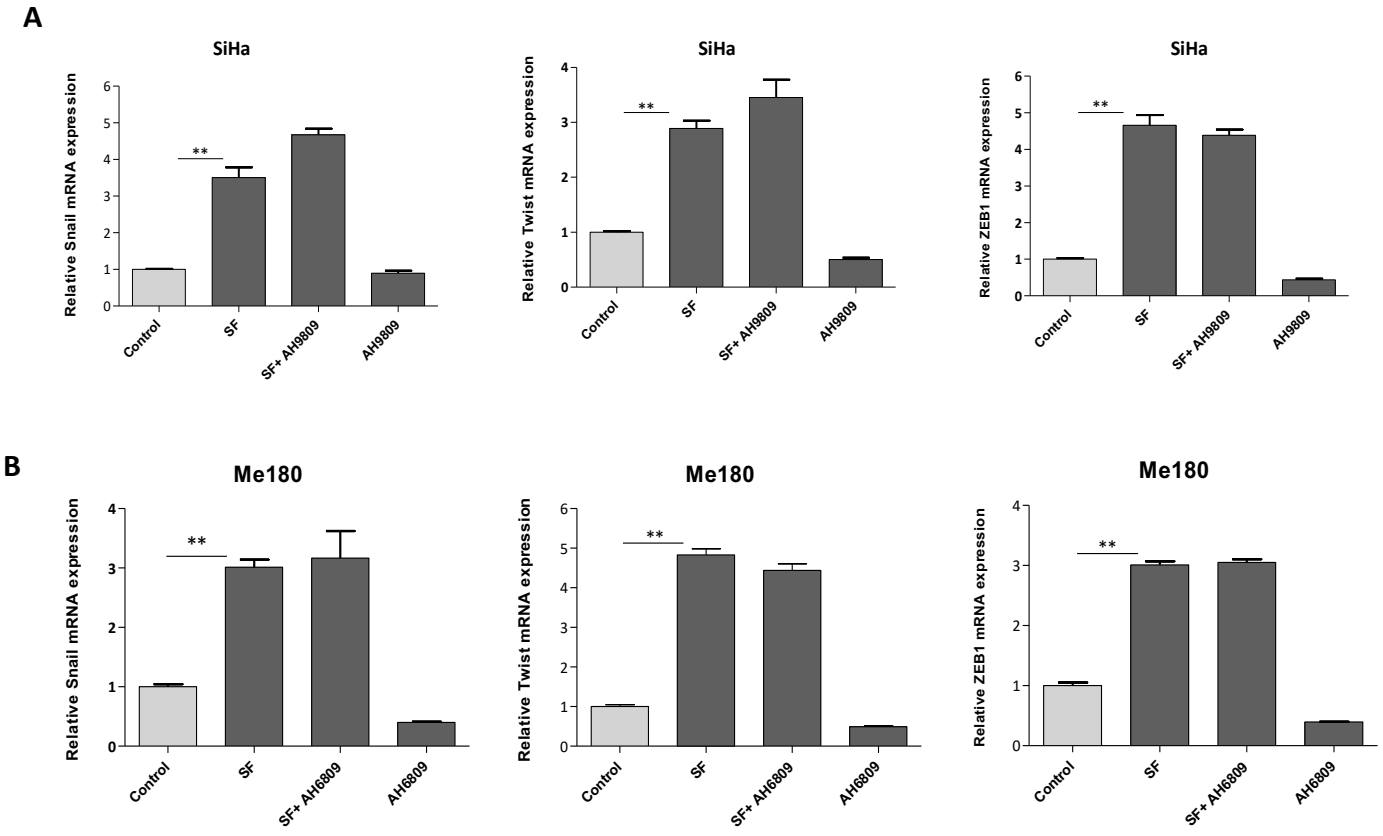


Figure 4.7. EP2 antagonist has no inhibitory effect on SF mediated Snail, Twist and ZEB1 upregulation. A) SiHa and **B)** Me180 cells were treated for 4hrs (Snail, ZEB1) or 8hrs (Twist) with SF (1:50) or control (DMSO) in the presence/absence of EP2 antagonist [AH6809; 20μM] and mRNA expression determined by RT-qPCR. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Data are represented as mean±SEM of experiment performed in triplicate and repeated three independent times. (**P<0,01).

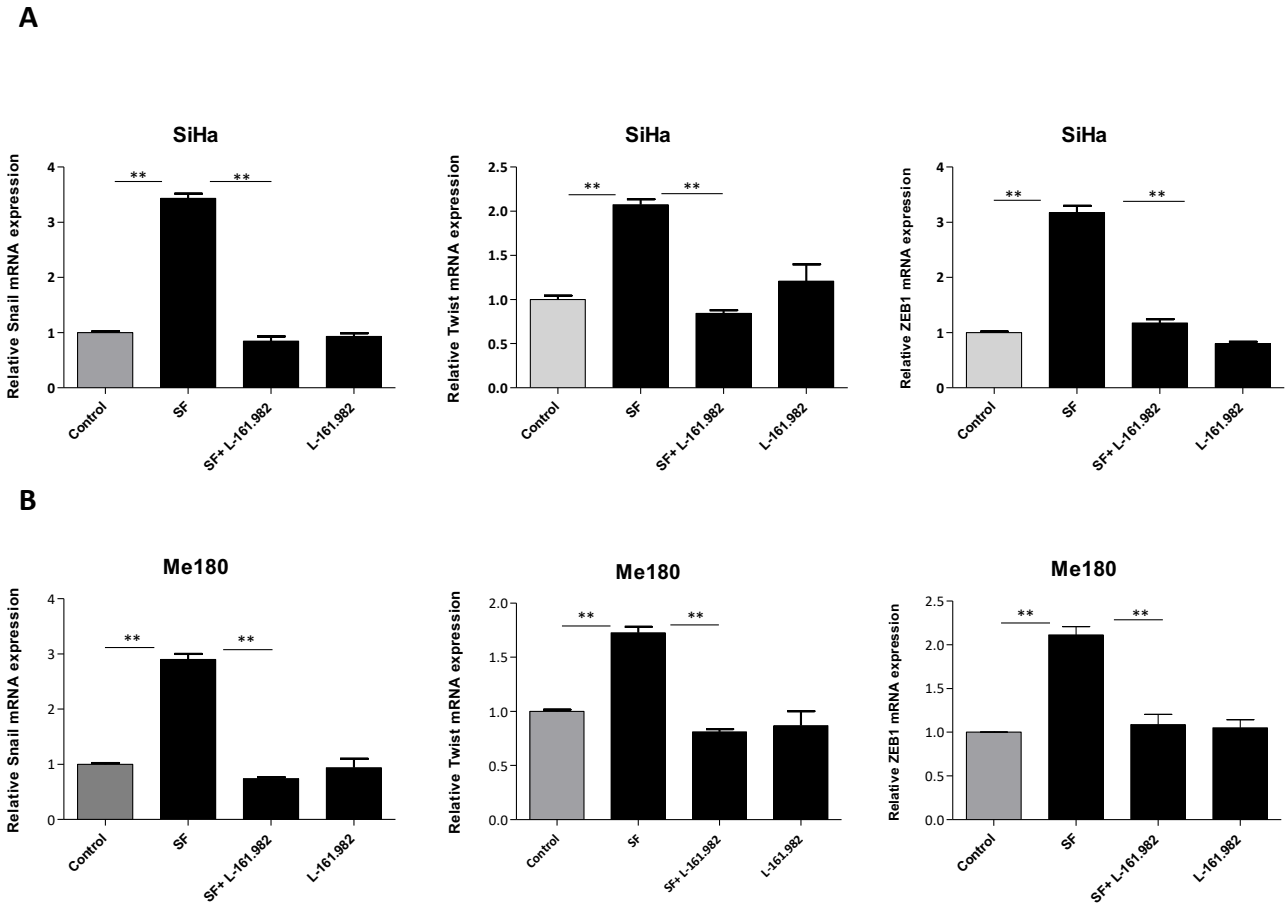


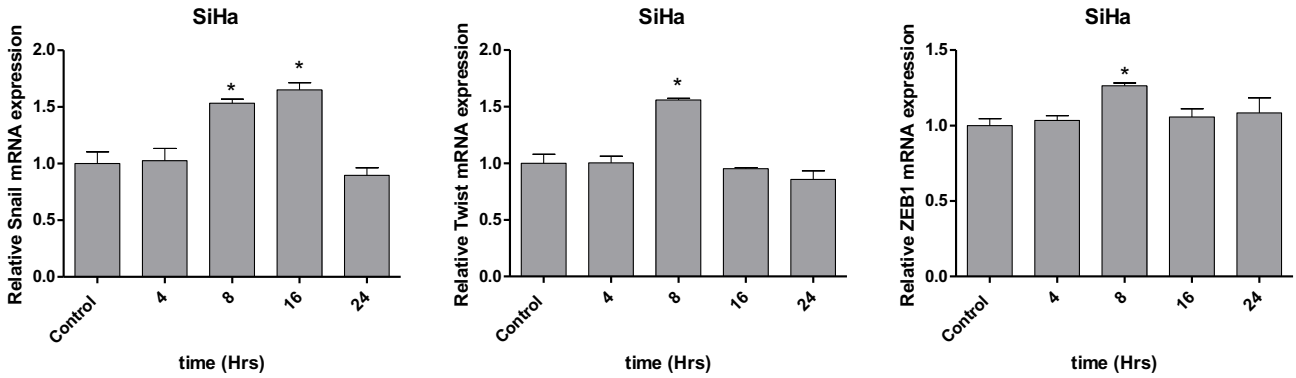
Figure 4.8. EMT-TFs mRNA expression is regulated by SF via EP4 receptor in cervical squamous carcinoma cells. **A)** SiHa and **B)** Me180 cells were treated for 4hrs (Snail, ZEB1) or 8hrs (Twist) with SF (1:50) or control (DMSO) in the presence/absence of EP4 antagonist [L-161.982; 20μM] and mRNA expression determined by RT-qPCR. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Data are represented as mean$\pm$ SEM of experiment performed in triplicate and repeated three independent times. (**P<0,01).

4.2.5 PGE2 induces the expression of Snail, Twist and ZEB1 mRNA expression in cervical squamous carcinoma cells

Having identified the role of the EP4 receptor in SF mediated EMT transcription factor induction, next we investigated whether PGE2 induced the upregulation of EMT-TFs Snail, Twist and ZEB1 similar to SF. Since the activation of E-series receptors such as EP4 occurs via PGE2 binding, it was hypothesized that PGE2 present in SF could be activating the signaling pathway via the EP4 receptor. With SF containing a host of biological molecules such as prostaglandins and growth factors, these molecules can bind receptors on target cells such as cervical carcinoma cells. In addition, PGE2 is abundant in SF with levels that are 10 000 times higher than that observed in tissue during an acute inflammatory response[349].

To investigate the role of PGE2 on Snail, Twist and ZEB1 mRNA expression; SiHa and Me180 cells were treated with vehicle or PGE2 [300nM] for 4, 8, 16 and 24hrs after which RT-qPCR was used to analyze gene expression. In SiHa cells, results showed that PGE2 significantly upregulated Snail, Twist and ZEB1 expression with peak induction observed at 8hrs for Twist and ZEB1, while peak induction was observed at 8 and 16hrs for Snail (Figure 4.9A). Similarly, PGE2 significantly upregulated Snail, Twist and ZEB1 mRNA expression in Me180 cells with peak induction observed at 8hrs (Figure 4.9B). Together this data suggests that PGE2 induces the mRNA expression of Snail, Twist and ZEB1 in cervical squamous carcinoma cells. For all future experiments, 8hr PGE2 treatment was used to further investigate EMT-TFs mRNA expression.

A



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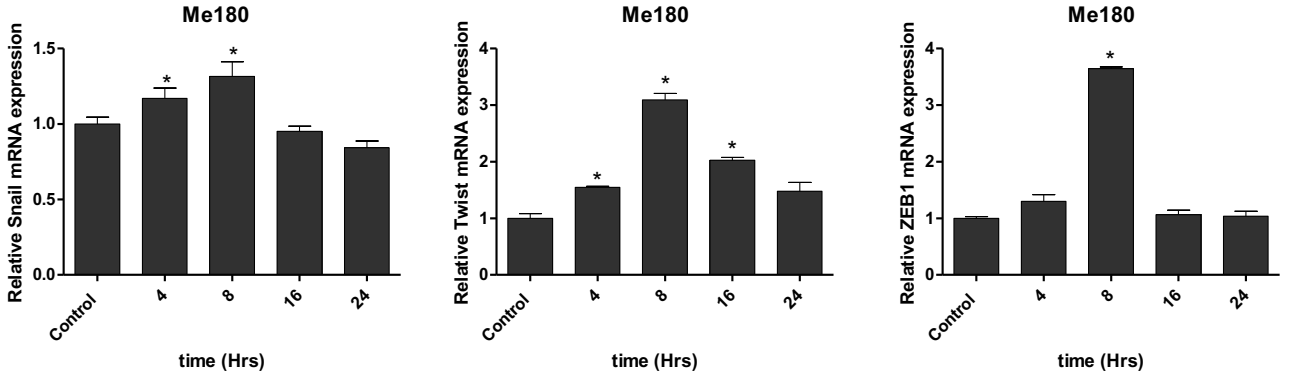


Figure 4.9. PGE2 treatment induces Snail, Twist and ZEB1 mRNA expression in cervical squamous carcinoma cells. Relative Snail, Twist and ZEB1 mRNA expression in **A)** SiHa and **B)** Me180 cells treated with PGE2 [300nM] for 4, 8, 16 and 24hrs compared to vehicle (PBS) as determined by RT-qPCR. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Results shown as mean ± SEM of experiments performed in triplicate and repeated three independent times. (*P<0,05).

4.2.6 PGE2-stimulated Snail, Twist and ZEB1 mRNA expression is via the EP4 receptor and mediated via the ERK1/2 signaling pathway

Our earlier results showed that the common signaling pathways involved in SF upregulation of Snail, Twist or ZEB1 were the COX-1 or COX-2 and ERK1/2 signaling pathways and that this effect was occurring via the EP4 receptor. It was also shown that PGE2 induces Snail, Twist and ZEB1 mRNA expression. Previous research in our laboratory showed that treatment of adenocarcinoma cells with SF or PGE2 activates ERK1/2 signaling via the EP4 receptor[273]. In this study, we investigated whether the EP4 receptor and ERK1/2 signal transduction pathways have a role in PGE2 mediated Snail, Twist and ZEB1 upregulation.

SiHa and ME180 cells were treated with PGE2 or vehicle in the absence/presence of an EP4 antagonist (L-161.982) or PD98059 (ERK1/2 inhibitor) for 8hrs and analysed by RT-qPCR. In both SiHa and Me180 cells, our results show that treatment with the EP4 antagonist (L-161.982) completely blocked PGE2 mediated Snail, Twist and ZEB1 upregulation. In addition, treatment with the inhibitor of ERK1/2 (PD98059) also blocked PGE2 induced Snail, Twist and ZEB1 upregulation in both SiHa and Me180 cells (Figure 4.10. A, B). This result shows that PGE2 upregulation of Snail, Twist and ZEB1 is via the EP4 receptor and is mediated by the ERK1/2 signaling pathway.

In summary, we postulate that SF induced Snail and Twist upregulation involves the EP4 receptor, ERK1/2 and COX-1 signaling pathways while ZEB1 upregulation involves EP4, EGFR, ERK1/2 and COX-1 or 2 signaling pathways and that endogenous PGE2 synthesized by COX enzymes and exogenous PGE2 present in SF can regulate Snail, Twist and ZEB1 expression in cervical squamous carcinoma cells.

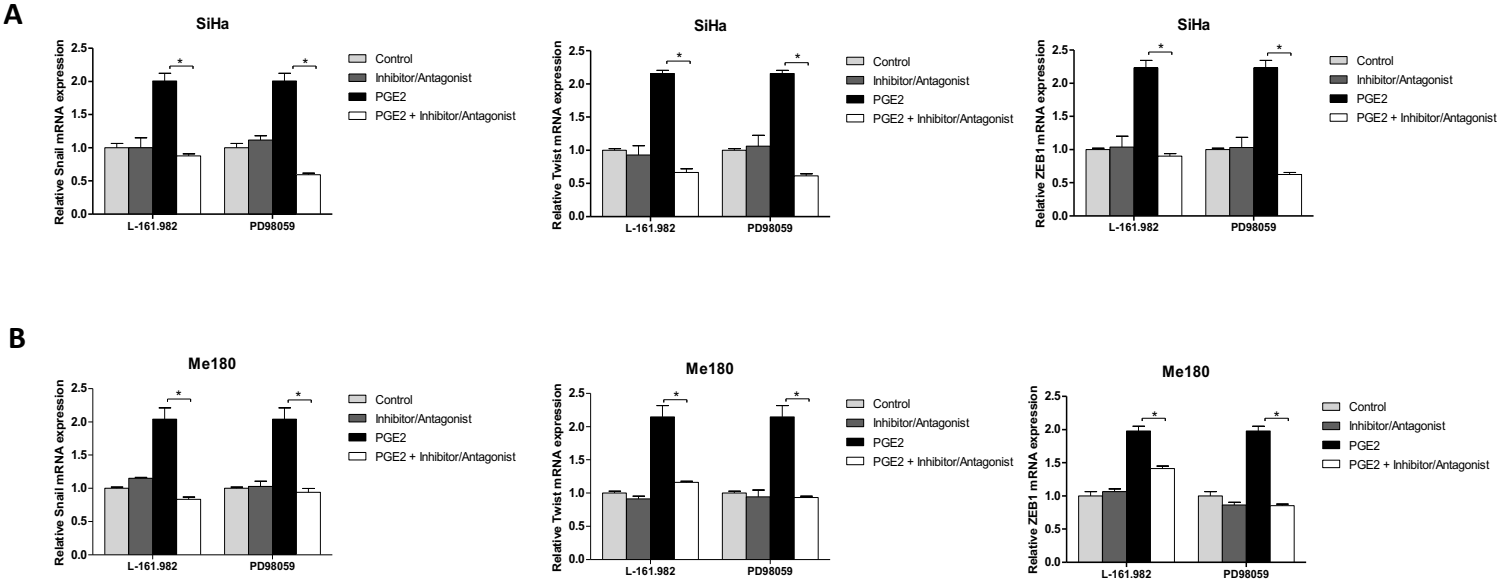


Figure 4.10. PGE2 mediated Snail, Twist and ZEB1 mRNA induction occurs via EP4 receptor and ERK1/2 signaling pathway in cervical squamous carcinoma cells. Relative Snail, Twist and ZEB1 mRNA expression in **A)** SiHa and **B)** Me180 cells treated with control (DMSO) or SF (1:50) in the absence/presence of the chemical inhibitor of ERK1/2 [PD98059; 50μM] and EP4 antagonist [L-161.982; 20μM] as determined by RT-qPCR. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Results shown as mean ± SEM of experiments plated in triplicate and repeated three independent times. (*P<0,05).

4.2.7. SF stimulated migration is mediated via the EGFR, ERK1/2 and COX-1/2 signaling pathways.

Here we investigated the signaling transduction pathways required for SF-stimulated directional migration by using previously investigated chemical inhibitors of COX-1 (SC560), COX-2 (NS398), EGFR (AG1478) and ERK1/2 (PD98059) and monitoring migration by transwell migration assay. The effect of cell death was excluded by exposing cells to chemical inhibitors for 8hrs and normalizing the quantified results to parallel MTT viability assay readings. Representative images of migrated cells were captured using phase contrast microscopy (Figure 4.11A). In SiHa cells, quantified results showed a significant reduction in SF mediated cell migration in cells treated with inhibitors of COX-1 (SC560), COX-2 (NS398), EGFR (AG1478) and ERK1/2 (PD98059). Similar results were observed in Me180 cells, where SF mediated directional migration was significantly inhibited by inhibitors of COX-1, COX-2, EGFR, and ERK1/2 (Figure 4.11B). No difference in cell migration was observed between control (DMSO) cells and cells treated with the chemical inhibitors in the absence of SF (Appendix II). Together these results suggest that SF mediated directional migration requires the activation of EGFR, ERK1/2 and COX-1/2 signal transduction pathways.

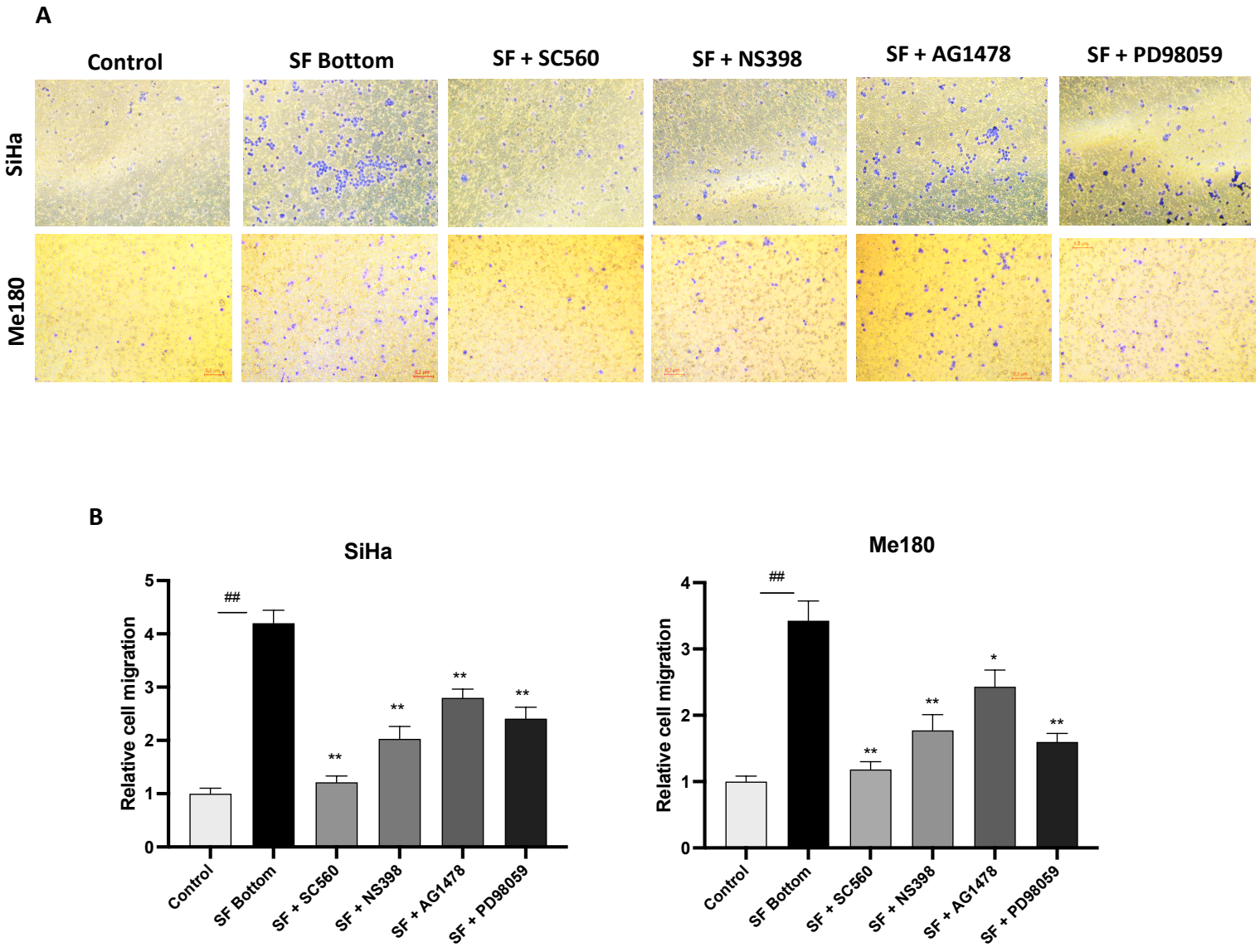


Figure 4.11. SF mediated directional migration occurs via the EGFR, ERK1/2 and COX-1/2 pathways. A) Representative images of transwell migration assay showing SiHa or Me180 cells that migrated through the membrane towards SF (1:50) following an 8hr pre-treatment with SC560 [10μM], NS398 [10μM], AG1478 [200nM] or PD98059 [50μM]. Magnification=x100, Scale bar=0,2μm. **B)** Quantification of transwell migration assay showing SiHa and Me180 cell migration towards SF post treatment with SC560, NS398, AG1478 and PD98059 treatment, normalized to MTT viability and plotted relative to control. Results shown are mean ±SEM of experiments performed in triplicate and repeated three independent times. (*P<0,05) and (**or$^{\#\#}$P<0,01).

4.3 DISCUSSION

Cancer is a disease characterized by genetic and epigenetic mutations that give cells the ability to proliferate uncontrollably and drive the hallmarks of cancer[103,47]. Many of these mutations result in a dysregulation of various signaling transduction pathways that control cell division, cell fate and cell motility[103]. Signaling pathways that have been shown to be dysregulated and implicated in cancer progression include the EGFR[350], COX-PG[334,351], PI3K[103] and ERK1/2[125] pathways. In addition to the dysregulated signaling pathways in cancer, cervical neoplastic cells can be further regulated by exogenous factors found in SF during coitus. Indeed, previous research in our laboratory showed that SF activated IL-8 and Gro-α chemokines through the EGFR, ERK1/2, COX-PG and NF-kB signaling pathways in cervical adenocarcinoma cells[278]. Muller et al. (2006) demonstrated that SF promoted the expression of tumorigenic and angiogenic genes in cervical adenocarcinoma cells through the activation of the EP4 receptor, EGFR and ERK1/2 signaling pathways[273]. Previous research has also shown that SF induces the EP2 receptor, EGFR and PI3K/AKT signaling pathways leading to the production of IL-1α in cervical adenocarcinoma cells[279]. Therefore, in our study we investigated whether signaling via EP receptors, EGFR, COX-1/2 and ERK1/2 shown to be activated by SF in adenocarcinoma cells, plays a role in SF mediated cell proliferation, actin rearrangement, EMT and migration in cervical squamous carcinoma cells.

Our study showed that SF induced cell proliferation is mediated via the COX-1 and ERK1/2 signaling pathways in both SiHa and Me180 cells. In contrast, EGFR and COX-2 were shown to not have a role in SF mediated cell proliferation. Previous work in our laboratory showed that SF (1:100) enhances adenocarcinoma cell proliferation via the COX-2 pathway where COX-1 inhibition showed marginal inhibition of cell proliferation[277]. Differences in results may be

attributed to cell line specific effects. The role of MAPK-ERK1/2 signaling in promoting cell proliferation and survival in cancer has been well established[352]. In line with our study, a study by Guo et al (2019) showed that SF significantly enhanced cell proliferation via the ERK1/2 signaling pathway in cervical adenocarcinoma cells[280]. Our study suggests that SF enhances cell proliferation through the activation of COX-1 and ERK1/2 oncogenic signaling pathways in cervical squamous carcinoma cells.

We showed that SF mediated actin rearrangement and morphological changes was mediated via the COX-1, EGFR and ERK1/2 signaling pathways, in both SiHa and Me180 cells. Inhibition of COX-2 had no inhibitory effect on SF induced morphological changes and stress fiber formation. In our study inhibition of COX-1 completely blocked the effects of SF on actin rearrangement while EGFR and ERK1/2 inhibition partially inhibited SF mediated actin rearrangement in cervical squamous carcinoma cells. This suggests that EGFR and ERK1/2 may be upstream of COX-1, though further investigation is needed to confirm this. Our study also suggests that in addition to enhancing cell proliferation, activation of COX-1 and ERK1/2 signaling by SF also leads to actin rearrangement with the additional involvement of EGFR. This actin cytoskeletal rearrangement is consequent of the activation of EMT.

Our data showed that SF mediated Snail upregulation involved the COX-1 and ERK1/2 signaling pathways. The transcription of Snail is regulated by multiple pathways. Various pathways such as TGF-β/Smad, NF-kB and MAPK/ERK have been shown to stimulate Snail transcription through direct promoter binding[353]. In hepatocellular carcinoma cells, the transcription of Snail was shown to be interrupted by interfering with Smad4 binding to the Snail promoter[354]. In a study that investigated the human Snail promoter region in tumor cells,

it was shown that Snail promoter activity was dependent on ERK1/2 signaling and that NF-kB p65 stimulated Snail transcription by binding regions upstream of the Snail promoter[355]. Previous research in our laboratory has shown the activation of NF-kB, COX1/2 and ERK1/2 signaling pathways by SF in adenocarcinoma cells[278]. Therefore, in the context of our study it is likely that in addition to COX-1 and ERK1/2 signaling, SF mediated Snail transcriptional upregulation could be regulated via other signaling pathways e.g., NF-kB.

Similar to Snail, our data showed that SF mediated Twist upregulation involved the COX-1 and ERK1/2 signaling pathways. In addition to being an EMT inducing transcription factor, Twist has been implicated in stemness, angiogenesis, invasion and chemo-resistance in a variety of carcinomas[356]. At a genetic level, the transcription of Twist is modulated by a host of upstream regulators which include NF-kB[357], STAT3[358], Smad[359], hypoxia inducible factor (HIF)-1α[360] amongst others, all of which bind the Twist promoter regions. These modulators are activated by key EMT signaling pathways such as TGF-β, Wnt, MAPK, PI3K and Notch[356]. Similar to what was observed in SF mediated Snail signaling, it is likely that SF induced Twist upregulation is mediated via multiple parallel signaling pathways that activate downstream modulators that bind directly to the Twist promoter. It is also possible that the ERK1/2 signaling pathway could be activating downstream modulators that activate Snail or Twist transcription. Further research into alternative pathways and downstream modulators is required to better understand the mechanism behind SF mediated Snail and Twist upregulation.

We showed that SF mediated ZEB1 was regulated via COX-1 or COX-2, EGFR and ERK1/2 signaling pathways. Complete inhibition was observed with EGFR and COX-1 or COX-2 inhibitors, highlighting a prominent role for EGFR and COX signaling in SF mediated ZEB1

upregulation. While a minor effect was observed for ERK1/2 signaling. In line with our observations, a study by Dohadwala et al (2006), showed the upregulation of ZEB1 mRNA in COX-2 silenced non-small cell lung carcinoma cells[361]. Another study by Bae et al. (2013) showed the activation of ZEB1 via EGFR-MEK/ERK signaling in lung carcinoma cells[362]. In a study using prostate carcinoma cells, ZEB1 activation by HGF was shown to be regulated by the ERK/MAPK signaling pathway[363]. This evidence highlights the role of EGFR, COX and ERK1/2 signaling pathways in ZEB1 transcriptional regulation. However, similar to other EMT transcription factors ZEB1 transcription is regulated by several signaling pathways such as TGF-β, NF-kB, PI3K/AKT, MAPK/ERK and YAP[364,365]. With SF containing an array of bioactive molecules such as cytokines, TGF-β and PGE2, all of which have been implicated as activators of EMT signaling pathways, our study identifies one possible signaling pathway involved in SF mediated EMT-TFs induction however it is feasible that SF may be inducing multiple pathways concurrently to achieve EMT induction.

The inhibition of SF mediated Snail, Twist and ZEB1 by either COX-1 or COX-2 inhibitors in our study suggested that the effects observed may be mediated via Prostaglandin E-series receptors (EP), since COX enzymes are responsible for the synthesis of PGE2 which exerts its effects via EP receptors. Our study confirmed the role of the EP4 receptor in SF mediated Snail, Twist and ZEB1 upregulation. Treatment with an EP4 antagonist completely abolished SF mediated Snail, Twist and ZEB1 upregulation in both SiHa and Me180 cells. This marked inhibition by the EP4 antagonist highlighted a possible role for PGE2 in SF mediated Snail, Twist and ZEB1 induction. Our study found that treatment with PGE2 significantly upregulated Snail, Twist and ZEB1 mRNA expression. These findings are in line with a study by Dohadwala et al (2006) that showed that PGE2 upregulated the expression of Snail and ZEB1 in a dose

dependent manner in non-small cell lung cancer cell lines[361]. In a study by Jang et al. (2009) it was shown that exposure of colon cancer cells to PGE2 resulted in an upregulation of Snail expression and enhanced cell motility [366]. Our data suggests that PGE2 present in SF and produced endogenously in the FMRT may act as a ligand to the EP4 receptor which in turn activates downstream signaling pathways that activate the transcription of Snail, Twist and ZEB1 in squamous carcinoma cells of the cervix.

Research has shown that EP4 receptor activation leads to the activation of downstream signaling pathways. Unlike the EP2 receptor which signals via the cAMP dependent protein kinase (PKA) only, EP4 also stimulates non-canonical downstream pathways such as PI3K/Akt which drives survival and ERK1/2 signaling which drives migration and proliferation[367]. Activation of the ERK1/2 signaling pathway has been shown to occur via the PI3 protein kinase[367]. Herein, we showed that PGE2 mediated Snail, Twist and ZEB1 upregulation was blocked by treatment with the EP4 antagonist and the ERK1/2 inhibitor in cervical squamous carcinoma cells. In our study, treatment with the ERK1/2 inhibitor completely blocked PGE2 mediated Snail, Twist and ZEB1 while SF mediated Snail, Twist and ZEB1 upregulation was slightly inhibited by the ERK1/2 inhibitor, highlighting that other factors in SF may be playing a role in SF mediated EMT-TFs induction. It is therefore likely that the EP4 receptors expressed on neoplastic cells can be directly activated by PGE2 present in SF and PGE2 produced endogenously by COX-1/2 enzymes to activate Snail, Twist and ZEB1 transcription via the ERK1/2 signaling pathway as shown schematically in Figure 4.12, however other signaling pathways are possibly involved.

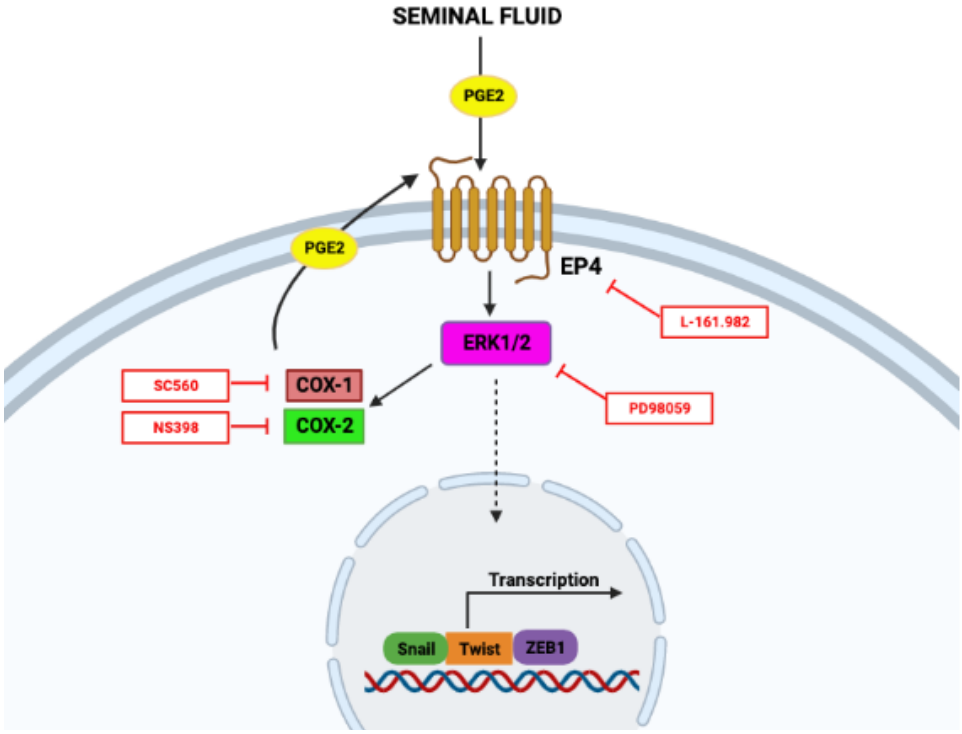


Figure 4.12. Schematic summary showing the role of SF and its constituent PGE2 in the activation of EMT transcription factors Snail, Twist and ZEB1 via the EP4, ERK1/2 and COX pathways in cervical squamous carcinoma cells.

In the present study we further showed that SF mediated directional migration requires the activation of EGFR, ERK1/2 and COX-1/2 signaling. Besides proliferation and survival, ERK1/2 signaling has been shown to play a role in cell migration and invasion in various cancers including breast[368], gastric[369], pancreatic[370] and colorectal cancers[371]. Similarly, research has shown the role of COX-2 signaling in migration and invasion of cancers[372–374].

In summary, this study found that SF mediated cervical squamous carcinoma cell proliferation is regulated via COX-1 and ERK1/2 signaling pathways. In addition, SF mediated actin cytoskeleton rearrangement was shown to be mediated via EGFR, ERK1/2 and COX-1 signaling pathways. Furthermore, SF mediated Snail and Twist induction was shown to involve COX-1 and ERK1/2 signaling while ZEB1 induction was regulated by EGFR, ERK1/2 and COX-1 or

COX-2 signaling and that this effect was mediated via the EP4 receptor. We further identified PGE2 as a ligand involved in SF mediated EMT-TFs induction. Lastly, SF induced directional migration was shown to be regulated by EGFR, ERK1/2 and COX-1/2 signaling pathways in cervical squamous carcinoma cells. To our knowledge, our study is a first to identify signaling pathways involved in SF mediated proliferation, actin rearrangement, EMT and migration in a cervical squamous carcinoma cell line model. From our data it is evident that SF activates different pleiotropic signaling pathways at varying extents to mediate proliferation, EMT induction and migration. With many of these signaling pathways being interconnected, crosstalk between different pathways is expected. Our study identified the PGE2/EP4/ERK1/2 and COX-1 or COX-2 signaling pathways as playing a role in SF mediated EMT induction. It is likely that other pathways are also involved since SF contains a plethora of bioactive molecules that can induce EMT in cervical neoplastic cells. Therefore, future studies using other inhibitors such as TGF-β, PI3K/AKT or NF-kB inhibitors may identify additional mechanisms associated with SF mediated EMT in cervical neoplastic cells.

In addition to EMT and motility, SF has also been implicated in cervical cancer inflammation and angiogenesis. Evidence has shown that the cytokines and chemokines induced by SF in cervical cancer cells may drive inflammation[375] and angiogenesis[278]. Interestingly, mediators of inflammation such NF-kB, STAT and AP-1 have been shown to be regulators of cytokine and chemokine production[376,377]. After nuclear import by Karyopherin proteins, these mediators activate the transcription of various genes that play a role in tumorigenesis[230]. Therefore, the correct nuclear localization of these mediators is required for the transcription of their target genes[378]. This localization is controlled by the nuclear-cytoplasmic transport system which has been shown to be dysregulated in cancer and identified as a mediator of inflammation[379,281].

Chapter 5:

Investigating the effect of a nuclear import inhibitor on seminal fluid regulated proinflammatory and angiogenic genes in cervical squamous carcinoma cell lines.

5.1 INTRODUCTION

Inflammation is a normal physiological process used to protect organisms from pathogens and irritants. Upon activation of the inflammatory process, chemokines and cytokines are released which activates the innate immune system. This is then followed by a recruitment of immune cells to the site of injury[228]. Once repair or signaling is complete the inflammatory process is disabled. However, inflammation can be seen as a double edged sword because the inflammatory response can also drive pathogenesis[375]. When inflammation is persistent and non-resolving it can become chronic. Indeed, it has been shown that chronic inflammation is a driving factor in a host of diseases including cardiovascular disease, cancer, diabetes, arthritis and autoimmune diseases[380]. In cancer, inflammation is accepted as a hallmark of cancer and the inflammatory response is a major feature of carcinogenesis[47]. In the tumour microenvironment (TME), inflammatory cells are present and play a role in releasing proinflammatory signals. In addition, cancer cells also release large amounts of inflammatory cytokines and chemokines which activate the recruitment of immune cells therefore further aggravating inflammation[247]. Key features in cancer related inflammation include the recruitment of inflammatory cells, the presence of inflammatory messengers such cytokines and chemokines; the occurrence of tissue remodelling and angiogenesis[381]. It is therefore assumed that the initiation and progression of cancer are promoted by an inflammatory environment[382].

In 1863, Rudolf Virchow was the first to suggest the link between chronic inflammation and cancer. Chronic inflammation is characterised by a persistent unresolved immune response resulting in repeated tissue damage and repair. This unresolved inflammation can lead to DNA damage and promote carcinogenesis[228,383]. In cervical cancer, the major pathogen shown to drive chronic inflammation is HPV. HPV infects cervical epithelial cells through sexual contact[228]. Though HPV infection can be cleared naturally, a subset of women experience persistent HPV infection putting them at risk of developing neoplastic lesions or invasive cervical cancer[16]. Chronic inflammation has been shown to drive carcinogenesis by initiating mechanisms which include an elevated generation of cytokines (IL-1, IL-6 and TNF-α)[384], chemokines, expression of oncogenes, cyclooxygenases (COX)[253] and increased expression of proinflammatory transcription factors- NF-kB[385], STAT3[386], and AP-1[387]. These mechanisms play a role in driving cell multiplication, angiogenesis, invasiveness, viability and resistance to cancer therapeutics[380].

In the FMRT, physiological processes such as menstruation, ovulation and implantation have been shown to display inflammatory responses[388]. This inflammatory response is characterized by an upregulation of cytokines, growth factors and lipids in the FMRT[256]. In normal conditions this response is tightly regulated, however unresolved inflammation in the FMRT has been shown to associate with pathologies such as cervical cancer, endometrial cancer and pelvic inflammatory disease [249]. In addition to the endogenous inflammatory response, the inflammatory response in the FMRT can be further regulated by external factors, such as exposure to seminal fluid (SF) [375,260,389]. SF has been shown to directly interact with cells in the FMRT. The inflammatory response elicited by SF in the FMRT has been shown to be essential for pregnancy, embryogenesis and implantation[256]. In the context of a well-regulated immune response, the inflammatory response induced by SF may be harmless,

however, in women with neoplastic lesions repeated exposure to SF during coitus may play a role in cervical tumorigenesis by creating an inflammatory environment.

Indeed, previous research in our laboratory has shown that SF upregulates proinflammatory cytokines such as IL-1α, IL-11 and IL-6 in cervical cancer cells and tissue explants[273,279]. SF has also been shown to activate master inflammatory pathways such as PGE2/COX[276] and NF-kB[278] in cervical cancer cells. In addition to proinflammatory factors, SF has been shown to upregulate angiogenic chemokines (IL-8 and growth regulated oncogene alpha (Gro-α)) and growth factors (VEGF) in cervical cancer cells[273]. In a study by Sales et al. (2012) it was shown that SF significantly upregulated IL-8 and Gro-α which occurred via activation of NF-kB pathway, implicating NF-kB as a possible regulator of angiogenesis[278]. In addition NF-kB has been shown to activate pro-angiogenic genes such as VEGF, CXCL1[390], IL-8 and MMP-9[391]. In a study by Xie et al. (2010), blockade of NF-kB activation was shown to suppress angiogenesis and tumour growth *in vivo* using human glioblastoma cells. Furthermore, blockade of NF-kB signaling was shown to inhibit IL-8 and VEGF expression *in vitro* and *in vivo* in glioblastoma cells[392]. In order for NF-kB to activate its target genes it requires nuclear localization[393]. Similarly, other transcription factors such as STAT3[394] and AP-1[395], that have been associated with activation of the inflammatory response, also require nuclear localization to initiate their transcription. This nuclear localization is achieved through nuclear import.

Nuclear-cytoplasmic transport of protein and transcription factors across the nuclear membrane is a tightly regulated process that is essential for cell function. Nuclear transportation is mediated by a class of proteins that belong to the Karyopherin family[396]. The Karyopherin beta superfamily is a major class of proteins that transport soluble proteins

through the nuclear pore complex (NPC)[235]. A prominent transport protein which transports cargoes into the nucleus via the NPC is Karyopherin Beta 1 (KpnB1), also known as Importin beta. KpnB1 imports specific cargoes into the nucleus independently (Non-classical) or with the assistance of an adaptor protein, Karyopherin alpha (Kpnα)[397–399] in the classical import pathway. Interestingly, research has shown that the nuclear-cytoplasmic transport system is dysregulated in cancer resulting in aberrant localization of various proteins including oncogenes[379]. KpnB1 expression has been shown to be upregulated in various cancers including prostate[242],ovarian, cervical[292] and gastric cancer[400]. Research has also shown that KpnB1 inhibition leads to cell death via apoptosis in cervical cancer cells[292]. KpnB1 is responsible for the nuclear import of various transcription factors such as NFAT[401], NF-kB[239], AP-1[241], cAMP response element binding protein (CREB)[241], STAT3[240] and SOX2[402]. As previously mentioned, NF-kB and AP-1 are major transcription factors that play a critical role in proinflammatory signaling. Chemokines and cytokines have been shown to directly activate NF-kB and AP-1 signaling. Activation of AP-1 has been shown to inflammatory target genes such as TNF-α, IL-1, IL-6, COX-2 and GM-CSF[225,403]. Similarly, the activation of NF-kB initiates the expression of inflammatory and angiogenic genes such as IL-1, IL-6, TNF-α, COX-2, IL-8, VEGF and MMPs[227,404–406]. Therefore, targeting the inhibition of these transcription factors may be a beneficial approach for inhibiting elevated levels of cytokine and chemokine production that is associated with cancer. The approach of inhibiting the nuclear entry of transcription factors as a mode of suppressing inflammatory cytokines has previously been shown in a study by Liu et al. (2000). In this study a cyclic peptide (cSN50) was used to inhibit NF-kB nuclear entry which in turn blocked the production of proinflammatory cytokines in murine mice challenged with lipopolysaccharide (LPS)[231]. This highlights the potential of

targeting nuclear import as an approach for regulating inflammatory and angiogenic cytokine production.

A novel small molecule inhibitor, INI-43, has been described to inhibit nuclear import pathways via KpnB1[234]. Previous work in our laboratory has also shown that INI-43 interferes with nuclear import of nuclear factor of activated T-cells (NFAT) in cervical cancer cells[281]. In addition, INI-43 has been shown to inhibit cervical cancer cell proliferation *in vitro* and *in vivo*, induce apoptosis, induce a G2/M cell cycle arrest and inhibit inflammatory gene induction[281]. As NF-kB and AP-1 require KpnB1 for their nuclear import, we hypothesized that INI-43 treatment may abrogate SF induced proinflammatory and angiogenic gene upregulation in cervical cancer cells. Previous research by Stelma et al. (2017) showed that inhibition of KpnB1 by siRNA and INI-43 inhibits IL-1, IL-6 and TNF-α expression as well as NF-kB and AP-1 transcriptional activity[238]. Therefore, in this study we investigated the effect of INI-43 on SF-induced proinflammatory and angiogenic gene expression. In addition, we investigated the effect of SF on NF-kB and AP-1 activity.

5.2 RESULTS

5.2.1 SF treatment upregulates proinflammatory gene expression in cervical squamous carcinoma cells

To investigate the effect of proinflammatory and angiogenic gene expression by SF, RT-qPCR was used to measure mRNA expression after SF treatment in SiHa and Me180 squamous carcinoma cell lines. Cells were treated with vehicle or SF for 4, 8, 16 and 24hrs after which the expression of proinflammatory genes including COX-1, COX-2, IL-6, IL-11 and IL-1α was measured. In SiHa cells, SF significantly upregulated COX-1 and IL-11 mRNA expression at 4hrs and 8hrs, with peak induction observed at 4hrs for both genes. The gene expression of IL-6 and IL-1α was upregulated at all time points and peak induction was observed at 4hrs SF treatment. In addition, SF induced COX-2 mRNA expression at 4 and 24hrs with the most prominent expression observed at 4hrs (Figure 5.1.A).

Similarly, in Me180 cells SF significantly induced expression of COX-1 and IL-6 at all time points and peak induction was seen at 24hrs and 4hrs respectively. SF significantly upregulated COX-2 expression at 4hrs and 24hrs with peak induction observed at 24hrs. Furthermore, SF significantly induced IL-11 and IL-1α expression with the most prominent upregulation observed at 4hrs (Figure 5.1.B). Similar to SiHa cells, peak induction of majority of the proinflammatory genes induced by SF in Me180 occurred at the 4hrs SF treatment timepoint Together these results suggest that SF significantly induces the expression of proinflammatory genes. For all future experiments 4hrs SF was used to investigate the effect of SF on proinflammatory genes.

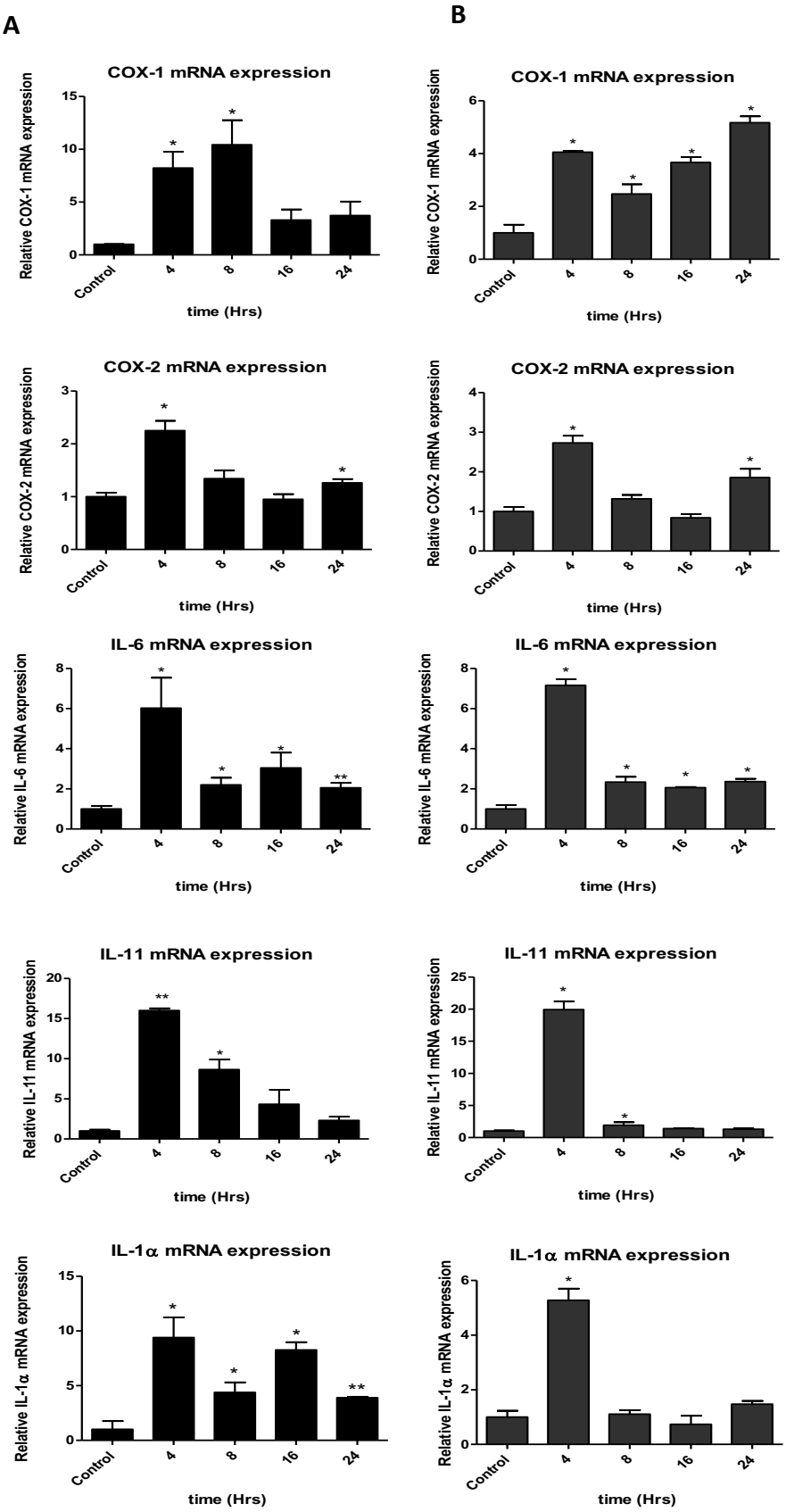


Figure 5.1. SF induces proinflammatory gene expression in cervical squamous carcinoma cells. Relative COX-1, COX-2, IL-6, IL-11, and IL-1α mRNA expression in **A)** SiHa and **B)** Me180 cells treated with SF (1:50) for 4, 8, 16 and 24hrs or vehicle (PBS) as determined by RT-qPCR. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Data presented as mean ± SEM from three independent experiments performed in triplicate. (*P<0,05).

5.2.2 SF treatment induces the expression of angiogenic genes in squamous carcinoma cells

The effect of SF on the expression of angiogenic genes (IL-8, VEGF-A and Gro-α) in SiHa and Me180 cells treated with vehicle or SF for 4, 8, 16 and 24hrs was monitored by RT-qPCR. In SiHa cells, results showed a significant induction of IL-8 at 4 and 8hrs SF treatment with peak induction observed at 4hrs. The gene expression of VEGF-A was significantly induced by SF at 24hrs. In addition, gene expression of Gro-α was significantly upregulated at all time point with peak induction occurring at 4hrs SF treatment (Figure 5.2.A).

In Me180 cells, similar results were observed where SF induced the gene expression of IL-8 at 4 and 8hrs with the most prominent upregulation occurring at 4hrs. Additionally, SF significantly induced the expression of VEGF-A at 24hrs. SF mediated Gro-α gene expression was upregulation at all time points with the peak induction occurring at 4hrs (Figure 5.2.B). Together, these results show that SF significantly upregulates the expression of angiogenic genes. For all future experiments 4hrs SF stimulation was used to investigate IL-8 and Gro-α while 24hrs was used for VEGF-A.

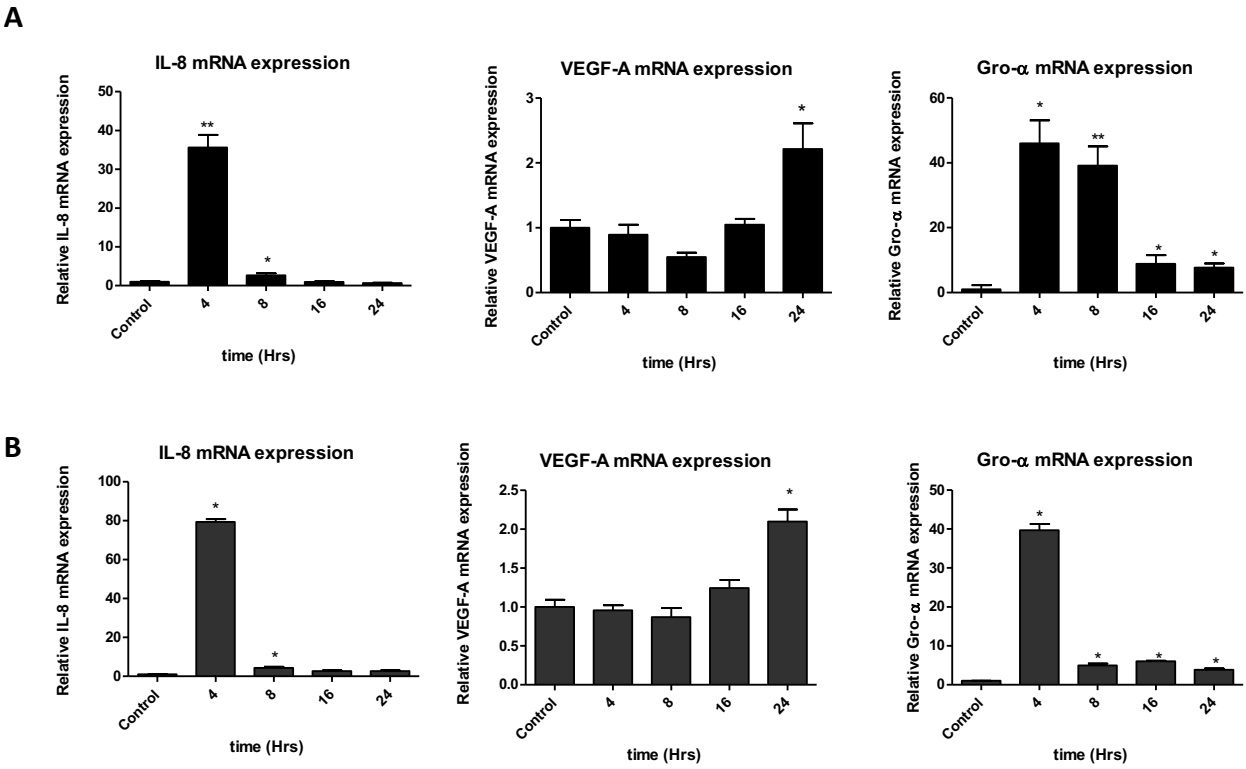


Figure 5.2. SF induces angiogenic gene expression in cervical squamous carcinoma cell lines. Relative IL-8, VEGF-A and Gro-α mRNA expression in **A)** SiHa and **B)** Me180 cells treated with SF (1:50) for 4, 8, 16 and 24hrs or vehicle (PBS) as determined by RT-qPCR and quantified relative to control. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Data presented as mean $\pm$ SEM from four independent experiments performed in triplicate. (*P<0,05).

5.2.3 The effect of SF on KpnB1 expression in cervical squamous carcinoma cells

Having identified that SF induces the expression of both proinflammatory and angiogenic genes, and knowing that KpnB1 mediated nuclear import plays a role in the inflammatory response, we hypothesized that inhibition of KpnB1 by the small molecule inhibitor INI-43 might abrogate the proinflammatory and angiogenic effects induced by SF. Previous research in our laboratory[292], as well as others[242,407,400] has shown that KpnB1 expression is elevated in various cancers, however the effect of SF on KpnB1 expression has not been determined. Therefore, experiments were first conducted to determine the effect of SF on KpnB1 mRNA and protein expression.

5.2.3.1 SF upregulates the expression of KpnB1 expression in cervical squamous carcinoma cells.

To investigate the effect of SF on KpnB1 mRNA expression, SiHa and Me180 cells were treated with vehicle or SF for 4, 8, 16 and 24hrs after which gene expression was analysed by RT-qPCR. Our results revealed a significant upregulation of KpnB1 mRNA expression in SiHa cells at 4, 8 and 24hrs SF treatment with peak induction observed at 8hrs (Figure 5.3.A). Similar results were observed in Me180 cells, where results showed a significant upregulation of KpnB1 mRNA expression after 8, 16 and 24hrs SF treatment, with peak induction occurring at 8hrs SF treatment (Figure 5.3.B).

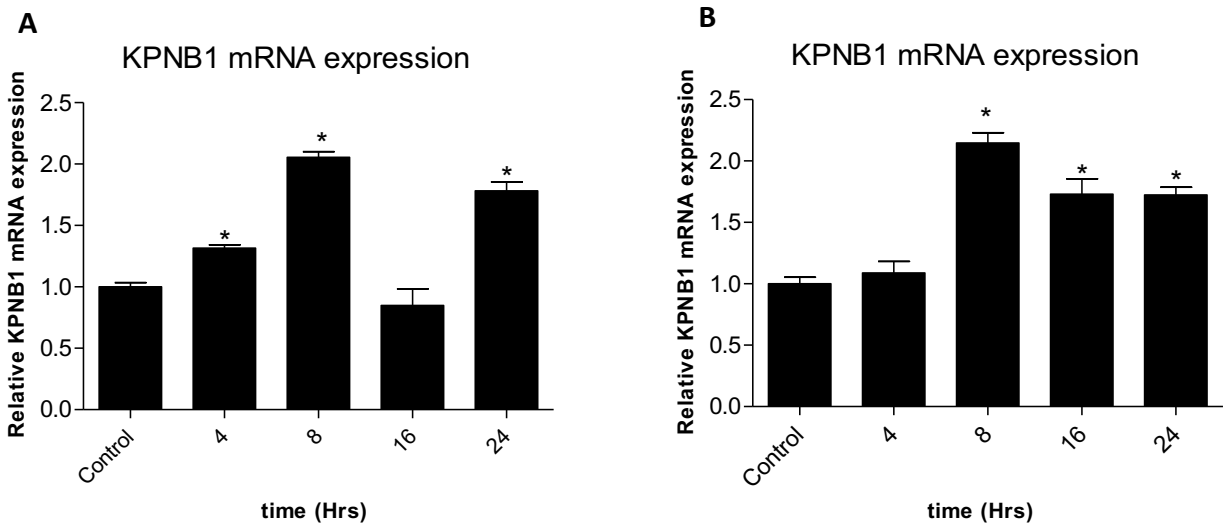


Figure 5.3. SF upregulates KpnB1 mRNA expression in cervical squamous carcinoma cells. A) SiHa and **B)** Me180 cells treated with SF (1:50) for 4, 8, 16 and 24hrs or control (PBS) as determined by RT-qPCR and quantified relative to control. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Data presented as mean ± SEM from three independent experiments performed in triplicate. (*P<0,05).

Having identified that SF upregulates the mRNA expression of KpnB1, next we investigated whether this upregulation is also seen in protein expression. Western blot analysis showed an increased expression of KpnB1 at 8hrs SF treatment for both SiHa and Me180 cells (Figure 5.4.A, B). This time point correlates with the time point where peak induction was observed for SF induced KpnB1 mRNA expression.

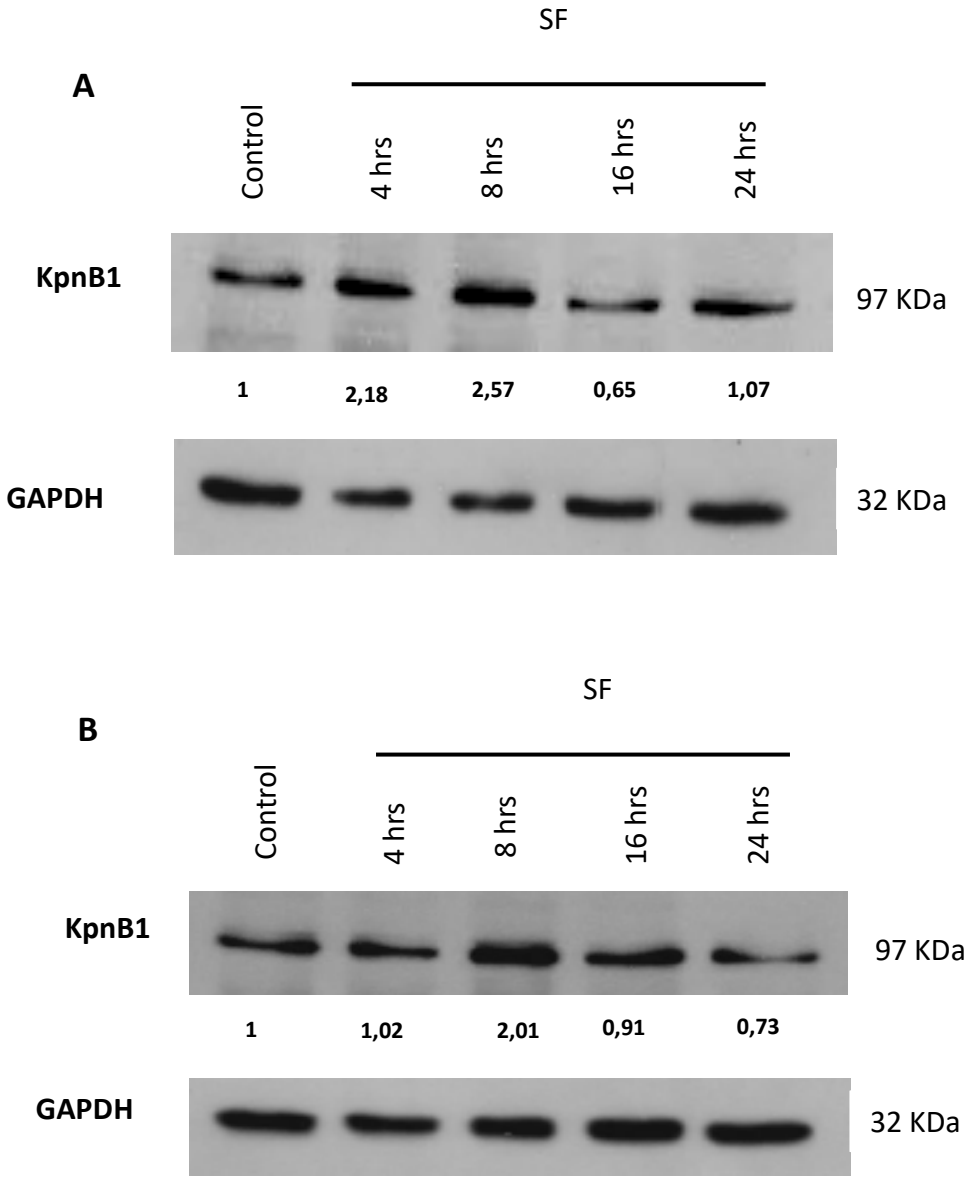


Figure 5.4. Western blot analysis of KpnB1 after SF treatment in cervical squamous carcinoma cells. A) SiHa and **B)** Me180 cells were treated with vehicle or SF (1:50) for 4,8,16 and 24hrs after which western blot analysis was used to monitor protein expression of KpnB1. GAPDH was used as a loading control and densitometry analysis was normalized to GAPDH. Results shown are representative images of experiments repeated at least three independent times.

5.2.4 INI-43 inhibits SF regulated proinflammatory and angiogenic gene expression in cervical squamous carcinoma cells

Inhibitor of nuclear import-43 (INI-43) is a small molecule inhibitor that inhibits KpnB1-mediated nuclear transport. This small molecule inhibitor was shown to inhibit nuclear import of KpnB1-cargoes such as NF-kB, AP-1 and NFAT in cervical cancer cells[281]. In addition, INI-43 was shown to inhibit NF-kB and AP-1 transcriptional activity as well as expression of target genes including IL-6, TNF-α and IL-1 in cervical cancer cells[238]. Therefore, here we investigated whether INI-43 could abrogate SF induced proinflammatory and angiogenic gene expression.

5.2.4.1 Determining the EC_{50} of INI-43 in SiHa and Me180 squamous carcinoma cell lines

Previous research in our laboratory identified INI-43 as an inhibitor of KpnB1 with an EC_{50} of approximately 10μM. Here we determined the EC_{50} concentration of INI-43 in SiHa and Me180 cells to be used in subsequent experiments intended to investigate the effect of SF on the expression of inflammatory and angiogenic genes. SiHa and Me180 cells were treated with a range of concentrations (0-30μM) of INI-43 and an MTT cell viability assay was used to analyse absorbance (OD_{595nm}). Absorbance values were then used to plot a sigmoidal dose-response curve (Hill plot) using graph pad prism and the EC_{50} value for INI-43 was determined (Figure 5.5.). The EC_{50} value for INI43 in SiHa was 9,77μM and in Me180 the determined INI-43 EC_{50} was 12,38μM in line with previous studies (Table 5). For all future experiments 5 and 10μM INI-43 as $\frac{1}{2}EC_{50}$ and EC_{50} were used to treat cervical cancer cells.

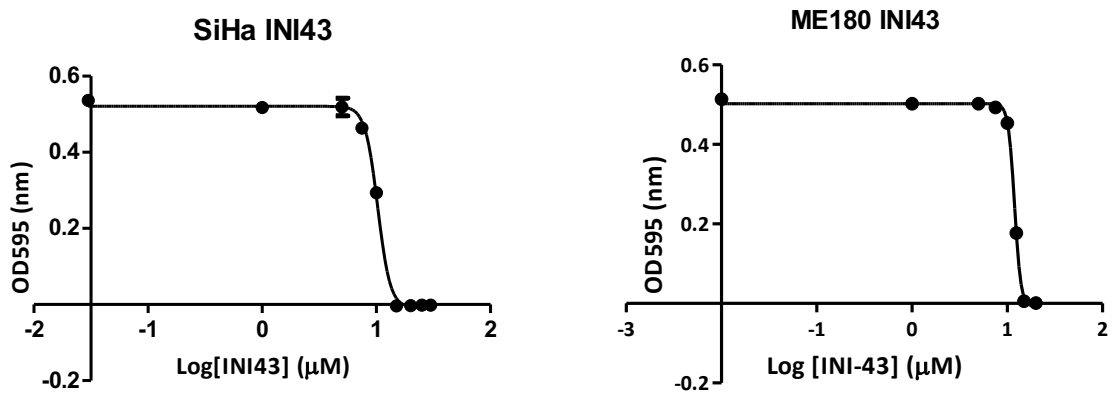


Figure 5.5. EC_{50} determination of INI-43 in cervical cancer cell line. SiHa and Me180 cells were seeded in triplicate into 96-well plates. Cells were treated with a range of INI-43 concentration and MTT was added after 48hrs. Absorbance was read at OD_{595nm} and transformed log[INI-43] graphs were plotted on graph pad prism where EC_{50} values were determined. Representative sigmoidal dose response curves are shown above. Results shown are the mean ±SEM of experiments performed in triplicate and repeated three independent times.

Table 5.1: EC_{50} values for INI-43

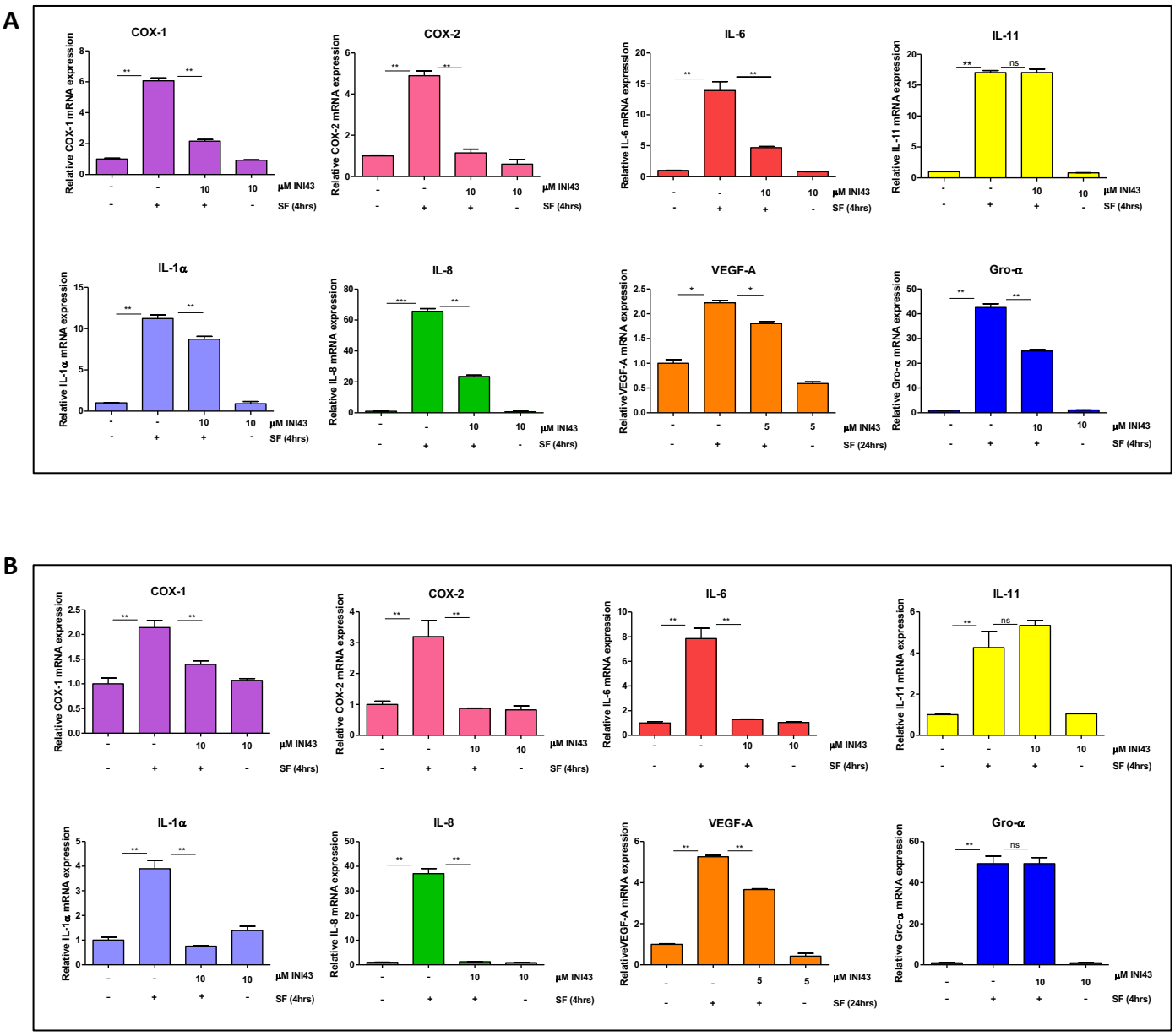
	EC_{50} (μM)	**95% Confidence interval**
SiHa	9,77	9,09 - 10,49
Me180	12,32	11,61 - 13,08

5.2.4.2. INI-43 abrogates SF regulated proinflammatory and angiogenic gene expression in cervical squamous carcinoma cells.

Having determined the EC_{50} of INI-43 in SiHa and Me180 cells, next we investigated the effect of INI-43 on SF induced proinflammatory and angiogenic genes. Previous research in our laboratory showed that treatment with 10μM INI-43 had a significant inhibitory effect while lower concentrations such as 5μM had little/no effect on the cell viability of cervical cancer cell lines[234]. Therefore, the INI-43 concentration used to investigate the expression of all proinflammatory and angiogenic genes, except for VEGF-A, was 10μM. To avoid the effects of cell toxicity, a concentration of 5μM INI-43 was used to investigate VEGF-A gene expression. To investigate the effect of INI-43 on SF induced genes, SiHa and Me180 cells were pre-treated

with 10μM INI-43 for 1hr followed by 4hrs of SF stimulation before gene expression was analysed by RT-qPCR. For VEGF-A, cells were co-treated with 5μM INI-43 and SF for 24hrs and gene expression was analysed. In SiHa cells, results showed that INI-43 significantly inhibited SF mediated induction of COX-1, IL-6 and IL-1α proinflammatory genes by 64%, 66% and 22% respectively. Treatment with INI-43 completely blocked SF induced COX-2 gene expression. For angiogenic genes, INI-43 significantly inhibited SF induced IL-8, Gro-α and VEGF-A gene expression by 64%, 41% and 19% respectively. INI-43 treatment had no inhibitory effect on SF-induced IL-11 mRNA expression in SiHa cells (Figure 5.6.A).

In Me180 cells, INI-43 treatment completely inhibited SF mediated COX-2, IL-6 and IL-1α expression. In addition, INI-43 significantly inhibited SF induced COX-1 upregulation with an inhibition of 60%. For angiogenic genes, INI-43 treatment significantly inhibited SF mediated VEGF-A expression by 30% and completely blocked IL-8 induction. Interestingly, INI-43 treatment had no inhibitory effect on SF-mediated Gro-α upregulation in Me180 cells. Similar to SiHa cells, INI-43 had no inhibitory effect on SF induced IL-11 mRNA expression (Figure 5.6.B). Together these results show that the KpnB1-associated nuclear import pathways plays a role in SF mediated induction of proinflammatory (COX-1, COX-2, IL-6 and IL-1α) and angiogenic (IL-8 and VEGF-A) genes in cervical squamous carcinoma cells.



5.6. Treatment with INI-43 blocks SF mediated proinflammatory and angiogenic gene expression in cervical squamous carcinoma cells. **A)** SiHa and **B)** Me180 cells were pre-treated with INI-43 (10μM) for 1hr followed by 4hrs stimulation with SF (1:50) or vehicle and mRNA expression was determined by RT-qPCR for COX-1, COX-2, IL-6,IL-11, IL-1α, IL-8 and Gro-α genes. Co-treatment with INI-43 (5μM) and SF for 24hrs was performed for VEGF-A after which gene expression was determined by RT-qPCR. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Data are represented as mean± SEM form three independent experiments. (*P<0.05).

5.2.5 SF activates the AP-1 (c-Jun) signaling pathway in squamous carcinoma cells.

Having observed the inhibition of SF-induced proinflammatory and angiogenic genes by INI-43, it was hypothesized that the inflammatory transcription factor, AP-1, reported to be a KpnB1 cargo[241] may play a role in SF mediated proinflammatory and angiogenic gene induction. AP-1 has been reported to induce the transcription of inflammatory genes such as IL-1, IL-6, COX-2, TNF-α and GM-CSF[225,403]. AP-1(Jun/Fos) is a dimeric transcription factor consisting of one member each from the Jun and Fos families[224]. When the AP-1 pathway is activated, the c-Jun N-terminal Kinase (JNK) phosphorylates c-Jun in the nucleus [408]. The c-Jun component of AP-1 contains a nuclear localization signal (NLS) that has been reported to be recognized by KpnB1 in the non-classical pathway[241], however, c-Jun nuclear import is not confined to KpnB1 only[409]. Phosphorylation of c-Jun is indicative of activated AP-1. In order to investigate the effect of SF on p-c-Jun expression, SiHa and Me180 cells were treated with vehicle or SF for 4, 8 and 16hrs and protein expression was analysed by western blotting.

Our results show that SF upregulates the phosphorylation status of p-c-Jun as early as 4hrs SF treatment in both SiHa and Me180 cells (Figure 5.7A, B). In Me180 cells, SF treatment upregulated p-c-Jun phosphorylation status at 4hrs albeit not to the same extent as observed in SiHa cells as compared to control. The phosphorylation in Me180 appeared sustained until 16hrs SF treatment (Figure 5.7B). In addition, the results showed an increase in expression of total c-Jun after 4hrs SF treatment in both SiHa and Me180 cells. AP-1 has been reported to autoregulate its own expression[410] and our results are in line with this. Taken together these results show that SF induces p-c-Jun expression therefore activates the AP-1 signaling pathway.

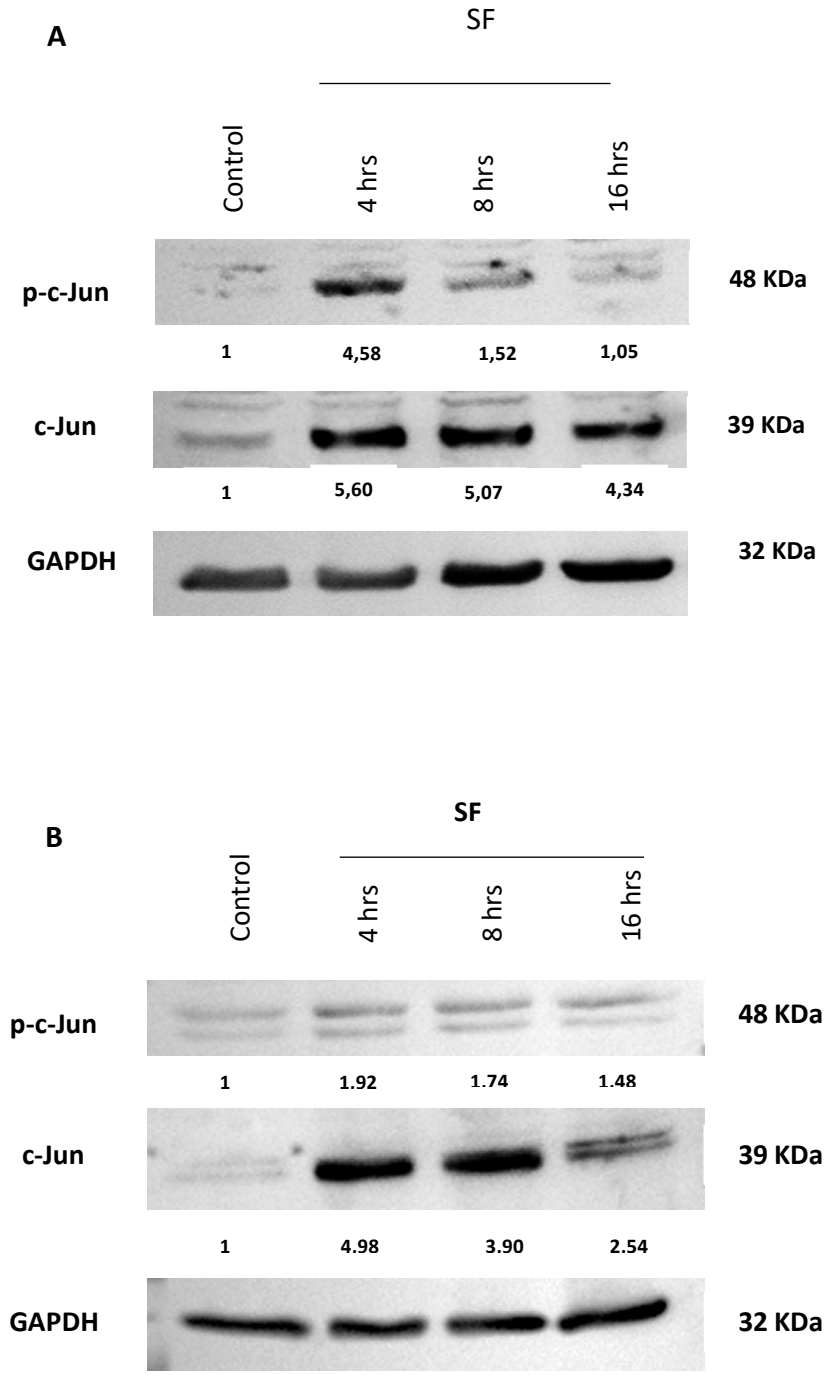


Figure 5.7. SF induces p-c-Jun and c-Jun protein expression in cervical squamous carcinoma cells A) SiHa and **B)** Me180 cells were treated with vehicle or SF (1:50) for 4, 8 and 16hrs after which western blot analysis was used to monitor protein expression. Results are a representative western blot showing p-c-Jun and c-Jun expression at varying time points of SF treatment. GAPDH was used as a loading control. Results shown are representative images of experiments repeated at least three independent times.

5.2.5.2 INI-43 does not inhibit SF induced AP-1 activation in cervical squamous carcinoma cells

To independently determine the activation of AP-1 by SF, AP-1 transcriptional activity was investigated under SF stimulation. In addition, the effect of INI-43 was tested to investigate whether KpnB1 inhibition abrogated SF induced AP-1 activity. SiHa and Me180 cells were transfected with a 4X-AP-1 luciferase promoter-reporter construct containing four AP-1 binding sites[282]. The following day, cells were pre-treated with INI-43 for 1hr followed by 4hrs SF stimulation after which firefly luciferase activity was measured and quantified relative to Renilla luciferase readings. Quantified results showed that SF significantly induced AP-1 transcriptional activity within 4hrs of SF treatment in both SiHa and Me180 cells (Figure 5.8.A, B). INI-43, however had no inhibitory effect on SF induced AP-1 transcriptional activity in SiHa and Me180 cells, suggesting that SF mediated AP-1 activation is not mediated via KpnB1 dependent nuclear import.

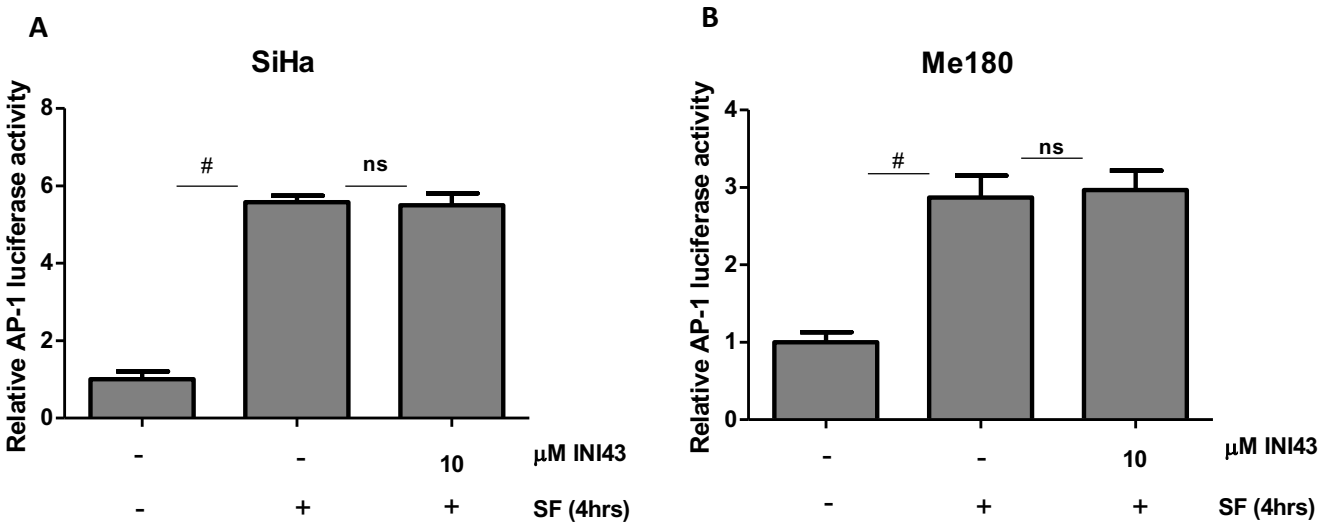


Figure 5.8 INI-43 does not block SF mediated AP-1 activation in cervical squamous carcinoma cells A) SiHa and **B)** Me180 were transfected with 4X-AP-1 Luc reporter construct containing multiple AP-1 binding sites. Cells were pre-treated with INI-43 (10μM) followed by a 4hr SF (1:50) stimulation and luciferase activity was measured. Results shown are the mean±SEM of experiments performed in quadruplicate and repeated three independent times. Luciferase activity was normalized to Renilla readings.(*P<0,05).

5.2.6 INI-43 inhibits SF activation of p65 NF-kB in cervical squamous carcinoma cells.

As the majority of the proinflammatory and angiogenic genes we investigated earlier in response to SF are also NF-kB target genes, we investigated the effect of SF on p65 NF-kB, which has been reported to be a KpnB1 cargo for nuclear entry. Previous research from our laboratory has shown that PMA stimulation of p65 NF-kB can be inhibited by INI-43[238], hence in this study we tested the effect of INI-43 on SF induced NF-kB activation. SiHa and Me180 cells were transfected with the NF-kB p65 luciferase promoter-reporter construct that contains five copies of p65 binding site[238]. The following day cells were pre-treated with IN-I43 for 1hr followed by SF stimulation for 4hrs. Fire fly luciferase activity was measured and quantified relative to Renilla luciferase readings. Our results showed that SF significantly induced p65 NF-kB transcriptional activity in both SiHa and Me180 cells. Furthermore, treatment with INI-43 significantly inhibited SF-induced p65 NF-kB transcriptional activity by 75% in SiHa cells and 69% in Me180 cells (Figure 5.9.A,B). This result suggests that SF mediated p65 NF-kB activation is occurring via KpnB1 nuclear import.

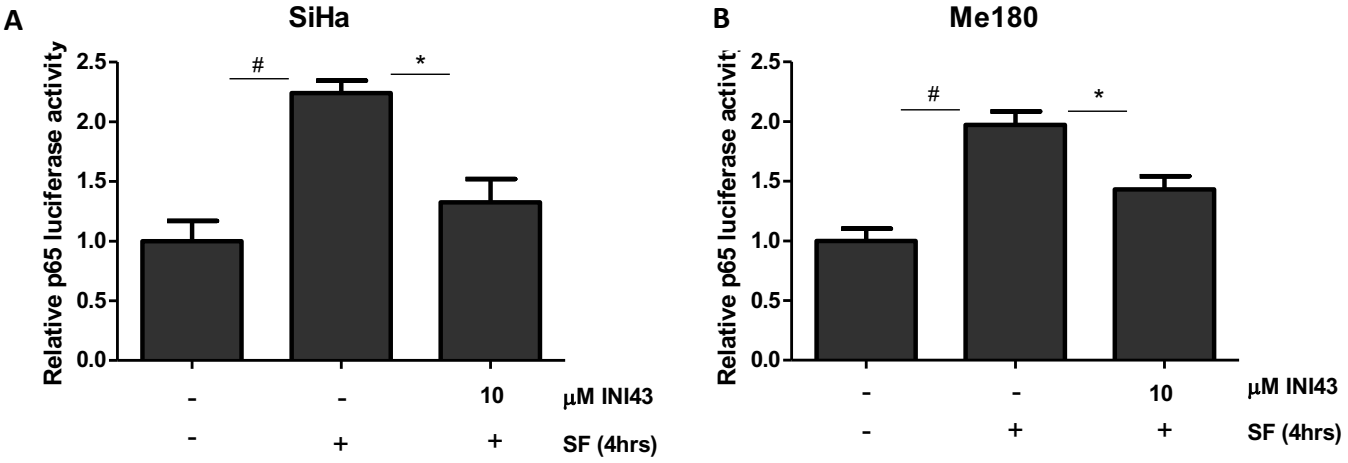


Figure 5.9. INI-43 inhibits SF mediated NF-kB transcriptional activity in squamous carcinoma cells. A) SiHa and **B)** Me180 were transfected with an p65 NF-kB or AP-1 luciferase reporter construct containing multiple NF-kB binding sites. Cells were pre-treated with INI-43 (10μM) for 1hr followed by a 4hr SF (1:50) stimulation and luciferase activity was measured. Results shown are the mean±SEM of experiments performed in quadruplicate and repeated three independent times. Luciferase activity was normalized to Renilla readings.($^{\#or*}$P<0,05).

5.2.7 INI-43 blocks SF mediated NF-kB p65 nuclear localization.

We also investigated whether SF had any effect on p65 NF-kB cellular localization. In its inactive state NF-kB localizes to the cytoplasm[411]. When cells are stimulated by extracellular stimuli such as lipopolysaccharides (LPS), phorbol ester (PMA), TNF-α, IL-1 or radiation[412], NF-kB is activated and transported into the nucleus for transcription of target genes[413]. As described earlier, KpnB1 is a nuclear import protein that has been reported to import p65 NF-kB into the nucleus. Therefore, we hypothesized that SF may have an effect on endogenous p65 NF-kB localization since the active NF-kB form is found in the nucleus.

To test this, SiHa and Me180 cells were pre-treated with INI-43 for 1hr followed by treatment with SF for 4hrs and p65 NF-kB localization was monitored using immunofluorescence. Localization of p65 NF-kB in cells was categorized into two categories; 1) predominantly nuclear or 2) predominantly cytoplasmic. Approximately 300 cells were scored per condition and results plotted. In SiHa cells, control cells showed predominantly cytoplasmic localization (98%) of p65 NF-kB. Upon stimulation with SF, there was a shift in p65 NF-kB localization where cells showed a 60% increase in nuclear localization and 40% cytoplasmic localization (Figure 5.10). A similar result was seen in Me180 cells where a shift in p65 NF-kB localization to 58% of cells showing nuclear and 42% showing cytoplasmic localization of p65 NF-kB (Figure 5.11). This indicates that SF treatment stimulates p65 NF-kB nuclear translocation. INI-43 treatment resulted in p65 NF-kB retention in the cytoplasm in both SiHa and Me180 cells. This indicates that INI-43 has an inhibitory effect on the SF-mediated nuclear transport of p65 NF-kB in SiHa and Me180 squamous carcinoma cells.

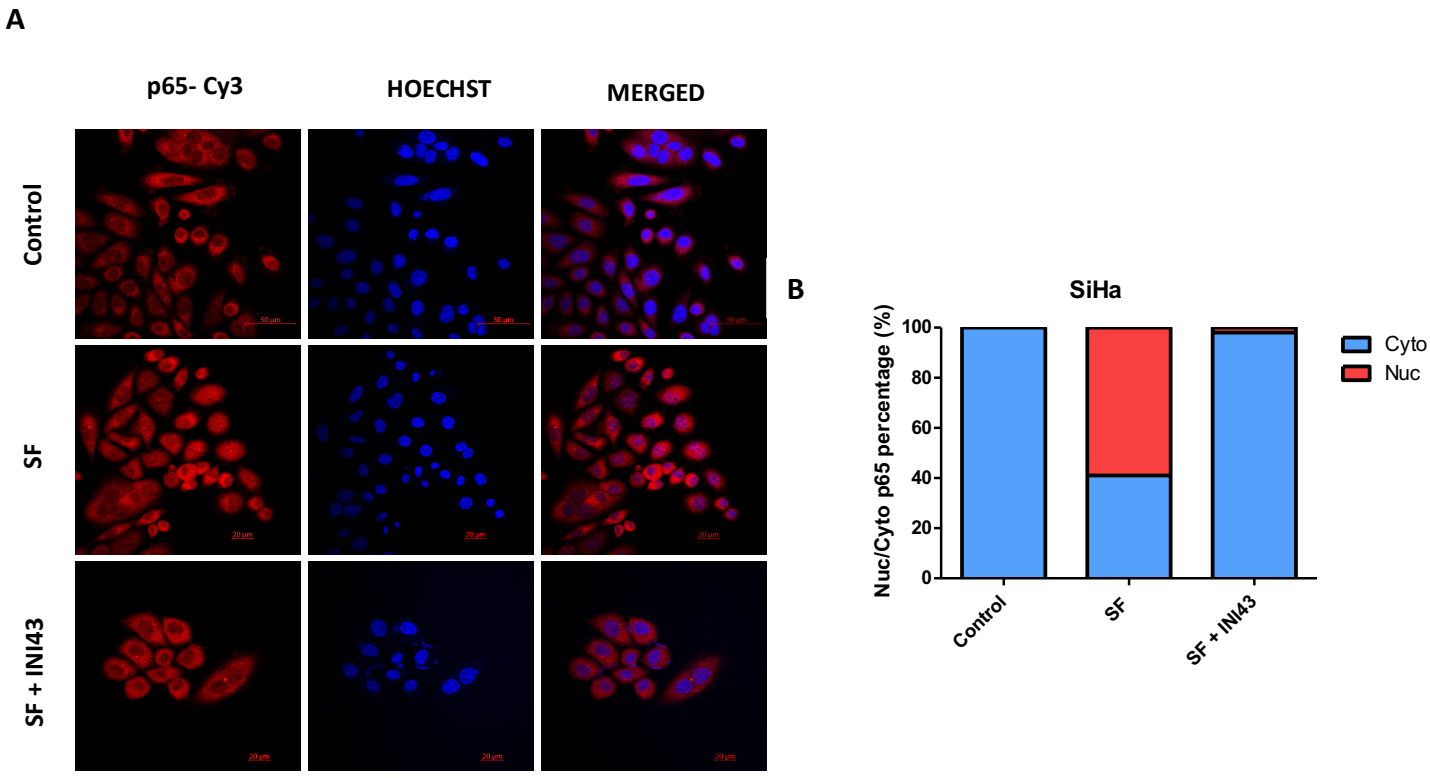


Figure 5.10. INI-43 inhibits SF mediated nuclear localization of endogenous NF-kB p65 in SiHa cells.. A) SiHa cells were plated on coverslips and pre-treated with INI43 for 1hr followed by stimulation with SF (1:50) for 4hrs in the presence of INI43. p65 NF-kB was labelled with cy3 (red) and the nuclei with Hoechst (blue). B) Immunofluorescence of p65 NF-kB was quantified by scoring 300 cells per condition across 40 images and categorized as showing "predominantly nuclear" or "predominantly cytoplasmic" staining. Graph showing percentage of cells with various p65 NF-kB localization category. Scale bar= 20μm, Magnification=400X. Experiments were repeated three independent times.

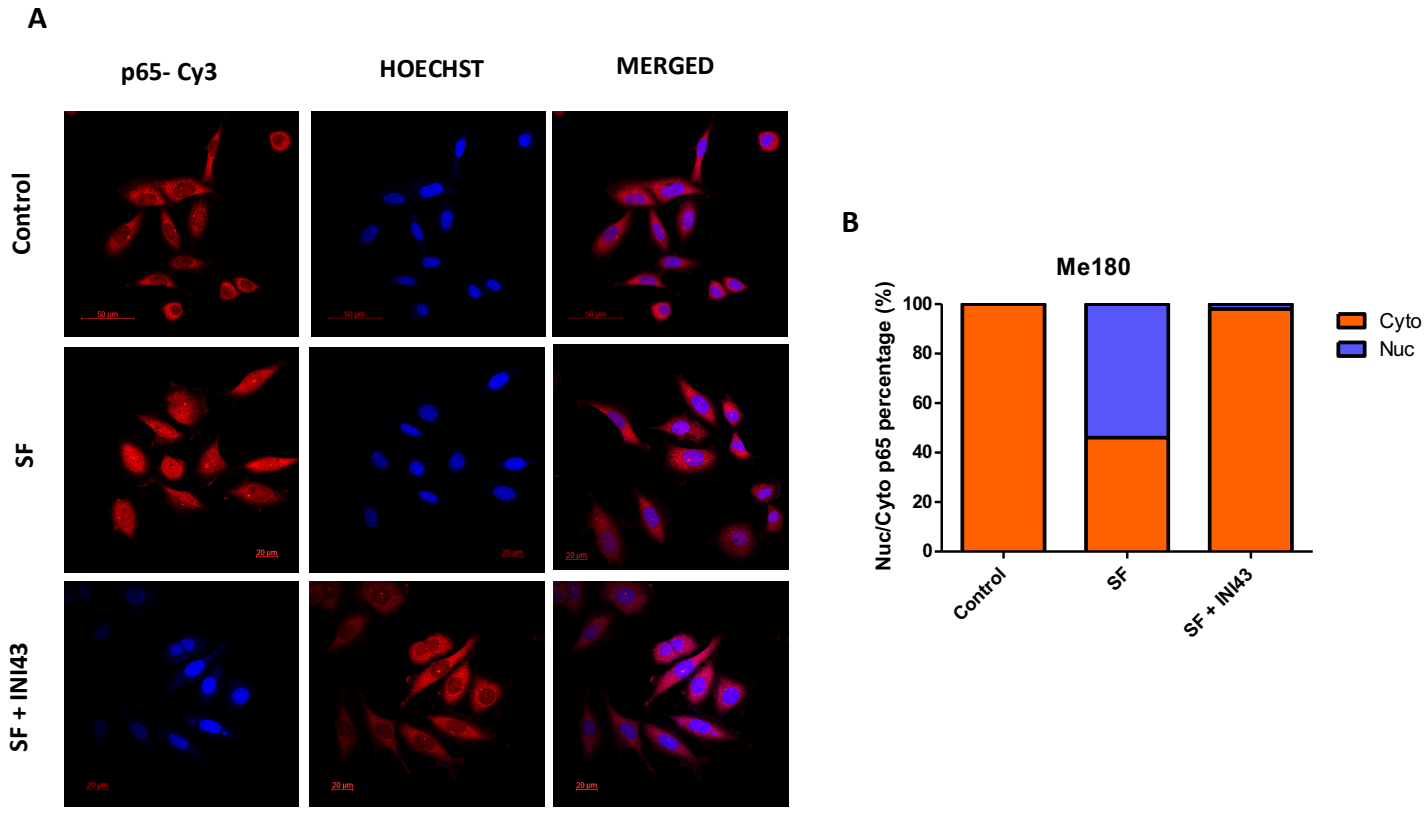


Figure 5.11. INI-43 inhibits SF mediated nuclear localization of endogenous NF-kB p65 in in Me180 cells. A) Me180 cells were plated on coverslips and pre-treated with INI43 for 1hr followed by stimulation with SF (1:50) for 4hrs in the presence of INI43. p65 NF-kB was labelled with cy3 (red) and the nuclei with Hoechst (blue). B) Immunofluorescence of p65 NF-kB was quantified by scoring 300 cells per condition across 40 images and categorized as showing "predominantly nuclear" or "predominantly cytoplasmic" staining. Graph showing percentage of cells with various p65 NF-kB localization category. Scale bar= 20μm, Magnification=400X. Experiments were repeated three independent times.

5.3 DISCUSSION

Cancer-related inflammation is considered a key hallmark of cancer[47]. This relationship was observed when the administration of non-steroidal anti-inflammatory drugs (NSAIDs),such as Aspirin, was shown to decrease the risk of developing cancer in various clinical trials[414,415]. Other studies have also shown that statins reduce cancer risk in multiple cancers such as breast cancer, colorectal cancer and hepatocellular cancer by exerting anti-inflammatory effects[416–418]. In cancer, chronic inflammation has been shown to facilitate tumour progression[178], highlighting the importance of an inflammatory environment in cancer development and progression.

The initiation of inflammation is characterized by the release of proinflammatory cytokines (e.g. IL-1, IL-6, TNF-α) ,chemokines (e.g. IL-8) and the recruitment of immune cells to the site of infection[384]. Unlike wound healing, the inflammation in cancer has been described as non-resolving and persistent. Many signaling pathways have been implicated as regulators of cancer related inflammation and they include NF-kB[385], JAK-STAT[189], MAPK/AP-1[419,420] and PGE2/COX[421]. Owing to the relationship between inflammation and tumorigenesis, research has aimed to target inflammatory regulators as anti-cancer therapeutics. One approach that has been investigated in our laboratory is targeting the localization of inflammatory transcription factors by inhibiting their nuclear-cytoplasmic transport.

Nuclear-cytoplasmic transport is an important aspect of normal cell function. The physical separation of the genome and the cytoplasm by the nuclear envelope creates a requirement for the shuttling of macromolecules across the nuclear membrane. Separating proteins or transcription factors from their site of action is a regulatory mechanism used to ensure

controlled cell signalling and inhibit aberrant activation[422]. While proteins or transcription factors less than 40KDa can freely shuttle between the nucleus and cytoplasm, larger molecules require active transport which is facilitated by transport proteins[422,423]. Many transcription factors reside in the cytoplasm in an inactive form. Upon stimulation, by various stimuli, these transcription factors are transported by importins/karyopherins into the nucleus where transcription of their target genes occurs[424]. This is indeed the case for transcription factors such as NF-kB[393], AP-1[395] and STAT3[394] which require nuclear localization for their transcription. NF-kB and AP-1 are key transcription factors implicated in tumorigenesis due to the activation of their target genes[225,425]. These target genes activate various signaling pathways which drive hallmarks of cancer. Activation of NF-kB and AP-1 occurs after nuclear entry which is achieved through nuclear import. A prominent nuclear import protein reported to transport AP-1 and NF-kB into the nucleus is Karyopherin beta 1 (KpnB1)[241,422]. Previous research by Stelma et al. (2017) has shown that inhibition of KpnB1 by siRNA or INI-43 significantly inhibited NF-kB and AP-1 transcriptional activity and nuclear entry. In addition, inhibition of KpnB1 significantly inhibited the expression NF-kB and AP-1 target genes[238,234]. This lead us to hypothesize that inhibition of KpnB1 by INI-43 could possibly abrogate SF mediated activation of genes associated with inflammation and angiogenesis. Our study investigated the effect of INI-43 on SF mediated proinflammatory and angiogenic gene regulation using cervical squamous carcinoma cells.

Our data showed that SF upregulated the expression of proinflammatory and angiogenic genes in cervical squamous carcinoma cells. This is in agreement with a study by Sales et al. (2002) that showed that SF significantly upregulated COX-2 expression in cervical adenocarcinoma cells[276]. In addition a study by Sharkey et el. (2007) showed that SF

upregulates inflammatory cytokine (IL-6, IL-8) expression in cervical and vaginal epithelial cells[260]. Similarly, Sutherland et al. (2012) showed the upregulation of IL-6, IL-11 and VEGF-A post SF treatment in cervical adenocarcinoma cells[277]. In a more recent study, SF was shown to induce the expression of IL-1β, IL-6, IL-8, VEGF and MCP-1 at 8hrs SF treatment in cervical adenocarcinoma cells[426]. Therefore, our study similar to adenocarcinoma cells supports that SF upregulates proinflammatory and angiogenic genes in cervical squamous carcinoma cells.

We further found that SF upregulates the mRNA and protein expression of KpnB1 in SiHa and Me180 cells. KpnB1 expression has been reported to be regulated by E2F in cervical cancer[427]. It is likely that bioactive molecules such as growth factors present in SF may stimulate E2F activation and E2F target genes such as KpnB1. Further research is required to confirm this association .

KpnB1 has been identified as inflammatory mediator due to its nuclear import of various inflammatory transcription factors such as AP-1[241], STAT[428], GATA-3[429] and NF-kB[239]. Our data showed that the small molecule INI-43, described as an inhibitor of KpnB1-associated nuclear import, significantly inhibited SF-induced COX-1, COX-2, IL-6, IL-1α, IL-8, VEGF-A and Gro-α expression in SiHa cells. Interestingly, no inhibitory effect was observed in SF-induced IL-11 expression in SiHa cells. In Me180 cells, inhibition of all genes except for Gro-α and IL-11 was observed after INI-43 treatment. This is in agreement with a study by Stelma et al. (2017), that showed that INI-43 treatment inhibited IL-1 and IL-6 gene expression, though the stimulation used in their study was PMA[238]. Therefore, our results suggest that SF mediated

proinflammatory and angiogenic gene expression is regulated via the KpnB1 nuclear import pathway.

Furthermore, we found that SF mediated AP-1 activity was not regulated by the KpnB1 nuclear import pathway. Our data also revealed an induction of p-c-Jun and c-Jun protein expression by SF treatment. Treatment with INI-43 had no inhibitory effect on SF mediated AP-1 activity. It is likely that SF mediated AP-1 activity in cervical cancer is regulated via other nuclear import proteins such as Transportin, Importin 7 and Importin 9, which have also been reported to import AP1[409]. Nonetheless, our study is the first to show the activation of the inflammatory mediator; AP-1, in cervical squamous carcinoma cells in response to SF.

In addition, this study showed that SF activates NF-kB signaling which was inhibited by treatment with INI-43. In line with our observation, a study by Sales et al. (2012) showed the activation of NF-kB signaling in cervical adenocarcinoma cells in response to SF[278]. Our study is in agreement with a study by Stelma et al (2017) who showed that INI-43 significantly inhibited NF-kB activity in cervical cancer cell lines, after PMA stimulation[238]. Another study by Yang et al.(2019) demonstrated that KpnB1 inhibition, using Importazole, a small molecule inhibitor of KpnB1, blocked NF-kB activity in pancreatic cancer cells[242]. This suggests that SF mediated NF-kB activation requires KpnB1 mediated nuclear import.

Our study further showed that SF induced the nuclear translocation of p65 NF-kB which was inhibited by INI-43 in both SiHa and Me180 cells. Previous research in our laboratory has also demonstrated the inhibition of p65 NF-kB nuclear localization by INI-43 treatment in cervical cancer cells[238,234]. In another study by Zhu et al (2018), p65 NF-KB nuclear translocation was shown to be inhibited by Importazole treatment in glioblastoma cells[407]. Taken together, our

study suggests that SF induced proinflammatory and angiogenic gene expression is regulated by the KpnB1 nuclear import pathways. In addition, it can be postulated that SF induced proinflammatory and angiogenic gene expression is associated with NF-kB signaling because inhibition of nuclear import by INI-43 blocked SF induced gene expression and NF-kB nuclear translocation.

To our knowledge, our study is a first to show that inhibition of KpnB1 associated activities by INI-43 abrogates SF mediated proinflammatory and angiogenic gene expression in cervical squamous carcinoma cells and that this inhibition may be associated with NF-kB signaling. We postulate that, in sexually active women, repeated exposure of neoplastic lesions to SF may induce an inflammatory response characterized by an upregulation of cytokines and chemokines which may be regulated via the nuclear import of NF-kB. It should be noted that INI-43 did not inhibit all SF mediated proinflammatory and angiogenic genes, which suggests that other inflammatory signaling pathways are involved in SF mediated inflammation. Given that inflammatory mediators such as NF-kB, AP-1 and STAT3 may be dependent on other importins for nuclear import, it would be worth considering the inhibition of multiple nuclear import proteins to regulate SF mediated gene expression. Additionally, SF is rich in an array of cytokines, chemokines, proteins and growth factors[258] that activate various signaling pathways in the FMRT, therefore further characterization of the effect of INI-43 on SF mediated inflammation and angiogenesis using *ex vivo* or 3D tissue models will shed more light on the role of SF in cancer related cellular processes.

Chapter 6:

Investigating the effect of seminal fluid on EMT, angiogenesis and inflammation in cervical tissue biopsies: an *ex vivo* model.

6.1 INTRODUCTION

The female reproductive tract (FMRT) can be divided into two regions namely the upper FMRT (endocervix, uterus and the ovaries) and the lower FMRT (ectocervix and vagina)[430]. The human cervix is mainly made up of two cell types and has three main regions: endocervix, transformation zone and the ectocervix. The ectocervix is covered in multiple layers of cells that are stratified and keratinized[10]. The two dominant cell types found in the ectocervix are the epithelial cells and stromal cells[431]. The stromal layer is below the basement membrane and mainly consists of fibroblasts, smooth muscle cells and immune cells embedded in a collagen rich ECM[432]. In the ectocervix, the keratinized stratified epithelial cells act as the physical barrier to potential foreign pathogens that infiltrate the FMRT. These epithelial cells are the frontline of the host defense as they come into direct contact with various foreign microorganisms, sexually transmitted infections (STI), and semen[433]. As previously described, SF interacts directly with cells in the cervix leading to an inflammatory response that penetrates through the stratified epithelial cell layer deep into the stroma[261]. This highlights that the effects of SF on cervix go further than the monolayer of epithelial cells and may regulate multiple cells in the cervical tissue environment. The complexity and diversity of human tissue is frequently oversimplified in traditional 2D *in vitro* cell models[434]. In order to better understand the effect of SF on the FMRT, specifically during tumorigenesis, adequate tissue models are needed.

While conventional 2D *in vitro* cell culture models are useful in many areas of cancer research, these models have limitations. Isolated cells are void of important aspects found in the tissue environment such as cell-cell interactions, cell heterogeneity and tissue architecture[435]. Advances in tumor biology have shown that the complex interaction between tumor cells and their adjacent microenvironment is mandatory for cancer progression[436]. This has been shown in the TME, which is made up of surrounding blood vessels, stromal cells, fibroblasts, immune cells, and the extracellular matrix all of which are constantly interacting with tumor cells[437]. Therefore, tissue explants and *ex vivo* tissue models function as a bridge between 2D *in vitro* cell culture models and *in vivo* models[434].

Patient derived explants have the advantage of preserving the tissue architecture. Other advantages of 3D tissue explants are the maintenance of tissue heterogeneity, cell-cell, and cell-stroma interactions as well as interaction with the ECM[10]. In vitro 3D cultures of tumor and stromal cells have been shown to better represent the tissue architecture and phenotypical features observed in tumors[438]. Cervical explants were first developed by Fink et al. (1973) who studied epithelium metaplasia using large tissue explants cultured on a thin agarose gel in serum-free medium[439]. Since then, other studies have used cervical tissue explants/biopsies to investigate microbicides or to study events of FMRT infection[440,441]. Previous research in our laboratory and others has investigated the effect of SF on cervical tissue biopsies. In a study by Sharkey et al. (2012) the effect of SF on the ectocervix tissue was investigated by collecting small needle tissue biopsies from ovulating women before and after vaginal intercourse (in the absence of a condom). This study revealed that SF exposure at coitus caused an accumulation of T cells and macrophages in the epithelial and stromal layers of the ectocervix and this was accompanied by an upregulation of IL-6, IL-8 and IL-1α[261].

Another study showed that treatment with SF significantly upregulated IL-1α in normal cervical tissue explants and cervical cancer biopsies[279].

In this study we aim to investigate the effect of SF on EMT transcription factors, MMPs and inflammatory and angiogenic gene expression in ectocervical tissue biopsies. Our earlier findings showed that SF upregulated EMT-TFs, MMPs, proinflammatory and angiogenic gene expression in two cervical squamous carcinoma cell line models. In addition, inhibition of KpnB1 with INI-43 was shown to inhibit SF mediated gene expression. Due to issues of access to readily available cervical cancer biopsies, normal cervical biopsies were used instead. Here, we used normal ectocervical biopsies obtained from women undergoing hysterectomies for non-cancerous reasons, as an *ex vivo* model to evaluate the effect of SF on EMT-TFs, MMPs and to investigate the effect of INI-43 on SF mediated gene expression.

6.2 RESULTS

6.2.1 SF induces Snail, Twist and ZEB1 mRNA expression in normal ectocervical tissue

To investigate the effect of SF on EMT-TFs induction, ectocervical tissue biopsies were cut into small pieces and equally divided into control and experimental groups. Biopsies were incubated with either vehicle (PBS) as control or SF for 24hrs after which mRNA extracted from eight tissue biopsies was analysed by RT-qPCR. Results showed that SF significantly induced the mRNA expression of Snail, Twist and ZEB1 mRNA expression in normal ectocervical tissue (Figure 6.1.). The extent of induction was similar across all EMT-TFs investigated. This result suggests that SF stimulates Snail, Twist and ZEB1 upregulation in normal cervical tissue.

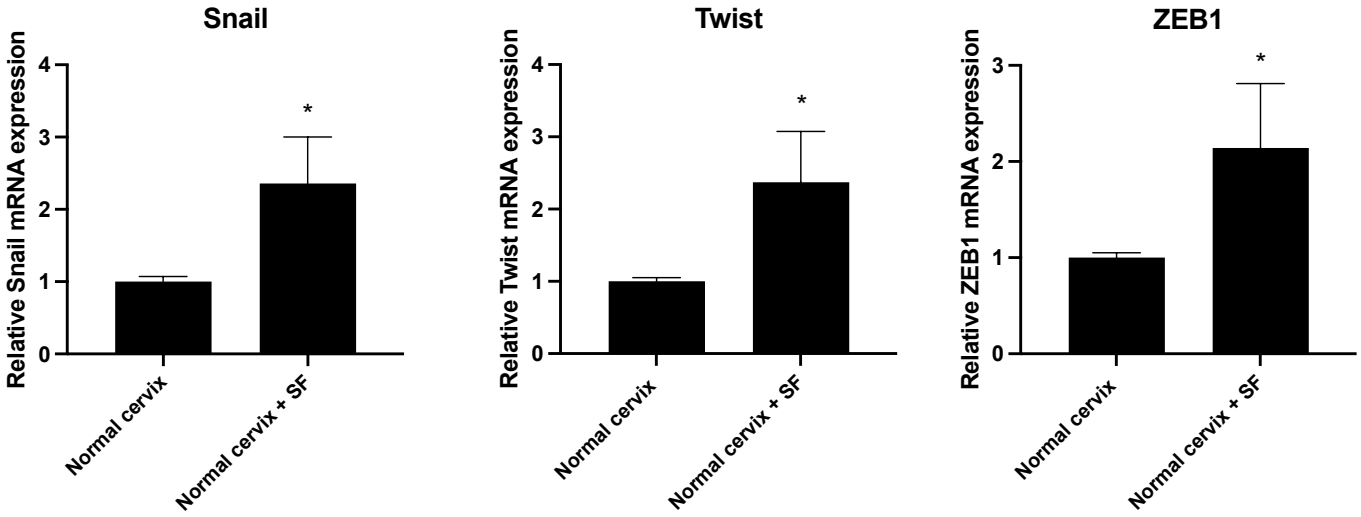


Figure 6.1 SF stimulates Snail, Twist and ZEB1 mRNA expression in normal ectocervical explants. Relative Snail, Twist and ZEB1 mRNA expression in normal ectocervical tissue treated with vehicle (PBS) or SF (1:50) for 24hrs as determined by RT-qPCR. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Data presented as mean ± SEM (n=8). (*P<0.05).

6.2.2 SF induces MMP-9 mRNA expression in normal ectocervical tissue

Having observed the induction of EMT-TFs in normal cervical tissue, next we investigated whether SF had an effect on the mRNA expression of gelatinases MMP-2 and MMP-9. Treatment of ectocervical tissue with SF showed an increase in MMP-2 mRNA expression, however results were not significant. In contrast, results showed that SF significantly upregulated MMP-9 mRNA expression (Figure 6.2.). This suggests that SF induces the mRNA expression of MMP-9 but possibly not MMP-2 in normal cervical tissue.

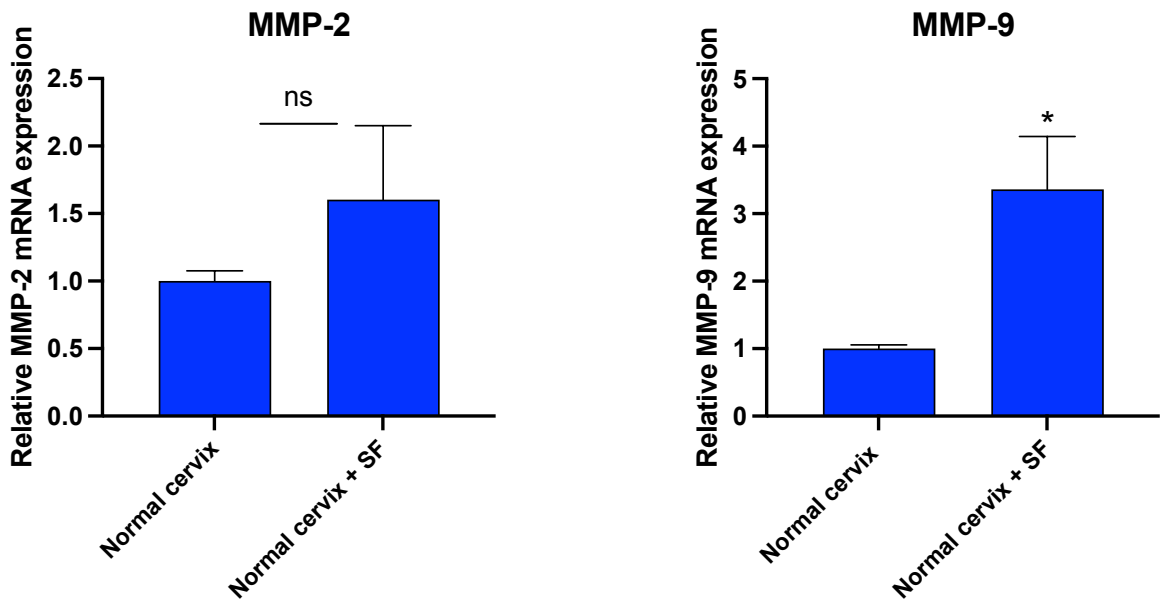


Figure 6.2 SF induces MMP-9 mRNA expression in normal ectocervical explants. Relative MMP-9 and MMP-2 expression in normal ectocervical tissue treated with vehicle (PBS) or SF (1:50) for 24hrs as determined by RT-qPCR. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Data presented as mean ± SEM (n=7). (*P<0.05).

6.2.3 INI-43 inhibits SF induced proinflammatory gene expression in ectocervical explants

Our *in vitro* results showed that INI-43 had an inhibitory effect on SF mediated proinflammatory gene expression. Here we investigated whether INI-43 had an effect on SF induced proinflammatory gene expression employing ectocervical tissue. Normal cervical tissue was stimulated with SF or vehicle in the absence/presence of INI-43 (10μM) for 24hrs, and gene expression was analyzed by RT-qPCR. Results showed that SF significantly induced COX-1, COX-2, IL-6 and IL-1α gene expression. Addition of INI-43 completely blocked SF mediated COX-2 and IL-1α gene expression and significantly blocked SF mediated COX-1 and IL-6 gene expression by 50% and 52% respectively (Figure 6.3.). These results show that INI-43 inhibits SF mediated proinflammatory gene expression in normal cervical tissue.

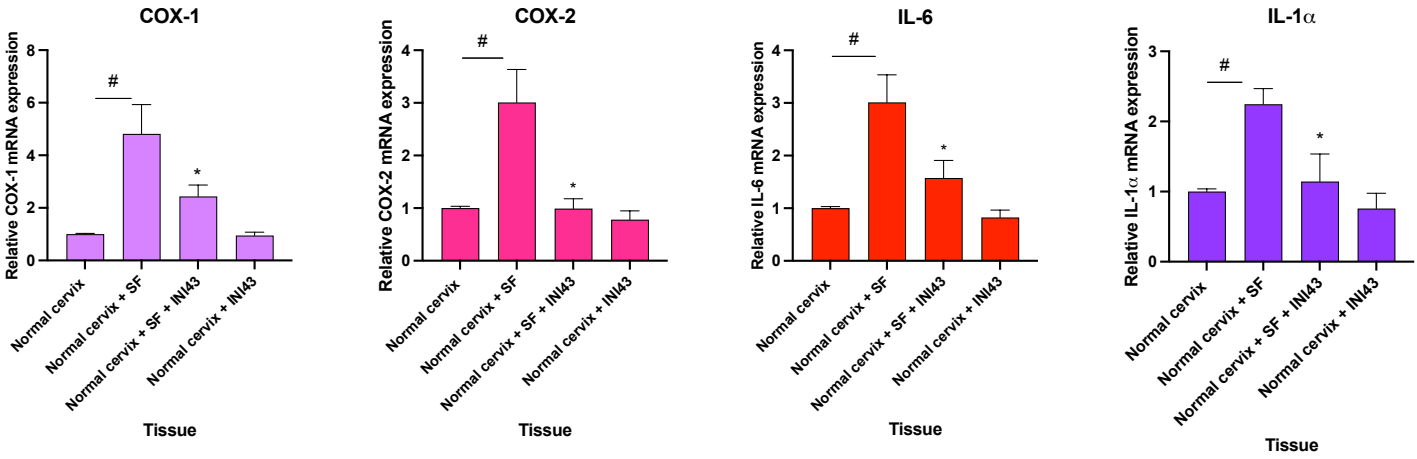


Figure 6.3 INI-43 inhibits SF induced COX-1, COX-2, IL-6 and IL-1α mRNA expression in normal ectocervical explants. Relative COX-1, COX-2, IL-6 and IL-1α mRNA expression in normal ectocervical tissue treated with vehicle (PBS) or SF (1:50) and INI-43 [10μM] for 24hrs as determined by RT-qPCR. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Data presented as mean ± SEM (n=6). (*P<0.05).

6.2.4 INI-43 inhibits SF mediated angiogenic gene expression in ectocervical tissue.

Previous research has shown the upregulation of angiogenic genes by SF stimulation. Here we investigate whether INI-43 has an inhibitory effect on SF mediated angiogenic gene expression employing ectocervical tissue biopsies. Normal cervical tissue was stimulated with SF or vehicle in the absence/presence of INI-43 (10μM) for 24hrs, and gene expression was analyzed by RT-qPCR. Results show that SF significantly upregulated IL-8, Gro-α and VEGF gene expression, with Gro-α showing the highest induction. Treatment with INI-43 completely inhibited SF mediated IL-8, Gro-α and VEGF-A mRNA upregulation (Figure 6.4.). Together these results show that INI-43 significantly inhibits SF mediated angiogenic gene expression in normal ectocervical tissue.

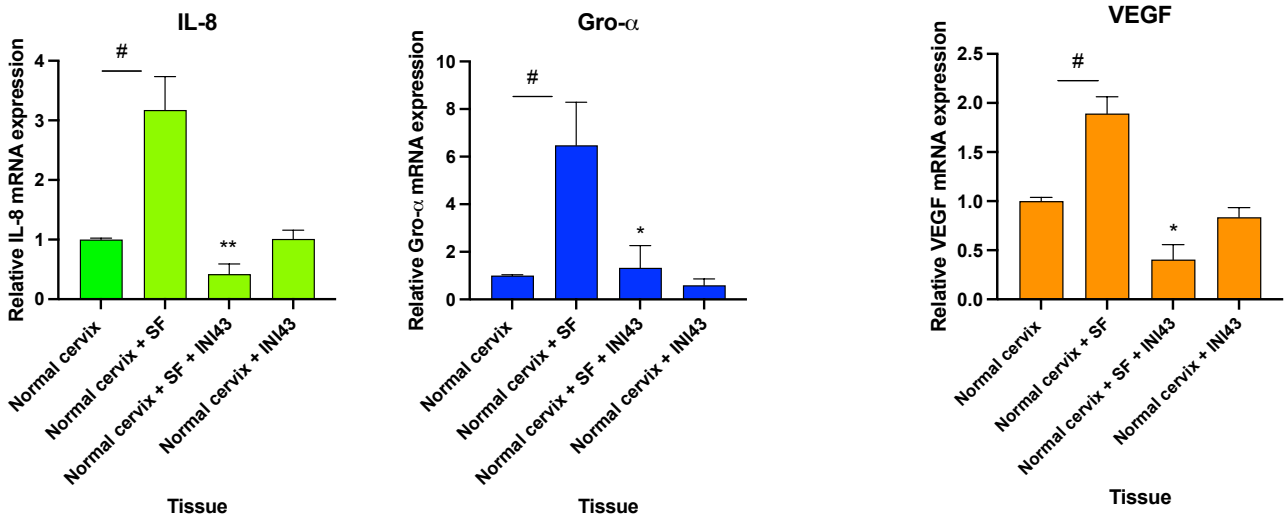


Figure 6.4 INI-43 inhibits SF mediated IL-8, Gro-α and VEGF mRNA expression in normal ectocervical explants. Relative IL-8, Gro-α and VEGF mRNA expression in normal ectocervical tissue treated with vehicle (PBS) or SF (1:50) and INI-43 [10μM] for 24hrs as determined by RT-qPCR. Samples were standardized to the house keeping gene B-glucuronidase (*GusB)*. Data presented as mean ± SEM (n=6).

6.3 DISCUSSION

Healthy functioning of the FMRT is maintained by a balanced and dynamic regulation that is coordinated by sex hormones during the menstrual cycle, pregnancy, and childbirth[442]. Vaginal intercourse is the main route of infection for women[10]. The cervix, which is part of the lower FMRT, plays an essential role in the immune response of the FMRT. Overall, the cervical epithelial cells and immune cells from the endo- and ectocervix protect the upper FMRT from infiltrating pathogens[443]. Therefore, these epithelial cells are seen as the first responders as they come into direct contact with pathogens, STIs and semen[433]. The ectocervix is lined by multiple layers of stratified epithelial cells that create a physical barrier to underlining basal cells and the stromal layer[10]. Though the stratified epithelial layer is able to protect the FMRT, micro abrasions caused by friction during coitus damage the epithelial layer[444] and therefore exposes underlying cells to potential pathogens such as HPV that can penetrates the epithelial layer and infects basal cells.

SF which is a biological fluid rich in an array of bioactive molecules, has been shown interact directly with epithelial cells of the cervix and elicit an immune response that penetrates into the stromal layer[256]. This immune response is characterized by a surge in inflammatory cytokines and chemokines as well as a recruitment of immune cells[261]. Increasing evidence has implicated SF in the progression of cervical cancer. Our laboratory previously showed the upregulation of inflammatory and angiogenic genes in adenocarcinoma HeLa cells in response to SF treatment[279,273]. SF was also shown to enhance cell proliferation *in vitro* and *in vivo* using HeLa cells[277]. Additionally, upregulation of angiogenic genes in response to SF stimulation, was shown to be regulated by key oncogenic signaling pathways which triggered phenotypic changes in cervical tissue such as the promotion of vasculature/angiogenesis[278]. This suggests

that bioactive molecules present in SF, that come into direct contact with target cells, may play an important role in modulating neoplastic cervical cells in sexually active women by initiating signaling cascades that may enhance certain hallmarks of cancer.

Despite the growing evidence that shows a link between cervical tumorigenesis and SF exposure, more data into the effects of SF on cervical tissue is needed. For many years, cancer biology research has faced a challenge of a translational gap between *in vitro* and *in vivo* models[10]. Although 2D monolayer cell culture models have long been the standard model in cancer biology research, their ability to represent the complexity of human tissue is limited[445]. Cancer progression has been shown to be dependent on the plasticity and complex cellular interactions in the TME[436]. The development of 3D and *ex vivo* models in cancer biology research has introduced models that better represent the architecture, interactions and molecular characteristics of tumors or tissue[435]. Evidence on the effect of SF on normal cervical tissue is limited hence the objective of this study was to investigate whether upregulated expression of EMT transcription factors, MMPs, proinflammatory and angiogenic genes observed in cervical carcinoma cells is also induced in normal cervical tissue in response to SF stimulation.

Here we showed that SF significantly upregulated Snail, Twist and ZEB1 gene expression in normal cervical tissue biopsies. In a study using endometrial tissue biopsies, 10% SF was shown to upregulate ZEB2 mRNA expression after 6hrs. Other mediators of EMT such as Snail 1 and 2 showed a trend of higher expression, although not significant due to high variability[319]. As previously described EMT transcription factors such as Snail, Twist and ZEB are regulated by multiple signaling pathways. Therefore, it is likely that the inflammatory response initiated by

SF in the FMRT can activate other phenotypes such as EMT by directly activating EMT signaling pathways. In a study that used breast and lung carcinoma cells, it was shown that EMT can be triggered by cytokines normally produced in an inflammatory environment (TGF-β, TNF-α and IF-γ)[446]. With SF being rich in an array of bioactive molecules such as TGF-β, cytokines, PGE2 and growth factors; these molecules may activate signal transduction pathways leading to the activation of EMT-TFs in cervical tissue as observed in our study.

We further showed that SF upregulated MMP-9 mRNA expression but did not significantly enhance MMP-2 expression. Upregulation of MMP-9 mRNA expression has been previously shown in adenocarcinoma cells in response to SF[280]. Our study shows the upregulation of MMP-9 in cervical squamous carcinoma cells and in normal cervical tissue in response to SF. In another study using cervical carcinoma cells, treatment with SF (1:50) significantly upregulated MMP-9 expression but did not enhance MMP-2 expression[329]. This in turn suggests that similar to cervical carcinoma cells, SF upregulates MMP-9 in normal cervical tissue and may likely induce MMP expression in neoplastic cervical tissue.

In our study SF induced the upregulation of proinflammatory and angiogenic genes in normal cervical tissue. In line with our study, previous research in our laboratory showed the upregulation of IL-1α in normal and cancerous cervical tissue in response to SF stimulation[256]. Previous research has also shown the upregulation of proinflammatory (IL-6, COX-1, COX-2,IL-11) and angiogenic (IL-8, Gro-α, VEGF) genes in normal cervical tissue after stimulation with SF[447]. The upregulation of COX-1 and COX-2 genes has also been shown in cervical cancer tissue[448]. These studies, together with results presented here suggests that SF upregulates genes associated with inflammation and angiogenesis in the cervix of sexually active women.

Furthermore, our study showed that INI-43 significantly inhibited SF mediated proinflammatory and angiogenic gene expression in normal cervical tissue. Inhibition of IL-6 and IL-1 mRNA expression by INI-43 has been previously shown in cervical cancer cell lines[238]. Our study is a first to show the inhibition of IL-1 and IL-6 using normal cervical tissue. This may suggest that INI-43 has the potential to inhibit SF mediated upregulation of inflammatory and angiogenic cytokines and chemokines in the cervix.

Taken together, this preliminary data presented using *ex vivo* non-cancerous cervical tissue shows that SF upregulates EMT transcriptions factors, MMP-9 and genes associated with inflammation and angiogenesis in normal cervical tissue. Additionally, treatment with INI-43 can block SF mediated inflammatory and angiogenic gene expression. It is likely that SF may enhance EMT transcription factors, MMP-9 and inflammation in cervical cancer tissue too. To further understand the role of SF in EMT and gene expression, biopsies from low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL) and invasive cervical cancer tissue can be used to investigate whether SF mediated phenotypes witnessed in 2D cell culture translate in cervical dysplasia or invasive cervical cancer. Due to issues of access to available LSIL, HSIL and invasive cervical cancer tissue this could not be explored in the current study.

Chapter 7: General Discussion & Conclusion

7.1 GENERAL DISCUSSION

Cervical cancer is the fourth most common cancer in women world-wide. The incidence of cervical cancer is the highest amongst women in Southern, Eastern and Western Africa[1]. In South Africa, cervical cancer is the second most common cancer and the leading cause of mortalities in women aged between 15-44[6]. Although cervical cancer is a preventable disease that is curable when diagnosed early, this disease remains deadly amongst women in low-to-middle income countries (LMICs). Due to screening and early detection not being readily available, the burden of cervical cancer in LMICs is the highest[449]. Approximately 85% of new cases and 90% of cervical cancer mortalities are in LMICs[5], highlighting cervical cancer as a major public health problem in LMICs.

The main causal factor for cervical cancer is persistent infection with high risk- HPV (hr-HPV)[9]. HPV is a common sexually transmitted virus that infects basal cells of the cervix by penetrating the epithelial layer through micro abrasions caused by friction during coitus[21]. Infection with HPV can be cleared naturally by most women, however a subset of women experience persistent infection with hr-HPV putting them at risk of developing pre-cancerous lesions that may progress to invasive cervical cancer[16]. HPV triggers the development of cervical neoplasia by directly integrating its DNA into the hosts epithelial cell DNA and using its oncoproteins to subsequently dysregulate the hosts intracellular signaling pathways, cell cycle and replication[25,21]. HPV has been implicated in 99% of cervical squamous carcinoma cases worldwide[24]. Adenocarcinomas of the cervix are related to HPV infection but correlation with HPV is not as prominent and is also age specific[21]. Although, persistent infection with hr-HPV

is the main etiology for cervical cancer, HPV alone is not enough for the development of malignant transformation. Various factors that work in conjunction with oncogenic HPVs play a role in the development of cervical lesions and invasive cervical cancer[8]. Risk factors that have been implicated in cervical cancer development include long-term contraceptive use, suppressed immunity, smoking and high parity[8]. Interestingly, mounting evidence has implicated seminal fluid in cervical cancer.

Seminal fluid (SF) is a biological fluid rich in an array of bioactive molecules. For many years, SF was merely seen as a medium that transport sperm however, accumulating evidence has shown an important role for SF in conception and embryo development[257]. It is known that SF elicits an inflammatory like response in the FMRT that is characterized by a surge of chemokines, cytokines and the recruitment of immune cells which is required for a successful pregnancy[261,263]. In the context of a normal functioning immune response SF mediated inflammation may not be of concern however, in sexually active women with cervical intraepithelial lesions or cervical neoplastic cells this inflammatory response elicited by SF may aggravate cervical tumorigenesis by creating an inflammatory environment in the cervix.

Cervical cancer is regarded as a chronic inflammatory disease[178]. Inflammation is a normal defense response to tissue damage caused by pathogens or other stimuli. Most infections or pathogens are known to activate a self-contained fast inflammatory response (acute) where immune activation and signaling is terminated after healing[182]. In the case of chronic inflammation, dysregulation of the immune response leads to a persistent and non-resolving inflammatory response which predisposes the host to diseases such as cancer[183,450]. Inflammation has been accepted as an enabling characteristic in carcinogenesis[47].

Inflammation has been shown to contribute to multiple hallmarks of cancer by producing signaling molecules such as growth factors that sustain cell proliferation, survival factors that inhibit cell death, extracellular matrix enzymes that facilitate angiogenesis, invasion and metastasis as well as stimuli that lead to the activation of EMT or other hallmark-driving programs[58–60,451]. A key feature of chronic inflammation is the upregulation of inflammatory mediators, cytokines and chemokines that act as signals that amplify the immune response and the recruitment of immune cells in the tissue[452]. This upregulation of cytokines and chemokines as well as immune cells in the tissue is the main factor that led to the implication of seminal fluid in cervical cancer in our laboratory.

Previous research in our laboratory as well as others, has shown an upregulation of a host of inflammatory cytokines in cervical cancer cells in response to SF [279,261,273,426]. In addition, SF was shown to enhance cell proliferation of adenocarcinoma cells in vitro and in vivo[277]. Furthermore, SF activated various oncogenic pathways such as NF-kB, EGFR, ERK1/2, COX-1/2 and PI3K/Akt in cervical adenocarcinoma cells which in turn led to the upregulation of tumorigenic genes, enhancement of angiogenic genes and activation of vasculature[279,278,273,276]. As previously described, inflammation has been identified as an enabling characteristic in tumorigenesis therefore, inflammation elicited within the cervical environment may also drive or enhance other hallmarks of neoplastic cells in the cervix.

Our study shows that SF stimulated other cervical cancer cell biological phenotypes such as cell proliferation, actin rearrangement, EMT, directional migration and invasion of cervical squamous carcinoma cells. EMT is a process that occurs during embryogenesis, chronic inflammation and fibrosis[64]. EMT can be initiated by various factors such TGF-β, TNF-α and

cytokines[446], which have also been shown to be present in SF. Activation of EMT results in cytoskeleton rearrangement that enables cell elongation and motility[70]. This was evident in our study, as SF induced morphological changes and directional migration & invasion in cervical squamous carcinoma cells. Our study further showed the upregulation of MMP-2 and MMP-9 in response to SF. The role of MMPs as proteolytic enzymes that remodel the ECM is well known. In addition, MMPs have been shown to be regulated by EMT factors. The EMT inducing transcription factor Snail has been reported to activate the expression of MMP-2 and MMP-9 in squamous carcinomas[453] and oral squamous carcinomas[454]. In particular, increased expression and activation of MMP-2 and MMP-9 has been associated with nodal metastasis and recurrence in cervical squamous carcinomas[455]. Together, this suggests that bioactive molecules in SF that interact with cervical carcinoma cells may elicit an inflammatory response and activate other cancer biology phenotypes such as cell proliferation, EMT, motility and invasion.

Little is known about the mechanism of SF mediated cell proliferation, actin rearrangement, EMT and migration in cervical cancer. In this study we showed that SF mediated cell proliferation was regulated via the COX-1 and ERK1/2 signaling pathways. Previous research in our laboratory has reported the upregulation of COX-1 in cervical cancer. In addition, the overexpression of COX-1 was associated with the upregulation of COX-2 and EP receptors in HeLa cells[253]. Enhanced PGE2 signaling has also been reported in cervical carcinoma together with upregulated expression of EP2 and EP4 receptors. The upregulation of COX enzymes, EP2 and EP4 receptors in response to SF stimulation in cervical adenocarcinoma cells has been reported[276]. A study using colon cancer cells showed enhanced cell proliferation and tumorigenic effects requires PGE2/EP4 signaling[456]. It is likely that a similar mechanism may

occur in cervical squamous carcinoma cells where cell proliferation could be enhanced via EP receptors. PGE2 has also been shown to enhance cell proliferation via the activation of the Ras-MAPK-ERK pathway and COX-2 in intestinal adenocarcinomas[457].It is possible that PGE2 is a ligand present in SF that binds receptors on neoplastic cells to activate cell proliferation. Our data together with other published studies suggests that proliferation induced in neoplastic cells of the cervix by SF could be mediated via the COX-1 and ERK1/2 signaling pathways. In our study these signaling pathways were also shown to play a role in actin rearrangement, EMT induction as well as SF mediated migration and invasion.

Induction of EMT leads to various phenotypic changes such as cytoskeletal rearrangement, loss in cell-cell interaction, polarity and increased motility[458]. EMT is induced by specific activators known as EMT inducing transcription factors (EMT-TFs)[318]. In our study we showed that the EMT-TFs Snail, Twist and ZEB1 were all differently regulated in response to SF. We showed that SF induced Snail, Twist and ZEB1 was occurring via PGE2/EP4, ERK1/2 and COX-1 or COX-2 signaling pathways. Since the transcription of each of these EMT-TFs is regulated by several signaling pathways such as TGF-β, TNF-α, NF-kB, MAPK, PI3K and others[355–357,364], it is likely that SF mediated EMT may occur via multiple parallel pathways. This suggests that EP4 receptors present on neoplastic cell membranes in the cervix can bind PGE2 present in SF and endogenously produced by COX enzymes to activate EMT-TFs which in turn activates EMT through ERK1/2 signaling as well as other EMT signaling pathways. Our study also showed the involvement of EGFR signaling in SF mediated actin rearrangement, migration and ZEB1 activation. A role of EGF in EMT and migration has been previously reported. In a study by Zhao et al (2017), EGF-stimulated EMT and migration was shown to occur via ZEB activation through PI3/Akt signaling in gastric carcinoma cells[459]. Therefore, highlighting

another possible signaling pathway that may play a role in SF mediated EMT induction in cervical carcinoma cells. Since EMT signaling pathways have been shown to be activated by inflammatory mediators (e.g TNF-α, TGF-β, NF-kB, cytokines), it is also likely that cytokines present in SF may trigger signaling pathways that activate inflammation and EMT concurrently in neoplastic cells in the cervix.

The link between inflammation and cervical cancer has been established. Previous research in our laboratory has shown that SF exacerbates inflammation and angiogenesis in the cervix by upregulating genes associated with inflammation and angiogenesis[279,277,273]. SF mediated upregulated angiogenic genes, such as IL-8 and Gro-α, have been shown to activate vasculature via the NF-kB signaling pathway in adenocarcinoma cells[278]. Therefore, SF may exacerbate the already upregulated inflammatory mediators present in the cervical tumour microenvironment.

The approach of blocking the nuclear entry of inflammatory transcription factors such as NF-kB, AP-1 and NFAT as an approach to control inflammation was proposed by Hawiger (2019) and co-workers[229]. They showed that blocking NF-kB nuclear import suppressed the production of proinflammatory cytokines (TNF-α and IF-γ) in mice challenged with LPS[460]. Since inflammatory mediators such as NF-kB and AP-1 require nuclear entry to activate a host of proinflammatory and angiogenic genes, in our study we proposed that INI-43 could abrogate SF mediated proinflammatory and angiogenic gene expression.

INI-43 is a novel small molecular inhibitor of KpnB1 that has been shown to inhibit the nuclear entry of KpnB1 cargoes such as NF-kB, AP-1 and NFAT in cervical cancer cells[238,234]. Previous

research in our laboratory showed that INI-43 treatment blocked the upregulation of proinflammatory genes such as IL-1, IL-6, GM-CSF and TNF-α in cervical cancer cells stimulated with PMA[238]. Considering this, we investigated the effect of INI-43 on SF mediated proinflammatory and angiogenic gene expression. Key findings generated in this study showed that SF upregulated the expression of KpnB1. Overexpression of KpnB1 has been reported in several carcinomas[292]. This overexpression has been linked to an increased rate of the KpnB1 mediated nuclear import of KpnB1 cargoes including AP-1 and NF-kB[461,462]. Therefore, in our study interaction of neoplastic cervical cells with SF may further upregulate the already overexpressed KpnB1 and increase nucleo-cytoplasmic transport. We showed that INI-43 inhibited SF mediated proinflammatory and angiogenic gene expression. It was proposed that SF mediated gene expression occurs via inflammatory transcription factors such as AP-1 and NF-kB. Our study showed that SF significantly activated AP-1 and NF-kB activity. Treatment with INI-43 blocked SF mediated NF-kB activity and nuclear import however, AP-1 activity was unaffected by INI-43 treatment. Taken together, we showed that SF mediated gene expression associates with KpnB1-mediated nuclear import of transcription factors such as NF-kB that ultimately has an effect on the expression of proinflammatory and angiogenic genes.

7.2 CONCLUSION

Our study provides evidence that SF regulates the activation of various signaling pathways that activate inflammation which in turn promote cancer development by activating EMT, motility and invasion via the induction of EMT inducing transcription factors, MMPs and angiogenic factors all of which play a role in modulating the cervical microenvironment. Moreover, we show that nuclear import pathways are needed for SF induced activation of proinflammatory and angiogenic factors.

This study suggests that repeated exposure of neoplastic cervical epithelial cells to SF may have an impact on inflammation and tissue remodelling and driving hallmarks of cancer such as proliferation, inflammation, angiogenesis and tumour invasion. Together this may aggravate cervical tumorigenesis in sexually active women.

7.3 FUTURE WORK

Recommended future work includes the use of a positive control e.g. TGF-β to investigate the extent of proliferation and EMT induced by SF in cervical squamous carcinoma cells. As we speculate and have data to suggest that SF induces EMT, further experiments to support this could include live cell imaging to examine the effects of SF on the cell morphology of live, unfixed cells.

One of the limitations present in this study is the limited number of inhibitors used to investigate the mechanistic pathway involved in SF induced EMT and migration. With SF containing a range of bioactive molecules, future experiments should include the use of other inhibitors such as TGF-β, PI3K/Akt or NF-kB inhibitors. In addition, future experiments can

include testing the chemical inhibitors on SF induced MMP-2 and MMP-9 mRNA expression to elucidate the signaling pathways involved in SF induced invasion.

Our data showed promising results of INI-43 inhibiting SF induced proinflammatory and angiogenic gene expression. Our data further showed that INI-43 inhibited SF induced NF-kB transcriptional activity. Therefore, we postulated that INI-43 was blocking SF induced proinflammatory and angiogenic gene expression by blocking the nuclear import of NF-kB. To confirm this, future experiments should include testing NF-kB inhibitors on SF induced inflammatory and angiogenic gene expression.

Due to a lack of access to cervical cancer biopsies, this study used normal ectocervical tissue biopsies to investigate the effect of SF on EMT, MMPs, inflammatory and angiogenic gene expression. Future experiments can include the use of neoplastic cervical biopsies (LSIL or HSIL) or invasive cervical cancer biopsies to investigate whether the SF-induced effects seen in cervical squamous carcinoma cells *in vitro* translates to cervical cancer tissue, which is more heterogenous in nature. In addition, patient derived cervical epithelial cells can be isolated form cervical cancer tissue and used to investigate the effect of SF on cell morphology, F-actin arrangement, proliferation, migration and EMT.

APPENDIX I: Buffers and solutions

Tissue culture solutions

Complete Media
500mL DMEM
50mL FCS (10%)
5mL Penicillin/Streptomycin

Serum-free Media
500mL DMEM
5mL Penicillin/Streptomycin

Cryopreservation Media
90% Complete Media
10% DMSO

10XPBS
1g KCL
1g KH_2PO_4
40g NaCl
5.75 $Na_2HPO_4.7H_2O$
Make up to 500mL with dH_2O.
Autoclave

MTT solution (5mg/mL)
100mg MTT powder
20mL 1XPBS
Vortex and incubate in 37C water for 15minutes.
Filter sterilize and store at 4°C

Solubilization Solution
25g SLS
76.6μL concentrated HCl
Make up to 250mL with dH_2O.

Crystal violet solution (0.5%)
500mg crystal violet
25mL Methanol
75mL dH_2O

Actin staining solutions.

16% Paraformaldehyde
8g Paraformaldehyde
50mL dH_2O
Stir and heat at 60°C until solution turns milky.
Add 0.1M NaOH dropwise until solution clears.
Cool to room temperature and store at -20°C

4% Paraformaldehyde
2.5mL 16% Paraformaldehyde
1mL 10X PBS
6.5mL dH_2O

0.04% PBS-Tween
12μL Tween
30mL 1XPBS

1% BSA
0.2g BSA
20mL 1XPBS

Mowial
9.6g Moviol 4-88
24mL Glycerol
24mL dH_2O
48mL 0.2M Tris (pH 8.5)

Immunofluorescent staining solutions

Permeabilization solution (0.25% Triton)
0.125mL Triton X-100
49.88mL 1XPBS

Blocking solution
0.5g BSA
1.126g Glycine
Make up to 50mL with PBS-Tween

RNA Solutions

10X MOPS
41.86g MOPS
16.6mL 3M Sodium acetate

20mL 0.5M EDTA

0.5M EDTA
95.1g EDTA
500mL dH_2O
pH to 8

RNA gel (1.5% gel)
0.75g Agarose
5mL 10X MOPS
42mL dH_2O
2.7mL Formaldehyde (37%)
2.5µL Ethidium bromide

RNA Loading dye.
160µL 10X MOPS
720µL Formamide
260µL Formaldehyde (37%)
40µL dH_2O
200µL 0.25% Bromophenol blue
100µL 80% Glycerol

Running Buffer
250mL 1X MOPS
2µL Ethidium bromide

Protein solutions

RIPA
1g Sodium deoxycholate (1%)
3mL 5M NaCl (150mM)
1mL Triton X-100
1mL 10% SDS (0.1%)
1mL 1M Tris pH7.4 (10mM)
94mL dH_2O
Store at 4°C

Complete RIPA
0.1mM Na_3VO_4
1X Protease Inhibitor (Roche)
Make up to desired volume with RIPA.

10% SDS
20g SDS
Make up to 200mL with dH_2O.

1M Tris
60.5g Tris
300mL dH_2O
Adjust pH to desired pH of 6.8 or 8.8

10X TBS
24.23g Tris
80.1g NaCl
Adjust pH to 7.6
Make up to 1L with dH_2O

1X TBST
100mL 10X TBST
900mL dH_2O
1mL Tween

10% APS
1g APS
1mL dH_2O

10X Running Buffer
20g Glycine
31.6g Tris
5g SDS
500mL dH_2O

10X Transfer Buffer
72g Glycine
19g Tris
500mL dH_2O

1X Transfer Buffer
700mL dH_2O
200mL Isopropanol
100mL 10X Transfer buffer.

4x Loading dye.
2.5mL 1M Tris (pH 6.8)
3mL 20% SDS
0.5mL 0.1% Bromophenol Blue
4mL Glycerol
60μL β-mercatoethanol/0.6mL

10% Separating gel.
2.75mL dH_2O
3.75mL 1M Tris (pH 8.8)
100µL 10% SDS
3.35mL Bis-acrylamide (30%)
200µL 10% APS
20µL TEMED

4% Stacking gel
3.65mL dH_2O
625µL Tris (pH 6.8)
650µL Bis-acrylamide (30%)
50µL 10% SDS
60µL 10% APS
6µL TEMED

Stripping buffer
37.54g Glycine (1M)
500mL dH_2O
pH to 2.5

5% Milk
1g skimmed milk powder.
20mL TBST

DNA Solutions

10X TBE Buffer
54g Tris
27.5 Boric acid
3.7g EDTA
Make up to 500mL with dH_2O.

DNA gel (1%)
0.5g Agarose
50mL 1xTBE
2.5µL Ethidium Bromide

APPENDIX II

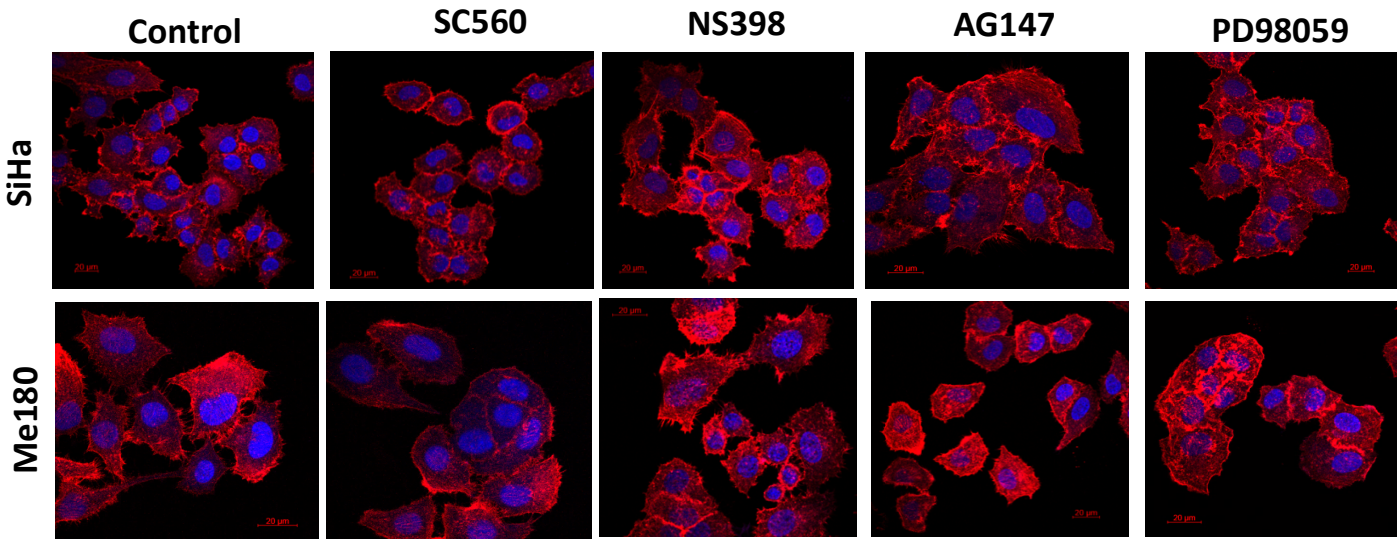


Figure A1: F-actin cytoskeleton rearrangement experiment: chemical inhibitor only controls. Representative immunofluorescent images of SiHa and Me180 cells stained with Phalloidin (cy3) for F-actin and Hoechst (blue) for the nuclei following treatment with SC560 [10μM], NS398 [10μM], AG1478[200nM] or PD98059[50μM] for 24hrs. Magnification=630x oil, scale bar=20μm.

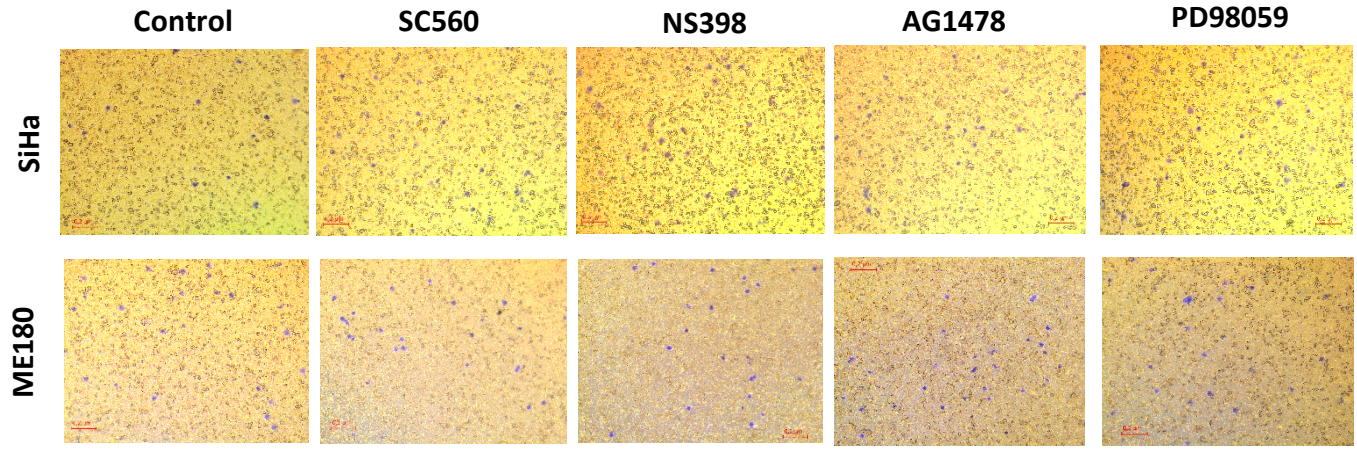


Figure A2: Transwell migration chemical inhibitor only controls. Representative images of transwell migration assay showing SiHa and Me180 cells that migrated through the membrane following an 8hr pre-treatment with SC560 [10μM], NS398 [10μM], AG1478[200nM] or PD98059[50μM]. Magnification x100, scale bar=0.2μm.

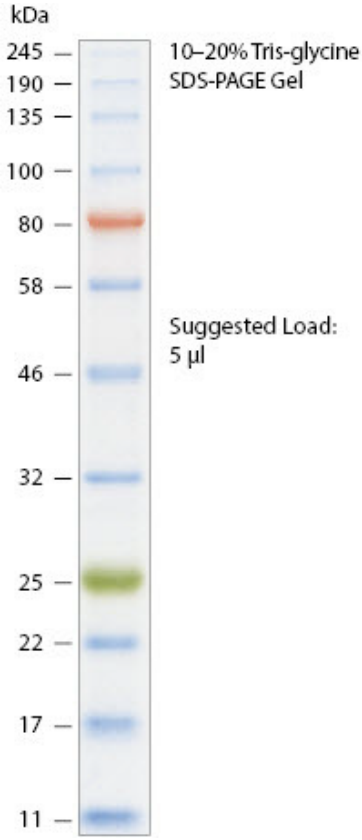


Figure A3: Prestained Color Protein Standard, Broad Range (11-245 KDa). Protein ladder used to determine protein band size on SDS-PAGE gels for western blotting.

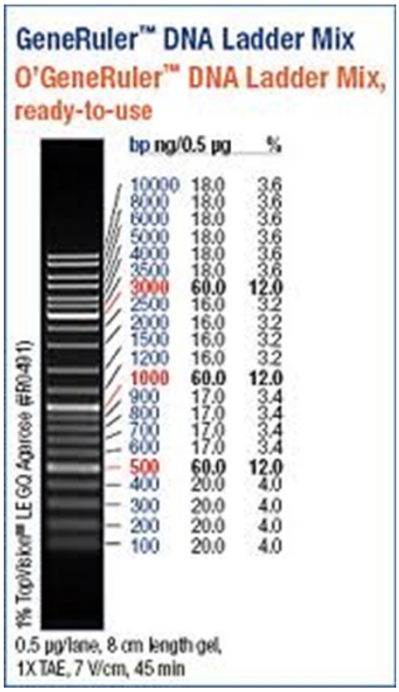


Figure A4: O'GeneRuler DNA Ladder Mix (100-1000 bps). DNA ladder used for size determination in agarose DNA gel.

REFERENCES

1. Sung, H. *et al.* Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA. Cancer J. Clin.* **71**, 209–249 (2021).
2. Gultekin, M., Ramirez, P. T., Broutet, N. & Hutubessy, R. World Health Organization call for action to eliminate cervical cancer globally. *Int. J. Gynecol. Cancer* **30**, 426 LP – 427 (2020).
3. Stelzle, D. *et al.* Estimates of the global burden of cervical cancer associated with HIV. *Lancet Glob. Heal.* **9**, e161–e169 (2021).
4. World Health Organization. *Global strategy to accelerate the elimination of cervical cancer as a public health problem and its associated goals and targets for the period 2020 – 2030*. *United Nations General Assembly* vol. 2 (2021).
5. Bhatla, N., Aoki, D., Sharma, D. N. & Sankaranarayanan, R. Cancer of the cervix uteri: 2021 update. *Int. J. Gynecol. Obstet.* **155**, 28–44 (2021).
6. Hopkins, K. L. *et al.* Assessing national cervical cancer screening guidelines: Results from an HIV testing clinic also screening for cervical cancer and HPV in Soweto, South Africa. *PLoS One* **16**, 1–13 (2021).
7. Tsu, V. D. & Levin, C. E. Making the case for cervical cancer prevention: what about equity? *Reprod. Health Matters* **16**, 104–112 (2008).
8. Burd, E. M. Human papillomavirus and cervical cancer. *Clin. Microbiol. Rev.* **16**, 1–17 (2003).
9. Vaccarella, S., Lortet-Tieulent, J., Plummer, M., Franceschi, S. & Bray, F. Worldwide trends in cervical cancer incidence: Impact of screening against changes in disease risk factors. *Eur. J. Cancer* **49**, 3262–3273 (2013).
10. Merbah, M. *et al.* Cervico-vaginal tissue ex vivo as a model to study early events in HIV-1 infection. *Am. J. Reprod. Immunol.* **65**, 268–278 (2011).
11. Deng, H., Hillpot, E., Mondal, S., Khurana, K. K. & Woodworth, C. D. HPV16-Immortalized Cells from Human Transformation Zone and Endocervix are More Dysplastic than Ectocervical Cells in Organotypic Culture. *Sci. Rep.* **8**, 1–13 (2018).
12. Rajendra, S. & Robertson, I. K. Similar immunogenetics of Barrett's oesophagus and cervical neoplasia: Is HPV the common denominator? *J. Clin. Pathol.* **63**, 1–3 (2010).
13. Iwasaki, A. Antiviral immune responses in the genital tract: Clues for vaccines. *Nat. Rev. Immunol.* **10**, 699–711 (2010).
14. Zur Hausen, H. Papillomaviruses and cancer: From basic studies to clinical application. *Nat. Rev. Cancer* **2**, 342–350 (2002).
15. Walboomers, J. *et al.* Human Papillomavirus Is a Necessary Cause. *J. Pathol.* **189**, 12–19 (1999).
16. Mishra, G. A., Pimple, S. A. & Shastri, S. S. An overview of prevention and early detection of cervical cancers. *Indian J. Med. Paediatr. Oncol.* **32**, 125–132 (2011).
17. Bernard, H., Burk, R. D., Chen, Z., Doorslaer, K. Van & Villiers, E. De. Classi fi cation of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. *Virology* **401**, 70–79 (2010).
18. McLaughlin-Drubin, M. E. Human papillomaviruses and non-melanoma skin cancer. *Semin. Oncol.* **42**, 284–290 (2015).
19. Roden, R. B. S. & Stern, P. L. Opportunities and challenges for human papillomavirus vaccination in cancer. *Nat. Rev. Cancer* **18**, 240–254 (2018).
20. Eide, M. L. & Debaque, H. HPV detection methods and genotyping techniques in screening for cervical cancer. *Ann. Pathol.* **32**, e15–e23 (2012).
21. McMurray, H. R., Nguyen, D., Westbrook, T. F. & McAnce, D. J. Biology of human papillomaviruses. *Int. J. Exp. Pathol.* **82**, 15–33 (2001).
22. Mirkovic, J. *et al.* Carcinogenic HPV infection in the cervical squamo-columnar junction. *J. Pathol.* **236**, 265–271 (2015).
23. Doorbar, J. *et al.* The biology and life-cycle of human papillomaviruses. *Vaccine* **30**, F55–F70

(2012).
24. Walboomers, J. *et al.* Human papilommavirus is a necessary vause of invasive cervical cancer worldwide. *J. Pathol.* **189**, 12–19 (1999).
25. Oyervides-Muñoz, M. A. *et al.* Understanding the HPV integration and its progression to cervical cancer. *Infect. Genet. Evol.* **61**, 134–144 (2018).
26. Moody, C. A. Human papillomavirus oncoproteins: pathways to transformation. *Nat. Rev. Cancer* **10**, 550–560 (2010).
27. Hopman, A. H. N. *et al.* Transition of high-grade cervical intraepithelial neoplasia to micro-invasive carcinoma is characterized by integration of HPV 16/18 and numerical chromosome abnormalities. *J. Pathol.* **202**, 23–33 (2004).
28. Rusan, M., Li, Y. Y. & Hammerman, P. S. Genomic Landscape of Human Papillomavirus – Associated Cancers. *Clin. Cancer Res.* **21**, 2009–2019 (2015).
29. Zhao, J. W. *et al.* HPV16 integration probably contributes to cervical oncogenesis through interrupting tumor suppressor genes and inducing chromosome instability. *J. Exp. Clin. Cancer Res.* **35**, 1–14 (2016).
30. McLaughlin-Drubin, M. E. & Münger, K. Oncogenic activities of human papillomaviruses. *Virus Res.* **143**, 195–208 (2009).
31. Rihet, S., Lorenzato, M. & Clavel, C. Oncogenic human papillomaviruses and ploidy in cervical lesions. *J. Clin. Pathol.* **49**, 892 LP – 896 (1996).
32. Sağnıç, S. Human Papillomavirus and Cervical Cancer. in (ed. Rajkumar, R.) Ch. 8 (IntechOpen, 2021). doi:10.5772/intechopen.98490.
33. Marei, H. E. *et al.* p53 signaling in cancer progression and therapy. *Cancer Cell Int.* **21**, 703 (2021).
34. DiMaio, D. & Mattoon, D. Mechanisms of cell transformation by papillomavirus E5 proteins. *Oncogene* **20**, 7866–7873 (2001).
35. Garland, S. M. *et al.* Natural history of genital warts: Analysis of the placebo arm of 2 randomized phase III trials of a quadrivalent human papillomavirus (types 6, 11, 16, and 18) Vaccine. *J. Infect. Dis.* **199**, 805–814 (2009).
36. World Health Organization. Human papillomavirus vaccines: WHO position paper, May 2017. *Relev. Epidemiol. Hebd.* **92**, 241–268 (2017).
37. Alrajjal A, Pansare V, Choudhury MSR, Khan MYA, S. V. Squamous intraepithelial lesions (SIL: LSIL, HSIL, ASCUS, ASC-H, LSIL-H) of Uterine Cervix and Bethesda System. *Cytojournal* **18**, 16 (2021).
38. Papanicolaou, G. N. Classes in oncology: George Nicholas Papanicolaou's new cancer diagnosis presented at the Third Race Betterment Conference. *CA Cancer J Clin* **23**, 174–179 (1973).
39. Jenkins, D. Histopathology and cytopathology of cervical cancer. *Dis. Markers* **23**, 199–212 (2007).
40. Spaans, V. M. *et al.* Precise classification of cervical carcinomas combined with somatic mutation profiling contributes to predicting disease outcome. *PLoS One* **10**, (2015).
41. Gien, L. T., Beauchemin, M. C. & Thomas, G. Adenocarcinoma: A unique cervical cancer. *Gynecol. Oncol.* **116**, 140–146 (2010).
42. Lee, J. Y. *et al.* Prognosis of adenosquamous carcinoma compared with adenocarcinoma in uterine cervical cancer: A systematic review and meta-analysis of observational studies. *Int. J. Gynecol. Cancer* **24**, 289–294 (2014).
43. Kaur, H. *et al.* Diagnosis, Staging, and Surveillance of Cervical Carcinoma. *Am. J. Roentgenol.* **180**, 1621–1631 (2003).
44. Muthusamy, R. K. & Mehta, S. S. Squamous Cell Carcinoma In situ of the Cervix with Superficial Intraepithelial Extension to the Endometrium of Lower Uterine Segment: A Rare Presentation. *Indian J. Med. Paediatr. Oncol.* **38**, 88–89 (2017).
45. Ashfaq, R. *et al.* ThinPrep Pap Test Accuracy for Glandular Disease. *Acta Cytol.* **43**, 81–85

(1999).
46. Bulk, S., Visser, O., Rozendaal, L., Verheijen, R. H. M. & Meijer, C. J. L. M. Cervical cancer in the Netherlands 1989-1998: Decrease of squamous cell carcinoma in older women, increase of adenocarcinoma in younger women. *Int. J. Cancer* **113**, 1005–1009 (2005).
47. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: The next generation. *Cell* **144**, 646–674 (2011).
48. Hanahan, D. & Weinberg, R. A. The Hallmarks of Caancer. *Cell* **100**, 57–70 (2000).
49. Feitelson, M. A. *et al.* Sustained proliferation in cancer: Mechanisms and novel therapeutic targets. *Semin. Cancer Biol.* **35**, S25–S54 (2015).
50. Sporn, M. B. & Roberts, A. B. Autocrine growth factors and cancer. *Nature* **313**, 745–747 (1985).
51. Bhowmick, N. A., Neilson, E. G. & Moses, H. L. Stromal fibroblasts in cancer initiation and progression. *Nature* **432**, 332–337 (2004).
52. Antignani, A. *et al.* Targeting Receptors on Cancer Cells with Protein Toxins. *Biomolecules* **10**, 1331 (2020).
53. Lococo, F. *et al.* Preliminary Evidence on the Diagnostic and Molecular Role of Circulating Soluble EGFR in Non-Small Cell Lung Cancer. *International Journal of Molecular Sciences* vol. 16 19612–19630 (2015).
54. Jiang, B.-H. & Liu, L.-Z. PI3K/PTEN signaling in angiogenesis and tumorigenesis. *Adv. Cancer Res.* **102**, 19–65 (2009).
55. Sudarsanam, S. & Johnson, D. E. Functional consequences of mTOR inhibition. *Curr. Opin. Drug Discov. Devel.* **13**, 31–40 (2010).
56. Downward, J. Mechanisms and consequences of activation of protein kinase B/Akt. *Curr. Opin. Cell Biol.* **10**, 262–267 (1998).
57. Singh, N. *et al.* Inflammation and cancer. *Ann Afr Med* **18**, 121–126 (2019).
58. DeNardo, D. G., Andreu, P. & Coussens, L. M. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev.* **29**, 309–316 (2010).
59. Grivennikov, S. I., Greten, F. R. & Karin, M. Immunity, inflammation, and cancer. *Cell* **140**, 883–899 (2010).
60. Qian, B.-Z. & Pollard, J. W. Macrophage diversity enhances tumor progression and metastasis. *Cell* **141**, 39–51 (2010).
61. Valastyan, S. & Weinberg, R. A. Tumor metastasis: Molecular insights and evolving paradigms. *Cell* **147**, 275–292 (2011).
62. Steeg, P. S. Tumor metastasis: Mechanistic insights and clinical challenges. *Nat. Med.* **12**, 895–904 (2006).
63. Son, H. & Moon, A. Epithelial-mesenchymal Transition and Cell Invasion. *Toxicol. Res.* **26**, 245–252 (2010).
64. Thiery, J. P., Acloque, H., Huang, R. Y. J. & Nieto, M. A. Epithelial-Mesenchymal Transitions in Development and Disease. *Cell* **139**, 871–890 (2009).
65. Raghu, K. & Weinberg, R. A. The basics of epithelial-mesenchymal transition. *J. Clin. Invest.* **119**, 1420–1428 (2009).
66. Yilmaz, M. & Christofori, G. EMT, the cytoskeleton, and cancer cell invasion. *Cancer Metastasis Rev.* **28**, 15–33 (2009).
67. Van Zijl, F., Krupitza, G. & Mikulits, W. Initial steps of metastasis: Cell invasion and endothelial transmigration. *Mutat. Res. - Rev. Mutat. Res.* **728**, 23–34 (2011).
68. Tam, W. L. & Weinberg, R. A. The epigenetics of epithelial-mesenchymal plasticity in cancer. *Nat. Med.* **19**, 1438–1449 (2013).
69. Dongre, A. & Weinberg, R. A. New insights into the mechanisms of epithelial–mesenchymal transition and implications for cancer. *Nat. Rev. Mol. Cell Biol.* **20**, 69–84 (2019).
70. Lamouille, S., Xu, J. & Derynck, R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat. Rev. Mol. Cell Biol.* **15**, 178–196 (2014).

71. Herranz, N. *et al.* Polycomb complex 2 is required for E-cadherin repression by the Snail1 transcription factor. *Mol. Cell. Biol.* **28**, 4772–4781 (2008).
72. Aigner, K. *et al.* The transcription factor ZEB1 (δEF1) promotes tumour cell dedifferentiation by repressing master regulators of epithelial polarity. *Oncogene* **26**, 6979–6988 (2007).
73. Miyoshi, A. *et al.* Snail and SIP1 increase cancer invasion by upregulating MMP family in hepatocellular carcinoma cells. *Br. J. Cancer* **90**, 1265–1273 (2004).
74. Matsuo, N. *et al.* Twist expression promotes migration and invasion in hepatocellular carcinoma. *BMC Cancer* **9**, 240 (2009).
75. Morel, A.-P. *et al.* Generation of Breast Cancer Stem Cells through Epithelial-Mesenchymal Transition. *PLoS One* **3**, e2888 (2008).
76. Mani, S. A. *et al.* The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133**, 704–715 (2008).
77. Giannoni, E. *et al.* Reciprocal Activation of Prostate Cancer Cells and Cancer-Associated Fibroblasts Stimulates Epithelial-Mesenchymal Transition and Cancer Stemness. *Cancer Res.* **70**, 6945–6956 (2010).
78. Pollard, T. D. & Cooper, J. A. Actin, a central player in cell shape and movement. *Science* **326**, 1208–1212 (2009).
79. Svitkina, T. M. Actin Cell Cortex: Structure and Molecular Organization. *Trends Cell Biol.* **30**, 556–565 (2020).
80. Haynes, J., Srivastava, J., Madson, N., Wittmann, T. & Barber, D. L. Dynamic actin remodeling during epithelial-mesenchymal transition depends on increased moesin expression. *Mol. Biol. Cell* **22**, 4750–4764 (2011).
81. Tojkander, S., Gateva, G. & Lappalainen, P. Actin stress fibers – assembly, dynamics and biological roles. *J. Cell Sci.* **125**, 1855–1864 (2012).
82. Wells, A., Grahovac, J., Wheeler, S., Ma, B. & Lauffenburger, D. Targeting tumor cell motility as a strategy against invasion and metastasis. *Trends Pharmacol. Sci.* **34**, 283–289 (2013).
83. Wells, A. Tumor Invasion: Role of Growth Factor-Induced Cell Motility. in (eds. Vande Woude, G. F. & Klein, G. B. T.-A. in C. R.) vol. 78 31–101 (Academic Press, 1999).
84. Klein, C. A. The Metastasis Cascade. *Science (80-.).* **321**, 1785–1787 (2008).
85. Roche, J. The Epithelial-to-Mesenchymal Transition in Cancer. *Cancers* vol. 10 (2018).
86. Friedl, P. & Gilmour, D. Collective cell migration in morphogenesis, regeneration and cancer. *Nat. Rev. Mol. Cell Biol.* **10**, 445–457 (2009).
87. Price, J. T., Wilson, H. M. & Haites, N. E. Epidermal growth factor (EGF) Increases the in vitro invasion, motility and adhesion interactions of the primary renal carcinoma cell line, A704. *Eur. J. Cancer* **32**, 1977–1982 (1996).
88. Shibata, T. *et al.* Enhancing Effects of Epidermal Growth Factor on Human Squamous Cell Carcinoma Motility and Matrix Degradation But Not Growth. *Tumor Biol.* **17**, 168–175 (1996).
89. Schelch, K. *et al.* EGF Induces Migration Independent of EMT or Invasion in A549 Lung Adenocarcinoma Cells . *Frontiers in Cell and Developmental Biology* vol. 9 (2021).
90. Lane, J., A. Martin, T. & G. Jiang, W. HGF and RhoGTPases in Cancer Cell Motility. *Current Signal Transduction Therapy* vol. 6 173–179 (2011).
91. Pollard, J. Macrophages define the invasive microenvironment in breast cancer. *J. Leukoc. Biol.* **84**, 623–630 (2008).
92. Day, P. M., Thompson, C. D., Schowalter, R. M., Lowy, D. R. & Schiller, J. T. Identification of a Role for the trans -Golgi Network in Human Papillomavirus 16 Pseudovirus Infection . *J. Virol.* **87**, 3862–3870 (2013).
93. Carmo, A. do *et al.* CXCL12/CXCR4 promotes motility and proliferation of glioma cells. *Cancer Biol. Ther.* **9**, 56–65 (2010).
94. Izumi, D. *et al.* CXCL12/CXCR4 activation by cancer-associated fibroblasts promotes integrin β1 clustering and invasiveness in gastric cancer. *Int. J. Cancer* **138**, 1207–1219 (2016).
95. Shin, S. Y., Nam, J.-S., Lim, Y. & Lee, Y. H. TNFα-exposed Bone Marrow-derived Mesenchymal

Stem Cells Promote Locomotion of MDA-MB-231 Breast Cancer Cells through Transcriptional Activation of CXCR3 Ligand Chemokines. *J. Biol. Chem.* **285**, 30731–30740 (2010).
96. Windmüller, C. *et al.* CXCR3 mediates ascites-directed tumor cell migration and predicts poor outcome in ovarian cancer patients. *Oncogenesis* **6**, e331–e331 (2017).
97. Cambien, B. *et al.* Organ-specific inhibition of metastatic colon carcinoma by CXCR3 antagonism. *Br. J. Cancer* **100**, 1755–1764 (2009).
98. Ma, X. *et al.* CXCR3 expression is associated with poor survival in breast cancer and promotes metastasis in a murine model. *Mol. Cancer Ther.* **8**, 490–498 (2009).
99. Bissell, M. & Hines, W. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nat Med* **17**, 320–329 (2011).
100. Gobin, E. *et al.* A pan-cancer perspective of matrix metalloproteases (MMP) gene expression profile and their diagnostic/prognostic potential. *BMC Cancer* **19**, 581 (2019).
101. Kessenbrock, K., Plaks, V. & Zena, W. Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. *Cell* **141**, 52–67 (2010).
102. Takeshima, H. & Ushijima, T. Accumulation of genetic and epigenetic alterations in normal cells and cancer risk. *npj Precis. Oncol.* **3**, 7 (2019).
103. Sever, R. & Brugge, J. S. *Signal transduction in cancer*. *Cold Spring Harbor perspectives in medicine* vol. 5 (Cold Spring Harbor Laboratory Press, 2015).
104. Nisar, S. *et al.* Exploring Dysregulated Signaling Pathways in Cancer. *Curr. Pharm. Des.* **26**, (2020).
105. Dassonville, O. *et al.* Expression of epidermal growth factor receptor and survival in upper aerodigestive tract cancer. *J. Clin. Oncol.* **11**, 1873–1878 (1993).
106. Nicholson, R. I., Gee, J. M. W. & Harper, M. E. EGFR and cancer prognosis. *Eur. J. Cancer* **37**, 9–15 (2001).
107. Wee, P. & Wang, Z. Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. *Cancers (Basel).* **9**, 52 (2017).
108. Marmor, M. D., Skaria, K. B. & Yarden, Y. Signal transduction and oncogenesis by ErbB/HER receptors. *Int. J. Radiat. Oncol.* **58**, 903–913 (2004).
109. Sasaki, T., Hiroki, K. & Yamashita, Y. The Role of Epidermal Growth Factor Receptor in Cancer Metastasis and Microenvironment. *Biomed Res. Int.* **2013**, 546318 (2013).
110. Suhardja, A. & Hoffman, H. Role of growth factors and their receptors in proliferation of microvascular endothelial cells. *Microsc. Res. Tech.* **60**, 70–75 (2003).
111. Marmiroli, S., Fabbro, D., Miyata, Y., Pierobon, M. & Ruzzene, M. Phosphorylation, Signaling, and Cancer: Targets and Targeting. *Biomed Res. Int.* **2015**, 601543 (2015).
112. Lu, Z., Jiang, G., Blume-Jensen, P. & Hunter, T. Epidermal Growth Factor-Induced Tumor Cell Invasion and Metastasis Initiated by Dephosphorylation and Downregulation of Focal Adhesion Kinase. *Mol. Cell. Biol.* **21**, 4016–4031 (2001).
113. Lu, Z., Ghosh, S., Wang, Z. & Hunter, T. Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of. *Cancer Cell* **4**, 499–515 (2003).
114. Clapéron, A. *et al.* EGF/EGFR axis contributes to the progression of cholangiocarcinoma through the induction of an epithelial-mesenchymal transition. *J. Hepatol.* **61**, 325–332 (2014).
115. Xu, Q. *et al.* EGF induces epithelial-mesenchymal transition and cancer stem-like cell properties in human oral cancer cells via promoting Warburg effect. *Oncotarget* **8**, 9557–9571 (2017).
116. Box, C., Peak, J., Rogers, S. & Eccles, S. EGFR signaling in invasion, angiogenesis and metastasis BT - EGFR Signaling Networks in Cancer Therapy. in (eds. Haley, J. D. & Gullick, W. J.) 245–264 (Humana Press, 2008). doi:10.1007/978-1-59745-356-1_18.
117. Van Meter, T. E., Broaddus, W. C., Rooprai, H. K., Pilkington, G. J. & Fillmore, H. L. Induction of membrane-type-1 matrix metalloproteinase by epidermal growth factor-mediated signaling in gliomas. *Neuro. Oncol.* **6**, 188–199 (2004).

118. O-Charoenrat, P. *et al.* Overexpression of epidermal growth factor receptor in human head and neck squamous carcinoma cell lines correlates with matrix metalloproteinase-9 expression and in vitro invasion. *Int. J. Cancer* **89**, 307–317 (2000).
119. Itoh, M. *et al.* Requirement of STAT3 activation for maximal collagenase-1 (MMP-1) induction by epidermal growth factor and malignant characteristics in T24 bladder cancer cells. *Oncogene* **25**, 1195–1204 (2006).
120. Tan, X. *et al.* Involvement of MMP-7 in invasion of pancreatic cancer cells through activation of the EGFR mediated MEK-ERK signal transauction pathway. *J. Clin. Pathol.* **58**, 1242–1248 (2005).
121. Majumder, A., Ray, S. & Banerji, A. Epidermal growth factor receptor-mediated regulation of matrix metalloproteinase-2 and matrix metalloproteinase-9 in MCF-7 breast cancer cells. *Mol. Cell. Biochem.* **452**, 111–121 (2019).
122. Rosenthal, E. *et al.* Role of the plasminogen activator and matrix metalloproteinase systems in epidermal growth factor- and scatter factor-stimulated invasion of carcinoma cells. *Cancer Res* **58**, 5221–30 (1998).
123. Wyckoff, J. *et al.* A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res.* **64**, 7022–7029 (2004).
124. Uribe, M. L., Marrocco, I. & Yarden, Y. Egfr in cancer: Signaling mechanisms, drugs and acquired resistance. *Cancers (Basel).* **13**, (2021).
125. Voisin, L., Duhamel, S. & Meloche, S. The ERK1/2 MAP Kinase Signaling Pathway in Tumor Progression and Metastasis BT - Signal Transduction in Cancer Metastasis. in (eds. Wu, W.-S. & Hu, C.-T.) 25–40 (Springer Netherlands, 2010). doi:10.1007/978-90-481-9522-0_3.
126. Khan, I. & Steeg, P. S. Metastasis suppressors: Functional pathways. *Lab. Investig.* **98**, 198–210 (2018).
127. Soares-Silva, M., Diniz, F. F., Gomes, G. N. & Bahia, D. The Mitogen-Activated Protein Kinase (MAPK) Pathway: Role in Immune Evasion by Trypanosomatids . *Frontiers in Microbiology* vol. 7 (2016).
128. Kyriakis, J. M. & Avruch, J. Mammalian MAPK Signal Transduction Pathways Activated by Stress and Inflammation: A 10-Year Update. *Physiol. Rev.* **92**, 689–737 (2012).
129. Owens, D. M. & Keyse, S. M. Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. *Oncogene* **26**, 3203–3213 (2007).
130. Roberts, P. J. & Der, C. J. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* **26**, 3291–3310 (2007).
131. Fukuda, M., Gotoh, Y. & Nishida, E. Interaction of MAP kinase with MAP kinase kinase: Its possible role in the control of nucleocytoplasmic transport of MAP kinase. *EMBO J.* **16**, 1901–1908 (1997).
132. Matsubayashi, Y., Fukuda, M. & Nishida, E. Evidence for Existence of a Nuclear Pore Complex-mediated, Cytosol-independent Pathway of Nuclear Translocation of ERK MAP Kinase in Permeabilized Cells*. *J. Biol. Chem.* **276**, 41755–41760 (2001).
133. Flores, K., Yadav, S. S., Katz, A. A. & Seger, R. The Nuclear Translocation of Mitogen-Activated Protein Kinases: Molecular Mechanisms and Use as Novel Therapeutic Target. *Neuroendocrinology* **108**, 121–131 (2019).
134. Plotnikov, A. *et al.* The nuclear translocation of ERK1/2 as an anticancer target. *Nat. Commun.* **6**, 6685 (2015).
135. Smorodinsky-Atias, K. *et al.* Intrinsically active variants of Erk oncogenically transform cells and disclose unexpected autophosphorylation capability that is independent of TEY phosphorylation. *Mol. Biol. Cell* **27**, 1026–1039 (2016).
136. Tai, C.-J. *et al.* Clinical-pathological correlation of K-Ras mutation and ERK phosphorylation in colorectal cancer. *Polish J. Pathol.* **63**, 93–100 (2012).
137. Theiry, J. P. Epithelial-mesenchymal transitions in tumor progression. *Nat. Rev. Cancer* **2**, 442–454 (2002).

138. Peinado, H., Olmeda, D. & Cano, A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat. Rev. Cancer* **7**, 415–428 (2007).
139. Janda, E. *et al.* Ras and TGFβ cooperatively regulate epithelial cell plasticity and metastasis: Dissection of Ras signaling pathways. *J. Cell Biol.* **156**, 299–313 (2002).
140. Peinado, H., Quintanilla, M. & Cano, A. Transforming growth factor β-1 induces Snail transcription factor in epithelial cell lines. Mechanisms for epithelial mesenchymal transitions. *J. Biol. Chem.* **278**, 21113–21123 (2003).
141. Chiu, L.-Y. *et al.* The ERK–ZEB1 pathway mediates epithelial–mesenchymal transition in pemetrexed resistant lung cancer cells with suppression by vinca alkaloids. *Oncogene* **36**, 242–253 (2017).
142. Weiss, M. B. *et al.* TWIST1 is an ERK1/2 effector that promotes invasion and regulates MMP-1 expression in human melanoma cells. *Cancer Res.* **72**, 6382–6392 (2012).
143. Wu, X. *et al.* Src promotes cutaneous wound healing by regulating MMP-2 through the ERK pathway. *Int. J. Mol. Med.* **37**, 639–648 (2016).
144. Tanimura, S., Asato, K., Fujishiro, S. H. & Kohno, M. Specific blockade of the ERK pathway inhibits the invasiveness of tumor cells: Down-regulation of matrix metalloproteinase-3/-9/-14 and CD44. *Biochem. Biophys. Res. Commun.* **304**, 801–806 (2003).
145. Sung, H. Y., Yang, S.-D., Ju, W. & Ahn, J.-H. Aberrant epigenetic regulation of GABRP associates with aggressive phenotype of ovarian cancer. *Exp. Mol. Med.* **49**, e335–e335 (2017).
146. Li, C. *et al.* Sulforaphane Inhibits Invasion via Activating ERK1/2 Signaling in Human Glioblastoma U87MG and U373MG Cells. *PLoS One* **9**, e90520 (2014).
147. Vial, E. & Pouysségur, J. Regulation of tumor cell motility by ERK mitogen-activated protein kinases. *Ann. N. Y. Acad. Sci.* **1030**, 208–218 (2004).
148. Vial, E., Sahai, E. & Marshall, C. J. ERK-MAPK signaling coordinately regulates activity of Rac1 and RhoA for tumor cell motility. *Cancer Cell* **4**, 67–79 (2003).
149. Huang, C., Jacobson, K. & Schaller, M. D. MAP kinases and cell migration. *J. Cell Sci.* **117**, 4619–4628 (2004).
150. Belton, O. & Fitzgerald, D. J. Cyclooxygenase isoforms and atherosclerosis. *Expert Rev. Mol. Med.* **5**, 1–18 (2003).
151. Pu, D. *et al.* Cyclooxygenase-2 Inhibitor: A Potential Combination Strategy With Immunotherapy in Cancer. *Front. Oncol.* **11**, 1–7 (2021).
152. Choi, S. H., Aid, S. & Bosetti, F. The distinct roles of cyclooxygenase-1 and -2 in neuroinflammation: implications for translational research. *Trends Pharmacol. Sci.* **30**, 174–181 (2009).
153. Parida, S. & Mandal, M. Inflammation induced by human papillomavirus in cervical cancer and its implication in prevention. *Eur. J. Cancer Prev.* **23**, 432–448 (2014).
154. Zhang, M. *et al.* Role of cancer-related inflammation in esophageal cancer. *Crit. Rev. Eukaryot. Gene Expr.* **23**, 27–35 (2013).
155. Knab, L. M., Grippo, P. J. & Bentrem, D. J. Involvement of eicosanoids in the pathogenesis of pancreatic cancer: The roles of cyclooxygenase-2 and 5-lipoxygenase. *World J. Gastroenterol.* **20**, 10729–10739 (2014).
156. Glover, J. A., Hughes, C. M., Cantwell, M. M. & Murray, L. J. A systematic review to establish the frequency of cyclooxygenase-2 expression in normal breast epithelium, ductal carcinoma in situ, microinvasive carcinoma of the breast and invasive breast cancer. *Br. J. Cancer* **105**, 13–17 (2011).
157. Kaminska, K., Szczylik, C., Lian, F. & Czarnecka, A. M. The role of prostaglandin E2 in renal cell cancer development: future implications for prognosis and therapy. *Futur. Oncol.* **10**, 2177–2187 (2014).
158. Pannunzio, A. & Coluccia, M. Cyclooxygenase-1 (COX-1) and COX-1 inhibitors in cancer: A review of oncology and medicinal chemistry literature. *Pharmaceuticals* **11**, 1–20 (2018).

159. Vane, J. R. Inhibition of Prostaglandin Synthesis as a Mechanism of Action for Aspirin-like Drugs. *Nat. New Biol.* **231**, 232–235 (1971).
160. Finetti, F. *et al.* Prostaglandin E2 and cancer: Insight into tumor progression and immunity. *Biology (Basel).* **9**, 1–26 (2020).
161. Thun, M. J., Namboodiri, M. M. & Heath, C. W. Aspirin Use and Reduced Risk of Fatal Colon Cancer. *N. Engl. J. Med.* **325**, 1593–1596 (1991).
162. Rouzer, C. A. & Marnett, L. J. Cyclooxygenases: structural and functional insights. *J. Lipid Res.* **50**, S29–S34 (2009).
163. Wilson, A. J. *et al.* Aberrant over-expression of COX-1 intersects multiple pro-tumorigenic pathways in high-grade serous ovarian cancer. *Oncotarget* **6**, 21353–21368 (2015).
164. Greenhough, A. *et al.* The COX-2/PGE 2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment . *Carcinogenesis* **30**, 377–386 (2009).
165. Wang, D. *et al.* Prostaglandin E2 promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor δ. *Cancer Cell* **6**, 285–295 (2004).
166. Krysan, K. *et al.* Prostaglandin E2 Activates Mitogen-Activated Protein Kinase/Erk Pathway Signaling and Cell Proliferation in Non–Small Cell Lung Cancer Cells in an Epidermal Growth Factor Receptor–Independent Manner. *Cancer Res.* **65**, 6275–6281 (2005).
167. Botha, J., Robinson, K., Ramchurren, N., Reddi, K. & RJ, N. Human esophageal carcinoma cell lines: prostaglandin production, biological properties, and behavior in nude mice. *J Natl Cancer Inst* **76**, 1053–6 (1986).
168. J., R., D., H. & A., F. Prostaglandin E2 EP receptors as therapeutic targets in breast cancer. *Cancer Metastasis Rev.* **30**, 449–463 (2011).
169. Majumder, M., Xin, X., Liu, L., Girish, G. V. & Lala, P. K. Prostaglandin E2 receptor EP4 as the common target on cancer cells and macrophages to abolish angiogenesis, lymphangiogenesis, metastasis, and stem-like cell functions. *Cancer Sci.* **105**, 1142–1151 (2014).
170. Kalinski, P. Regulation of Immune Responses by Prostaglandin E 2 . *J. Immunol.* **188**, 21–28 (2012).
171. Cheuk, I. W. *et al.* Association of EP2 receptor and SLC19A3 in regulating breast cancer metastasis. *Am. J. Cancer Res.* **5**, 3389–3399 (2015).
172. Robertson, F. *et al.* Molecular and pharmacological blockade of the EP4 receptor selectively inhibits both proliferation and invasion of human inflammatory breast cancer cells. *J Exp Ther Oncol* **7**, 299–312 (2008).
173. Nandi, P. *et al.* PGE2 promotes breast cancer-associated lymphangiogenesis by activation of EP4 receptor on lymphatic endothelial cells. *BMC Cancer* **17**, 1–17 (2017).
174. Tsujii, M., Kawano, S. & Dubois, R. N. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 3336–3340 (1997).
175. Dohadwala, M. *et al.* Non-small cell lung cancer cyclooxygenase-2-dependent invasion is mediated by CD44. *J. Biol. Chem.* **276**, 20809–20812 (2001).
176. Déry, M. C. *et al.* Oxytocin increases invasive properties of endometrial cancer cells through phosphatidylinositol 3-kinase/AKT-dependent up-regulation of cyclooxygenase-1, -2, and X-linked inhibitor of apoptosis protein. *Biol. Reprod.* **85**, 1133–1142 (2011).
177. Balkwill, F. & Mantovani, A. Inflammation and cancer: back to Virchow? *Lancet* **357**, 539–545 (2001).
178. Elinav, E. *et al.* Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. *Nat. Rev. Cancer* **13**, 759–771 (2013).
179. Colotta, F., Allavena, P., Sica, A., Garlanda, C. & Mantovani, A. Cancer-related inflammation, the seventh hallmark of cancer: Links to genetic instability. *Carcinogenesis* **30**, 1073–1081 (2009).
180. Coussens, L. M. & Werb, Z. Inflammation and cancer. *Nature* **420**, 860–867 (2002).
181. Grivennikov, S. I., Greten, F. R. & Karin, M. Immunity, inflammation and cancer. *Cell* **140**, 883–

899 (2010).
182. Liu, J., Lin, P. C. & Zhou, B. P. Inflammation fuels tumor progress and metastasis. *Curr. Pharm. Des.* **21**, 3032–3040 (2015).
183. Nathan, C. & Ding, A. Nonresolving Inflammation. *Cell* **140**, 871–882 (2010).
184. Dvorak, H. F. Tumors: Wounds That Do Not Heal. *N. Engl. J. Med.* **315**, 1650–1659 (1986).
185. Hussain, S. P. & Harris, C. C. Inflammation and cancer: An ancient link with novel potentials. *Int. J. Cancer* **121**, 2373–2380 (2007).
186. Bockerstett, K. A. & DiPaolo, R. J. Regulation of Gastric Carcinogenesis by Inflammatory Cytokines. *Cmgh* **4**, 47–53 (2017).
187. Ng, J. & Wu, J. Hepatitis B- and hepatitis C-related hepatocellular carcinomas in the United States: similarities and differences. *Hepat. Mon.* **12**, e7635–e7635 (2012).
188. Parkin, D. M., Hämmerl, L., Ferlay, J. & Kantelhardt, E. J. Cancer in Africa 2018: The role of infections. *Int. J. Cancer* **146**, 2089–2103 (2020).
189. Zhao, H. *et al.* Inflammation and tumor progression: signaling pathways and targeted intervention. *Signal Transduct. Target. Ther.* **6**, (2021).
190. Tan, Z. *et al.* The Role of Tumor Inflammatory Microenvironment in Lung Cancer. *Front. Pharmacol.* **12**, 1–13 (2021).
191. HELSON, L., GREEN, S., CARSWELL, E. & OLD, L. J. Effect of tumour necrosis factor on cultured human melanoma cells. *Nature* **258**, 731–732 (1975).
192. Wang, X. & Lin, Y. Tumor necrosis factor and cancer, buddies or foes? *Acta Pharmacol. Sin.* **29**, 1275–1288 (2008).
193. Waters, J. P., Pober, J. S. & Bradley, J. R. Tumour necrosis factor and cancer. *J. Pathol.* **230**, 241–248 (2013).
194. Di Paolo, N. C. & Shayakhmetov, D. M. Interleukin 1α and the inflammatory process. *Nat. Immunol.* **17**, 906–913 (2016).
195. Voronov, E. *et al.* Unique Versus Redundant Functions of IL-1α and IL-1β in the Tumor Microenvironment. *Front. Immunol.* **4**, 177 (2013).
196. Malik, A. & Kanneganti, T.-D. Function and regulation of IL-1α in inflammatory diseases and cancer. *Immunol. Rev.* **281**, 124–137 (2018).
197. Alheim, K., McDowell, T. L., Symons, J. A., Duff, G. W. & Bartfai, T. An AP-1 site is involved in the NGF induction of IL-1α in PC12 cells. *Neurochem. Int.* **29**, 487–496 (1996).
198. Bailly, S., Fay, M., Israël, N. & MA, G.-P. The transcription factor AP-1 binds to the human interleukin 1 alpha promoter. *Eur Cytokine Netw* **7**, 125–128 (1996).
199. León, X. *et al.* Expression of IL-1α correlates with distant metastasis in patients with head and neck squamous cell carcinoma. *Oncotarget* **6**, 37398–37409 (2015).
200. Tomimatsu, S., Ichikura, T. & Mochizuki, H. Significant correlation between expression of interleukin-1α and liver metastasis in gastric carcinoma. *Cancer* **91**, 1272–1276 (2001).
201. Bent, R., Moll, L., Grabbe, S. & Bros, M. Interleukin-1 beta—A friend or foe in malignancies? *Int. J. Mol. Sci.* **19**, (2018).
202. Apte, R. N. *et al.* Effects of micro-environment- and malignant cell-derived interleukin-1 in carcinogenesis, tumour invasiveness and tumour–host interactions. *Eur. J. Cancer* **42**, 751–759 (2006).
203. Tao, L. *et al.* IL-1β promotes cervical cancer through activating NF-κB/CCL-2. *Int. J. Clin. Exp. Pathol.* **14**, 426–433 (2021).
204. Jin, S. *et al.* Non-canonical Notch signaling activates IL-6/JAK/STAT signaling in breast tumor cells and is controlled by p53 and IKKα/IKKβ. *Oncogene* **32**, 4892–4902 (2013).
205. Eskiler, G. G. *et al.* IL-6 mediated JAK/STAT3 signaling pathway in cancer patients with cachexia. *Bratisl Lek List.* **66**, 819–826 (2019).
206. Wang, L. *et al.* Cancer-associated fibroblasts enhance metastatic potential of lung cancer cells through IL-6/STAT3 signaling pathway. *Oncotarget* **8**, 76116–76128 (2017).
207. Wei, L.-H. *et al.* Interleukin-6 promotes cervical tumor growth by VEGF-dependent

angiogenesis via a STAT3 pathway. *Oncogene* **22**, 1517–1527 (2003).
208. Lazennec, G. & Richmond, A. Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends Mol. Med.* **16**, 133–144 (2010).
209. Allavena, P., Germano, G., Marchesi, F. & Mantovani, A. Chemokines in cancer related inflammation. *Exp. Cell Res.* **317**, 664–673 (2011).
210. Maini, R. N. & Taylor, P. C. Anti-Cytokine Therapy for Rheumatoid Arthritis. *Annu. Rev. Med.* **51**, 207–229 (2000).
211. Xie, K. Interleukin-8 and human cancer biology. *Cytokine Growth Factor Rev.* **12**, 375–391 (2001).
212. Xu, L. & Fidler, I. J. Interleukin 8: An autocrine growth factor for human ovarian cancer. *Oncol. Res.* **12**, 97–106 (2000).
213. Jia, L. *et al.* IL-8 is upregulated in cervical cancer tissues and is associated with the proliferation and migration of HeLa cervical cancer cells. *Oncol. Lett.* **15**, 1350–1356 (2018).
214. Karin, M. Nuclear factor-κB in cancer development and progression. *Nature* **441**, 431–436 (2006).
215. Yu, H., Pardoll, D. & Jove, R. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat. Rev. Cancer* **9**, 798–809 (2009).
216. Qiao, Y. *et al.* AP-1 Is a Key Regulator of Proinflammatory Cytokine TNFα-mediated Triple-negative Breast Cancer Progression. *J. Biol. Chem.* **291**, 5068–5079 (2016).
217. Karin, M. Nuclear factor-κB in cancer development and progression. *Nature* **441**, 431–436 (2006).
218. Greten, F. R. *et al.* IKKβ Links Inflammation and Tumorigenesis in a Mouse Model of Colitis-Associated Cancer. *Cell* **118**, 285–296 (2004).
219. Xia, Y., Shen, S. & Verma, I. M. NF-κB, an active player in human cancers. *Cancer Immunol. Res.* **2**, 823–830 (2014).
220. Del Prete Annalisa, Allavena Paola , Santoro Giuseppe , Fumarulo Ruggiero , Corsi Massimi liano M, M. A. Molecular pathways in cancer-related inflammation. *Biochemia-medica.* **21**, 264–275 (2011).
221. Kortylewski, M. *et al.* Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nat. Med.* **11**, 1314–1321 (2005).
222. Lee, H. *et al.* Persistently activated Stat3 maintains constitutive NF-kappaB activity in tumors. *Cancer Cell* **15**, 283–293 (2009).
223. Grivennikov, S. *et al.* IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell* **15**, 103–113 (2009).
224. Vesely, P. W., Staber, P. B., Hoefler, G. & Kenner, L. Translational regulation mechanisms of AP-1 proteins. *Mutat. Res. Mutat. Res.* **682**, 7–12 (2009).
225. Ye, N., Ding, Y., Wild, C., Shen, Q. & Zhou, J. Small Molecule Inhibitors Targeting Activator Protein 1 (AP-1). *J. Med. Chem.* **57**, 6930–6948 (2014).
226. Macián, F., López-Rodríguez, C. & Rao, A. Partners in transcription: NFAT and AP-1. *Oncogene* **20**, 2476–2489 (2001).
227. Ji, Z., He, L., Regev, A. & Struhl, K. Inflammatory regulatory network mediated by the joint action of NF-kB, STAT3, and AP-1 factors is involved in many human cancers. *Proc. Natl. Acad. Sci.* **116**, 9453–9462 (2019).
228. Zhou, Z.-W. *et al.* Therapeutic Effects of Natural Products on Cervical Cancer: Based on Inflammatory Pathways. *Front. Pharmacol.* **13**, 1–19 (2022).
229. Hawiger, J. & Zienkiewicz, J. Decoding inflammation, its causes, genomic responses, and emerging countermeasures. *Scand. J. Immunol.* **90**, e12812–e12812 (2019).
230. Torgerson, T. R., Colosia, A. D., Donahue, J. P., Lin, Y.-Z. & Hawiger, J. Regulation of NF-κB, AP-1, NFAT, and STAT1 Nuclear Import in T Lymphocytes by Noninvasive Delivery of Peptide Carrying the Nuclear Localization Sequence of NF-κB p50. *J. Immunol.* **161**, 6084 LP – 6092 (1998).

231. Yan Liu, X. *et al.* Peptide-directed Suppression of a Pro-inflammatory Cytokine Response. *J. Biol. Chem.* **275**, 16774–16778 (2000).
232. Chook, Y. M. & Süel, K. E. Nuclear import by karyopherin-βs: recognition and inhibition. *Biochim. Biophys. Acta* **1813**, 1593–1606 (2011).
233. Mosammaparast, N. & Pemberton, L. F. Karyopherins: From nuclear-transport mediators to nuclear-function regulators. *Trends Cell Biol.* **14**, 547–556 (2004).
234. Van Der Watt, P. J. *et al.* Targeting the nuclear import receptor Kpnβ1 as an anticancer therapeutic. *Mol. Cancer Ther.* **15**, 560–573 (2016).
235. Marfori, M. *et al.* Molecular basis for specificity of nuclear import and prediction of nuclear localization. *Biochim. Biophys. Acta - Mol. Cell Res.* **1813**, 1562–1577 (2011).
236. Miyamoto, Y., Yamada, K. & Yoneda, Y. Importin α: a key molecule in nuclear transport and non-transport functions. *J. Biochem.* **160**, 69–75 (2016).
237. Bernhofer, M. *et al.* NLSdb—major update for database of nuclear localization signals and nuclear export signals. *Nucleic Acids Res.* **46**, D503–D508 (2018).
238. Stelma, T. & Leaner, V. D. KPNB1-mediated nuclear import is required for motility and inflammatory transcription factor activity in cervical cancer cells. *Oncotarget* **8**, 32833–32847 (2017).
239. Liang, P. *et al.* KPNB1, XPO7 and IPO8 Mediate the Translocation ofNF-κB/p65 into the Nucleus. *Traffic* **14**, 1132–1143 (2013).
240. Cimica, V., Chen, H. C., Iyer, J. K. & Reich, N. C. Dynamics of the STAT3 transcription factor: Nuclear import dependent on ran and importin-β1. *PLoS One* **6**, (2011).
241. Forwood, J. K., Lam, M. H. C. & Jans, D. A. Nuclear Import of Creb and AP-1 Transcription Factors Requires Importin-β1 and Ran but Is Independent of Importin-α. *Biochemistry* **40**, 5208–5217 (2001).
242. Yang, J. *et al.* Inhibition of Karyopherin beta 1 suppresses prostate cancer growth. *Oncogene* **38**, 4700–4714 (2019).
243. Castle, P. E. *et al.* An association of cervical inflammation with high-grade cervical neoplasia in women infected with oncogenic human papillomavirus (HPV). *Cancer Epidemiol Biomarkers Prev* **10**, 1021–1027 (2001).
244. Hawes, S. E. & Kiviat, N. B. Are Genital Infections and Inflammation Cofactors in the Pathogenesis of Invasive Cervical Cancer? *JNCI J. Natl. Cancer Inst.* **94**, 1592–1593 (2002).
245. Hammes, L. S. *et al.* Macrophages, inflammation and risk of cervical intraepithelial neoplasia (CIN) progression—Clinicopathological correlation. *Gynecol. Oncol.* **105**, 157–165 (2007).
246. Yeung, Y. T., Aziz, F., Guerrero-Castilla, A. & Arguelles, S. Signaling Pathways in Inflammation and Anti-inflammatory Therapies. *Curr. Pharm. Des.* **24**, 1449–1484 (2018).
247. Hemmat, N. & Bannazadeh Baghi, H. Association of human papillomavirus infection and inflammation in cervical cancer. *Pathog. Dis.* **77**, ftz048 (2019).
248. Sales, K. J. *et al.* Cyclooxygenase-2 expression and prostaglandin E(2) synthesis are up-regulated in carcinomas of the cervix: a possible autocrine/paracrine regulation of neoplastic cell function via EP2/EP4 receptors. *J. Clin. Endocrinol. Metab.* **86**, 2243–2249 (2001).
249. Adefuye, A., Katz, A. A. & Sales, K. J. The Regulation of Inflammatory Pathways and Infectious Disease of the Cervix by Seminal Fluid. *Patholog. Res. Int.* **2014**, 748740 (2014).
250. Subbaramaiah, K. & Dannenberg, A. J. Cyclooxygenase-2 transcription is regulated by human papillomavirus 16 E6 and E7 oncoproteins: Evidence of a corepressor/coactivator exchange. *Cancer Res.* **67**, 3976–3985 (2007).
251. Kim, M.-K. *et al.* Human papillomavirus type 16 E5 oncoprotein as a new target for cervical cancer treatment. *Biochem. Pharmacol.* **80**, 1930–1935 (2010).
252. Narumiya, S. & FitzGerald, G. A. Genetic and pharmacological analysis of prostanoid receptor function. *J. Clin. Invest.* **108**, 25–30 (2001).
253. Sales, K. J. *et al.* Cyclooxygenase-1 is up-regulated in cervical carcinomas: autocrine/paracrine regulation of cyclooxygenase-2, prostaglandin e receptors, and angiogenic factors by

cyclooxygenase-1. *Cancer Res.* **62**, 424–432 (2002).
254. Senba, M. & Mori, N. Mechanisms of virus immune evasion lead to development from chronic inflammation to cancer formation associated with human papillomavirus infection. *Oncol. Rev.* **6**, e17–e17 (2012).
255. Kemp, T. J. *et al.* Elevated systemic levels of inflammatory cytokines in older women with persistent cervical human papillomavirus infection. *Cancer Epidemiol. Biomarkers Prev.* **19**, 1954–1959 (2010).
256. Schjenken, J. E. & Robertson, S. A. The female response to seminal fluid. *Physiol. Rev.* **100**, 1077–1117 (2020).
257. Bromfield, J. J. Seminal fluid and reproduction: Much more than previously thought. *J. Assist. Reprod. Genet.* **31**, 627–636 (2014).
258. Maegawa, M. *et al.* A repertoire of cytokines in human seminal plasma. *J. Reprod. Immunol.* **54**, 33–42 (2002).
259. Perry, J. C., Sirot, L. & Wigby, S. The seminal symphony: How to compose an ejaculate. *Trends Ecol. Evol.* **28**, 414–422 (2013).
260. Sharkey, D. J., Macpherson, A. M., Tremellen, K. P. & Robertson, S. A. Seminal plasma differentially regulates inflammatory cytokine gene expression in human cervical and vaginal epithelial cells. *Mol. Hum. Reprod.* (2007) doi:10.1093/molehr/gam028.
261. Sharkey, D. J., Tremellen, K. P., Jasper, M. J., Gemzell-Danielsson, K. & Robertson, S. A. Seminal Fluid Induces Leukocyte Recruitment and Cytokine and Chemokine mRNA Expression in the Human Cervix after Coitus. *J. Immunol.* **188**, 2445–2454 (2012).
262. Robertson, S. A. & Sharkey, D. J. Seminal fluid and fertility in women. *Fertil. Steril.* **106**, 511–519 (2016).
263. Thompson, L. A., Barratt, C. L. R., Bolton, A. E. & Cooke, I. D. The leukocytic reaction of the human uterine cervix. *Am. J. Reprod. Immunol.* **28**, 85–89 (1992).
264. Seshadri, S., Bates, M., Vince, G. & Jones, D. I. L. The role of cytokine expression in different subgroups of subfertile men. *Am. J. Reprod. Immunol.* **62**, 275–282 (2009).
265. Naz, R. K. & Stanley, E. R. Enhanced Levels of Colony Stimulating Factor-1 (Csf-1) in Sera and Seminal Plasma of Antisperm Antibody-Positive Infertile Men. *Arch. Androl.* **35**, 5–11 (1995).
266. Chang, H., Brown, C. W. & Matzuk, M. M. Genetic analysis of the mammalian transforming growth factor-β superfamily. *Endocr. Rev.* **23**, 787–823 (2002).
267. Chu, T. M. & Kawinski, E. Plasmin, substilisin-like endoproteases, tissue plasminogen activator, and urokinase plasminogen activator are involved in activation of latent TGF-β1 in human seminal plasma. *Biochem. Biophys. Res. Commun.* **253**, 128–134 (1998).
268. Robertson, S. A. Seminal plasma and male factor signalling in the female reproductive tract. *Cell Tissue Res.* **322**, 43–52 (2005).
269. Tremellen, K. P., Seamark, R. F. & Robertson, S. A. Seminal Transforming Growth Factor β1, Stimulates Granulocyte-Macrophage Colony-Stimulating Factor Production and Inflammatory Cell Recruitment in the Murine Uterus1. *Biol. Reprod.* **58**, 1217–1225 (1998).
270. Szczykutowicz, J., Kałuza, A., Kaźmierowska-Niemczuk, M. & Ferens-Sieczkowska, M. The potential role of seminal plasma in the fertilization outcomes. *Biomed Res. Int.* **2019**, (2019).
271. Denison, F. C., Grant, V. E., Calder, A. A. & Kelly, R. W. Seminal plasma components stimulate interleukin-8 and interleukin-10 release. *Mol. Hum. Reprod.* **5**, 220–226 (1999).
272. Joseph, T., Zalenskaya, I. A., Sawyer, L. C., Chandra, N. & Doncel, G. F. Seminal Plasma Induces Prostaglandin-Endoperoxide Synthase (PTGS) 2 Expression in Immortalized Human Vaginal Cells: Involvement of Semen Prostaglandin E2 in PTGS2 Upregulation1. *Biol. Reprod.* **88**, 1-10,13 (2013).
273. Muller, M., Sales, K. J., Katz, A. A. & Jabbour, H. N. Seminal plasma promotes the expression of tumorigenic and angiogenic genes in cervical adenocarcinoma cells via the E-series prostanoid 4 receptor. *Endocrinology* **147**, 3356–3365 (2006).
274. Battersby, S., Sales, K. J., Williams, A. R., Anderson, R. A. & Gardner, S. Seminal plasma and

prostaglandin E 2 up-regulate fibroblast growth factor 2 expression in endometrial adenocarcinoma cells via E-series prostanoid-2 receptor-mediated transactivation of the epidermal growth factor receptor and ext. *Hum. Reprod.* **22**, 36–44 (2007).
275. García-Quiroz, J., Vázquez-Almazán, B., García-Becerra, R., Díaz, L. & Avila, E. The Interaction of Human Papillomavirus Infection and Prostaglandin E2 Signaling in Carcinogenesis: A Focus on Cervical Cancer Therapeutics. *Cells* vol. 11 (2022).
276. Sales, K. J., Katz, A. A., Millar, R. P. & Jabbour, H. N. Seminal plasma activates cyclooxygenase-2 and prostaglandin E 2 receptor expression and signalling in cervical adenocarcinoma cells. *Mol. Hum. Reprod.* **8**, 1065–1070 (2002).
277. Sutherland, J. R., Sales, K. J., Jabbour, H. N. & Katz, A. A. Seminal plasma enhances cervical adenocarcinoma cell proliferation and tumour growth in vivo. *PLoS One* **7**, 1–12 (2012).
278. Sales, K. J., Sutherland, J. R., Jabbour, H. N. & Katz, A. A. Seminal plasma induces angiogenic chemokine expression in cervical cancer cells and regulates vascular function. *Biochim. Biophys. Acta - Mol. Cell Res.* **1823**, 1789–1795 (2012).
279. Adefuye, A. O., Sales, K. J. & Katz, A. A. Seminal plasma induces the expression of IL-1α in normal and neoplastic cervical cells via EP2/EGFR/PI3K/AKT pathway. *J. Mol. Signal.* **9**, 1–16 (2014).
280. Guo, Z.-Q. *et al.* Semen affects the biological behavior of HeLa cells by altering ERK signaling. *Archives of medical science : AMS* vol. 16 915–923 (2019).
281. van der Watt, P. J. *et al.* Targeting the Nuclear Import Receptor Kpn 1 as an Anticancer Therapeutic. *Mol. Cancer Ther.* **15**, 560–573 (2016).
282. Maritz, M. F., Van Der Watt, P. J., Holderness, N., Birrer, M. J. & Leaner, V. D. Inhibition of AP-1 suppresses cervical cancer cell proliferation and is associated with p21 expression. *Biol. Chem.* **392**, 439–448 (2011).
283. Cooper, T. G. *et al.* The transcription factor activator protein-1 is activated and interleukin-6 production is increased in interleukin-1beta-stimulated human enterocytes*‡. *Hum. Reprod. Update* **16**, 231–245 (2010).
284. Jeremias, J., David, S. S., Toth, M. & Witkin, S. S. Induction of messenger RNA for the 70 kDa heat shock protein in HeLa cells and the human endocervix following exposure to semen: implications for antisperm antibody production and susceptibility to sexually transmitted infections. *Hum. Reprod.* **12**, 1915–1919 (1997).
285. Sales, K. J., Milne, S. A., Williams, A. R. W., Anderson, R. A. & Jabbour, H. N. Expression, Localization, and Signaling of Prostaglandin F2α Receptor in Human Endometrial Adenocarcinoma: Regulation of Proliferation by Activation of the Epidermal Growth Factor Receptor and Mitogen-Activated Protein Kinase Signaling Pathways. *J. Clin. Endocrinol. Metab.* **89**, 986–993 (2004).
286. Sales, K. J., Grant, V. & Jabbour, H. N. Prostaglandin E2 and F2alpha activate the FP receptor and up-regulate cyclooxygenase-2 expression via the cyclic AMP response element. *Mol. Cell. Endocrinol.* **285**, 51–61 (2008).
287. Keightley, M. C., Brown, P., Jabbour, H. N. & Sales, K. J. F-Prostaglandin receptor regulates endothelial cell function via fibroblast growth factor-2. *BMC Cell Biol.* **11**, 8 (2010).
288. Sales, K. J., Adefuye, A., Nicholson, L. & Katz, A. A. CCR5 expression is elevated in cervical cancer cells and is up-regulated by seminal plasma. *Mol. Hum. Reprod.* **20**, 1144–1157 (2014).
289. Cherukuri, D. P. *et al.* The EP4 receptor antagonist, L-161,982, blocks prostaglandin E2-induced signal transduction and cell proliferation in HCA-7 colon cancer cells. *Exp. Cell Res.* **313**, 2969–2979 (2007).
290. Fontana, F. *et al.* Epithelial-To-Mesenchymal Transition Markers and CD44 Isoforms Are Differently Expressed in 2D and 3D Cell Cultures of Prostate Cancer Cells. *Cells* **8**, 143 (2019).
291. Liu, J. *et al.* PI3K/Akt/HIF-1α signaling pathway mediates HPV-16 oncoprotein-induced expression of EMT-related transcription factors in non-small cell lung cancer cells. *J. Cancer* **9**, 3456–3466 (2018).

292. Van Der Watt, P. J. *et al.* The karyopherin proteins, Crm1 and Karyopherin β1, are overexpressed in cervical cancer and are critical for cancer cell survival and proliferation. *Int. J. Cancer* **124**, 1829–1840 (2009).
293. Park, H. *et al.* Effect of Sorbus commixta on the invasion and migration of human hepatocellular carcinoma Hep3B cells. *Int. J. Mol. Med.* **40**, 483–490 (2017).
294. Jedy-Agba, E. *et al.* Trends in cervical cancer incidence in sub-Saharan Africa. *Br. J. Cancer* **123**, 148–154 (2020).
295. International Agency for Research on Cancer (IARC). *GLOBOCAN* http://gco.iarc.fr/tomorrow/home (2018).
296. Arbyn, M. *et al.* Estimates of incidence and mortality of cervical cancer in 2018: a worldwide analysis. *Lancet Glob. Heal.* **8**, e191–e203 (2020).
297. Pezzatini, M., Marino, G., Conte, S. & Catracchia, V. Oncology: A forgotten territory in Africa. *Ann. Oncol.* **18**, 2046–2047 (2007).
298. Yang, J. & Weinberg, R. A. Epithelial-Mesenchymal Transition: At the Crossroads of Development and Tumor Metastasis. *Dev. Cell* **14**, 818–829 (2008).
299. Lo, U.-G., Lee, C.-F., Lee, M.-S. & Hsieh, J.-T. The Role and Mechanism of Epithelial-to-Mesenchymal Transition in Prostate Cancer Progression. *International Journal of Molecular Sciences* vol. 18 (2017).
300. Lamouille, S., Xu, J. & Derynck, R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat. Rev. Mol. Cell Biol.* **15**, 178–196 (2014).
301. Sun, B. O., Fang, Y., Li, Z., Chen, Z. & Xiang, J. Role of cellular cytoskeleton in epithelial-mesenchymal transition process during cancer progression. *Biomed. reports* **3**, 603–610 (2015).
302. Svitkina, T. The actin cytoskeleton and actin-based motility. *Cold Spring Harb. Perspect. Biol.* **10**, (2018).
303. Babaei, G., Aziz, S. G. G. & Jaghi, N. Z. Z. EMT, cancer stem cells and autophagy; The three main axes of metastasis. *Biomed. Pharmacother.* **133**, 110909 (2021).
304. Zhou, S. & Peng, F. Patterns of metastases in cervical cancer: a population-based study. *Int. J. Clin. Exp. Pathol.* **13**, 1615–1623 (2020).
305. Tan, Z. *et al.* The Role of Tumor Inflammatory Microenvironment in Lung Cancer . *Frontiers in Pharmacology* vol. 12 (2021).
306. Feitelson, M. A. *et al.* Sustained proliferation in cancer: therapeutic targets. *Semin. Cancer Biol.* **35**, 25–54 (2016).
307. Witsch, E., Sela, M. & Yarden, Y. Roles for Growth Factors in Cancer Progression. *Physiology* **25**, 85–101 (2010).
308. Pollard, T. D. & Wu, J. Q. Understanding cytokinesis: Lessons from fission yeast. *Nat. Rev. Mol. Cell Biol.* **11**, 149–155 (2010).
309. Basu, A. *et al.* Statistical parametrization of cell cytoskeleton reveals lung cancer cytoskeletal phenotype with partial EMT signature. *Commun. Biol.* **5**, (2022).
310. Apte, S. S. & Parks, W. C. Metalloproteinases: A parade of functions in matrix biology and an outlook for the future. *Matrix Biol.* **44–46**, 1–6 (2015).
311. Cui, N., Hu, M. & Khalil, R. A. Chapter One - Biochemical and Biological Attributes of Matrix Metalloproteinases. in *Matrix Metalloproteinases and Tissue Remodeling in Health and Disease: Cardiovascular Remodeling* (ed. Khalil, R. A. B. T.-P. in M. B. and T. S.) vol. 147 1–73 (Academic Press, 2017).
312. Bromfield, J. J. *et al.* Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 2200–2205 (2014).
313. Khan, K. N. *et al.* Effect of human seminal fluid on the growth of endometrial cells of women with endometriosis. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **149**, 204–209 (2010).
314. Matossian, M. D. *et al.* NEK5 activity regulates the mesenchymal and migratory phenotype in breast cancer cells. *Breast Cancer Res. Treat.* **189**, 49–61 (2021).

315. Morris, H. T. & Machesky, L. M. Actin cytoskeletal control during epithelial to mesenchymal transition: Focus on the pancreas and intestinal tract. *Br. J. Cancer* **112**, 613–620 (2015).
316. He, X. *et al.* RhoC is essential in TGF-β1 induced epithelial-mesenchymal transition in cervical cancer cells. *Oncol Lett* **10**, 985–989 (2015).
317. Saito, Y. *et al.* Aldolase A promotes epithelial-mesenchymal transition to increase malignant potentials of cervical adenocarcinoma. *Cancer Sci.* **111**, 3071–3081 (2020).
318. Nieto, M. A., Huang, R. Y.-J., Jackson, R. A. & Thiery, J. P. EMT: 2016. *Cell* **166**, 21–45 (2016).
319. Ibrahim, M. G. *et al.* Seminal plasma (SP) induces a rapid transforming growth factor beta 1 (TGFβ1)—independent up-regulation of epithelial–mesenchymal transdifferentiation (EMT) and myofibroblastic metaplasia-markers in endometriotic (EM) and endometrial cells. *Arch. Gynecol. Obstet.* **299**, 173–183 (2019).
320. Ji, H. *et al.* C-phycocyanin inhibits epithelial-to-mesenchymal transition in Caski cells. *Cancer Cell Int.* **20**, 292 (2020).
321. Wang, S. *et al.* Programmed death ligand 1 promotes lymph node metastasis and glucose metabolism in cervical cancer by activating integrin β4/SNAI1/SIRT3 signaling pathway. *Oncogene* **37**, 4164–4180 (2018).
322. Zhou, Y. *et al.* ΔNp63α exerts antitumor functions in cervical squamous cell carcinoma. *Oncogene* **39**, 905–921 (2020).
323. Araki, K. *et al.* E/N-cadherin switch mediates cancer progression via TGF-β-induced epithelial-to-mesenchymal transition in extrahepatic cholangiocarcinoma. *Br. J. Cancer* **105**, 1885–1893 (2011).
324. Aleskandarany, M. A. *et al.* Epithelial mesenchymal transition in early invasive breast cancer: an immunohistochemical and reverse phase protein array study. *Breast Cancer Res. Treat.* **145**, 339–348 (2014).
325. Mrozik, K. M., Blaschuk, O. W., Cheong, C. M., Zannettino, A. C. W. & Vandyke, K. N-cadherin in cancer metastasis, its emerging role in haematological malignancies and potential as a therapeutic target in cancer. *BMC Cancer* **18**, 939 (2018).
326. Roussos, E. T., Condeelis, J. S. & Patsialou, A. Chemotaxis in cancer. *Nat. Rev. Cancer* **11**, 573–587 (2011).
327. Biswenger, V. *et al.* Characterization of EGF-guided MDA-MB-231 cell chemotaxis in vitro using a physiological and highly sensitive assay system. *PLoS One* **13**, e0203040 (2018).
328. Iida, Y. *et al.* Lysophosphatidylserine stimulates chemotactic migration of colorectal cancer cells through GPR34 and PI3K/Akt pathway. *Anticancer Res.* **34**, 5465–5472 (2014).
329. Jeremias, J. & Witkin, S. S. Effect of human seminal fluid on production of messenger ribonucleic acid for metalloproteinase 2 and metalloproteinase 9 in cervical epithelial carcinoma cells. *Am. J. Obstet. Gynecol.* **181**, 591–595 (1999).
330. Adefuye, A. O. The role of seminal plasma in the regulation of inflammation and inflammatory pathways in the cervix: potential for cervical cancer progression and HIV transmission in South African women. (2014).
331. Ahmadi, H. *et al.* Composition and effects of seminal plasma in the female reproductive tracts on implantation of human embryos. *Biomed. Pharmacother.* **151**, 113065 (2022).
332. Parida, S. & Mandal, M. Inflammation induced by human papillomavirus in cervical cancer and its implication in prevention. *Eur. J. Cancer Prev.* **23**, (2014).
333. Gasparini, G., Longo, R., Sarmiento, R. & Morabito, A. Inhibitors of cyclo-oxygenase 2: A new class of anticancer agents? *Lancet Oncol.* **4**, 605–615 (2003).
334. Pannunzio, A. & Coluccia, M. Cyclooxygenase-1 (COX-1) and COX-1 Inhibitors in Cancer: A Review of Oncology and Medicinal Chemistry Literature. *Pharmaceuticals (Basel).* **11**, 101 (2018).
335. Hwang, D., Byrne, J., Scollard, D. & Levine, E. Expression of Cyclooxygenase-1 and Cyclooxygenase-2 in Human Breast Cancer. *JNCI J. Natl. Cancer Inst.* **90**, 455–460 (1998).
336. Kirschenbaum, A. *et al.* Expression of cyclooxygenase-1 and cyclooxygenase-2 in the human

prostate. *Urology* **56**, 671–676 (2000).
337. Roskoski, R. ERK1/2 MAP kinases: Structure, function, and regulation. *Pharmacol. Res.* **66**, 105–143 (2012).
338. Albeck, J. G., Mills, G. B. & Brugge, J. S. Frequency-Modulated Pulses of ERK Activity Transmit Quantitative Proliferation Signals. *Mol. Cell* **49**, 249–261 (2013).
339. Mittal, V. Epithelial Mesenchymal Transition in Tumor Metastasis. *Annu. Rev. Pathol. Mech. Dis.* **13**, 395–412 (2018).
340. Desouza, M., Gunning, P. W. & Stehn, J. R. The actin cytoskeleton as a sensor and mediator of apoptosis. *Bioarchitecture* **2**, 75–87 (2012).
341. Shankar, J. & Nabi, I. R. Actin cytoskeleton regulation of epithelial mesenchymal transition in metastatic cancer cells. *PLoS One* **10**, 1–12 (2015).
342. Craene, B. De & Berx, G. Regulatory networks defining EMT during cancer initiation and progression. *Nat. Rev. Cancer* **13**, 97–110 (2013).
343. Jiang, Y.-G. *et al.* Role of Wnt/β-catenin signaling pathway in epithelial-mesenchymal transition of human prostate cancer induced by hypoxia-inducible factor-1α. *Int. J. Urol.* **14**, 1034–1039 (2007).
344. Wang, Z., Li, Y., Kong, D. & Sarkar, F. H. The role of Notch signaling pathway in epithelial-mesenchymal transition (EMT) during development and tumor aggressiveness. *Curr. Drug Targets* **11**, 745–751 (2010).
345. Gurpinar, E., Grizzle, W. E. & Piazza, G. A. COX-independent mechanisms of cancer chemoprevention by anti-inflammatory drugs. *Front. Oncol.* **3 JUL**, (2013).
346. Fujino, H., Xu, W. & Regan, J. W. Prostaglandin E2 induced functional expression of early growth response factor-1 by EP4, but not EP2, prostanoid receptors via the phosphatidylinositol 3-kinase and extracellular signal-regulated kinases. *J. Biol. Chem.* **278**, 12151–12156 (2003).
347. Jabbour, H. N., Milne, S. A., Williams, A. R. W., Anderson, R. A. & Boddy, S. C. Expression of COX-2 and PGE synthase and synthesis of PGE2 in endometrial adenocarcinoma: a possible autocrine/paracrine regulation of neoplastic cell function via EP2/EP4 receptors. *Br. J. Cancer* **85**, 1023–1031 (2001).
348. Jabbour, H. N. & Sales, K. J. Prostaglandin receptor signalling and function in human endometrial pathology. *Trends Endocrinol. Metab.* **15**, 398–404 (2004).
349. Kelly, R. W. Prostaglandins in primate semen: biasing the immune system to benefit spermatozoa and virus? *Prostaglandins, Leukot. Essent. Fat. Acids* **57**, 113–118 (1997).
350. Yarden, Y. & Pines, G. The ERBB network: at last, cancer therapy meets systems biology. *Nat. Rev. Cancer* **12**, 553–563 (2012).
351. Mazhar, D., Gillmore, R. & Waxman, J. COX and cancer. *QJM An Int. J. Med.* **98**, 711–718 (2005).
352. Guo, Y.-J. *et al.* ERK/MAPK signalling pathway and tumorigenesis. *Exp. Ther. Med.* **19**, 1997–2007 (2020).
353. Baulida, J., Díaz, V. M. & Herreros, A. G. de. Snail1: A Transcriptional Factor Controlled at Multiple Levels. *J. Clin. Med.* **8**, 757 (2019).
354. Wang, M. *et al.* AGO1 may influence the prognosis of hepatocellular carcinoma through TGF-β pathway. *Cell Death Dis.* **9**, 324 (2018).
355. Barberà, M. J. *et al.* Regulation of Snail transcription during epithelial to mesenchymal transition of tumor cells. *Oncogene* **23**, 7345–7354 (2004).
356. Zhao, Z., Rahman, M., Chen, G. & Shin, D. Multiple biological functions of Twist1 in various cancers. *Oncotarget* **8**, (2017).
357. Jiang, J., Kosman, D., Ip, Y. T. & Levine, M. The dorsal morphogen gradient regulates the mesoderm determinant twist in early Drosophila embryos. *Genes Dev.* **5**, 1881–1891 (1991).
358. Ling, X. & Arlinghaus, R. B. Knockdown of STAT3 Expression by RNA Interference Inhibits the Induction of Breast Tumors in Immunocompetent Mice. *Cancer Res.* **65**, 2532–2536 (2005).

359. Thuault, S. *et al.* Transforming growth factor-beta employs HMGA2 to elicit epithelial-mesenchymal transition. *J. Cell Biol.* **174**, 175–183 (2006).
360. Yang, M.-H. *et al.* Direct regulation of TWIST by HIF-1α promotes metastasis. *Nat. Cell Biol.* **10**, 295–305 (2008).
361. Dohadwala, M. *et al.* Cyclooxygenase-2-dependent regulation of E-cadherin: Prostaglandin E 2 induces transcriptional repressors ZEB1 and snail in non-small cell lung cancer. *Cancer Res.* **66**, 5338–5345 (2006).
362. Bae, G.-Y. *et al.* Loss of E-cadherin activates EGFR-MEK/ERK signaling, which promotes invasion via the ZEB1/MMP2 axis in non-small cell lung cancer. *Oncotarget* **4**, 2512–2522 (2013).
363. Han, Y. *et al.* Hepatocyte growth factor increases the invasive potential of PC-3 human prostate cancer cells via an ERK/MAPK and Zeb-1 signaling pathway. *Oncol. Lett.* **11**, 753–759 (2016).
364. Bruneel, K., Verstappe, J., Vandamme, N. & Berx, G. Intrinsic Balance between ZEB Family Members Is Important for Melanocyte Homeostasis and Melanoma Progression. *Cancers (Basel).* **12**, 2248 (2020).
365. Seo, J., Ha, J., Kang, E. & Cho, S. The role of epithelial–mesenchymal transition-regulating transcription factors in anti-cancer drug resistance. *Arch. Pharm. Res.* **44**, 281–292 (2021).
366. Jang, T., Jeon, K. & Jung, K. Cyclooxygenase-2 Expression Is Related to the Epithelial-to-Mesenchymal Transition in Human Colon Cancers. *Yonsei Med. J.* **50**, 818–824 (2009).
367. Regan, J. W. EP2 and EP4 prostanoid receptor signaling. *Life Sci.* **74**, 143–153 (2003).
368. Li, J. *et al.* AKR1B10 promotes breast cancer cell migration and invasion via activation of ERK signaling. *Oncotarget* **8**, 33694–33703 (2017).
369. Jia, S. *et al.* MAGI1 inhibits migration and invasion via blocking MAPK/ERK signaling pathway in gastric cancer. *Chin. J. Cancer Res.* **29**, 25–35 (2017).
370. Ono, H., Basson, M. D. & Ito, H. PTK6 Promotes Cancer Migration and Invasion in Pancreatic Cancer Cells Dependent on ERK Signaling. *PLoS One* **9**, e96060 (2014).
371. Fan, L., Wu, Y., Wang, J., He, J. & Han, X. Sevoflurane inhibits the migration and invasion of colorectal cancer cells through regulating ERK/MMP-9 pathway by up-regulating miR-203. *Eur. J. Pharmacol.* **850**, 43–52 (2019).
372. Kim, M.-J. *et al.* NDRG2 controls COX-2/PGE_2-mediated breast cancer cell migration and invasion. *Mol. Cells* **37**, 759–765 (2014).
373. Lau, M.-T., Wong, A. S. T. & Leung, P. C. K. Gonadotropins Induce Tumor Cell Migration and Invasion by Increasing Cyclooxygenases Expression and Prostaglandin E2 Production in Human Ovarian Cancer Cells. *Endocrinology* **151**, 2985–2993 (2010).
374. Wang, W. & Wang, J. Toll-Like Receptor 4 (TLR4)/Cyclooxygenase-2 (COX-2) Regulates Prostate Cancer Cell Proliferation, Migration, and Invasion by NF-κB Activation. *Med. Sci. Monit.* **24**, 5588–5597 (2018).
375. Adefuye, A. O., Adeola, H. A., Sales, K. J. & Katz, A. A. Seminal Fluid-Mediated Inflammation in Physiology and Pathology of the Female Reproductive Tract. *J. Immunol. Res.* **2016**, (2016).
376. Li, Q. & Verma, I. M. NF-κB regulation in the immune system. *Nat. Rev. Immunol.* **2**, 725–734 (2002).
377. Fan, Y., Mao, R. & Yang, J. NF-κB and STAT3 signaling pathways collaboratively link inflammation to cancer. *Protein Cell* **4**, 176–185 (2013).
378. Ajayi-Smith, A., Watt, P. & Leaner, V. Interfering with nuclear transport as a means of interrupting transcription factor activity in cancer cells. *Crit. Rev. Eukaryot. Gene Expr.* **29**, (2019).
379. Çağatay, T. & Chook, Y. M. Karyopherins in cancer. *Curr. Opin. Cell Biol.* **52**, 30–42 (2018).
380. Islam, M. S., Morshed, M. R., Babu, G. & Khan, M. A. The role of inflammations and EMT in carcinogenesis. *Adv. Cancer Biol. - Metastasis* **5**, 100055 (2022).
381. Colotta, F., Allavena, P., Sica, A., Garlanda, C. & Mantovani, A. Cancer-related inflammation,

the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis* **30**, 1073–1081 (2009).
382. Demaria, S. *et al.* Cancer and Inflammation: Promise for Biologic Therapy. *J. Immunother.* **33**, (2010).
383. Yamanishi, Y. *et al.* Regional analysis of p53 mutations in rheumatoid arthritis synovium. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 10025–10030 (2002).
384. Zhang, J.-M. & An, J. Cytokines, Inflammation, and Pain. *Int. Anesthesiol. Clin.* **45**, (2007).
385. Taniguchi, K. & Karin, M. NF-κB, inflammation, immunity and cancer: coming of age. *Nat. Rev. Immunol.* **18**, 309–324 (2018).
386. Jarnicki, A., Putoczki, T. & Ernst, M. Stat3: linking inflammation to epithelial cancer - more than a 'gut' feeling? *Cell Div.* **5**, 14 (2010).
387. Young, M. R., Yang, H.-S. & Colburn, N. H. Promising molecular targets for cancer prevention: AP-1, NF-κB and Pdcd4. *Trends Mol. Med.* **9**, 36–41 (2003).
388. Goswami, B., Rajappa, M., Sharma, M. & Sharma, A. Inflammation: Its role and interplay in the development of cancer, with special focus on gynecological malignancies. *Int. J. Gynecol. Cancer* **18**, 591–599 (2008).
389. Gutsche, S., von Wolff, M., Strowitzki, T. & Thaler, C. J. Seminal plasma induces mRNA expression of IL-1β, IL-6 and LIF in endometrial epithelial cells in vitro. *Mol. Hum. Reprod.* **9**, 785–791 (2003).
390. Lee, C. H. & Kim, S. Y. NF-κB and therapeutic approach. *Biomol. Ther.* **17**, 219–240 (2009).
391. Huang, S., Pettaway, C. A., Uehara, H., Bucana, C. D. & Fidler, I. J. Blockade of NF-κB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. *Oncogene* **20**, 4188–4197 (2001).
392. Xie, T.-X., Xia, Z., Zhang, N., Gong, W. & Huang, S. Constitutive NF-κB activity regulates the expression of VEGF and IL-8 and tumor angiogenesis of human glioblastoma. *Oncol Rep* **23**, 725–732 (2010).
393. Wan, F. & Lenardo, M. J. The nuclear signaling of NF-kappaB: current knowledge, new insights, and future perspectives. *Cell Res.* **20**, 24–33 (2010).
394. Martincuks, A. *et al.* Nuclear translocation of STAT3 and NF-κB are independent of each other but NF-κB supports expression and activation of STAT3. *Cell. Signal.* **32**, 36–47 (2017).
395. Srivastava, M. & Baig, M. S. NOS1 mediates AP1 nuclear translocation and inflammatory response. *Biomed. Pharmacother.* **102**, 839–847 (2018).
396. Nachmias, B. & Schimmer, A. D. Targeting nuclear import and export in hematological malignancies. *Leukemia* **34**, 2875–2886 (2020).
397. Kau, T. R., Way, J. C. & Silver, P. A. Nuclear transport and cancer: from mechanism to intervention. *Nat. Rev. Cancer* **4**, 106–117 (2004).
398. Mosammaparast, N. & Pemberton, L. F. Karyopherins: from nuclear-transport mediators to nuclear-function regulators. *Trends Cell Biol.* **14**, 547–556 (2004).
399. Stewart, M. Molecular mechanism of the nuclear protein import cycle. *Nat. Rev. Mol. Cell Biol.* **8**, 195–208 (2007).
400. Zhu, J. *et al.* Upregulation of KPNβ1 in gastric cancer cell promotes tumor cell proliferation and predicts poor prognosis. *Tumor Biol.* **37**, 661–672 (2016).
401. Soderholm, J. F. *et al.* Importazole, a small molecule inhibitor of the transport receptor importin-β. *ACS Chem. Biol.* **6**, 700–708 (2011).
402. Yasuhara, N. *et al.* Triggering neural differentiation of ES cells by subtype switching of importin-α. *Nat. Cell Biol.* **9**, 72–79 (2007).
403. Wu, K. K., Liou, J.-Y. & Cieslik, K. Transcriptional Control of COX-2 via C/EBPβ. *Arterioscler. Thromb. Vasc. Biol.* **25**, 679–685 (2005).
404. De Luca, A., Lamura, L., Gallo, M., Maffia, V. & Normanno, N. Mesenchymal stem cell-derived interleukin-6 and vascular endothelial growth factor promote breast cancer cell migration. *J. Cell. Biochem.* **113**, 3363–3370 (2012).

405. Szabowski, A. *et al.* c-Jun and JunB antagonistically control cytokine-regulated mesenchymal-epidermal interaction in skin. *Cell* **103**, 745–755 (2000).
406. Kang, H. *et al.* N-(4-hydroxyphenyl)retinamide inhibits breast cancer cell invasion through suppressing NF-KB activation and inhibiting matrix metalloproteinase-9 expression. *J. Cell. Biochem.* **113**, 2845–2855 (2012).
407. Zhu, Z.-C., Liu, J.-W., Li, K., Zheng, J. & Xiong, Z.-Q. KPNB1 inhibition disrupts proteostasis and triggers unfolded protein response-mediated apoptosis in glioblastoma cells. *Oncogene* **37**, 2936–2952 (2018).
408. Morton, S., Davis, R. J., McLaren, A. & Cohen, P. A reinvestigation of the multisite phosphorylation of the transcription factor c-Jun. *EMBO J.* **22**, 3876–3886 (2003).
409. Waldmann, I., Wälde, S. & Kehlenbach, R. H. Nuclear Import of c-Jun Is Mediated by Multiple Transport Receptors* . *J. Biol. Chem.* **282**, 27685–27692 (2007).
410. Angel, P., Hattori, K., Smeal, T. & Karin, M. The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. *Cell* **55**, 875–885 (1988).
411. Hayden, M. & Ghosh, S. Signaling to NF-kB. *Genes Dev.* **18**, 2195–2224 (2004).
412. HELLWEG, C. E., ARENZ, A., BOGNER, S., SCHMITZ, C. & BAUMSTARK-KHAN, C. Activation of Nuclear Factor κB by Different Agents. *Ann. N. Y. Acad. Sci.* **1091**, 191–204 (2006).
413. Xia, L. *et al.* Role of the NFκB-signaling pathway in cancer. *Onco. Targets. Ther.* **11**, 2063–2073 (2018).
414. Cuzick, J. *et al.* Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: an international consensus statement. *Lancet Oncol.* **10**, 501–507 (2009).
415. Rothwell, P. M. *et al.* Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet* **377**, 31–41 (2011).
416. Bonovas, S., Filioussi, K., Tsavaris, N. & Sitaras, N. M. Use of Statins and Breast Cancer: A Meta-Analysis of Seven Randomized Clinical Trials and Nine Observational Studies. *J. Clin. Oncol.* **23**, 8606–8612 (2005).
417. Maisonneuve, P. & Lowenfels, A. B. Statins and the risk of colorectal cancer. *N. Engl. J. Med.* **353**, 952–954 (2005).
418. Bonovas, S., Nikolopoulos, G. & Sitaras, N. M. Statins and Reduced Risk of Hepatocellular Carcinoma in Patients With Hepatitis C Virus Infection: Further Evidence Is Warranted. *J. Clin. Oncol.* **31**, 4160 (2013).
419. Hungness, E. S. *et al.* The transcription factor activator protein-1 is activated and interleukin-6 production is increased in interleukin-1beta-stimulated human enterocytes. *Shock* **14**, (2000).
420. Veres, B. *et al.* Regulation of Kinase Cascades and Transcription Factors by a Poly(ADP-Ribose) Polymerase-1 Inhibitor, 4-Hydroxyquinazoline, in Lipopolysaccharide-Induced Inflammation in Mice. *J. Pharmacol. Exp. Ther.* **310**, 247 LP – 255 (2004).
421. Koehne, C.-H. & Dubois, R. N. COX-2 inhibition and colorectal cancer. *Semin. Oncol.* **31**, 12–21 (2004).
422. Hill, R., Cautain, B., De Pedro, N. & Link, W. Targeting nucleocytoplasmic transport in cancer therapy. *Oncotarget* **5**, 11–28 (2014).
423. Fried, H. & Kutay, U. Nucleocytoplasmic transport: taking an inventory. *Cell. Mol. Life Sci. C.* **60**, 1659–1688 (2003).
424. Zanella, F., dos Santos, N. R. & Link, W. Moving to the Core: Spatiotemporal Analysis of Forkhead Box O (FOXO) and Nuclear Factor-κB (NF-κB) Nuclear Translocation. *Traffic* **14**, 247–258 (2013).
425. Giridharan, S. & Srinivasan, M. Mechanisms of NF-κB p65 and strategies for therapeutic manipulation. *J. Inflamm. Res.* **11**, 407–419 (2018).
426. Rametse, C. L. *et al.* Inflammatory Cytokine Profiles of Semen Influence Cytokine Responses of Cervicovaginal Epithelial Cells . *Frontiers in Immunology* vol. 9 2721 (2018).
427. van der Watt, P. J., Ngarande, E. & Leaner, V. D. Overexpression of Kpnβ1 and Kpnα2 Importin Proteins in Cancer Derives from Deregulated E2F Activity. *PLoS One* **6**, e27723

(2011).
428. Fagerlund, R., Melén, K., Kinnunen, L. & Julkunen, I. Arginine/Lysine-rich Nuclear Localization Signals Mediate Interactions between Dimeric STATs and Importin α5*. *J. Biol. Chem.* **277**, 30072–30078 (2002).
429. Maneechotesuwan, K. *et al.* Regulation of Th2 Cytokine Genes by p38 MAPK-Mediated Phosphorylation of GATA-3. *J. Immunol.* **178**, 2491 LP – 2498 (2007).
430. Bekhtereva, I. A. & Dorosevich, A. E. [Histophysiology of epithelial and connective tissue components of the vaginal portion of the uterine cervix]. *Morfologiia* **136**, 90–96 (2009).
431. De Tomasi, J. B., Opata, M. M. & Mowa, C. N. Immunity in the Cervix: Interphase between Immune and Cervical Epithelial Cells. *J. Immunol. Res.* **2019**, 7693183 (2019).
432. Anton, L., DeVine, A., Sierra, L.-J., Brown, A. G. & Elovitz, M. A. miR-143 and miR-145 disrupt the cervical epithelial barrier through dysregulation of cell adhesion, apoptosis and proliferation. *Sci. Rep.* **7**, 3020 (2017).
433. Wira, C. R., Rodriguez-Garcia, M. & Patel, M. V. The role of sex hormones in immune protection of the female reproductive tract. *Nat. Rev. Immunol.* **15**, 217–230 (2015).
434. Jubelin, C. *et al.* Three-dimensional in vitro culture models in oncology research. *Cell Biosci.* **12**, 155 (2022).
435. Hoarau-Véchot, J., Rafii, A., Touboul, C. & Pasquier, J. Halfway between 2D and Animal Models: Are 3D Cultures the Ideal Tool to Study Cancer-Microenvironment Interactions? *International Journal of Molecular Sciences* vol. 19 (2018).
436. Ungefroren, H., Sebens, S., Seidl, D., Lehnert, H. & Hass, R. Interaction of tumor cells with the microenvironment. *Cell Commun. Signal.* **9**, 18 (2011).
437. Zhang, Y. S. *et al.* Bioprinting the Cancer Microenvironment. *ACS Biomater. Sci. Eng.* **2**, 1710–1721 (2016).
438. Hickman, J. A. *et al.* Three-dimensional models of cancer for pharmacology and cancer cell biology: Capturing tumor complexity in vitro/ex vivo. *Biotechnol. J.* **9**, 1115–1128 (2014).
439. Fink, C. G., Thomas, G. H., Allen, J. M. & Jordan, J. A. METAPLASIA IN ENDOCERVICAL TISSUE MAINTAINED IN ORGAN CULTURE–AN EXPERIMENTAL MODEL. *BJOG An Int. J. Obstet. Gynaecol.* **80**, 169–175 (1973).
440. E., C. J. *et al.* Preclinical Testing of Candidate Topical Microbicides for Anti-Human Immunodeficiency Virus Type 1 Activity and Tissue Toxicity in a Human Cervical Explant Culture. *Antimicrob. Agents Chemother.* **51**, 1770–1779 (2007).
441. Saba, E. *et al.* HIV-1 sexual transmission: early events of HIV-1 infection of human cervico-vaginal tissue in an optimized ex vivo model. *Mucosal Immunol.* **3**, 280–290 (2010).
442. Chumduri, C. & Turco, M. Y. Organoids of the female reproductive tract. *J. Mol. Med.* **99**, 531–553 (2021).
443. Barrios De Tomasi, J., Opata, M. M. & Mowa, C. N. Immunity in the Cervix: Interphase between Immune and Cervical Epithelial Cells. *J. Immunol. Res.* **2019**, 7693183 (2019).
444. Mk, N., Gi, B. & Rj, T. Investigation of microtrauma after sexual intercourse. *J. Reprod. Med.* **29**, 269–271 (1984).
445. Salinas-Vera, Y. M. *et al.* Three-Dimensional 3D Culture Models in Gynecological and Breast Cancer Research . *Frontiers in Oncology* vol. 12 (2022).
446. Ricciardi, M. *et al.* Epithelial-to-mesenchymal transition (EMT) induced by inflammatory priming elicits mesenchymal stromal cell-like immune-modulatory properties in cancer cells. *Br. J. Cancer* **112**, 1067–1075 (2015).
447. Sutherland, J. R. The role of seminal plasma in cervical carcinoma. (University of Cape Town, 2010).
448. Sales, K. J. Expression and functional role of cyclooxygenase enzymes in cervical carcinoma. (University of Cape Town, 2001).
449. Burt, L. M. *et al.* Cervix Cancer in Sub-Saharan Africa: An Assessment of Cervical Cancer Management. *JCO Glob. Oncol.* 173–182 (2021) doi:10.1200/GO.20.00079.

450. Greten, F. R. & Grivennikov, S. I. Inflammation and Cancer: Triggers, Mechanisms, and Consequences. *Immunity* **51**, 27–41 (2019).
451. Karnoub, A. E. & Weinberg, R. A. Chemokine Networks and Breast Cancer Metastasis. *Breast Dis.* **26**, 75–85 (2007).
452. Kany, S., Vollrath, J. T. & Relja, B. Cytokines in Inflammatory Disease. *Int. J. Mol. Sci.* **20**, 6008 (2019).
453. Yokoyama, K. *et al.* Increased invasion and matrix metalloproteinase-2 expression by Snail-induced mesenchymal transition in squamous cell carcinomas. *Int J Oncol* **22**, 891–898 (2003).
454. Vilen, S.-T., Salo, T., Sorsa, T. & Nyberg, P. Fluctuating Roles of Matrix Metalloproteinase-9 in Oral Squamous Cell Carcinoma. *Sci. World J.* **2013**, 920595 (2013).
455. Sheu, B.-C. *et al.* Increased expression and activation of gelatinolytic matrix metalloproteinases is associated with the progression and recurrence of human cervical cancer. *Cancer Res.* **63**, 6537–6542 (2003).
456. Sheng, H., Shao, J., Washington, M. K. & DuBois, R. N. Prostaglandin E2 Increases Growth and Motility of Colorectal Carcinoma Cells*. *J. Biol. Chem.* **276**, 18075–18081 (2001).
457. Wang, D., Buchanan, F. G., Wang, H., Dey, S. K. & DuBois, R. N. Prostaglandin E2 Enhances Intestinal Adenoma Growth via Activation of the Ras-Mitogen-Activated Protein Kinase Cascade. *Cancer Res.* **65**, 1822–1829 (2005).
458. Kalluri, R. & Weinberg, R. A. The basics of epithelial-mesenchymal transition. *J. Clin. Invest.* **119**, 1420–1428 (2009).
459. Zhao, L. *et al.* Src promotes EGF-induced epithelial-to-mesenchymal transition and migration in gastric cancer cells by upregulating ZEB1 and ZEB2 through AKT. *Cell Biol. Int.* **42**, 294–302 (2018).
460. Hawiger, J. Innate immunity and inflammation: A transcriptional paradigm. *Immunol. Res.* **23**, 99–109 (2001).
461. Kuusisto, H. V, Wagstaff, K. M., Alvisi, G., Roth, D. M. & Jans, D. A. Global enhancement of nuclear localization-dependent nuclear transport in transformed cells. *FASEB J.* **26**, 1181–1193 (2012).
462. He, S. *et al.* Upregulation of nuclear transporter, Kpnβ1, contributes to accelerated cell proliferation- and cell adhesion-mediated drug resistance (CAM-DR) in diffuse large B-cell lymphoma. *J. Cancer Res. Clin. Oncol.* **142**, 561–572 (2016).