Investigating pathways in tumour cell migration and invasion in response to novel isoquinolinone compounds

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Abstract

The ability of cancer cells to disseminate from a primary tumour and invade via the breaching of surrounding tissue vasculature is a hallmark of the metastatic phenotype. Cancer cells are highly dynamic adopting a wide range of mechanisms in order to accomplish this enhanced migrational activity. This study looks at the mechanisms behind enhanced migration and invasion *in vitro* in two highly aggressive cell lines in response to the heterocyclic aromatic organic Isoquinolinone compounds. Here we show that the structurally similar A5 and A3 compounds reduce adhesion of MDA-MB-231 cells to Fibronectin, Matrigel, and Type I collagen which was paralleled in A5 treated HT1080 cells on these matrix components. Compound A5 also induced 2D migration of MDA-MB-231 cells on all matrix substrates. Cell movement of MDA-MB-231 cells through 3D matrices was modestly enhanced by A5 and significantly enhanced by A3. Using gene expression arrays we were able to identify fifteen genes whose expression was regulated by the isoquinolinone compounds and heavily implicated in the process of cell motility. Moreover, we established that these genes are differentially regulated by the compounds over a 24 hour period with expression typically being more enhanced at the earlier time points of 4- and 8- hours and suppressed at 24 hours.

The main findings presented in this thesis reveal many proteins involved in cell motility that have not been studied in MDA-MB-231 or HT1080 cells. These results lend support that compound treated cell lines activate several pathways in cell migration and invasion that warrant further investigation to elucidate the precise mechanism of these proteins involved and to develop novel inhibitors for some of the more obscurely studied, but heavily implicated proteins such as RhoQ.

Table of contents

List of tables

List of figures

Acknowledgements

Abbreviations

Arf6 A   ADP-ribosylation factor 6
Arp2/3   Actin-related proteins 2/3 (Arp2/3) complex
ATP   Adenosine tri-phosphate
CAFs   Cancer associated fibroblasts
CTGF   Connective tissue growth factor
CYR61   Cysteine-rich angiogenic inducer 61
DMSO   Dimethyl sulfoxide
DNA   Desoxyribonucleic acid
ECM   Extracellular matrix
EGF   Epidermal growth factor
EGFR   Epidermal growth factor receptor
EGR1   Early growth response 1
EMT   Epithelial to mesenchymal transition
EPHA2   Ephrin type-A receptor 2
ERK   Extracellular signal-related protein kinase
ETS1   V-ets avian erythroblastosis virus E26 oncogene homolog 1
EVL   Ena-VASP-Like
FAK   Focal adhesion kinase
FPR1   Formyl peptide receptor 1
GEP100   Guanine nucleotide-exchange factor
JNK   Jun N-terminal kinase
MAPK   Mitogen-activated protein kinase
MDM2   Murine double minute 2
Mena   Mammalian Enabled
MMP   Matrix metalloproteinase
mRNA   messenger RNA
mTOR   Mammalian target of ramamycin
NFE2L3   Nuclear factor erythroid 2-related factor 3
PI3K   Phosphatidylinositol 3-kinase
SAA1   Serum amyloid A1
Scar   Suppressor of cAMP receptor
Src   Sarcoma kinase
TGF-α   Transforming growth factor alpha
TGF-β   Transforming growth factor beta
VASP   Vasodilator-Stimulated Phosphoprotein
WASP   Wiskott-Aldrich Syndrome Protein
Wave   WASP Verprolin-homologous
WIP   WASP interacting proteins

Chapter 1 Introduction

1.0 Cancer

The human body contains a complex ecosystem of roughly 10^{13} cells that interact with each other and their environment through physical stimulation or chemical cues (Frank and Nowak, 2004). The interacting network of cells however remains far from flawless, and may allow for neoplastic transformation for a subset of cells which are then capable of proliferating independently of internal or external signals, and are often immortal (Seger et al, 2002). Neoplasia involves stable cellular changes spontaneously or when a cell is exposed to a carcinogen. This is the initiation of carcinogenesis, and are essentially irreversible changes in predisposed somatic cells and their subsequent progeny (Seger et al, 2002; Frank and Nowak, 2004). Clonal events leading to somatic mutations affect genetic mosaicisim, and in turn cause alterations in oncogenes, microRNA genes, and tumour-suppressor genes which ultimately results in chromosome instability and loss of heterozygosity (Nigg, 2006). Whilst genetic and epigenetic alterations are heavily associated with this type of defined abnormality in cell behaviour, cancer etiology has provided identification of several other contributing factors such as hormones, chemical exposure, radiation, infections, diet, and lifestyle (Silverstein et al, 2006).

The human cell has evolved cellular regulatory mechanisms to deter the fostering of a cancerous phenotype. To prevent defects such as aneuploidy, cell cycle checkpoints exist to ensure faithful transmission of chromosomes from the mother cell to the daughter cells is accomplished (Zhou and Giannakakou, 2005). The cell cycle is characterised by distinct phases throughout its progression, this includes the Gap 0 (G_0) phase, Gap 1 (G_1) phase, Synthesis (S) phase, Gap 2 (G_2) phase, and Mitosis (M) phase (Schafer, 1998). DNA damage checkpoints exist at every phase to protect the cell from exogenous and endogenous genotoxic agents, with the exception of the G_0 phase which is when the cell is in a quiescent state (Mulumbres and Barbacid, 2009).   Alterations in DNA are sensed by biochemical signalling pathways which inhibit the enzymatic activity of cyclic dependent kinases (CDKs), the master regulators of mitotic entry, and ultimately result in cell arrest (Mulumbres and Barbacid, 2009). If the DNA damage is too excessive, or repair is hindered through genetically defective checkpoints and DNA repair machinery, cells reprogram to enter a state of senescence, or undergo apoptosis (Mulumbres and Barbacid, 2009).

The cancer associated phenotype comes into fruition when vital tumour suppressor genes that normally function at checkpoints in the cell cycle harbour mutations. The retinoblastoma

protein (pRb), and protein 53 (p53) are two well-known and thoroughly studied tumour suppressor genes that lose their ability to function in many types of sporadic cancers through frameshifts, deletions and premature stop codons (Poznic, 2009). In the case of pRb, cell arrest between the G_1 to S transition is executed when an interaction is established with the family of E2F transcription factors. When E2F transcriptional inactivation is alleviated due to a mutated Rb protein, E2F-controlled genes such as *c-myc* involved in the progression of the cell cycle are constantly transcribed (Poznic, 2009). This dysfunction causes further unbalanced regulation by crippling the expression of CDK inhibitors protein 15 (p15^{INK4B}) and protein 21 (p21$^{WAF1/CIP1}$) which normally act to inhibit cyclin E-cdk2, a protein that phosphorylates pRb to promote progression (Moustakas and Kardassis, 1998). Tumour suppressor genes can also be suppressed from targeting by oncogenes and their resulting oncoprotein. In response to stress signals at G_1 and G_2 to M phase, p53 would normally induce arrest, however the upregulated oncogene murine double minute (*mdm2*) binds the N-terminal transactivation domain, blocking p53 transcription (McCoy et al, 2003). All these factors combined lead to uncontrolled cell proliferation and the classical phenotype associated with many forms of cancers.

1.1 The Extracellular matrix

In humans, and every other multicellular animal organism, all tissues and organs are surrounded by the extracellular matrix (ECM) (Rovensky, 2011). This complex network of interconnected extracellular macromolecules provides essential physical scaffolding for cellular constituents whilst delivering vital biomechanical and biochemical cues that generate comprehensive tensile strength and elasticity for surrounding organs (Frantz et al, 2010). More precisely, the ECM includes the interstitial matrix and basement membrane, and is the defining feature of connective tissue that supports, separates or connects differing tissues or organs (Figure 1-1), (Rovensky, 2011).

Through dynamic reciprocal biochemical and biophysical dialogue between cellular components and evolving microenvironment, each tissue in the body has a unique ECM that is heterogeneous in composition and topology (Frantz et al, 2010). The composition of the ECM can be characterised by an interlocking mesh of two main classes of macromolecules known as the proteoglycans and fibrous proteins. In tissue, proteoglycans make up the majority of the extracellular interstitial space and function to provide force-resistance, hydration through unique buffering action, and binding through surface receptors (Hynes, 2012: Frantz et al, 2010). There are several families of proteoglycans characterised by their core proteins, localisation and nature of the covalently attached glycosaminoglycan (GAG) side chains.

GAGs can be divided into sulphated and non-sulfated, in which the former includes chondroitin sulphates, keratin sulphates, heparan sulphates, dermatan sulphates, and the latter includes hyaluronic acid (Rovensky, 2011). The fibrous proteins of the ECM, dubbed glycoproteins, provide a structural supportive role that allows a stable rigidity between major tissues to be maintained. They include collagens type I-XII, Elastin, Fibronectin, Laminin, Nidogens, Tenascin, and fibulin (Rovensky, 2011).

Tissue organisation and maintenance depends on resting fibroblasts embedded in a surrounding stroma which act to secrete and organise a repertoire of the previously mentioned fibrous proteins and proteoglycans in the ECM (Frantz et al, 2010). An example is the essential glycoprotein Fibronectin, which directly assists with the organisation of the interstitial ECM by mediating cell attachment and migration along the substrata. Through exposure of its synergy site, tensed fibronectin modulates the adhesion assembly of the integrin $\alpha5\beta1$. Integrins are obligate heterodimeric cell surface receptors that mediate cell-ECM adhesion through their specialised structures termed focal contacts or focal adhesions. They contain two distinct chains termed α subunit and β subunit which penetrate the plasma membrane and consist of a large extracellular domain, a single membrane spanning region, and a small cytoplasmic domain (Juliano, 2002). Integrins are also involved in cross talk with various growth factors via membrane-proximal interactions, or by intrinsic domains within the ECM proteins that act as ligands for canonical growth factor receptors (Hynes, 2012). These interactions spatially integrate specific signals in a certain microenvironment within the ECM, and are important in controlling cell behaviour, survival, proliferation and differentiation (Brizzi et al, 2012).   Orchestrated remodelling is dynamically regulated and crucial to normal function. A loss in tissue organisation and aberrant behaviour of cellular components generates pleiotropic effects which can lead to a stiffer matrix and ultimately tumourigenicity (Campbell et al, 2010).
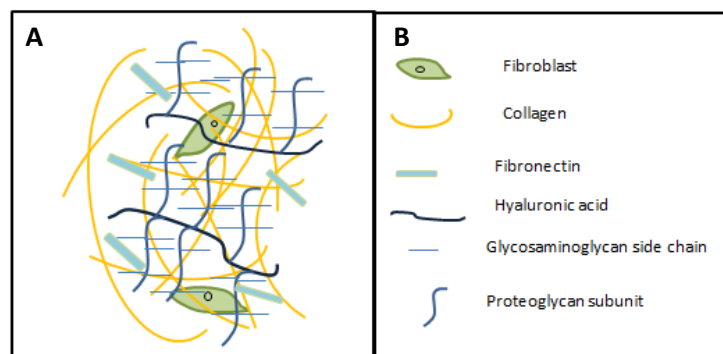
Figure 1-1 the extracellular matrix
A. Depiction of the extracellular matrix surrounding lymphatic or blood vessels.
B.A key representing the individual components of the extracellular matrix.

1.2 Tumour metastasis

1.2.1 Metastasis

In both health and disease the anatomical status quo, or tissue homeostasis, is reliant on a complex physicochemical process known as cell migration (Fridel et al, 2011; Schwab et al, 2012). The process of cell migration attains a central importance which is adopted by a plethora of organisms ranging from humans to plants, and even single celled organisms such as the amoeba (Ridley, 2003). In humans, cell migration orchestrates many essential physiological and pathological events such as morphogenesis throughout embryonic development, tissue repair, and immune surveillance (Ridley, 2003). Generally from early development spatial arrangements of tissues from different organs of the body are established via complex programming. The temporal stability of tissue organisation is then dependent on several mechanisms that act to restrain the intrinsic locomotory capabilities of cells (Wakefield and Hunter, 2007). In the mammalian body, cells primarily migrate within three dimensional (3D) tissues altering their shapes, kinetics and stringency with the surrounding environment to carry out the process (Friedl and Wolf, 2009). The transient migration of single cells from a primary tissue is driven in a directional manor through the coupling of the cells motility machinery to a motogenic signal (Petrie et al, 2009). Cells can undergo chemotaxis, durotaxis, electrotaxis and haptotaxis which is the response to soluble cues, environmental mechanical signals, electric fields and gradient of substrate bound chemoattractants on the ECM or existence of local adhesion gradients respectively (Petrie et al, 2009).

Chemical exposure, genomic instability and modified microenvironment signals can alter the migratory behaviour of native cells, which in turn may lead to the onset of many diseases, such as chronic inflammatory disease, vascular disease, mental retardation, tumour formation and metastasis (Ridley, 2003). Metastasis is a poorly understood component of cancer pathogenesis which currently accounts for one in four deaths in the UK each year , with an overall recorded death toll of 157,000 people in 2010(Chaffer and Weinberg, 2011; Cancer research UK, 2012). It involves the local dissemination of neoplastic tissue from primary tumours into the space of surrounding tissue whilst causing a disorganisation of planar boundaries between contiguous tissues in the process (Yamaguchi et al, 2005).

1.2.2 Stromal cells in metastasis

Fibroblasts are a main component of solid tumour stroma and are responsible for the growth of primary tumours that will eventually metastasize to distant tissue vasculature. They become generally known as cancer associated fibroblasts (CAFs) and can aid tumour cell proliferation, angiogenesis, and metastasis (Chen et al, 2012). One well known example is CAFs aid in the heterogeneous disease breast cancer. Breast cancers form within the breast ducts where they progress from a normal pathology to a ductal carcinoma *in situ*, in which cancerous cells have proliferated to fill the lobules, and finally an invasive ductal carcinoma, which is the breach of the basement membrane and invasion into surrounding stroma (McSherry et al, 2007). CAFs constitutively secrete a major source of the chemokine stromal cell-derived factor 1/C-X-C motif ligand 12 (SDF-1/CXCL12) which is essential in promoting tumour growth. This chemokine binds and activates the G protein-coupled receptor CXCR4 located on breast cancer cells which activates signalling pathways such as AKT (protein kinase B) or Mitogen-activated protein kinase (MAPK) which are involved in the survival and proliferation of cells (Luker et al, 2012). SDF-1 also plays a part in mediating the recruitment of endothelial progenitor cells which participate in pathologic angiogenesis. Secretion of a mitogenic factor called platelet-derived growth factor C (PDGF-C) by CAFs provides resistance to anti-angiogenic therapy ensuring a secure environment for malignant progression (Chen et al, 2012). In addition, hepatocyte growth factor (HGF) is another important fibroblast secreted protein that combines with the cell surface c-Met receptor tyrosine kinase on cancer cells and promotes angiogenesis and proliferation. Interestingly HGF also increases clonogenic survival of breast cancer cells though phosphorylating Met which provides subsequent resistance to inhibitors of the epidermal growth factor receptor which is essential for cell migration (Mueller et al, 2012). It is believed that breast cancer cells instruct their surrounding fibroblasts to secrete HGF via paracrine signalling, however the exact mechanism of how this occurs is not quite fully understood (Tyan et al, 2011).

Macrophages are myeloid lineage cells that are also present in the breast tumour microenvironment and play a role in tumour metastatic progression. Up-regulated autocrine CXCL12 signalling has been indicated to induce the differentiation of monocytes into macrophages increasing their presence in the surrounding stroma (Boimel et al, 2012). At some stage during tumour progression cancer cells produce chemoattractants such as colony stimulating factor 1 (CSF-1) and C-C motif chemokine ligand-2 (CCL2) which induces macrophage infiltration and activation in the tumour (Lin and Pollard, 2007). Macrophages

comprise up to 50% of the tumour cell mass in breast cancer and have been shown to be essential for regulating the 'angiogenic switch' creating a high-density vascular network through the secretion of angiogenic factors such as vascular endothelial growth factor (VEGF), angiogenin (ANG)1 and ANG2 (Lin and Pollard, 2007). It has been suggested that macrophages become trophic for tumour cells because of the cytokine context of the microenvironment which prevents maturation of antigen-presenting cells (McSherry et al, 2007). Macrophages also secrete several cytokines including transforming growth factor-beta (TGF-β) which initiates the dissemination of breast cancer cells in the early stages of metastasis (Williams et al, 2001). TGF-β is a potent inducer of epithelial -mesenchymal transition (EMT) which is the change from an apical-basal polarized epithelial phenotype to a more motile mesenchymal phenotype with no distinct polarity (Vervoort et al, 2013). During this differentiation cells lose classical markers of an epithelial phenotype such as E-cadherin, claudins, desmoplakins, cytokeratins, and zonula occludens (ZO)-1. These are substituted for markers typical of a mesenchymal phenotype such as N-cadherin and Vimentin (Vervoort et al, 2013). TGF-β-induced EMT phenotypic changes are exerted through alterations in the expression of several transcriptional regulators such as basic helix-loop-helix domain protein Twist1, E-box repressor Snail, Slug, zinc-finger E-box binding homeobox (ZEB)-1, ZEB2, Forkhead box protein C (FOXC)-1 and FOXC2 (Zhang et al, 2012). These transcription factors can transcriptionally repress *E-cadherin* expression either directly or indirectly which results in a loss of epithelial cell-cell adhesion and maintenance of the tissue architecture. Other transcriptional factors targeted by TGF-β such as *SOX4* (SRY-related HMG box) induce the expression of mesenchymal markers N-cadherin, vimentin, and fibronectin (Zhang et al, 2012; Vervoort et al, 2013).   Once breast cancer cells adopt a mesenchymal phenotype and are liberated from the primary tumour colony there migration is directed by the cytokine epidermal growth factor (EGF) which is secreted by macrophages. Tumour cells and macrophages co-migrate through a CSF-1/EGF paracrine loop which mutually accelerates their potential motility (McSherry et al, 2007).

1.3 Proteases in Cancer

To migrate effectively cells must overcome many complex molecular parameters including the organisation of cytoskeletal architecture, type of interactions between the cell and the matrix, and the structural modification of directly surrounding tissue in consequence to migrating cells (Friedl and Wolf, 2010). The ECM matrix hereby acts as an underlying substratum for cells to migrate on but also a physical barrier towards advancing tumour cells that needs to be overcome (Friedl and Wolf, 2003). Breast cancer cells utilise an actin-driven translocation method that uses proteolytic machinery to migrate on, or through tissue substrate (Friedl and Wolf, 2009).

1.3.1 Matrix Metalloproteinases in tumour cell invasion.

During cell ingress breast cancer cells transverse through tissue barriers whilst remodelling the collagen matrix in a protease-dependent manner by members of the matrix metalloproteinase (MMP) family (Hotary et al, 2000). MMPs are zinc-dependent endopeptidases characterised by a pro-peptide and catalytic domain linked to a hemopexin-like C-terminal domain via a flexible hinge region (Kessenbrock et al, 2010). MMPs are synthesised as pepro-enzymes and secreted as inactive zymogens due to an interaction between a cysteine residue of the pro-domain with the zinc ion of the catalytic domain (Kessenbrock et al, 2010). This latency is maintained until proteolytic cleavage of a consensus sequence in the pro-domain by convertases occurs. Once active, the hempexin domain allows collagenases to cleave triple helical interstitial collagens (Kessenbrock et al, 2010). Several classes of proteases are up-regulated in metastasis including MMPs, cathepsins, and serine proteases, of which the latter two have only received attention in the more recent research (Friedl and wolf, 2008). They drive accelerated branching tubulogenesis that is associated with unconventional cell invasion.

Bartsch et al showed that several MMPs are constitutively expressed in highly aggressive breast cancer cell lines including MMP-1, MMP-7 (Martilysin), MMP-8, MMP-10 (stromelysin-2), MMP-11 (Stromelysin-3), MMP-13, MMP-14 (MT1-MMP), MMP-15 (MT2-MMP), and MMP-16 (MT3-MMP), (Bartsch et al, 2003). Other MMPs such as MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are up-regulated through integrin-ligand binding that transduces signals from the ECM to activate specific regulatory signalling pathways that induces there expression (Baum et al, 2007: Maity et al 2011). Most MMPs are secreted as inactive pro-enzymes that are activated when cleaved by extracellular proteinases. The exceptions are MT1-MMP, MT2-MMP, and MT3-MMP which contain a transmembrane domain signifying these proteins are

expressed at the surface rather than secreted (Ellerbroek and Stack, 1999). Sabeh et al and Weiss have shown that the membrane-anchored metalloproteinases are absolutely essential in negotiating the ECM. Inhibition of MT1-MMP heavily restricts the cancer cells ability to transmigrate through a heavily cross-linked network of type I collagen fibrils (Sabeh et al, 2009: Ota et al, 2009). Other important proteolytic enzymes in tumour induced invasion include the cysteine proteases such as cathepsins B (Radashanda et al, 2012).

1.4 Cytoskeletal changes when the cell is navigating the extracellular matrix

1.4.1 Lamellipodia and Filopodia formation

The cell motility cycle usually commences with a migration-promoting agent such as a chemokine or through adhesion receptor mediated signalling which causes the cell to polarize and extend leading protrusions in the form of a pseudopod in the direction of migration (Ridley, 2003). This mode of action is dynamically carried out by the cytoskeleton which is what cancer cells use to orchestrate protrusive migration (Machesky, 2008).   In the cell there are three types of cytoskeletal proteins including microtubules, intermediate filaments, and actin filaments. Metastatic cancer cells use the latter to underpin protrusions that allow them to invade through the surrounding vasculature (Stevenson et al, 2012). Individual actin molecules are globular proteins, termed G-actin, which are 43 kilodalton (KDa) in size with three isotypes (α, β, and γ) that readily polymerize concurrently with the hydrolysis of adenosine triphosphate (ATP) to form thin flexible fibres called filamentous actin (F-actin),(Jiang et al, 2009). Research has shown functional polarity in the growth of these filaments which can be defined by a slow-growing pointed end, referred to as the minus (-) end, and a fast-growing barded end which is commonly referred to as the plus (+) end (Mullins et al, 1998).   Actin filament assembly is regulated by heterodimeric capping proteins such as gelsolin and CapZ which inhibit the addition or loss of actin subunits at the barbed ends ensuring a well-defined cell shape for motility is achieved (Cooper and Sept, 2008).

Actin assembly and dendritic nucleation is mainly controlled by the seven subunit protein complex, the actin-related proteins 2/3 (Arp2/3) complex. The Arp2/3 complex is composed of two actin-related proteins, Arp2 and Arp3, and also five structural subunits referred to as p41-Arc, P34-Arc, p21-Arc, p20-Arc, and p16-Arc (Welch et al, 1997). The activity of the Arp2/3 complex itself is controlled by actin-nucleation promoting factors such as WASP (Wiskott-Aldrich Syndrome Protein) and SCAR/WAVE (suppressor of cAMP receptor/WASP Verprolin-homologous), (Sossey-Alaoui, 2005). The WASP protein is expressed exclusively in hematopoietic cells; however N-WASP, a ubiquitously expressed homolog of WASP carries out many of the same functions in cancer cells (Westerberg et al, 2012). The SCAR/WAVE subfamily contains three members called WAVE1, WAVE2, and WAVE3 which all attain non-redundant roles in cell migration (Sossey-Alaoui, 2005). Members of the SCAR/WAVE and WASP family share a tripartite VCA (Verprolin homology, Cofilin homology, and Acidic) domain and that activity is governed by upstream Rho GTPases involved in the regulation of the actin cytoskeleton (Sossey-Alaoui, 2005).The Rho GTPases are a subgroup of the Ras superfamily of GTPases with three family members, including Rac1, RhoA, and Cdc42, which have been well studied in cellular locomotion (Jiang et al, 2009). Rho GTPases act as sensitive molecular switches that cycle between an inactive GDP-bound state and an active GTP-bound state. This process is carried out by GTPase activating enzymes (GAPs) and Guanine nucleotide exchange factors (GEFs) respectively, and allows active Rho GTPases to interact with a plethora of different downstream effectors (Parri and Chiarugi, 2010).

Protrusions can take form as large broad lamellipodia, or if microspikes within the lamellipodia spread beyond its own frontier, they are termed filopodia (Ridley et al, 2003). Lamellipodia formed by the initial propulsion are the main organelle for cell locomotion .These sheet like protrusions are structurally defined by long unbranched actin filaments at the base of the lamellipodium which progress into a highly lateral branched actin network as shown in Figure 1-2 (Machesky, 2008).   Metastatic cells are generally rich in filopodia-like structures which can be described as rod-like extensions formed by cross-linked actin filaments that penetrate into the cell wall as shown in Figure 1-2. These structures detect signals from the surrounding environment such as chemoattractants or nutrients acting as a sensory organ for the cell (Yimaz and Christofori, 2009).

Members of the WAVE family function downstream of Rac and are involved in the formation of lamellipodia. Members of the WASP subfamily are downstream effectors of Cdc42 and are thought to be involved in the formation of filopodia, however their role in this is much less

clear (Sossey-Alaoui, 2005). RhoA is known to regulate the actin cytoskeleton in the formation of stress fibres through its downstream effector protein ROCK1 (Rho-associated, coiled-coil containing protein kinase 1), (Jiang et al, 2009).

N-WASP exists in an auto-inhibited conformation due to an occluded C-terminus which occurs as a result of interacting with the proteins N-terminus (Pollitt and Insall, 2009). Unlike N-WASP, SCAR/WAVE proteins do not exist in an auto-inhibited form but rather a pentameric complex consisting of four additional subunits, namely, Sra1/PIP121, Abi1/2, Nap1 and HSPC300 (Pollitt and Insall, 2009).   The WASP subfamily's auto-inhibition is released by the competitive binding of Cdc42 and the phospholipid PIP_2. Whilst the SCAR/WAVE proteins are activated and regulated by Rac indirectly through intermediate molecules such as the insulin receptor substrate IRSp53 which brings the proteins together (Yamaguchi and Condeelis, 2007). The nucleation promoting factors then activate the Arp2/3 complex through the binding of their VCA domain which brings Arp 2 and Arp3 subunits together forming an active, pseudo-actin dimer (Pollitt and Insall, 2009). This allows profilin bound G-actin monomers to bind to the plus end of Arp3 to complete a trimeric nucleus that reduces the kinetic barrier of actin nucleation and promotes rapid polymerization. In the context of lamellipodia formation, new actin filaments branch at a 70°angle from mother filaments generating a cross-linked, anisotropic meshwork of actin that pushes the plasma membrane forward and expands it laterally (Pollitt and Insall, 2009).

The two dominating cell periphery organelles are interchangeable through the Ena/VASP family of proteins, which includes the mammalian homologues Mena (Mammalian Enabled), VASP (Vasodilator-Stimulated Phosphoprotein), and EVL (Ena-VASP-Like), (Applewhite et al, 2007). Ena/VASP proteins are localised at sites of dynamic actin turnover such as focal adhesions, the leading edge of protruding lamellipodia, and the tips of filopodia. They interplay with other key proteins such as Diaphanous-related formins (mDia) and fascin to initiate this membrane shaping. mDia is relieved from intramolecular auto-inhibition by RhoA GTPase and then acts by sequestering Ena/VASP from storage sites close to the leading edge, so that it may act at the leading edge(Homem and Peifer, 2009). Fascin, on the other hand is an actin-cross linker that compacts actin filaments into straight, rigid bundles with distinct mechanical stiffness orientated orthogonally to the membrane (Yang et al, 2012). Ena/VASP proteins are essential in actin-filament elongation and are hypothesised to function by antogonizing actin-filament capping proteins (Bear and Gertler, 2009). They also contain a polyproline-rich motif

which binds with a high affinity to profiling-G-actin complexes providing a mainstream of actin monomers for polymerisation (Bear and Gertler, 2009).

There are several other Rho GTPases have been implicated in having a role in cell migration, but have been less well studied. Examples include RhoQ/TC10 and RhoJ/TCL which can also interact with WASP proteins and stimulate actin polymerisation (Ridley, 2001). This highlights a potentially more complex mechanism in cell migration to un-fold with future studies.
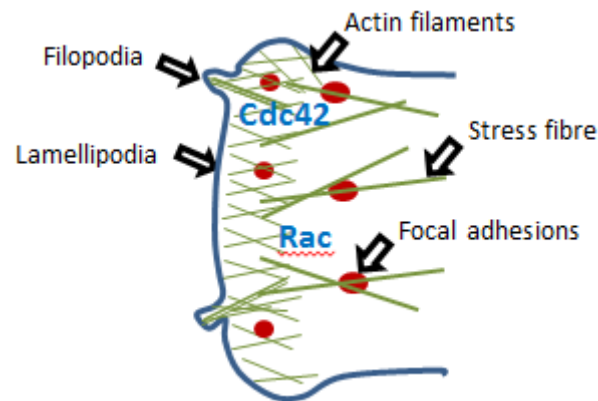
Figure 1-2 Morphology of the leading edge of a migrating cell
Diagram depicts a basic visual representation of both the lamellipodia and filopodia in migrating cells.

1.4.2 Invadopodia

Invadopodia are described as actin-rich ventral membrane protrusions found specifically on cancer cells that allow them to move into, or through a three-dimensional matrix (Yilmaz and Christofori, 2009). Mechanisms regulating invadopodium assembly and maturation are still not fully understood. Invadopodia are composed of many components that help facilitate its structure with the initiation of its formation being through Integrin-mediated adhesion to the ECM. This causes Src to become activated and allows for phosphorylation of the PIP_2 recruited scaffold protein Tks5. Phosphorylated Tks5 generates a specific binding site to the SH2 domains of Nck adaptor proteins, Nck 1 and Nck2. This allows for indirect association of Tks5 with N-WASP which is bound to the Arp2/3 complex. Cortactin also associates with N-WASP and the Arp2/3 complex forming an active complex through WASP interacting protein (WIP) and dynamin. Together these proteins compose a multi-subunit actin-regulatory complex that regulates invadopodia formation (Stylli etal, 2009).

Cofilin is a small ubiquitous protein that is also essential in regulating actin dynamics in invadopodia formation. It functions to depolymerize filaments to generate free actin that can be recycled for another round of polymerization. Typically cofilin accomplishes this by virtue of its severing activity which increases the number of free pointed and barbed ends (Yamaguchi and condeelis, 2007). In the initiation of invadopodia formation cortactin is bound to cofilin inhibiting its severing activity. Phosphorylation of cortactin leads to the dissociation of cofilin and allows for the generation of barbed ends from severed actin filaments to support Arp2/3-dependent actin polymerization (Oser et al, 2009). After barbed end formation cortactin is subsequently dephosphorylated which blocks the severing ability of cofilin and thereby enables invadopodia stabilization and degradative capacity (Figure 1-3), (Murphy and Courtneidge, 2009).
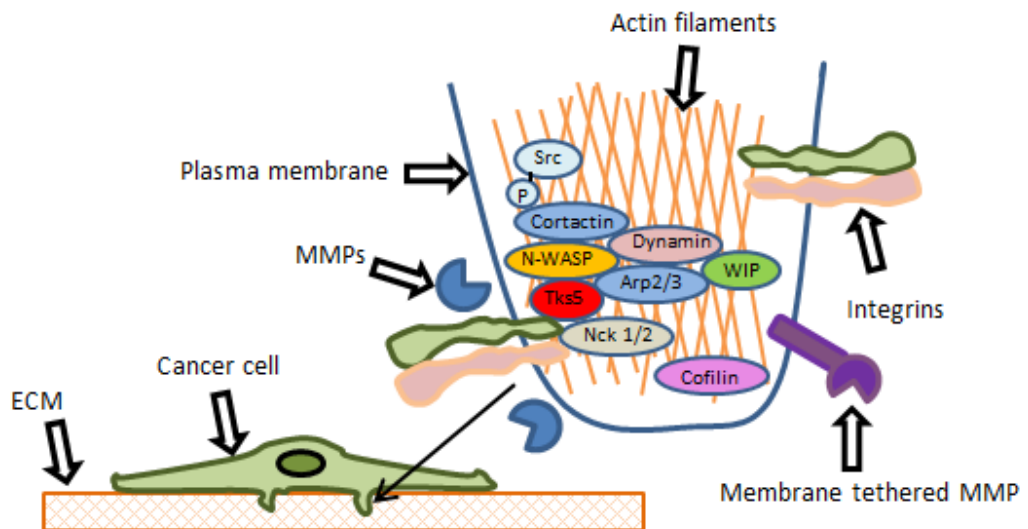
Figure 1-3 Structure of a mature invadopodium in the ECM

Formation occurs through recruitment and activation of the Arp2/3 complex and WIP. Phosphorylation of Tks5 and cortactin by Src causes binding of Nck 1 and Nck2 to Tks5. Subsequently cortactin and Tks5 bind N-Wasp and the Arp2/3 complex which generate a complex with WIP and dynamin. Cortactin phosphorylation also leads to cofilin dissociation and actin polymerisation. Presentation of MTI-MMP at the tip allows for the activation of secreted MMP2 enabling local degradation of the ECM.

1.5 Adhesion, cell migration, and Invasion

1.5.1 Adhesion formation

For migration to occur, the protrusion must anchor to the ECM through integrins that form a cluster of anterior focalised adhesion complexes (Friedl and Wolf, 2009). Without adhesion, the protruding membrane folds back to the dorsal surface of the cell resulting in membrane ruffles without distinct polarity. Integrin extracellular domains bind to ECM proteins through their sequence specific motifs (Parsons et al, 2010). This binding of extracellular ligands induces a conformational change that exposes integrin short cytoplasmic tails allowing linkage to the actin cytoskeleton. This intracellular interaction is mediated by several actin-binding proteins such as α-actinin, talin, and vinculin (Takahashi, 2001). Binding of these proteins triggers the indirect recruitment of several other essential scaffold and signalling proteins such as paxillin, FAK, and PI3K (Figure 1-4A) (Parsons et al, 2010). Clustering of these proteins leads to a transition between nascent adhesions at the protruding cell edge that are tension independent, to mature focal adhesions that serve as traction points to pull the cell body forward (Lawson et al, 2012). Short-lived nascent adhesions are not associated with the contractile actin stress fibre network and form independently of nonmuscle myosin II, a major component in actomyosin-mediated cell contraction. This prevents premature contraction from occurring during cell migration (Choi et al, 2008). Due to the multivalent nature of the ECM, the initial engagement typically forms many small-scale clusters of early maturing adhesions called focal complexes which are larger than nascent adhesions (Ridley et al, 2003). The rate of adhesion formation is coupled intimately to actin polymerization and organization (Huttenlocher and Horwitz, 2011). Between the lamellum and lamellipodium a transition zone for actin organization where adhesions in this region elongate centripetally along thin actin filament bundles is observed (Huttenlocher and Horwitz, 2011). The initial stages of this maturation process is dependent on the actin crosslinking activity of myosin II and α-actinin which then go on to form thick actin filament bundles and focal adhesions at the later stages of maturation (Choi et al, 2008). The Arp2/3 complex which drives membrane expansion also transiently localises to sites of new adhesions where it is involved in proper adhesion assembly, with its loss leading to disrupted global adhesion alignment (Wu et al, 2012).

Alterations of the integrin expression profile during migration govern the nature of the cells movement on the matrix. A balance between Rac and RhoA is crucial for spatiotemporal coordination of cytoskeletal dynamics and is modulated by the expression of different integrins (Huveneers and Danen, 2009). Integrin α5β1 expression mediates RhoA-induced

contractility, whereas αVβ3 is recruited in a Rac-dependent manner and promotes persistent cell migration characterised by a broad lamellipod with static adhesions. Conversely, there also exists an 'inside-out' paradigm during cell migration where integrins regulate the Rho GTPases and vice versa. RhoA induces the formation of focal adhesions whilst Rac and Cdc42 induce the formation of peripheral focal contacts. Integrins in resting cells exist in low-affinity and low-avidity states which then under the control of these Rho GTPases cluster into oligomers via an interaction with the integrin tails which affects their conformation allowing for enhanced access to the ECM ligands (Schwartz and Shattil, 2000).

Integrin endocytosis and recycling is important in influencing their function during cell migration. The trafficking of integrins is regulated by the small GTPases Ras-associated binding (Rab) family or proteins which act as molecular switches regulating vesicle transport (Shin et al, 2011). The major endocytosis mechanisms are through clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolin-independent endocytosis. It is assumed that depending on the type of integrin and its location on the cell will determine the route of internalisation. Internalised integrins localise to early endosomes and then are recycled back to the plasma membrane either quickly in a Rab4-dependent manner, or further trafficked and then recycled in a Rab11 dependent manner (Shin et al, 2011: Kawauchi, 2012). This recycling allows for minute-to-minute control over integrin function and in cancer is effective for the delivery of ECM receptors to the invadopodia in a dynamically changing environment (Figure 1-4B) (Shin et al, 2011: Kawauchi, 2012).
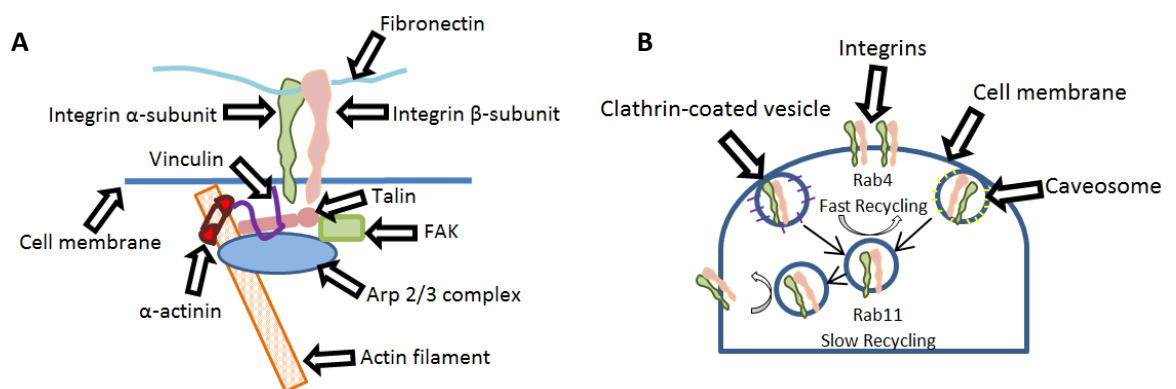
Figure 1-4 Integrin based adhesion and recycle in cancer cells
A) Integrins bind the ECM and link them to polymerised actin filaments through adaptor proteins vinculin, talin, α-actinin. FAk is recruited and is instrumental in the development of adhesions.   B) Recycling of integrins through clathrin-coated or caveolin dependent mechanisms. Rapid recycling (short loop) to the cell surface is through Rab4 whilst prolonged recycling (long loop) is through Rab11.

1.5.2 Migration and invasion

In order to migrate the cell body must modify its shape and stiffness in order to interact with the surrounding ECM, reducing physical stress and creating gaps and tracks (Figure 1-5), (Friedl and Wolf, 2009). The cortical actin cytoskeleton branches and expands the cell body pushing against ECM fibers being aided by proteases that perform local pericellular proteolysis by sequentially cleaving perpendicular fibres to generate space for the nucleus to slide across the ECM (Friedl and Wolf, 2009). The sheer traction associated with focal adhesions can deform the elastomeric substrate, increasing matrix stiffness, and thus playing a vital role in effective motility (Balaban et al, 2001).

Integrin attachment to the ECM promotes the expression of several MMPs which are recruited to sites of adhesion (Murphy and Courtneidge, 2011). For example, Src phosphorylation of the cytoplasmic tail of MT1-MMP facilitates an interaction with the FAK-p130[cas] complex which targets MT1-MMP to focal adhesions (Wang and McNiven, 2012). This then allows jointly enforced compartmentalization and activation of other MMPs such as MMP-2 by MT1-MMP and integrins that facilitates more proficient degradation (Yue et al, 2012). MT1-MMP binds to native type I Collagen and various other ECM components via its hemopexin C domain and cleaves it into ¾ and ¼ fragments which are then subsequently degraded by the gelatinases MMP-2 and MMP-9 (Friedl and Wolf, 2003).

During invadopodia-led migration of cells through the basement membrane perforation occurs as a result of localisation of MMPs to the invadopodia which is key in the structures maturation (Murphy and Courtneidge, 2011). There have been numerous ways identified in the method of delivery of MT1-MMP to the invadopodia. Endocytic recycling of MT1-MMP regenerates a significant fraction of the active enzyme to the cell surface. Through clathrin- and caveolae-mediated endocytosis MT1-MMP is successfully internalized via its 20-amino-acid cytoplasmic domain which binds the μ2 subunit of the AP-2-clathrin adaptor complex. Src phosphorylation of MT1-MMP impedes binding to the AP-2-clathrin adaptor complex and prevents endocytosis. Additional phosphorylation of endophilin A2 by the Src-FAK complex of also reduces endocytosis of MT1-MMP by reducing endophilin A2 affinity with GTPase dynamin 2, a protein involved in fission of endocytic membranes. The Rab8-dependent pathway which mobilizes MT1-MMP from the secretory pathway and delivers it from a storage compartment to collagen contact sites also occurs and acts as a delivery mechanism for the MMP in invadopodia (Poincloux et al, 2009). A more recent model suggests N-WASP recruitment of MT1-MMP from Rab7 late endosome cargo vesicles to actin rich regions

generated by N-WASP. Once resident in the invadopodia, N-WASP stabilizes MT1-MMP through direct tethering of its cytoplasmic tail to F-actin networks allowing for effective receptor targeting of degradiative machinery (Yu et al, 2012). There also exists an important role between the coordinated actions of the actin cytoskeleton and exocytic machinery. Both Cdc42 and RhoA regulate association of IQGAP1, a protein that links microtubules and actin cytoskeleton networks, with the exocyst vesicle-docking complex. Together these proteins control docking of MT1-MMP transport vesicles to the invadopodial plasma membrane (Murphy and Courtneidge, 2011). As the cell moves there is a generation of loose ends of ECM fibres by focal proteolytic cleavage. Secondary remodelling through secretion of ECM components creates an orientated, realigned ECM scaffold that is stable and linear. This new track takes form of a hollow tube that is approximately the diameter of a single cell and supports relatively persistent forward gliding of subsequent follower cells (Friedl and Wolf, 2009).

During migration mechanosensitive focal adhesions localise to to a myosin II containing F-actin network where F-actin from the leading edge has been transported to the cell centre in a myosin II dependent process called retrograde actin flow (Stricker et al, 2009). The myosin II complex is comprised of two nonmuscle myosin II heavy chains, two essential light chains, and two regulatory light chains. Mysoin II activity is mainly controlled through phosphorylation of the myosin light chain which is mainly regulated by myosin light chain kinase, a Ca^{2+}/Calmodulin (CaM)-dependent effector. Mysoin II has also been shown to be regulated by Rho-kinases and myosin phosphatase as the downstream effector of RhoA, ROCK, phosphorylates myosin light chain kinase (Niggli et al, 2006). In some breast cancer cell lines there are two isoforms of myosin II expressed termed IIA and IIB. The myosin IIB isoform primarily attains a role in the mechanics of lamellar protrusive events, whilst the myosin IIA isoform is important for normal migration (Betapudi et al, 2006). The C-terminal domain of myosin integrates into filaments allowing their head domains to tether actin filaments and through hydrolyses of ATP exert filament sliding (Vicente-Manzanares et al, 2009). This process is what primarily controls translocation of the cell body across the matrix.

Adhesive release at the rear of migrating cells involves disassembly of integrins with the ECM or severing of integrin linkages with the cytoskeleton (Huttenlocher and Horwitz, 2011). The rearmost adhesion sometimes strongly tethers the cell to the substratum which results in a long tail that generates enough physical tension to disrupt the linkage (Ridley et al, 2003). This retraction is spatially regulated by myosin II which aids in directing traction forces centripetally

and allowing for detachment at the rear. Intracellular calcium-dependent protease calpains also plays a role in rear end detachment by proteolytically cleaving several proteins found in focal adhesions such as paxillin, talin and FAK (Huttenlocher and Horwitz, 2011). There is also evidence to suggest that Src-FAK signalling mediates adhesion disassembly at the rear as well as the front of the cell through phosphorylation of myosin light chain kinase and ERK (Webb et al, 2004). In breast cancer the RhoA-ROCK-mysoin II cascade at the cell rear has been shown to exert substantial traction forces on the ECM through $\beta 1$ integrins that a significant propulsion through the matrix is generated (Poincloux et al, 2011).
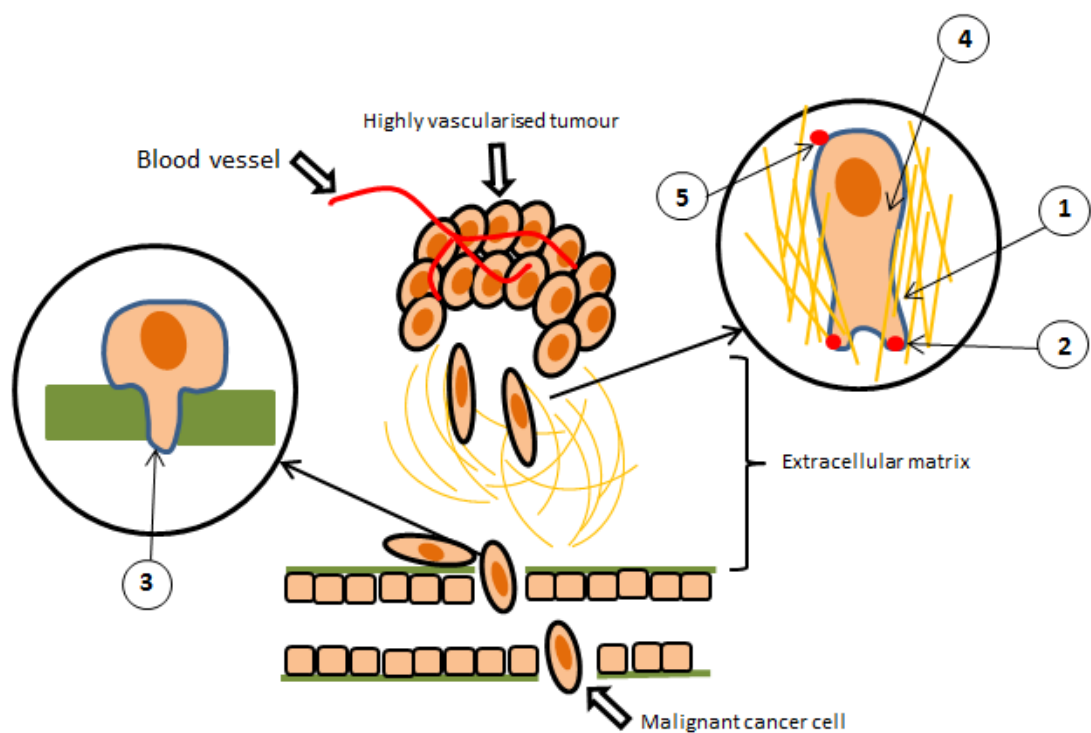
Figure 1-5 Cell migration: a five step process
Figure represents individual cell migration and invasion. Protrusion of the anterior pseudopod *(Step 1)* occurs which binds the surrounding ECM through focal contacts generating traction force *(Step 2)*. When cells invade the additional step of ventral membrane protrusions which focally cleave and remodel the surrounding collagen substratum slightly backward of leading adhesion sites *(Step 3)*. The generation of actomyosin contractions allow for cell body translocation in the direction of migration *(Step 4)* which is followed by the disassembly of focal contacts at the cellular rear *(Step 5).*

1.6 Cell migration and Invasion is governed by dynamic signalling pathways

Intracellular signalling is necessary for many processes such as proliferation, survival, migration and invasion. The aberrant behaviour of complex and highly regulated network of pathways leads to the adoption of a malignant and invasive phenotype. This provides a heavy incentive to understand the mechanisms behind delineating pathways of this process identifying whether they have become hyper-activated or attenuated as a result of genetic lesions (Ellerbroek and Stack, 1999). The current literature has already identified many signalling pathways that in some way enhance breast cancer cell motility and invasive potential.

1.6.1 The PI3K/AKT-mTOR signalling pathway

The phosphatidylinositol 3-kinase (PI3K)- AKT- mammalian target of rapamycin (mTOR) pathway plays a key regulatory function in cell proliferation, metabolism, survival, apoptosis, and migration (Hernandez-Aya and Gonzalez-Angulo, 2011). The PI3K are a family of lipid kinases that generate lipid secondary messengers by phosphorylating phosphoinositides at the D-3 position of the inositol head group (Yamaguchi et al, 2011). In mammals the PI3Ks are subdivided into three classes based on their substrate specificity, regulation, enzyme domain structure and sequence homology (Cain and Ridley, 2009). Class IA P13Ks (PI3K-Cα, PI3K-Cβ, and PI3K-Cδ) are the most studied in cancer and consist of a heterodimeric catalytic subunit (p110) with a regulatory subunit (p85), (Hernandez-Aya and Gonzalez-Angulo, 2011). Class II PI3Ks contain three isoforms PI3K-C2α, PI3K-C2β, and PI3K-C2γ, and finally class III PI3Ks contain only a single isoform called Vps34 (Yamaguchi et al, 2011).In breast cancer there are elevated levels of the p85 regulatory subunit with mutations in the Src homology domain 2 (SH2) region and amplifications of the p110α catalytic subunit. Together this and the loss the pathway suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN) can lead to aberrant overexpression of the PI3k signalling pathway (Arboleda et al, 2003). Price et al showed this pathway was primarily activated upon EGF stimulation of the EGFR receptor on breast cancer cells (Price et al, 1999). Class IA PI3Ks p85 regulatory subunit becomes tyrosine phosphorylated and relieves the intermolecular inhibition of the p110 catalytic subunit that then phosphorylates 4,5-phosphoinositide (4,5-PIP$_2$) which in turn generates the biologically active second messenger 3,4,5-phosphatidylinositol triphosphate (PtdIns (3,4,5) P$_3$), (Yamaguchi et al, 2011: Geltz and Augustine, 1998). PIP$_3$ plays a key role in downstream signalling of several effector proteins such as AKT and phosphoinoside-dependent

protein kinase-1 (PDK1). Generated PIP_3 binds and recruits PDK1 through its pleckstrin-homology (PH) domain to the cell membrane where it co-localises with AKT and partially activates it by phosphorylation. AKT is then further phosphorylated by PDK2, or a variety of other potential kinases, which allows it to become fully activated. Active AKT translocates to distinct areas within the cytosol or into the nucleus where it phosphorylates a host of downstream substrates (Yamaguchi et al, 2011:Hernandez-Aya and Gonzalez-Angulo, 2011). AKT has been found to be dysregulated in a variety of ways in human breast cancers. There are three isoforms of AKT identified, AKT 1-3, with AKT2 predominately promoting the most cell transforming properties in human breast cancer (Cain and Ridley, 2009). During EMT, TGFβ phosphorylates and activates PI3K which stimulates AKT2 phosphorylation (Figure 1-6). AKT2 has been shown to play a role in the sequestering and delocalisation of E-cadherin and tight junction protein ZO-1 in early metastasis (Larue and Bellacosa, 2005). The P13K-AKT pathway, along with other signalling pathways, is also involved in the expression, activation, and secretion of MMP-9 as a consequence of fibronectin-integrin binding during migration (Maity et al, 2011). This allows breast cancer cells to migrate more readily as type IV Collagen, an abundant protein of the basement membrane, is degraded by MMP-9 (Kohrmann et al, 2009). AKT2 overexpression in cells is usually localised adjacent to collagen IV in the matrix and has been associated with the expression of β1 integrins indicating its role further in invasion (Arboleda et al, 2003). More recent research has shown that TGFβ induced PI3K-AKT signalling plays an important role in invadopodia biogenesis. PIP_2 and PIP_3 recruits essential podosome components Tks5 and N-WASP which allows more rapid actin polymerisation. Furthermore, whilst the full mechanism has still not been disclosed, it has been shown that the class IA p110α subunit is a critical regulator of F-actin cores and cortactin structures and that its inhibition leads to blocked formation of invadopodia (Yamaguchi et al, 2011). AKT up-regulates many cell proliferation and survival protein targets such as Bad, tuberin, GSK3b, forkhead transcription factors and mammalian target of rapamycin (mTOR). mTOR exists a two multiprotein complexes mTORC1 and mTORC2 in which the formers activation is dependent of AKT and the latter is not (Hernandez-Aya and Gonzalez-Angulo, 2011). The mechanism behind mTORC2 activation is poorly understood however it is theorised it may be through 80S ribosome binding in response to growth factor stimulated PI3K signalling (Zinzalla et al, 2011). Whilst mTORC1 function is primarily involved in protein synthesis, proliferation, and survival, mTORC2 functions as an important regulator of the cytoskeleton (Sarbassov et al, 2004). How mTORC2 drives F-actin reorganisation and cell motility is still to be fully elucidated. It is thought that mTORC2 signals to small GTPases RhoA ,Rac1 and Cdc42 which induces the

formation of actin stress fibres, lamellipodia membrane ruffles and actin microspikes associated with filopodia formation (Liu et al, 2010).
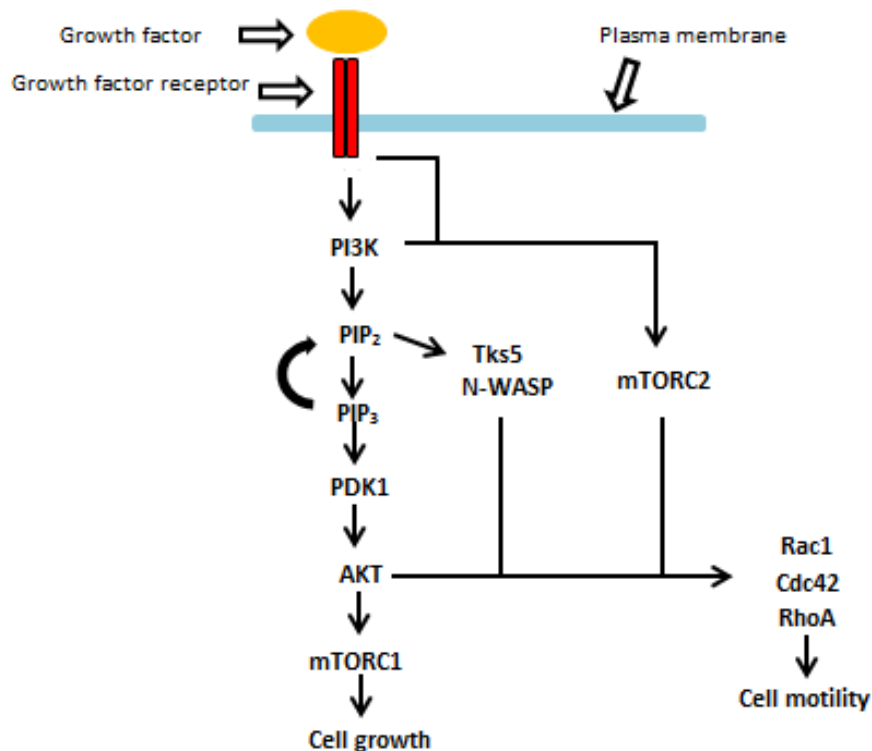
Figure 1-6 schematic diagram of the PI3K-AKT signalling pathway
PI3K is activated downstream of growth factor receptor. The P110 subunit of PI3K phosphorylates and activates PIP_2 which activates PIP_3 and leads to the activation of AKT. AKT can activate mTORC1 which leads to cell growth or along with mTORC2 regulate the small RhoGTPases involved in cell migration and invasion.

1.6.2 The Src-FAK signalling pathway

The Sarcoma Kinase-Focal adhesion kinase (Src-FAK) signalling pathway is another well-defined system that contributes to cancer cell metastasis and tumourgenesis. Src is one of nine members of the Src family of kinases that regulates intracellular signalling induced by transmembrane growth factors and cytokine receptors such as EGFR, IGF1R, and VEGFR (Sanchez-Bailon et al, 2012). Src has a modular structure that consists of an N-terminal SH3 domain, a central SH2 domain, and a tyrosine kinase domain that attains an ATP binding site and auto-phosphorylation residue (Sanchez-Bailon et al, 2012). Src is involved in the formation of focal adhesion complexes during migration and invasion. FAK is an intracellular non-

receptor tyrosine kinase and major scaffolding protein composed of an N-terminal FERM domain, central kinase domain, a focal adhesion targeting sequence, and C-terminal domain that has two proline-rich motifs which serve as binding sites for SH3 domain-containing proteins (Luo and Guan, 2010). FAK is phosphorylated and activated by cellular responses such as integrin- ECM engagement, or a variety of extracellular stimuli such as EGF, PDGF and HGF. Binding of the Src-SH2 domain with an auto-phosphorylated FAK creates a complex in which Src undergoes a conformational change from a non-activated state to an activated state and phosphorylates FAK at several sites (Sanchez-Bailon et al, 2012). The Src-FAK complex is recruited to sites of integrin clustering where it is acts as a major mediator of signal transductions by cell surface receptors and regulates adhesion, migration, invasion, survival and proliferation (Figure 1-7). Silencing of FAK in breast cancer cells diminished integrin clustering and the formation of focal adhesions to the matrix indicating its importance in cell migration. Several studies show that Src-FAK regulates focal adhesion dynamics and the cytoskeleton networks they connect with. Upon EGF stimulation the scaffolding function of FAK allows Src to phosphorylate several signalling molecules bound to other FAK sites including the adaptor protein p130^{cas} (Carry et al, 1998). p130^{cas} couples with another adaptor molecule called CrkII which induces the recruitment of the guanine exchange factor (GEF) DOCK180 to the cell membrane where it activates the small GTPase Rac1 involved in generation of membrane ruffles (Zhang et al, 2003). The phosphorylation of paxillin by Src-FAK also allows it to couple with CrkII which relocalises it to focal complexes at the cell periphery of lamellipodia. Here paxillin associates with an adaptor molecule called paxillin kinase linker (PKL)which links it to p21-activated protein kinase (PAK) through intermediary Coll/PIX Cdc42/Rac GEF. PAK is a major translation point in Cdc42, Rac1, and RhoA signalling to the cytoskeleton and nucleus (Brown et al, 2002). PAK is recruited to RhoA focal adhesions where it triggers turnover and also to the leading edge of cells enriched with nascent Cdc42/Rac1 induced focal complexes where it may mediate maturation into RhoA focal adhesions (Brown et al, 2005).   In addition, FAK promotes the functional assembly of a complex composed of Src, FAK, Calpain-2 and p42 ERK that is also important in focal adhesion turnover, migration and invasion. The extracellular regulated kinase (ERK)/mitogen activated protein kinase (MAPK) is transported to focal adhesions where it induces the activation of the protease calpain-2 involved in the disassembly of focal adhesions through cleavage of focal complex components such as β3 integrin and paxillin (Westhoff et al, 2004). Moreover integrin-induced expression of MMP-9 is through cross talk between downstream activated p42 ERK by FAK and the PI3K-Akt signalling pathway (Meng et al, 2009). Other metalloproteinase such as MMP-2

are up-regulated by FAK signalling through enhanced activation of c-Jun N-terminal kinase (JNK). Additionally, the Src-FAK complex phosphorylates Endophilin A2 which is implicated in the endocytosis of the membrane tethered MT1-MMP at the cytoplasmic face of cell membranes. Normally Endophilin A2 allows cells to control MT1-MMP dependent proteolytic activity; however its phosphorylation leads to increased accumulation of MT1-MMP on tumour cell surfaces (Wu et al, 2005). N-WASP phosphorylation by FAK allows it to maintain its cytoplasmic localisation and in turn encourage the facilitation of actin polymerisation through the Arp2/3 complex (Zhao and Guan, 2009).   The Src-FAK complex also cross-talks with PI3K through binding of its p85 subunit to trigger a cell survival response mediated through PI3K-AKT signalling (Reiske et al, 1999). Src-FAK mediated PI3K-AKT signalling induces the expression of inhibitors of apoptosis through NFκB which protects the cell from various forms of treatment including ultraviolet irradiation (Sonoda et al, 2000).
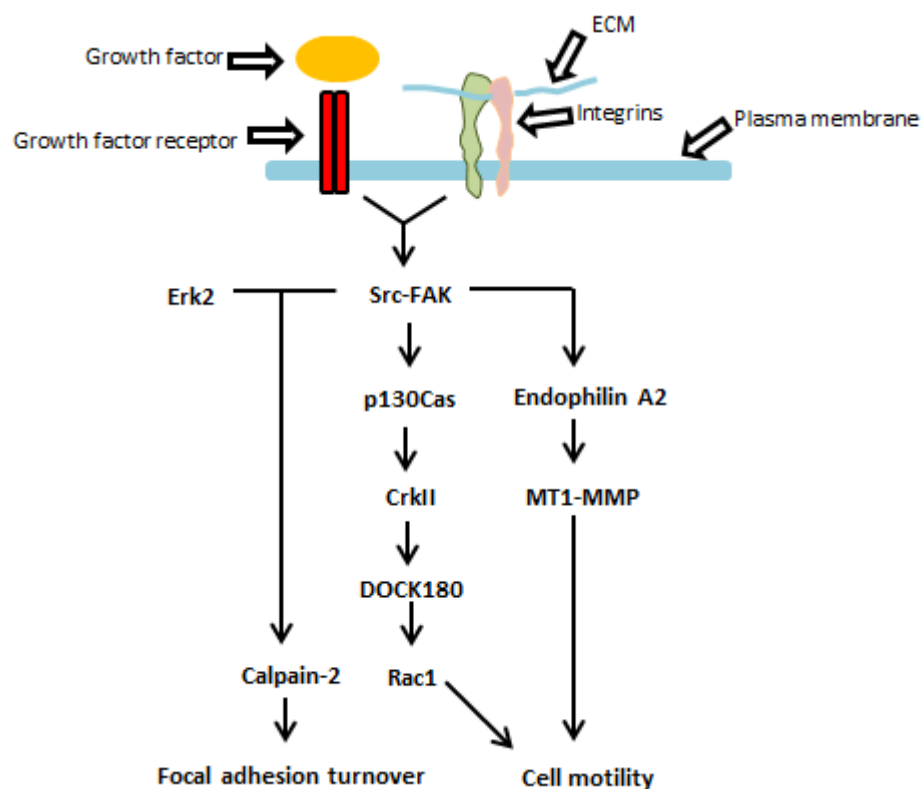
Figure 1-7 schematic diagram of the Src-FAk signalling pathway
Integrin or growth factor activated FAK binds to Src forming a complex. Src-FAK complex binds p130Cas which couples with CrkII and recruits DOCK180 to the cell membrane to activate Rac1. Src FAK phosphorylates Endophilin A2 preventing endocytosis of MT1-MMP. Src-FAK also forms a complex with ERK2 which activates and further complexes with calpain-2 leading the regulation of focal adhesion turnover during cell migration.

1.6.3 The EGFR-GEP100-Arf6-AMAP1 signalling pathway

Another more recently discovered pathway that has been implicated specific to breast cancer invasion and metastasis is the EGFR-GEP100-Arf6-AMAP1 signalling pathway (Figure 1-8), (Sabe et al, 2008). The activation of this pathway is fully dependent of exogenous ligand stimulation of the EGFR by tumour-associated macrophages stationed in the microenvironment (Sabe et al, 2009). Phosphorylated EGFR binds to the PH domains of a guanine nucleotide-exchange factor GEP100. Through this binding, GEP100 via its Sec7 domain induces the activation of ADP-ribosylation factor 6 (Arf6) (Morishige et al, 2008). Arf6 is from a small Ras superfamily of GTPases that regulate membrane trafficking and remodelling. Arf GTPases are highly conserved throughout eukaryotic evolution and consist of 6 isoforms, Arf 1-6, with Arf 2 being the only variant lost in humans (Sabe et al, 2009). Arfs 1-5 are primarily involved in intracellular secretory processes at the Golgi apparatus within cells. Arf6 on the other hand is involved in a variety of different cellular processes at the cell periphery such as regulating endocytosis, and recycling back plasma membrane components and cell surface receptors (Donaldson, 2003). The significance of Arf6 in cancer has been shown by its ubiquitous expression in several different tissues and organs in adults. In its active form, Arf6 transmits downstream signals through the interaction with a variety of proteins called effectors. AMAP1, also called DDEF1 in humans, is an effector protein that binds directly to GTP-Arf6 via its ArfGAP domain. Typically, AMAP1 is recruited by Arf6 to activation sites such as the plasma membrane or the cytoplasmic large vesicles. However in cancer cells under the stimulation of EGF AMAP1 co-loacalises with Arf6 at sites of invadopodia formation (Hashimoto et al, 2005). Both Arf6 and AMAP1 are abnormally overexpressed in highly invasive breast tumour cell lines to levels between 10- to 20-fold. The precise mechanism of how enhanced translation of the mRNA products encoded by the genes of these proteins is still to be elucidated, nether the less they act as viable predictive biomarkers for cancer (Hashimoto et al, 2004: Onodera et al, 2005) . In non-cancerous cells a Skp-Cullin-F-box (SCF) complex composed of Skp1, Cullin 1, and Fbx8 acts as a suppressor of Arf6 activity through ubiquitination, ultimately interfering with its ability to bind GEFs and GAPs. However, the expression of an essential protein of this complex, Fbx8, is typically found to be impaired in many breast cancer cell lines (Yano et al, 2008). In tumour cell migration and invasion the EGFR-GEP100-Arf6-AMAP1 signalling pathway is involved in many essential processes that contribute to passing through the basement membrane. Increased Arf6 expression induces actin polymerisation at the cell surface. It is suggested that reorganisation of the actin cytoskeleton occurs when Arf6 synergistically interacts with partner of Rac1 (POR1) and ADP-

ribosylation factor-interacting protein 2 partner of Rac1 (arfaptin-2). Cooperatively, these two proteins mediate the binding of Arf6 to Rac1 and this consequently allows membrane ruffling to occur (Shin and Exton, 2001). During the formation of invadopodia, AMAP1 forms a trimeric complex with actin cytoskeletal remodelling proteins cortactin and paxillin through the binding of their SH3 via its proline-rich sequence (Hashimoto et al, 2006). AMAP1 is recruited to its binding partners by Arf6 which co-localises with AMAP1 to the plasma membrane. Here, Arf6 aids in the formation of Fcγ receptor-mediated phagosomes and facilitates endosomal recycling which is essential for membrane trafficking and the phagocytosis of ECM components bound to integrins(Hashimoto, 2004). AMAP1 also localises at Fcγ receptor-mediated phagocytic cups where it interacts with several endocytic proteins such as amphiphysin IIm and intersectin (sabe et al, 2009). Amphiphysin IIm is localised to sites of Arf6 activation where it is release from prebound AMAP1 and itself recruits dynamin to the invadopodia. Dynamin is a small GTPase responsible for mediating membrane constriction and the fission reaction of vesicles polymerised in the donor membrane by vesicle coat proteins COPI, COPII, and clathrin. Coincident with this reaction, AMAP1 through its Bar domain aids in the invagination of the membrane into buds where coat proteins are then shed allowing vesicles to be delivered to an acceptor compartment (Nie et al, 2006). In addition to invadopodia formation, Arf6 also plays a pivotal role in the assembly and disassembly of E-cadherin cell-cell adhesions. Downregulation of Arf6 results in blockage of E-cadherin endocytosis leading to significant cytoplasmic accumulation of the protein in the plasma membrane. However in cancer, overexpressed Arf6 upregulates phosphatidylinositol(4) phosphate 5 kinase lγ (PIPKlγ) which increases E-cadherin exocytosis (Figueiredo et al, 2011). Whilst little is known about the molecular mechanisms involved, both GEP100 and AMAP1 have been shown to regulate β1 integrin's during invasion. GEP100 selectively activates Arf6 which regulates endocytosis and internalisation of the β1 integrin (Dunphy et al, 2006). AMAP1 binds directly to protein kinase D2 (PRKD2) which forms a complex with the cytoplasmic tail of the β1 subunit of β1integrins such as α2β1 and α3β1. The binding of AMAP1 to PRKD2 is mediated by Ras-related protein 5C (Rab-5C) which promotes intracellular association of the two proteins (Onodera et al, 2012).This complex is recruited to the plasma membrane by Arf6 where it is internally recycled.
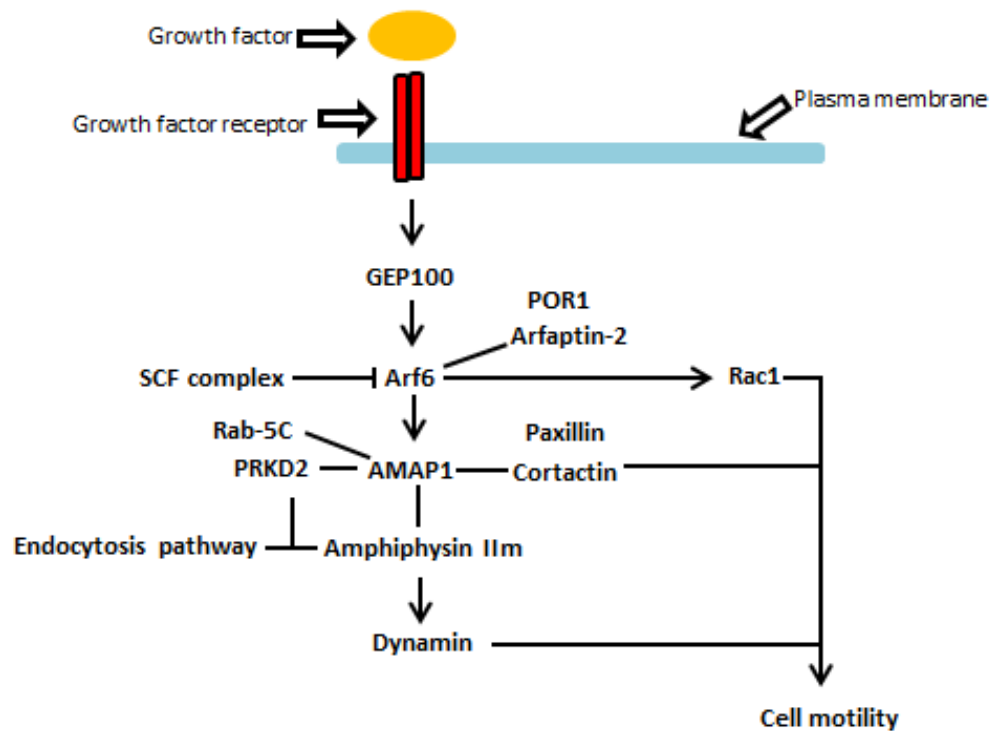
Figure 1-8 schematic diagram of the EGFR-GEP100-Arf6-AMAP1 signalling pathway
EGF ligand stimulation activates GEP100 which phosphorylates Arf6. Arf6 interacts with
POR1 and arfaptin-2 which couple it to Rac1 to induce membrane ruffling. Arg6 also
activates AMAP1 which can interact with Rab-5C which couples it to PRKD2. AMAP1
additionally interacts with amphiphysin IIm and together with PRKD2 is involved in
endocytosis of integrin and ECM components. AMAP1 also interacts with paxillin and
cortactin which are involved in the formation of invadopodia along with dynamin which is
recruited by amphiphysin IIm.

1.6.4 MAPK pathways

The mitogen-activated protein kinases (MAPK) are an evolutionary conserved family of
serine/threonine kinase modules where many extracellular signals converge in order to
activate machinery that controls fundamental processes such as migration, growth, and
proliferation (Dhillon et al, 2007: Huang et al, 2004). MAPKs are activated through a kinase
cascade and can be divided into three major groups depending on the differences in the motifs
within their activation loop. This includes the extracellular signal-related protein kinase
(ERK/MAPK), Jun N-terminal kinase (JNK), and the p38 MAPK (Huang et al, 2004). In cancer,
abnormalities in MAPK signalling impinge on most processes that are critical in tumour
progression.

The most extensively studied of all the mammalian MAPK pathways is the ERK pathway which is deregulated in many human cancers (Figure 1-9). Typically upon the binding of extracellular ligands such as EGF to EGFR, phosphorylation and dimerization of receptors occurs leading to a signalling cascade. Specific tyrosine phosphorylation isrecognised by the SH2 domain of growth factor receptor bound protein 2 (Grb2) which localises and docks at the receptors cytoplasmic domain. Grb2 then binds to a protein called son of sevenless (SOS) through its SH3 domain which associates with the G protein Ras removing bound GDP and allowing GTP to bind causing activation of the protein. Ras then activates the mitogen-activated protein kinase kinases (MKKK), Raf-1, which then phosphorylates and activates the MKK, MEK 1/2, which activates MAPK ERK 1/2 (Sitaramayya, 2010). ERK have several cytoplasmic and nuclear target substrates. ERK has been shown to phosphorylate MLCK and regulate focal adhesion formation and disassembly at the front of polarized cells (Webb et al, 2004). In addition ERK also phosphorylates FAK and paxillin which enhances paxillin and FAK association and the formation of a complex. It is speculated in this complex that ERK may be involved in modulating focal adhesion turnover and defining a region for Rac activation (Provenzano et al, 2009: Ishibe et al, 2004). Furthermore, ERK phosphorylation of calpain stimulates an interaction with FAK and causes degradation of cytoskeletal proteins and adhesion disassembly (Huang et al, 2004).

JNK can be activated by various extracellular stimuli such as EGF which activates MEKK1 through Src-FAK or Rac. MEKK1 then phosphorylates MKK4 and MKK7 which then phosphorylates JNK (Figure 1-9). It is speculated that JNK phosphorylates paxillin which may disrupt its interaction with binding partners such as FAK and lead to focal adhesion disassembly (Haung et al, 2003). JNK has been shown to crosstalk with ERK and through an ERK-Fra-1/JNK/*slug* axis induce breast cancer cell migration through the regulation of integrins (Chen et al, 2009). Furthermore, JNK acts as a physiological regulator of MMP-2 and MT1-MMP in conjunction with the PI3K signalling pathway during migration (Ispanovic and Haas, 2006).

Many growth factors, cytokines, and chemotactic substances can stimulate p38 activity (Figure 1-9). These factors activate MAPKKKs which then phosphorylate MKK3 or MKK6. Then subsequent phosphorylation by MKK3 and MKK6 activates p38 MAPK (Huang et al, 2004). The activation of p38 has been linked to the phosphorylation of MAPK-activated protein kinase-2 and -3. These proteins then phosphorylate heat shock protein 27 (HSP27), which modulates cytoskeletal remodelling and F-actin polymerisation. Additionally, p38 activates stathmin, a

microtubule destabilizer protein involved in cytoskeletal reorganisation. H-Ras activation of p38 through Rac and MKK3/6 leads to a marked induction of MMP-2 expression leading to a more invasive phenotype (Kim et al, 2003). Constitutive p38 activity is also important in the protein expression of uPA and uPAR and inhibition of p38 abrogates the cells ability to invade through the matrix (Huang et al, 2000).
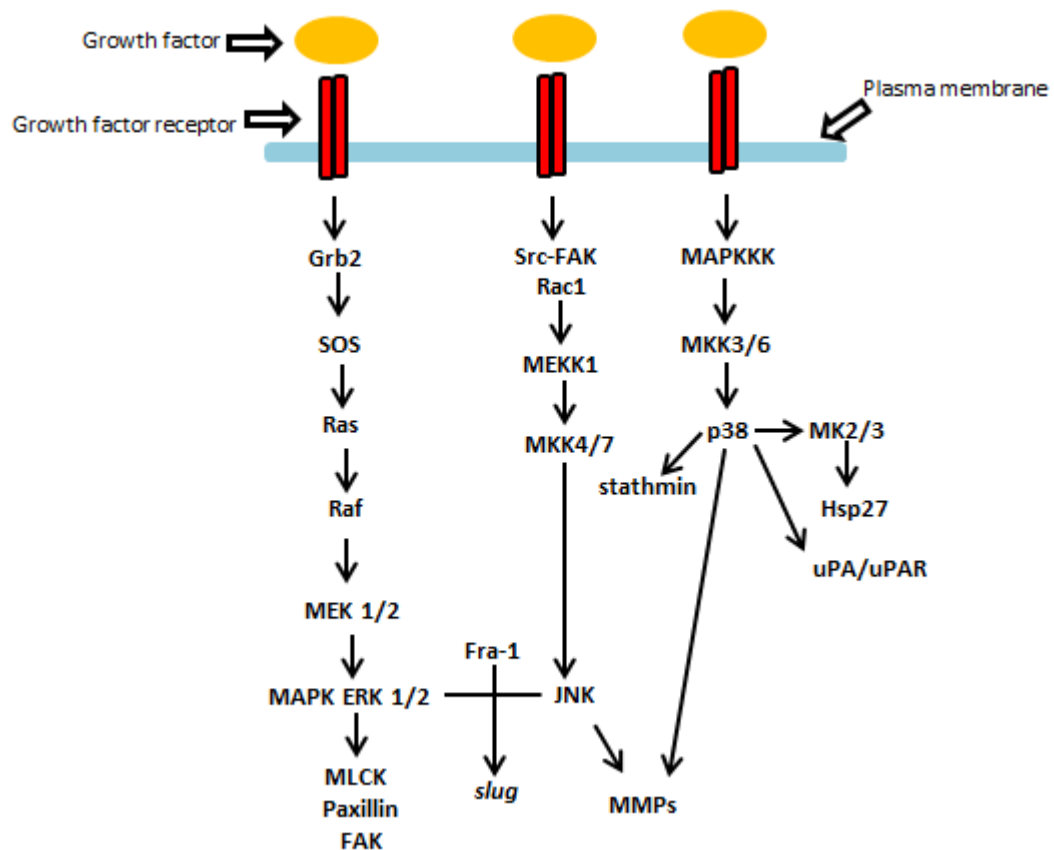
Figure 1-9 schematic diagram of the MAPK signalling pathways
Activation of ERK pathway (left) through Grb2 which binds SOS and phosphorylates Ras. Ras then activates Raf which is followed by a subsequent activation of MEK1/2 and finally ERK1/2. ERK1/2 can activate proteins involved in cell migration such as myosin light chain kinase, paxillin, and FAK. In the JNK pathway (centre) growth factor activation of Src-FAK or Rac leads to phosphorylation of MEKK1. MEKK1 then activates MKK4/7 which then phosphorylates JNK. JNK can regulate MMPs and form a complex with ERK and Fra-1 to modulate *slug* expression. The p38 pathway (right) is activated through growth factor/cytokines which phosphorylate MAPKKK. MAPKKK then activates MKK3/6 which then activate p38. P38 can phosphorylate MK2 and -3 which regulate Hsp27; it can also regulate MMPs and activate uPA/uPAR expression.

1.7 Targeting pathways in cancer with isoquinolinones

1.7.1 Isoquinolinones

Isoquinolinones are a class of fused nitrogen containing heterocycles that constitute an integral part of many biologically active alkaloids (Chuang and Wu, 2006). The syntheses of these compounds have remained important in both synthetic and medicinal chemistry research for the potential use as therapeutic end-points (Chuang and Wu, 2006). Isoquinolinones can have diverse chemical structures that allow them to carry out several functions which is one of the reasons why defining a mechanism for them has remained difficult. Research has shown they can target DNA repair machinery, inhibit Rho-kinases and antagonise integrins, angiotensin II and the $5-HT_3$ receptor (Chiarugi et al, 2003: Plettenburg et al, 2012: Seitz et al, 2003: Chakravartz et al, 1992: Matsui et al, 1992). There is also emerging research regarding the effects if Isoquinolinones on metastatic progression of certain types of cancers. It is well documented that the human p53 tumour suppressor protein is mutated in several cancer lines. p53 typically acts to maintain the integrity of the genome and mutations can lead to a loss of function, or even a gain in function (Rothweiler et al, 2008). In cancers that still obtain a wild-type p53 there have been several strategies to regulate its activity more proficiently which could lead to apoptosis. p53 is involved in an autoregulatory feedback loop with murine double minute 2 (MDM2) which acts to suppress it through the binding of its N-terminal transactivation domain and target it for degradation. The Isoquinolinone structure has been loosely designed on the *cis*-imidazoline compound Nutlin which disrupts the MDM2-p53 complex causing the dissociation of p53 (Rothweiler et al, 2008: Diarmuid and Maki, 2010). In theory this should therefore induce the suppression of a migratory and invasive phenotype of cancer cells as p53 overexpression has been shown to decrease actin stress fibre levels, focal adhesions and the small GTPase Cdc42 responsible for filopodia and invadopia formation (Moran and Maki, 2010).
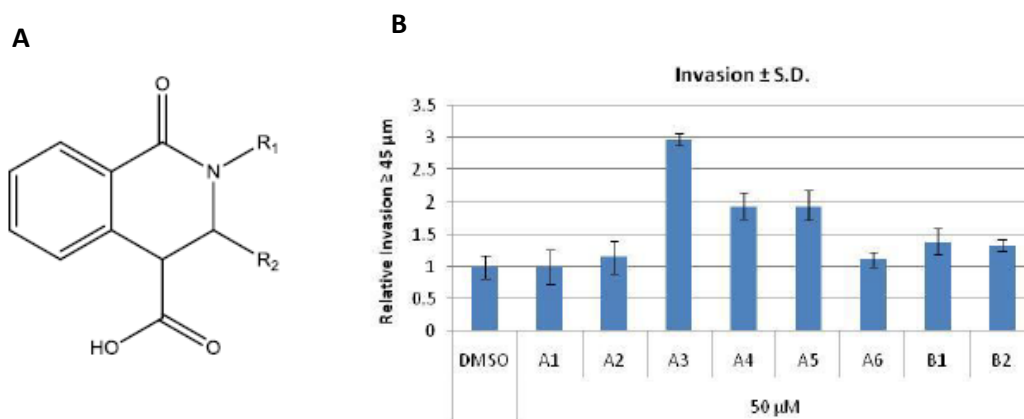
Figure 1-10 Isoquinolinones in cancer research
(A) Generalised chemical structure of the Isoquinolinone library. (B) HT1080 inverted 3D invasion assay using several Isoquinolinone derivatives at a concentration of 50µM for 72 hours. Image and data from unpublished work by Howe, J.D., Payerne, E.,Woon, E.C.Y., Searcey, M.,

Studies have shown that Isoquinolinones can induce apoptosis and an anti-migratory behaviour in cancer cells, however there is also evidence to suggest that they can enhance a metastatic phenotype. A study performed in the Gavrilovic lab showed that several compound derivatives increased invasion of a fibrosarcoma cell line HT1080 though a Matrigel plug as can be seen in Figure 1-10 (unpublished observations by Howe, J.D., Payerne, E.,Woon, E.C.Y., Searcey, M., and Gavrilovic, J). As mentioned previously, Isoquinolinones have multiple targets with more to be identified. This raises the interesting proposition of investigating some of the compounds used in the study such as A3 to elucidate a mechanism by which they trigger enhanced migratory and invasive phenotype. This may also open up the avenues to the finding of novel signalling pathways that are being triggered during cancer cell invasion, and therefore has the end potential for the development of a new therapeutic intervention.

1.7.2 Global approaches taken to explore mechanisms underlying tumour cell migration and invasion

Invasion of cancer cells into the surrounding tissue is a crucial step that requires the cyclic process of cell migration. Advances in microscopic imaging techniques such as phase contrast imaging, fluorescent live cell imaging, and confocal laser scanning microscopy has allowed for spatiotemporally resolved imaging that details important processes of cell migration such as adhesion turnover dynamics (Le Devedec et al, 2010). Breast cancer is a heterogeneous disease and several studies have tried to look at the disparities on a molecular level between differing therapeutic groups. Recent developments of high information content platforms such as gene expression DNA microarrays, protein arrays, DNA methylation arrays, and reverse phase protein arrays allow identifying therapeutically tractable genomic aberrations that drive tumour cell invasion (Cancer Genome Atlas Network). The use of proteomic analysis is also increasing in the study of breast cancer due to the high-throughput, simultaneous analysis of large numbers of proteins at once and the potential identification of true cancer-specific proteins (Qin and Ling, 2012). Other studies have used the phosphoprotein array assay in breast cancer to simultaneously examine multiple phosphorylation events in several upstream and downstream molecules occurring in multiple signalling pathways (Chergui et al, 2009). Altogether this equates to fairly comprehensive study in breast cancer cells which will hopefully unveil the mechanism behind motility in a complete entirety.

1.8 Project Aims

Metastasis is attributed as the main cause of death by cancer in people plagued by the disease. To date, it is unclear how the isoquinolinone compounds affect the motility of carcinoma cell lines.Therefore this project sets out to address the question by studying how the isoquinolinones alter the behaviour of cancer cells, breast in particular, in tumour cell migration in 2D and 3D microenvironments. Moreover, we aimed to investigate genes regulated by the isoquinolinones in order to identify any novel potential inducers of enhanced migrational activity.

Chapter 2 Materials and Methods

2.1 *Cell lines and culture*

Human breast adenocarcinoma cell line MDA-MB-231 and human fibrosarcoma cell line HT1080 were cultured in T175 and T25 corning cell culture flasks respectively. Both cell lines were grown in low glucose Dulbecco's modified Eagle's medium (DMEM) (HT1080 in 4.5ml; MDA-MB-231 in 12ml) maintained at 37˚C, 5% CO_2 and supplemented with 2mM L-glutamine, 1mM sodium pyruvate, 10% Fetal calf serum (FCS),10U/ml penicillin and 10mg/ml streptomycin.

2.2 *Passaging cells*

Cells were split once reaching 85% confluence or the day before an experiment was carried out. Modified DMEM media in the flasks was dispensed and cells were washed with PBS, which was dispensed and followed by the addition of trypsin; then flasks were incubated at 37˚C, 5% CO_2 for 5 minutes. Flasks were checked under the microscope to ensure detachment of cells, and then DMEM (10% FCS) was added and cells which were placed in 15ml conical centrifugation tubes and spun down in the centrifuge for 5 minutes at 1000rcf. Media in tubes was dispensed and the pellet of cells at the bottom of the tube was resuspended in DMEM (10%FCS) at differing volumes depending on the desired split. Then cells were pipetted back into Corning cell culture flasks made up to the original volumes and placed in 37˚C, 5% CO_2 incubation.

2.3 *Cell adhesion assays*

Cell adhesion assays were performed essentially as described Messent et al and Stringa et al (Messent et al, 1998: Stringa et al 2000). Multi-well tissue culture plates (96 wells, Nunc) were coated with serial dilutions of Fibronection (Sigma), Matrigel, and type I collagen (BD Biosciences) at a final concentration of 0.1μg/ml-10μg/ml, 100μl/per well and placed in a 4°C cold room overnight. Wells were washed twice with PBS (100μl/per well), followed by blocking of nonspecific binding sites with 1% BSA 100μl/well, with the exception of wells coated with DMEM and DMEM (10%FCS) at 100μl/per well. Plates were then incubated at 4˚C either for 1 hour or overnight. Cells split from previous day were trypsinized, incubated for 5 minutes at 37˚C, 5% CO_2, and centrifuged down at 1000 rcf for 5 minutes. Supernatant was removed and non-adherent cells were resuspended in DMEM serum-free medium. Haemocytometer count was carried out ensuring 60,000 cells/100μl HT1080 cells and 50,000 cells/100μl MDA-MB-231 cells were added to each well and then the plates were incubated at 37˚C, 5% CO_2 for 1 hour. A3 and A5 compounds were added to wells at a concentration of

100μM. After incubation non-adherent cells were removed by flicking and then adherent cells were fixed down using EtOH (100μl/per well) and left at 4˚C overnight. Cells are stained with 100μl/well 1% Methylene blue which is then released by cell lysis from adding 100μl/well 1:1 EtOH HCL. The absorbance of the samples was measured using the FLUOstar Omega multi-mode microplate reader at 630nm and then analysed using MARS software. Results were expressed as a mean ± S.E.M. The paired student's *t*-test or ANOVA test was used to determine the *P*-value which if less than 0.05 attested a significant difference between means.

2.4 3D Invasion assays

Inverted invasion assays were performed essentially as described in Hennigan et al and modified in Scott et al (Hennigan et al, 1994:Scott et al, 2004). Transwell cell culture chambers containing polyvinylpyrrolidone-free polycarbonate porous membrane filters (8μm Millipore diameter) were used to measure MDA-MB 231 and HT1080 tumour cell invasion. Chambers were transferred into wells of a BD Falcon^{TM} TC Companion plate then coated with 100μl/well of either rat tail tendon type I collagen (2.8mg/ml) or Matrigel (4.2mg/ml) (with or without 10μg/ml Fibronectin) and then incubated at 37˚C, 5% CO_2 for 1 hour. Cells split from previous day were trypsinized, incubated for 5 minutes at 37˚C, 5% CO_2, and centrifuged down at 1000 rcf for 5 minutes. Supernatant was removed and non-adherent cells were resuspended in DMEM (10% FCS) serum. Seeding density per chamber was 100,000 cells per 100μl quantified by a haemocytometer and seeded to the underside of each filter. Each well of another BD Falcon^{TM} TC Companion plate had 1ml DMEM serum-free medium inserted for washing; both plates were placed in 37˚C, 5% CO_2 incubator for 3-4 hours. Transwells were then washed twice in wash plate and placed in final wells containing 1ml DMEM serum-free medium or with 0.2% FCS. Chemoattractants DMEM 20%FCS, DMEM 10%FCS, 100 ng/ml CCL2/CCL5 (R&D systems), and 10ng/ml EGF in serum- free DMEM (1ml) were added to the upper section of the transwells. A3 and A5 compounds (50μm) were added to bijou tubes containing 2.5 ml of cells in serum-free DMEM or with 0.2% FCS. Controls were filled with serum –free DMEM. Plates were left for 72 hours in 37˚C, 5% CO_2. Each assay was performed in duplicate and after incubation period cells in transwell chambers were stained with 1ml calcein acetoxymethyl ester (4mg/ml) diluted in serum-free DMEM. Transwell chambers were placed on a thin coverslip and read using the LEICA LSM microscope at an excitation/emission wavelength of 530nm. Three fields per filter were counted scanning 15 layers (225microns) for each one. Results were expressed as a mean plus the standard error. The paired student's *t*-test was

used to determine the P value which if less than 0.05 attested a significant difference between means.

2.5 Random 2D migration and scratch wound assay

Thermo Scientific nunc 24 well plates were coated with serial dilutions of Fibronectin, Matrigel, or type I collagen at a final concentration of 10μg/ml and placed in a 4°C cold room overnight. Cells split from previous day were trypsinized, incubated for 5 minutes at 37˚C, 5% CO_2, and centrifuged down at 1000 rcf for 5 minutes. Supernatant was removed and non-adherent cells were resuspended in DMEM (2 % FCS) serum. A cell density of 30,000 cells per well was determined by a haemocytometer count for both MDA-MB-231 and HT1080 cells. Plates are then incubated for 3 hours at 37˚C, 5 % CO_2 to ensure binding. In the case of a scratch assay a vertical wound was introduced by scratching with a 1 ml pipette tip. Just before imaging chemoattractants EGF (10 ng/ml), CCL2 and CCL5 (20 ng/ml) are diluted in DMEM 2% FCS and added to the wells. Compound A5 (50 μM) was also diluted in DMEM 2% FCS and added just before imaging. Random migration and wound closure was monitored at 10 minute intervals for 17 hours using the Zeiss Axiovert time-lapse microscope. Each condition was performed in triplicate with images for each condition taken from one distinct field with five cells tracked per field. Migration patterns form experiments were analysed using the chemotaxis tool from the ImageJ cell tracking sortware. Results were expressed as a mean ± S.E.M. The paired student's t-test was used to determine the P-value which if less than 0.05 attested a significant difference between means.

2.6 A5 treated MDA-MB-231 24 hour time-course

 We coated 12 wells of three Thermo Scientific nunc 24 well plates labelled 4 hours, 8 hours, and 24 hours with Matrigel mixed with Fibronectin at a final concentration of 10μg/ml which were placed in a 4°C cold room overnight. Cells split from previous day were trypsinized, incubated for 5 minutes at 37˚C, 5% CO_2, and centrifuged down at 1000 rcf for 5 minutes. Supernatant was removed and non-adherent cells were resuspended in DMEM (10% FCS) serum. A cell density of 70,000 cells per well was determined by a haemocytometer count and plates were left to incubate overnight at 37˚C, 5% CO_2. Cells were checked to be ~80% confluent and then media was pipetted out of each well followed by a wash with PBS. Pipetted 500 μl serum-free DMEM, 0.5% DMSO and A5 50 μM (diluted in serum-free DMEM) into respective wells (Fig 2-1) and then plates were placed in 37˚C, 5% CO_2 Incubator.
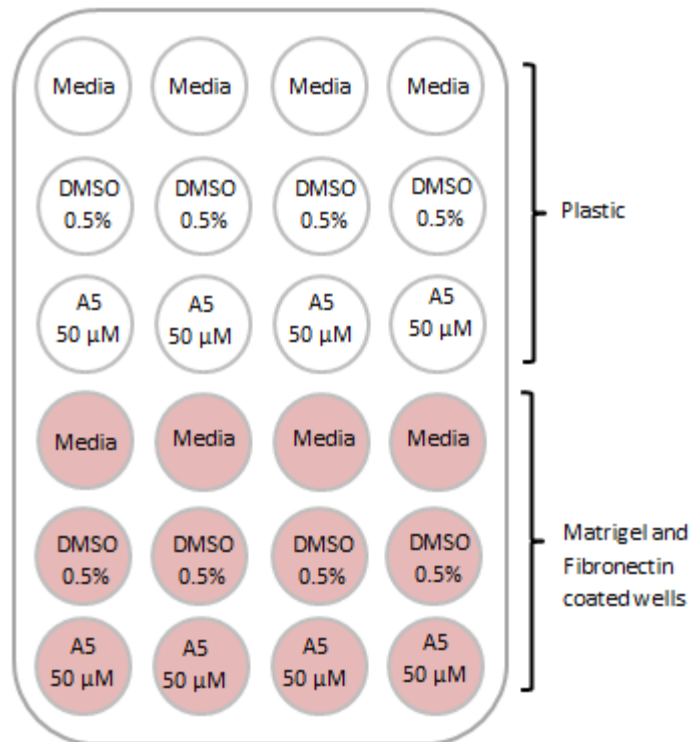
Figure 2-1 Schematic diagram depicting plate used in A5-treated MDA-MB-231 time-course The top six wells (white) consist of cells grown on plastic and bottom six wells (Pink) consist of cells grown on Matrigel plus Fibronectin. Wells 1-4 and 13-16 had no treatment. Wells 5-8 and 17-20 were treated with 0.5% DMSO. Wells 9-12 and 21-24 were treated with 50µM A5 compound.

2.7 RNA extraction

At each time point the media of each well was pipetted out into 1.5 ml eppendorf tubes and placed in -20°C freezer. RNA was isolated from MDA-MB-231 cells as instructed by the manufacturer's instructions using the RNeasy mini kit (Qiagen). Purified RNA samples were snap-frozen on dry ice and stored at -20°C.

2.8 Reverse transcription

Samples were nanodropped to determine concentration and purity followed by diluting in differing concentrations of nuclease-free H_2O depending on the concentration of RNA in each sample to end up with 1000 ng in 15 µl. Then 2 µl of random primers (Invitrogen) was added to each tube and all samples were placed in the PCR machine for 10 minutes at 70˚C and placed on Ice directly after. Following this 8 µl mastermix (Table 2-1) was added to the annealing RNA samples and run on the PCR machine for 60 minutes at 42˚C and then 10 minutes at 70˚C. cDNA samples were stored at -20˚C until needed.

Table 2-1 –Mastermix for primer extension during reverse transcription

Reagent	Volume per sample
5 x M-MLV buffer (Promega)	5 µl
125 x dNTPs (Bioline)	0.2 µl
M-MLV (Promega)	1 µl
H$_2$O	1.8 µl

2.9 Quantitative real time PCR (Taqman)

To each well of a Taqman array plate (life technologies) 5 µl of cDNA (1 ng/µl) was added followed by 15 µl of the reaction mix (Table 2-2)*.* Plates were then run on the Taqman 7500 system as directed by the instructions in Table 2-3. This was performed for each of our 15 candidate genes as depicted in Table 2-4. Data extracted was used to determine changes in gene expression between conditions according to the $\Delta\Delta C_t$ method described by Livak and Schmittgen (Livak and Schittgen, 2001). This method takes loading variations into account by normalising target gene expression to our reference 18s gene (Fig2-2 and Fig 2-3).

Table 2-2–Reaction mix composition for Taqman.

Reaction mix	Volume per sample
2 x Master mix (Applied Bioscience)	8.3 µl
Inventorized ABI primer sets 20 x (Consists of forward primer, probe and reverse primer)	1 µl
H$_2$O	5.7 µl

Table 2-3 –Thermal cycling conditions for Taqman run on 7500 system

Temperature	Time (minutes)
Hold	-
50˚C	2
Hold	-
95 ˚C	10
40 cycles of	
95 ˚C	0.15
60 ˚C	1

Table 2-4 – Genes ordered from applied biosciences Invitrogen with their product code. 18s taken from the universal primers and probe library. All probes and primers designed to target *Homo sapien* genes.

Gene	Primers and probes/ABI ID
18s	Forward primer 5'-ACCGGCGCAAGACGGA-3' Reverse primer 5'-GCCGCTAGAGGTGAAATTCTTG-3' Probe 5'-CATTCTTGGCAAATGCTTTCG-5'
CCL2	Hs00234140_m1
CCL5	Hs00174575_m1
CTGF	Hs01026927_g1
CYR61	Hs00998500_g1
EGR1	Hs00152928_m1
EPHA2	Hs00171656_m1
ETS1	Hs00428293_m1
FPR1	Hs04235426_s1
HBEGF	Hs00181813_m1
IL-8	Hs01567912_g1
MYC	Hs00153408_m1
NFE2L3	Ha00852569_g1
RHOQ	Hs00817629_g1
SAA1	Hs00761940_s1
TGFA	Hs00608187_m1

A

B
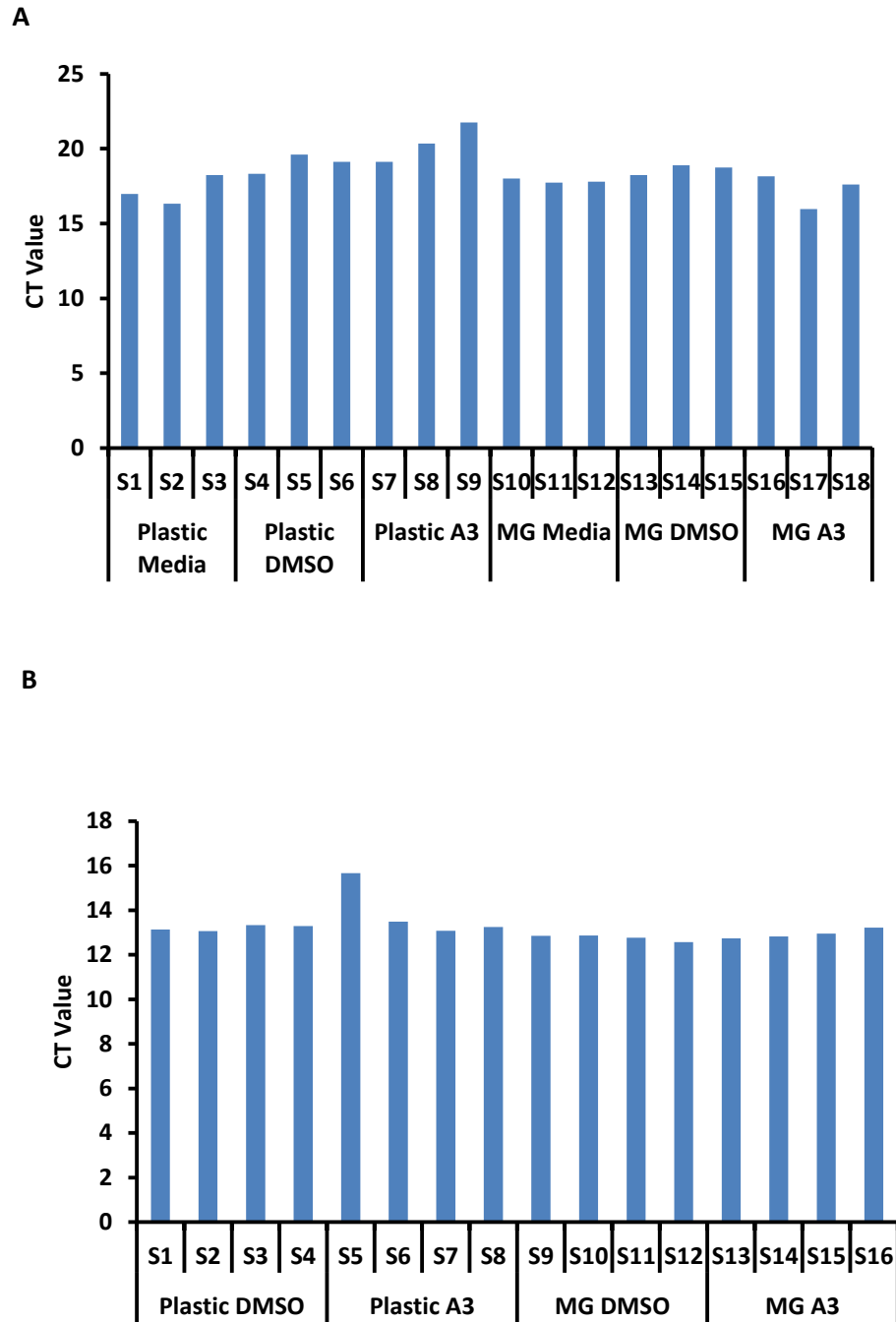
Figure 2-2– Reference 18s gene C_t values for HT1080 cells treated with DMSO and A3 at A) 4 hours and B) 8 hours. Each sample is abbreviated as 'S' and Matrigel as 'MG'.
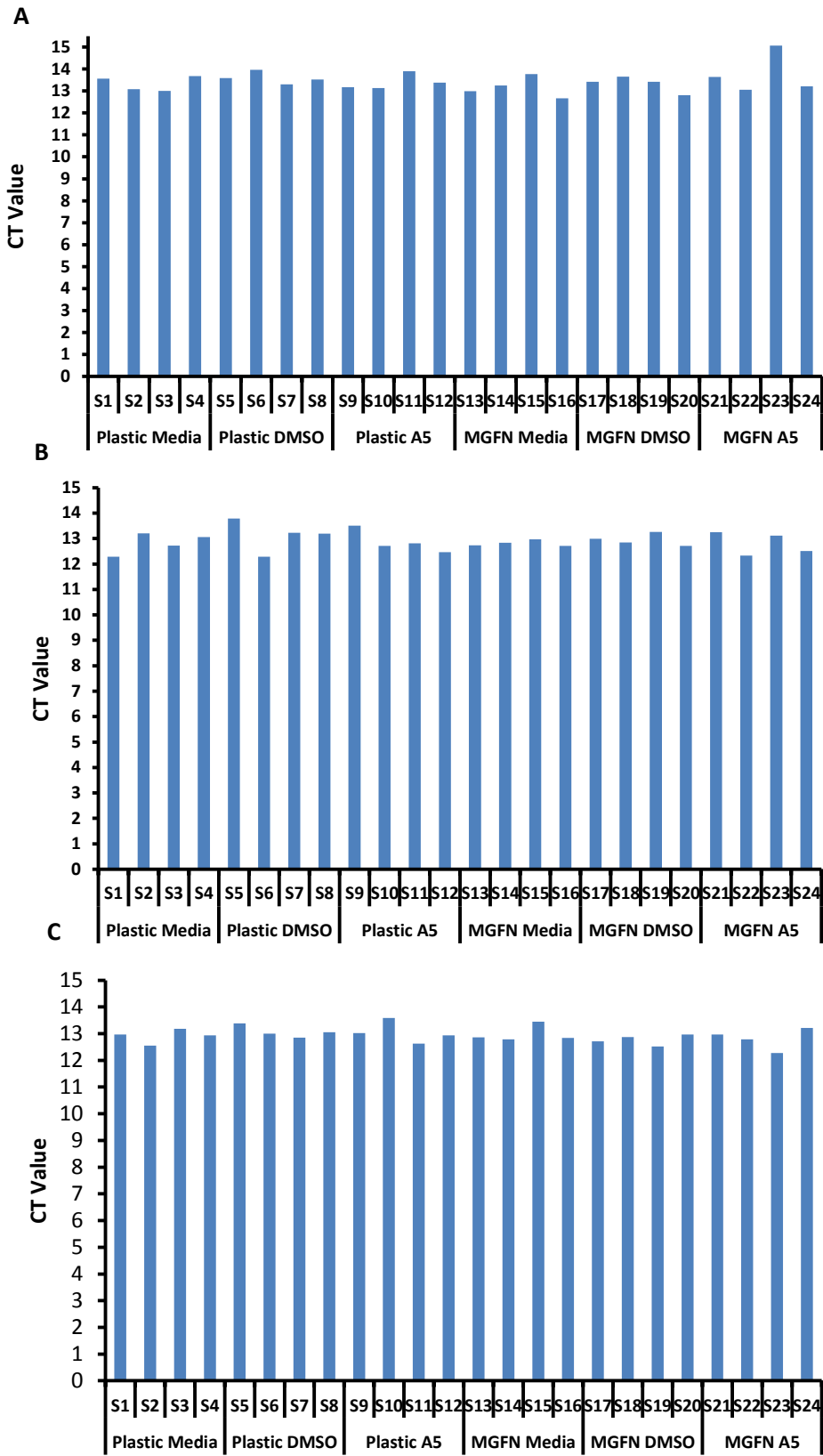
Figure 2-3 – Reference 18s gene C_t values for MDA-MB-231 cells treated with DMSO and A5 at A) 4 hours, B) 8 hours and C) 24 hours. Each sample is abbreviated as 'S' and Matrigel plus Fibronectin as 'MGFN'.

2.10 DNA microarray

Experiment was carried out by Dr Damon Bevan and Liam Pudwell. Briefly, 40,000 HT1080 cells were seeded into wells with or without Matrigel and left overnight in 37°C incubator with 5% CO_2. Media was replaced with serum-free media supplemented with either 0.5% DMSO or (50µM) A3 compound and left for 24 hours before RNA extraction. RNA was nanodropped and diluted according to the specifications of the Cambridge genomic services. Experiment was performed in quadruplicate but triplicate samples were sent and analysed using the Illumia WG-GEX HumanHT-12 bead chip array. Quality control analysis of all samples was perfomed by Illumina. Raw data is subjected to quantile normalisation to remove technical variation in data and log2 transformation (Du et al, 2008). In our analysis of this data we segregated genes according to three filtering parameters. They had to be significantly expressed according to the adjusted P-value ≤0.05 which takes correction of multiple testing into account. The B statistic set at ≥1, to ensure the genes are truly differentially expressed, and a fold change of ≥1.3.

Chapter 3: Isoquinolinones affect cell adhesion

3.1 Introduction

Cell behaviour is mediated by interactions with the extracellular matrix which provides the structural support for cells to adhere to. Dynamic reciprocity elicits many regulatory cues that are essential in the progression of physiological and pathological processes such as cell proliferation, cell growth, cell survival, differentiation, and metastasis (Nelson and Bissell, 2005). Adherent cell types will attach to matrix components through the major transmembrane integrin receptors. The specificity of the binding is determined by the extracellular domain of integrins which recognize a plethora of matrix ligands. An example of Integrin recognition of several matrix proteins in vertebrates includes integrins α5β1, αvβ3, and α4β1 which all recognise the dimeric glycoprotein fibronectin though through different interactions (Huttenlocher and Horwitz, 2011).

Contact with the ECM causes the cytoskeleton of the cell to induce clustering of integrins which causes individual integrins to lock in a conformation that allows them to more tightly bind the ligand causing the formation of a strong aggregate bond. During metastasis, the turnover of focal adhesions composed of clustered integrins is important in allowing the cell to migrate over the substrate. The overexpression of focal adhesions can lead to an anti-migratory phenotype in many cancers. An example includes the carrier protein insulin-like growth factor binding protein 5 (IGFBP5) which has been shown to increase binding of breast cancer cells to ECM components even when expressing low levels of specific integrins to those components. This altered cell adhesion restricts fibrosis and prevents migration (Sureshbabu et al, 2012).

The ECM is composed of a complex tapestry of interwoven proteins that cancer cells migrate through during metastasis. Fibronectin is a ubiquitous glycoprotein of the ECM that is essential in all living organisms. Fibronectin can be subdivided into two isoforms, the soluble promoter plasma Fibronectin, and the less-soluble polymeric fibrillar Fibronectin that is assembled into the ECM (Pankov and Kenneth, 2002). An overexpression of Fibronectin has been observed in a variety of different carcinoma cell lines and through integrin binding triggers intracellular signalling cascades that lead to an invasive phenotype (Maity et al, 2010).

Many research studies regarding the migratory and invasive potential of carcinoma cells have been undertaken using Matrigel to mimic a suitable ECM environment. Matrigel is a solubilised tissue basement membrane extract derived from Engelbreth Holm-Swarm (EHS) mouse

tumour. The main components that make up the composition of Matrigel include laminin, type IV Collagen, entactin, and heperan sulphate binding proteoglycans (Mullen et al, 1996).

In vertebrates type I collagen provides the mechanical stability in tissues and is most abundant ECM protein found in mammals. Type I collagen is composed of three polypeptide chains which self-associate to form a triple helical structure (Messent et al, 1998).   In the ECM cells will have to navigate their way through a heavily cross-linked meshwork of type I collagen fibrils (Sabeh et al, 2009). Since there are many different collagen subtypes in the ECM cells express integrin type collagen receptors that have the ability to recognize distinct collagens (Jokinen et al, 2004).

The size and distribution of adhesions depend on the cell type and pliability of the matrix. Since the speed of the migrating cell can be directly correlated with the rate of focal adhesion turnover it is important to understand how the isoquinolinone compounds may effect integrin expression and recycling of matrix components found in physiological models.

In this chapter we look at the adhesive potential of both MDA-MB-231 and HT1080 on certain matrix components and what effect the isoquinolinones have on this adhesion.

3.2 Results

3.2.1 Cell adhesion to Fibronectin

Investigation of cell adhesion to differing concentrations of the matrix component Fibronectin was carried out in order to find the optimal binding concentration for our chosen cell lines. In this experiment, and subsequent dose-response curves, 96 well plates were coated with the appropriate immobilized extracellular matrix component that increased by a factor of 10 with each subsequent concentration. Both MDA-MB-231 and HT1080s adhere to Fibronectin in a dose dependent manner (Fig 3-1). The shape of the curves is also similar in both MDA-MB-231 and HT0180 graphs. HT1080 and MDA-MB-231 adhesion continues to increase at 10 µg/ml but the data does suggest a plateau would be reached at higher concentrations. Additionally the half-maximal attachment for both MDA-MB-231 and HT1080s to fibronectin appears to be at about 0.5 µg/ml.

3.2.2 Cell adhesion to Matrigel

The attachment of cells to Matrigel was explored as it emulates the underlying basement membrane that influences cellular behaviour. Similar to Fibronection, adhesion of both MDA-MB-231 and HT1080s to Matrigel is dose dependent with the optimal concentration of Matrigel at 1 µg/ml (Fig 3-2). The half maximal attachment for both cell lines was approximately at 0.5 µg/ml. Maximal adhesion to Matrigel was greater in HT1080s than in MDA-MB-231, however both cell lines had a rapid incline at 0.001 µg/ml followed by a decline at 0.01 µg/ml.

3.2.3 Cell adhesion to Type I Collagen

As with Fibronectin and Matrigel, MDA-MB-231 and HT1080s adhere to type I collagen in a dose dependent manner (Fig3-3). There appears to be a much more sigmoidal shape with MDA-MB-231 cells compared to the HT1080 curve which seems to take on the form of a consistent incline at each increasing concentration. Both MDA-MB-231 and HT1080 adhesion to type I collagen reaches a plateau at 0.1 µg/ml with the half-maximal attachment of both cell lines occurring at about 0.05 µg/ml.

A

MDA-MB-231 cells

B
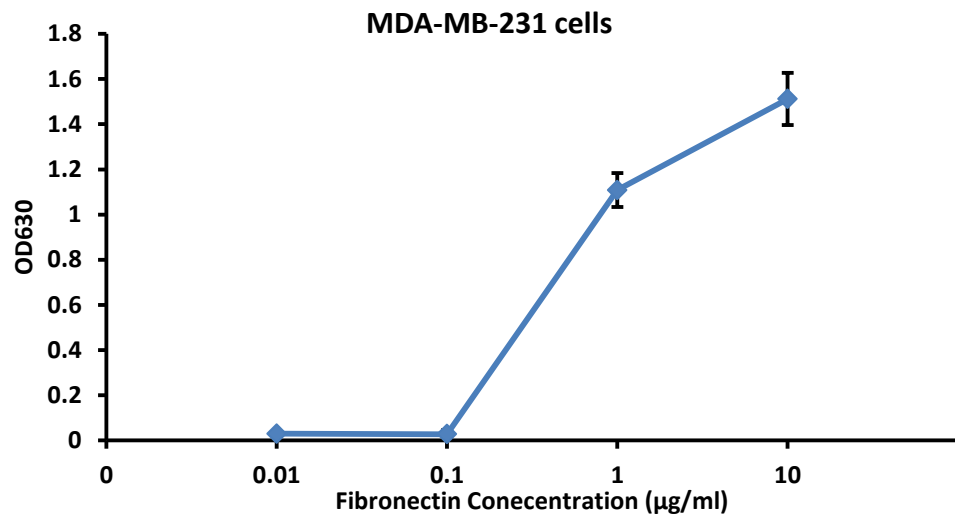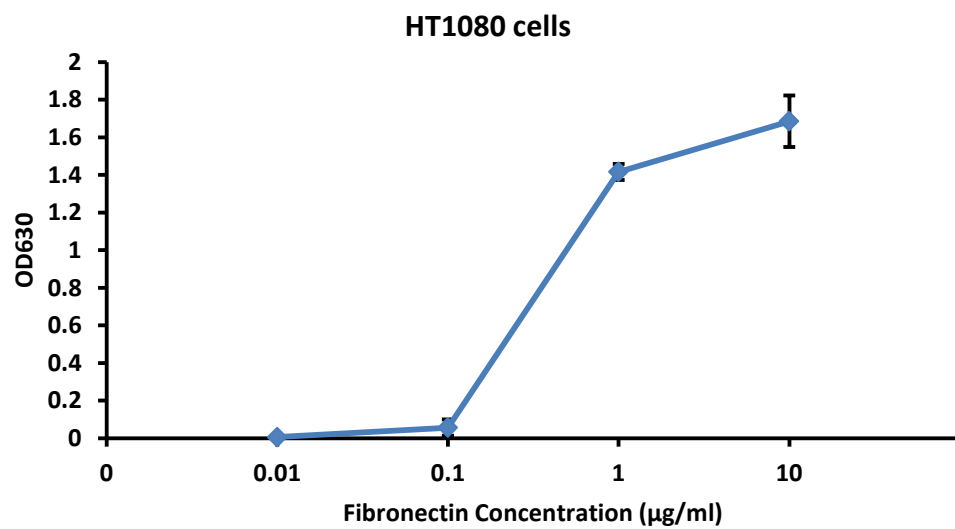
HT1080 cells

Figure 3-1: Adhesion of MDA-MB-231 (A) and HT1080 (B) to 96 mutli-well plates coated with increasing concentrations of immobilised Fibronectin. Cells in each well were quantified at OD630 using the Omegastar plate reader. Error bars represent the mean ± S.E.M. This experiment was perfomed twice and a representative experiment is shown.

A

MDA-MB-231 cells

B
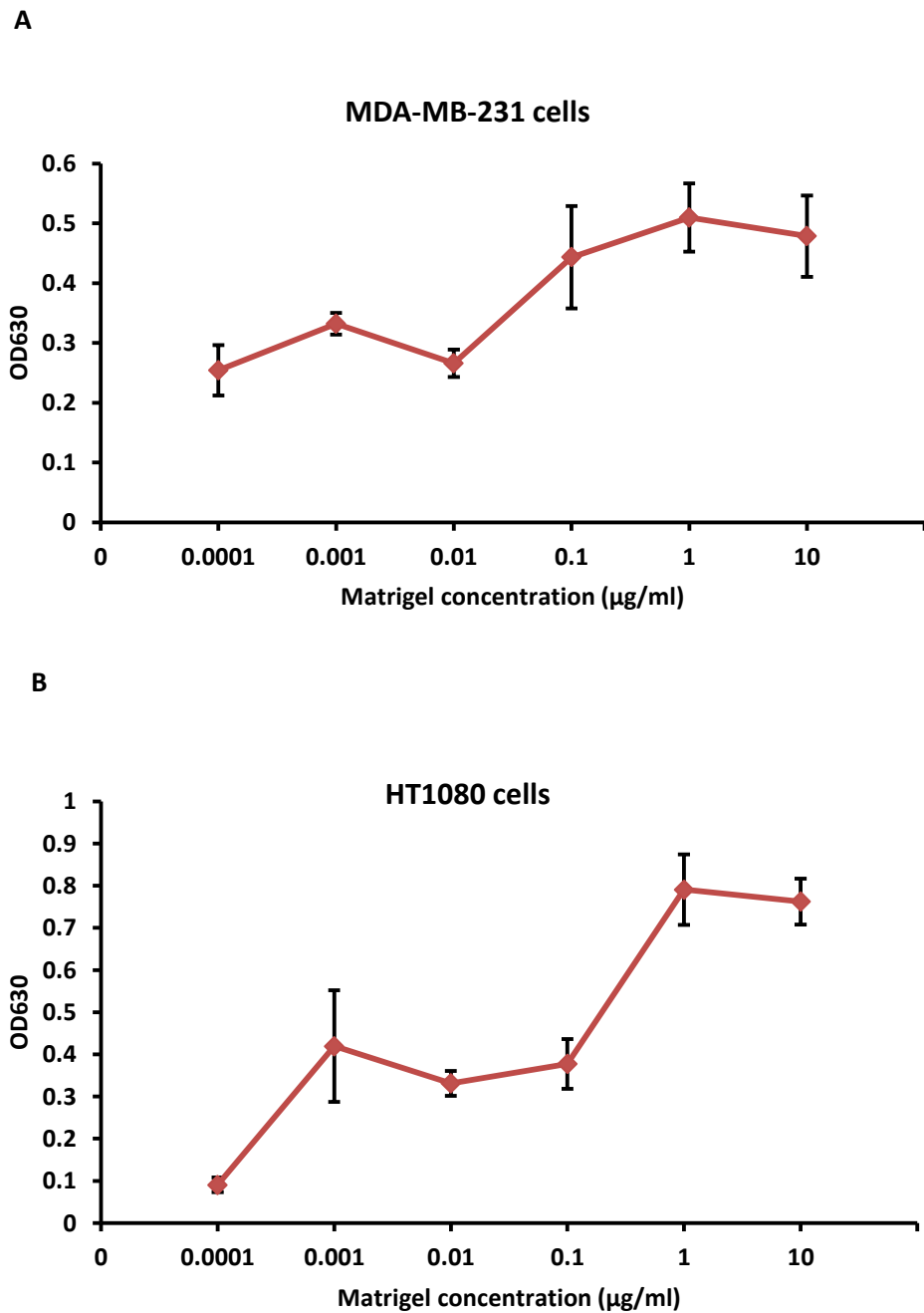
HT1080 cells

Figure 3-2: Adhesion of MDA-MB-231 (A) and HT1080 (B) to 96 mutli-well plates coated with increasing concentrations of immobilised Matrigel. Cells in each well were quantified at OD630 using the Omegastar plate reader. Error bars represent the mean ± S.E.M. This experiment was perfomed twice and a representative experiment is shown.

A

MDA-MB-231 cells

B
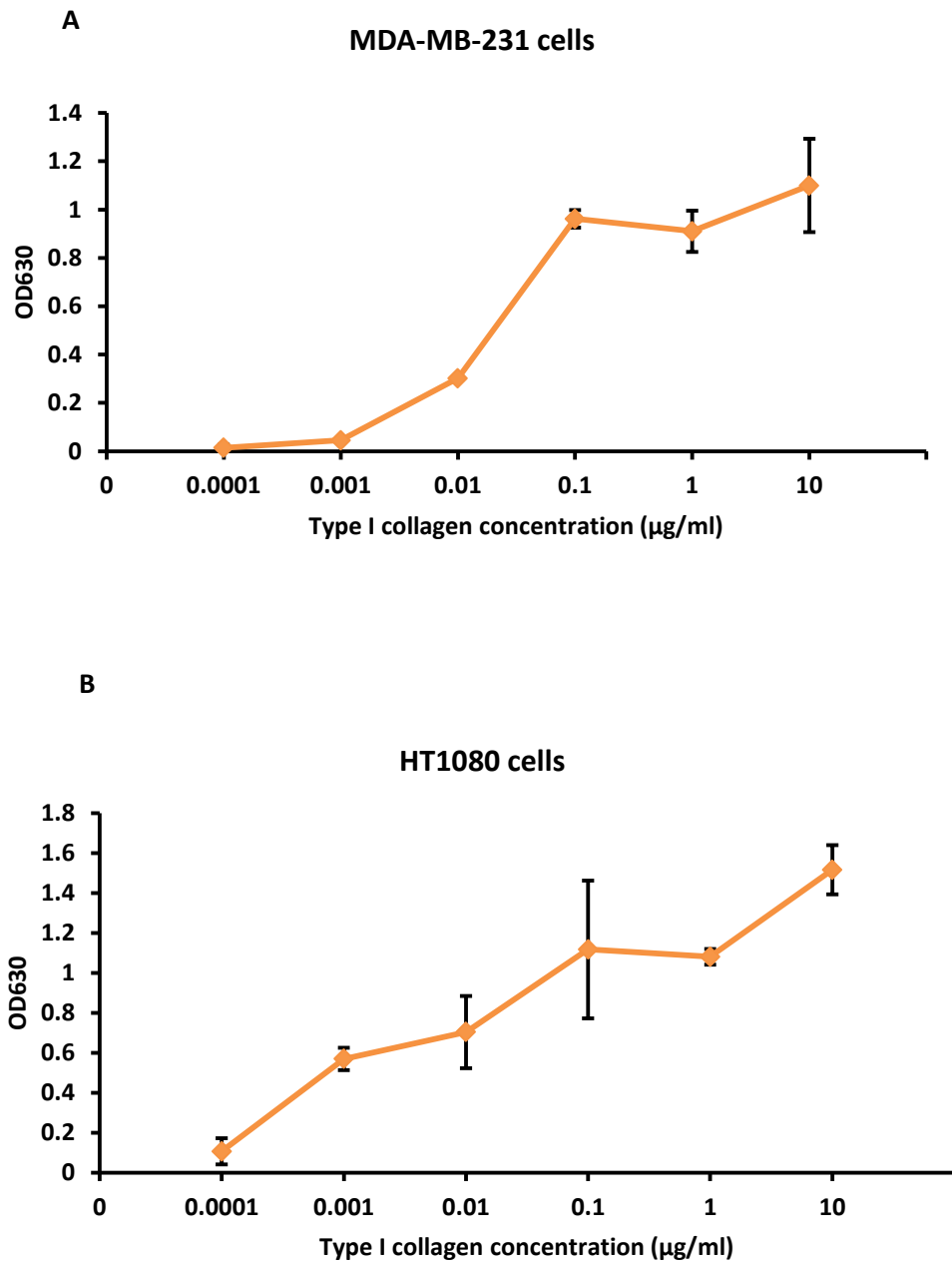
HT1080 cells

Figure 3-3: Adhesion of MDA-MB-231 (A) and HT1080 (B) to 96 mutli-well plates coated with increasing concentrations of immobilised type I collagen. Cells in each well were quantified at OD630 using the Omegastar plate reader. Error bars represent the mean ± S.E.M. This experiment was perfomed twice and a representative experiment is shown.

3.2.4 A5 and A3 dose response assay

To identify the optimal compound concentration to use in adhesion assay experiments a dose response was performed using MDA-MB-231 cells grown on Fibronectin (Fig 3-4). For both A3 and A5 there was a dose dependent decrease observed in MDA-MB-231 adhesion. In both graphs there is a noticeable decrease in adhesion from 5 μg/ml, however then not much difference between 10- and 35 μg/ml. Then at 50 μg/ml there is another noticeable decrease from 35 μg/ml in A5 and A3 treated cells. In both A5 and A3 treated cells, 100 μg/ml showed a great decrease in adhesion to Fibronectin. Initially, due to difficulty of handling large numbers of samples in the assay run DMSO was omitted.

3.2.5 A5 adhesion assay

The effect the isoquinolinone compounds have on cell attachment to ECM components was explored. The same cell adhesion biological assay technique as previously described was used with the exception of coating the wells with our cell lines half-maximal binding concentration to those substrates. For both A5 and A3 experiments, the trend of adhesion reduction appears similar between replicate experiements, however due to differences in absolute levels of adhesion, as often observed; only representative experiements are shown to clarify an effect (Jelena Gavrilovic, personal communication).

Results indicate no clear effect of the A5 isoquinolinone compound on the matrix components for either HT1080 or MDA-MB-231 (Fig 3-5). DMSO was used as our vehicle to dissolve the A5 compound as it is not readily soluble in other carriers such as water. The results in both cell lines seem to suggest that 1% DMSO is having an effect on the cells ability to bind to the matrix. On type I collagen there was a 31% and 43% reduction in adhesion of HT1080 and MDA-MB-231 respectively in response to DMSO (Fig 3-5). Whilst on Fibronectin there was a 34% and drastic 82% reduction in the adhesion of MDA-MB-231 and HT1080 respectively to the substrate in response to DMSO (Fig 3-5). Even though there is a reduction in the adhesion of both cell lines to the matrix components in response to A5, we cannot confirm any induced effect due to the reduction rates being identical to DMSO for all the substrates.

A

B
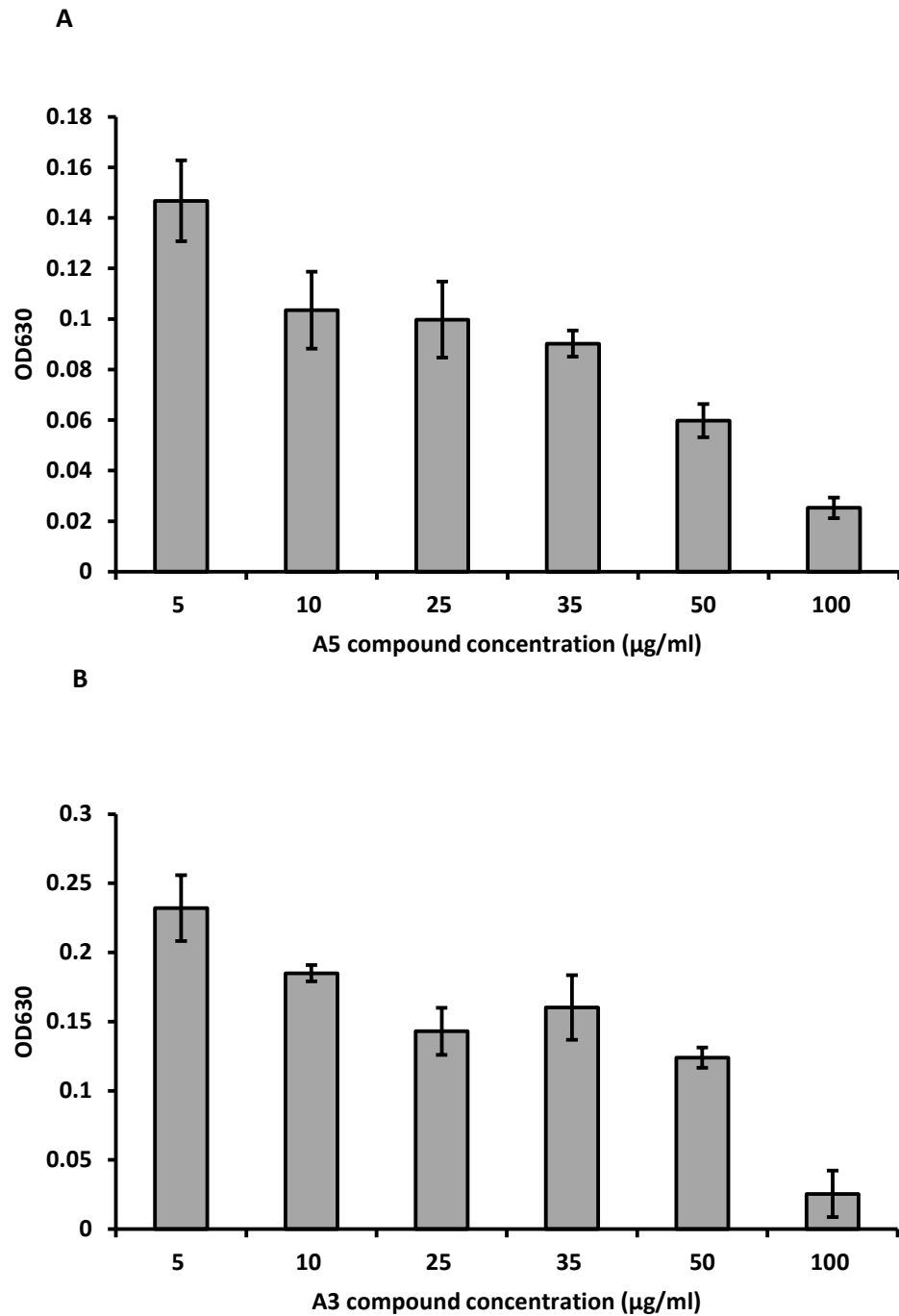
Figure 3-4: A5 and A3 dose response assay. MDA-MB-231 cells were seeded on Fibronectin (10 µg/ml) coated wells of 96 well nunc plate and then treated with increasing doses of (A) A5 compound or (B) A3 compound. Error bars represent the mean ± S.E.M. This experiment was perfomed once.

A

B
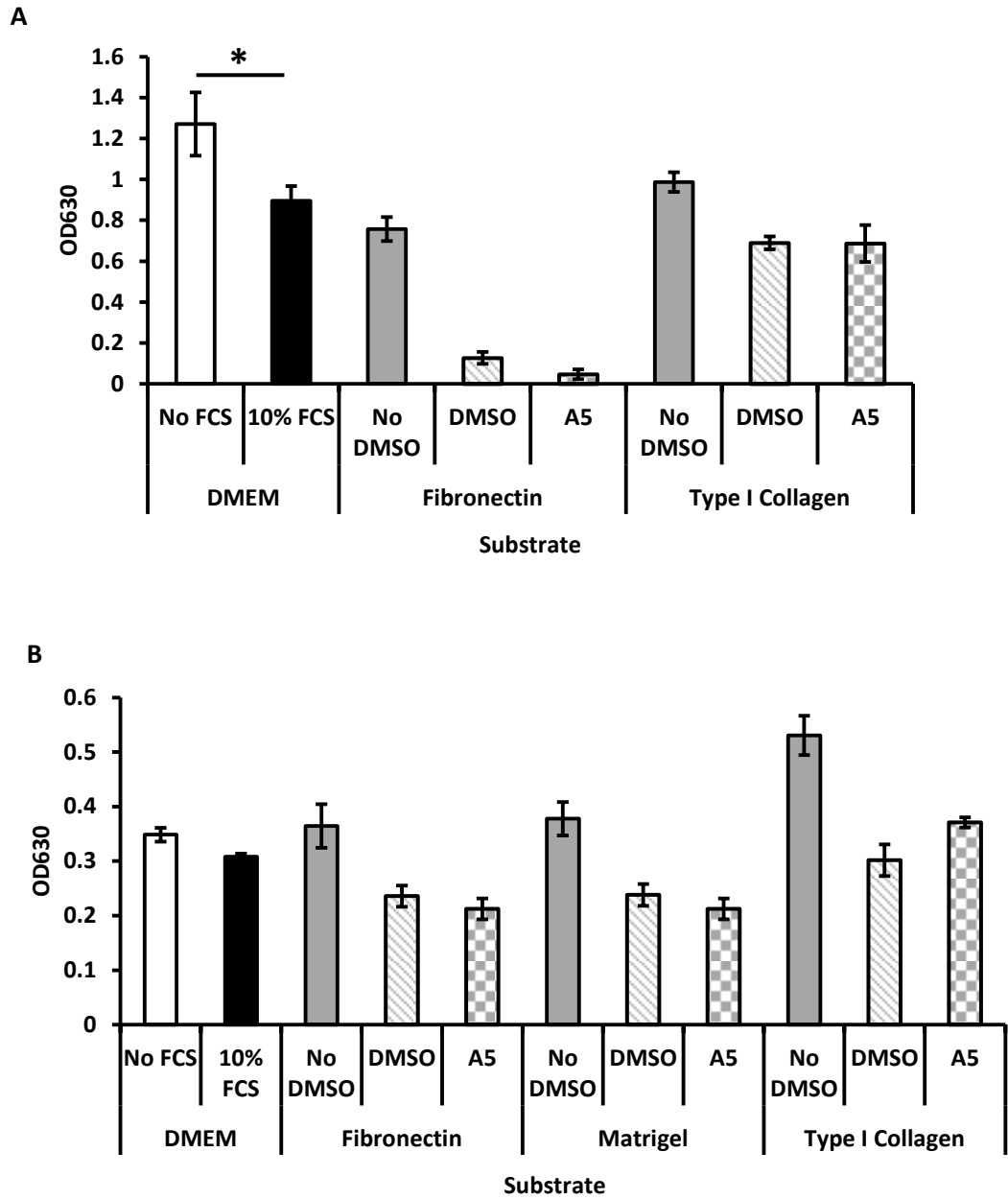
Figure 3-5: Adhesion of HT1080(A) and MDA-MB-231 (B) to 96 mutli-well plates coated with increasing concentrations of immobilised matrix components in response to 100 µM A5. Cells in each well were quantified at OD630 using the Omegastar plate reader. Error bars represent the mean ± S.E.M of quadruplicate samples. Statistical significance was measured using the Anova *Tukey test* *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. This experiment was perfomed once.

3.2.6 Effect of DMSO on cell adhesion

Due to an apparent effect of 1 % DMSO from prior results the possibility of DMSO having a toxic effect on MDA-MB-231 cells at lower concentrations of our matrix components was tested. Contrary to what was observed in our previous experiments these results on Fibronectin and Matrigel showed that DMSO has no adverse effect on the MDA-MB-231 cell's ability to bind (Fig 3-6A and B). In fact on Fibronectin, DMSO increased adhesion at 0.5 µg/ml and 1 µg/ml by 53% and 35% respectively (Fig 3-6A). However, on type I Collagen it appears as though there may be some toxic effect of DMSO (Fig 3-6C). At 1 µg/ml there was a 25% decrease in adhesion, and whilst not significant, a 9% decrease in adhesion at 0.1 µg/ml. Even though these results showed that 1 % DMSO did not affect cell adhesion to Fibronectin and Matrigel, research has shown that at a 1% concentration DMSO has been known to induce cell death and a loss of attachment (Pal et al, 2012). Since the A5 compound is dissolved in 1% DMSO for the desired concentration of our experiments, this prevents us from altering the concentration. However, we deduce that using a higher concentration for our matrix, 1 µg/ml for Matrigel and Fibronectin, and 0.1 µg/ml for type I collagen may provide some protective effects against DMSO.
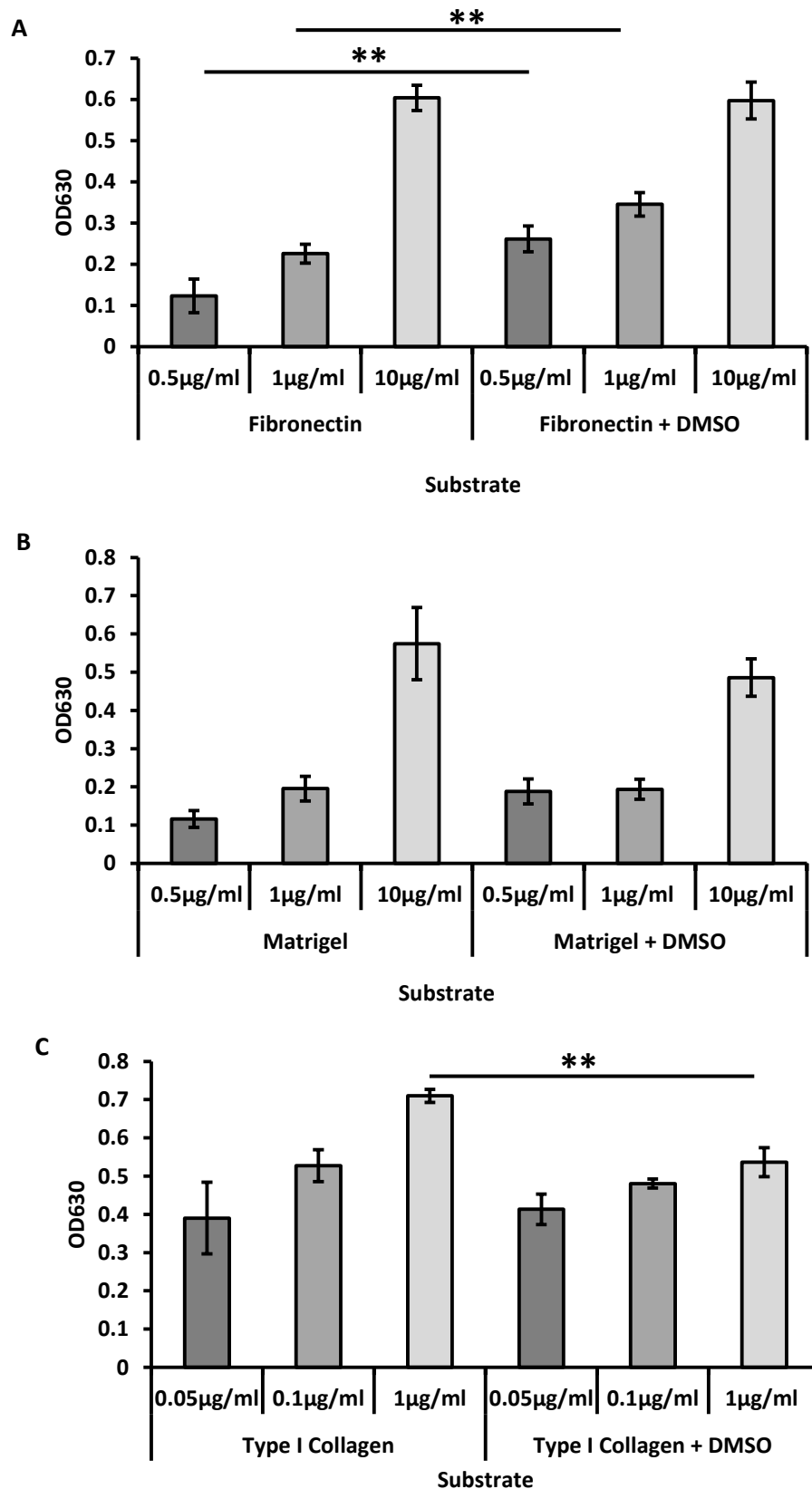
Figure 3-6: MDA-MB-231 adhesion to immobilised Fibronectin (A), Matrigel (B), and type I collagen (C) in the presence and absence of 1% DMSO. Cells in each well were quantified at OD630 using the Omegastar plate reader. Error bars represent the mean ± S.E.M of quadruplicate samples. Statistical significance was measured using the Anova *Tukey test* *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. This experiment was perfomed once.

3.2.7 Cell adhesion in response to A3

The effects of the structurally similar A3 compound was also tested in the adhesion of MDA-MB-231 and HT1080 cells to matrix components (Fig 3-7). In MDA-MB-231 cells adhesion was significantly reduced by 55-, 70-, and 49% on 1 μg/ml Fibronectin, Matrigel, and type I collagen respectively in response to 100 μM A3 (Fig 3-7A). In HT1080 cells the effect of the A3 compound was tested 1 μg/ml and 10 μg/ml Fibronectin. At both concentrations the A3 compound at 100 μM was ineffective at reducing or increasing adhesion of HT1080 cell to Fibronectin (Fig 3-7B).

3.2.8 Cell adhesion in response to A5

Following the findings above adhesion in response to A5 was performed with adjusted variations of matrix components. The A5 compound was found to reduce cell adhesion in both MDA-MB-231 and HT1080 cells (Fig 3-8). Following treatment with the A5 compound there was an 80% and 66% reduction in adhesion on 0.5 μg/ml and 1 μg/ml Fibronectin respectively when compared to DMSO treatment (Fig 3-8B). There was also a reduction in HT1080 adhesion by 39% on 1 μg/ml Matrigel (Fig 3-8C); however no observable effect was seen at 0.5 μg/ml were DMSO was inhibiting.

As mentioned, the A5 compound also reduced adhesion of MDA-MB-231 cells (3-8A). There was a significant decrease in adhesion by 73-,80-, and 49% on Fibronectin, Matrigel, and type I collagen respectively in response to 100 μM A5.

A
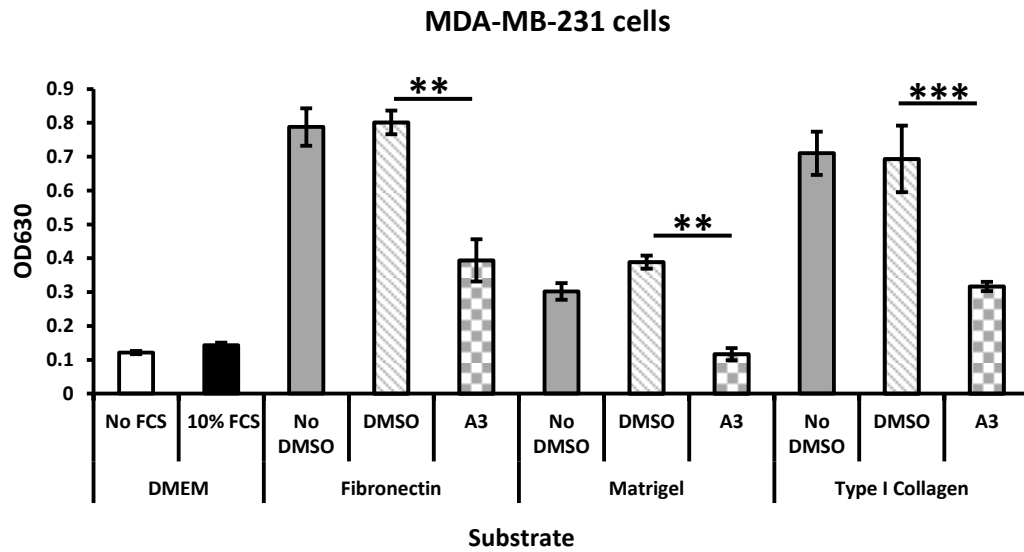
B
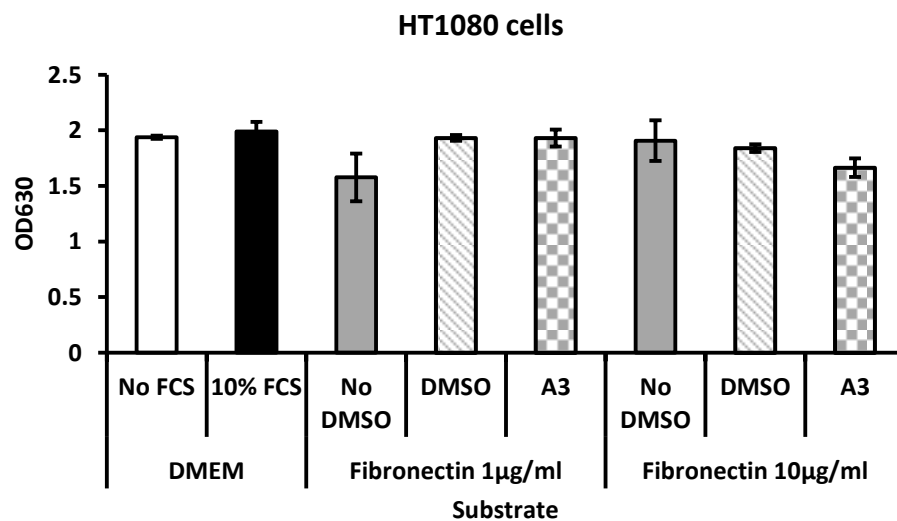
Figure 3-7: (A) MDA-MB-231 adhesion assay to matrix components at 1 μg/ml in response to 100 μM A3. (B) HT1080 adhesion to immobilised Fibronectin at 1- or 10 μg/ml in response to 100μM A3. Cells in each well were quantified at OD630 using the Omegastar plate reader. Error bars represent the mean ± S.E.M. Statistical significance was measured using the Anova *Tukey test* *$p{\leq}$0.05, **$p{\leq}$0.01, ***$p{\leq}$0.001. These experiments were perfomed twice with one representative experiment of each shown.

A

MDA-MB-231 cells

B

HT1080 cells

C
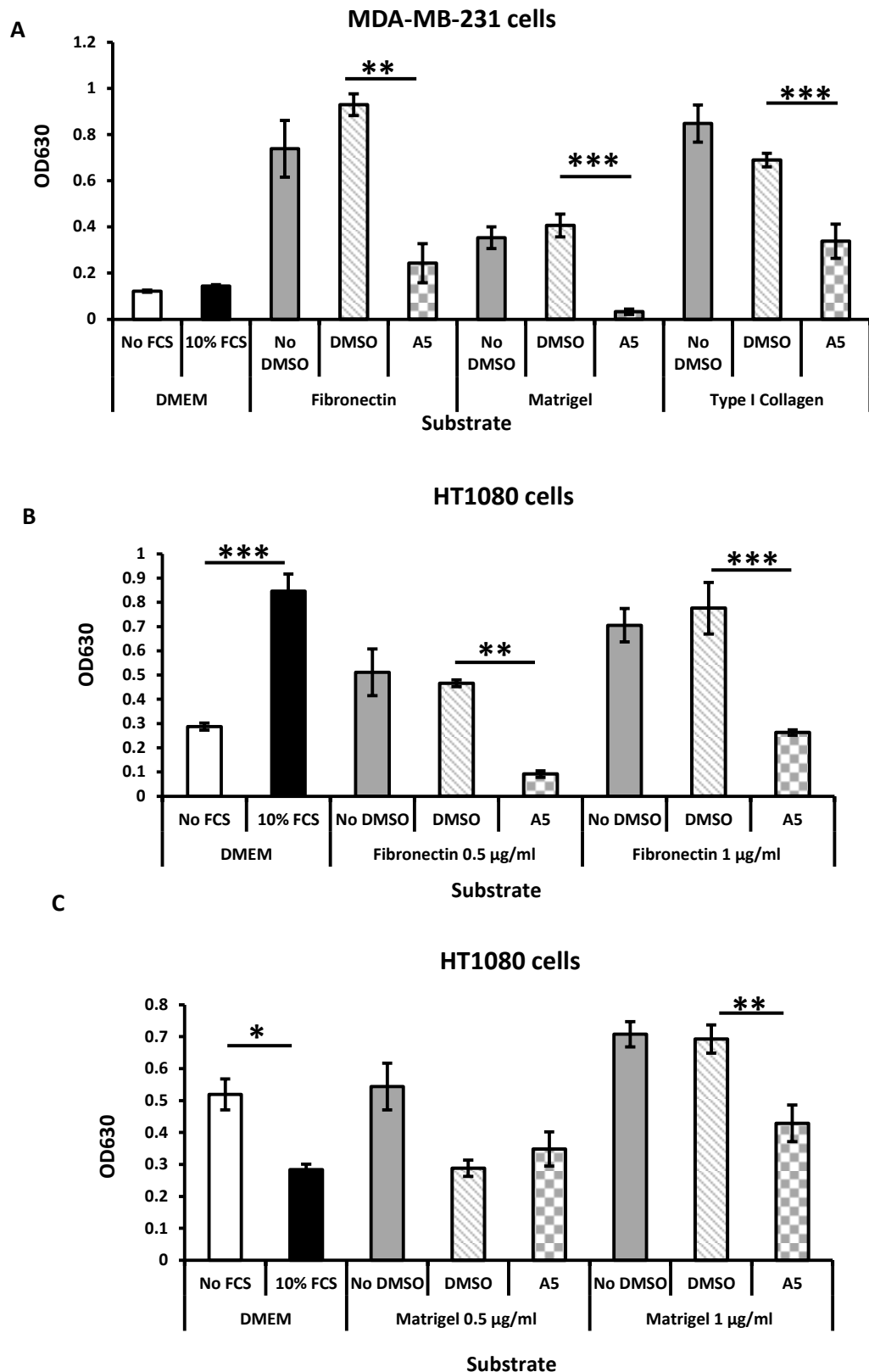
HT1080 cells

Figure 3-8: (A) MDA-MB-231 adhesion assay to matrix components at 1 μg/ml in response to 100 μM A5. (B) HT1080 adhesion to immobilised Fibronectin and (C) Matrigel at 0.5- or 1 μg/ml in response to 100μM A5. Cells in each well were quantified at OD630 using the Omegastar plate reader. Error bars represent the mean ± S.E.M. Statistical significance was measured using the Anova *Tukey test* *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. These experiments were perfomed twice with one representative experiment of each shown.

3.3 Discussion

The aim of this chapter was to explore the effects of the structurally identical A5 and A3 isoquinolinones on MDA-MB-231 and HT1080 adhesion to major components that make up the ECM. To date there is no research ascribing the mechanistic behaviour of these compounds and how they may, or may not be involved in cell adhesion of breast cancer cells. In this chapter we provide evidence that the A5 and A3 isoquinolinone compound suppress cell adhesion to the ECM.

3.3.1 Cell adhesion is dose-dependent

As previously mentioned, Fibronectin is a major component of the ECM and its up-regulation in many invasive carcinoma make it a potential prognostic marker in patients (Bae et al, 2013). Both MDA-MB-231 and HT1080 showed an identical curve in response to increasing doses of Fibronectin. The optimal binding of the cell lines to the substrate occurred at 1 μg/ml as observed in other reports (Bartsch et al, 2003). This suggests that MDA-MB-231 and HT1080 cells express a similar level of receptors for fibronectin. Both cell lines highly express the $\alpha5\beta1$ integrins which is the main receptor for fibronectin (Mierke et al, 2010: Grenz et al, 1993). A similar trend in optimal binding was observed in both cell lines on the basement membrane extract Matrigel. Matrigel is composed of a variety of components with the most abundant protein in the extract is laminin. Both MDA-MB-231 and HT1080 express a similar of the $\alpha6\beta1$ integrin when cultured on purified laminin (Berno, et al, 2005: Zuber, et al 2008). Moreover work has been done showing that MDA-MB-231 adhere to laminin-1 in a dose-dependent manner and begin to plateau at roughly at 1 μg/ml (Kousuke, et al, 1999). Binding of MDA-MB-231 and HT1080 to type I collagen was also dose-dependent and beginning to plateau at 0.1 μg/ml for both cells lines. Similar results have been obtained in studies for MDA-MB-231 and HT1080 cell adhesion to type I collagen (Vuoriluoto et al, 2008 : Messent et al, 1998). HT1080 cells bind collagen through the $\alpha2\beta1$ receptor whilst MDA-MB-231 have been shown to bind collagen through both $\alpha2\beta1$ and $\alpha1\beta1$ integrins (Grenz et al, 1993: Ibaragi et al, 2011).

3.3.2 DMSO effect on cell adhesion

It is well-known that a lot of synthesised compounds are highly lipophilic and so encounter the problem of being poorly-water soluble. Alternative means of delivery have since been discovered and utilised in order to allow administration of drugs. DMSO is a popular organic solvent that has been widely used in conventional medicine and biological research as a carrier molecule. Whilst co-solvents such as DMSO have been shown to greatly increase solubility, they also have some irremediable cytotoxic effects (Violante, et al 2002). In our studies we

found that DMSO disrupted the adhesion of HT1080s and MDA-MB-231 cells on all matrix components. This led us to evaluate the effect of DMSO on the cells seeded onto different concentrations of each substrate. Our study showed that DMSO was in fact having no suppressive effect on the cells at any of the tested concentrations. Moreover, on Fibronectin we even saw an increase of cell binding to the matrix which is an effect that has been reported in melanoma cells in response to DMSO (Lampugnani et al, 1987). Despite this, in subsequent experiments at lower concentrations of coated matrix we still saw a decrease in adhesion in response to DMSO. More testing would have to be conducted in order to fully clarify the effect of DMSO on both breast and fibrosarcoma cell lines. As previously mentioned studies have shown a significant deformation in cell activity at 1% DMSO (Pal et al, 2012).

3.3.3 A3 and A5 compound in cell adhesion

In our experiments both the A3 and A5 compound reduced cell adhesion in MDA-MB-231 cells, however only the A5 compound altered the adhesive properties of HT1080s to Matrigel, Fibronectin, or type I collagen. This contrasts previous results showing that the A3 compound reduces cell reduces HT1080 adhesion to the matrix   (Howe,J.D., Payerne, E., Woon, E.C.Y., Searcey, M., and Gavrilovic, J). A plausible explanation for this may be because the compound used in the HT1080 experiment is three years old and so its biological activity may have been diminished. Evidence for this claim was observed in previous adhesion assays with MDA-MB-231 cells treated with the same A3 compound as the HT1080 cells which also failed to show any effect on adhesion (data not shown).

Reduced adhesion to the matrix in response to A5 has been observed in a prior study using HT1080 cells (Howe,J.D., Payerne, E., Woon, E.C.Y., Searcey, M., and Gavrilovic, J unpublished observations). Here the A5 compound was shown to have a similar effect on the breast carcinoma cell line. This similarity in the compounds effects supports the idea of a similar function in both cell lines. Isoquinolinones are still being explored in research and the sparse studies available suggest they have a wide-range of targets (Cheon, et al, 2001). One study in particular has shown that isoquinolinones act as integrin receptor antagonists (Seitz et al, 2007). This most likely provides an explanation for the results obtained in these experiments. It is also worth noting that the reduction of adhesion for both cell lines was greatest on Fibronectin. Studies with the isoquinolinones have shown that differing structures of compound derivatives can alter the selectivity to certain integrins (Seitz et al, 2007).

3.3.4 Summary

In this chapter we identified optimal binding concentrations for cell adhesion of HT1080 and MDA-MB-231 cells to Fibronectin, Matrigel, and type I collagen. Furthermore we found that compound A3 reduces adhesion of MDA-MB-231 cells to matrix componenets which is paralleled by compound A5 in both MDA-MB-231 cells and HT1080 cells. In the next chapter we will investigate what affect the compounds have on cell motility both in 2D and 3D.

Chapter 4: Isoquinolinones in cancer cell migration and 3D invasion assay

4.1 Introduction

Cancer is a major health problem that kills patients essentially on the basis of their migratory nature which allows cells to scatter and invade into the surrounding tissue (Debeir et al, 2008). It is therefore important to understand the biological mechanisms driving cancer cell migration and investigate the effects of pharmaceutical compounds which constitute the current needs in oncology (Debeir et al, 2008). By doing this it allows for the exploration of developing anti-migratory drugs that delay tissue invasion and move away from solely trying to trigger cell death which has up to now, proven unsuccessful and cytotoxic to healthy cells (Debeir et al, 2008).

The onset of migration in cancer is reliant on contextual extracellular cues which promotes the activation of migratory machinery. As mentioned previously, integrins are bidirectional signalling receptors that are coupled molecularly to cell protrusions. Not only do they contribute to the stability of membrane protrusions, but engagement of integrins at the lamella initiates 'outside- in' signal cascades which activate many effector proteins such as Rac and Cdc42 (Legate et al, 2009). As well as a major contribution of the integrin-matrix signalling there is an abundance of motogenic proteins such as EGF which can induce chemokinesis and chemotaxis (Wang et al, 2004).

Isoquinolinones have been shown to have varying *in vitro* anticancer activity depending on their structure and the cancer cell line (Cheon et al, 2001). Whilst there is no literature reviewing their effects on the migratory or invasive capacity of cancer cells, we do know that they can target integrins. This currently leaves the field open to interpretation on exactly how they may alter typical cell migration (Seitz et al, 2007).

In the last chapter we found that the A5 isoquinolinone was able to reduce adhesion of MDA-MB-231 and HT1080 to the matrix. It is well known that protrusions and adhesion are molecularly coupled. It therefore remains important to understand how the isoquinolinones may affect the migratory and invasive potential of cancer cells. In this chapter we use random 2D migration and the inverted 3D invasion assay to investigate what effect the isoquinolinones have on cancer cell migration and invasion using matrix components that emulate typical physiological conditions.

4.2 Results

4.2.1 MDA-MB-231 migration in response to EGF using the scratch wound healing assay

To assess qualitative migration in response to a chemotactic agent we used a widely adopted method known as the scratch wound healing assay. Here cells were seeded in a 24-well plate and then an artificial wound was created by physical scratching following the addition of the growth factor EGF (Fig 4-1). The measurement of cellular repopulation into the disrupted area is indicative of the migratory potential of cells with or without treatment (Lal et al, 2002). We decided to use EGF in our studies as it is known to activate many well elucidated signalling pathways involved in cell migration such as MAPK and PI3K (Price et al, 1999: Azios and Dharmawardhane, 2005). Furthermore, this allows us to construct an assessment in subsequent experiments on the effectiveness of the compounds ability to induce migration and whether they may be activating the same pathways. On Matrigel, Fibronectin, and the combination of Matrigel and Fibronectin there was no significant increase in the speed of cell migration in response to EGF (Fig 4-2A). However, it is worth noting that in the presence of EGF on these three conditions there was a modest increase in speed. The speed between cells migrating on Fibronectin, Matrigel, or Matrigel with Fibronectin did not vary much either. Matrigel and Fibronectin combined did see the greatest difference in speed with EGF treatment showing an 18% increase as opposed to 13% on Matrigel and 6% on Fibronectin. MDA-MB-231 migrational speed was significantly upregulated by 32% on plastic in response to EGF. Cell migrating on plastic in the presence and absence of EGF showed the greatest and lowest overall speed (Fig 4-2A). The forward migration index (FMI) was also calculated as it is a measurement of wound sensing and the cell's efficiency to migrate forward (Fig 4-2B). FMI did not change upon the addition of EGF to cell's migrating on plastic, Fibronectin, and Matrigel mixed with Fibronectin. However, on Matrigel there was a significant 45% increase in the forward direction of migration (Fig 4-2C).

A Plastic

-EGF   +EGF

0 hours

17 hours

B Fibronectin

-EGF   +EGF

C Matrigel

-EGF   +EGF

0 hours

17 hours

D Matrigel + Fibronectin

-EGF   +EGF
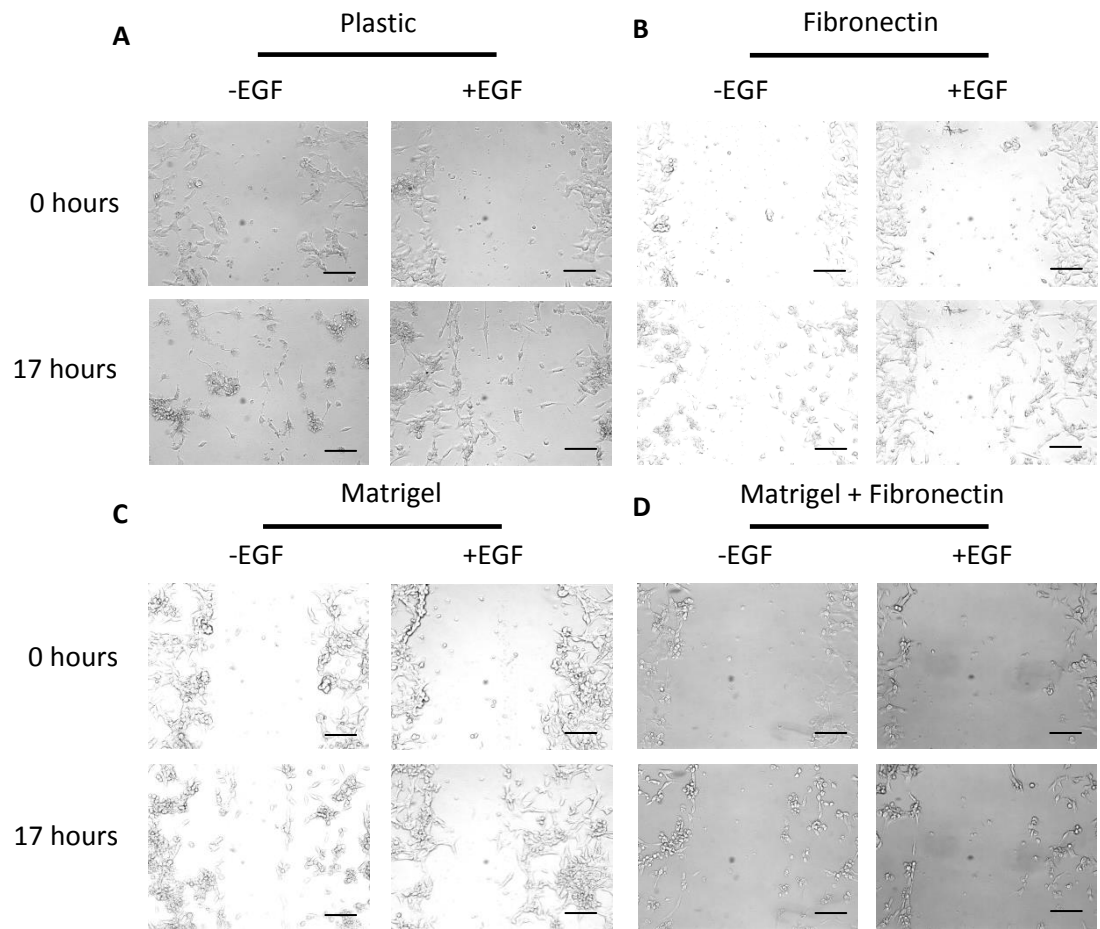
Figure 4-1: In wound healing assay, MDA-MB-231 cells were treated with 100ng/ml EGF and seeded on either (A) plastic, (B) Fibronectin, (C) Matrigel, (D) or the combination of Matrigel and Fibronectin. The monolayer was scratched using a sterile pipette tip and cell migration was observed using time-lapse microscopy for 17 hours. Representative images are shown at given time points. Scale bar is equal to 160µm.

A

B

$$Directionality = \frac{Euclidean\ distance}{Accumulated\ path\ length}$$

$$FMI = \frac{Perpendicular\ distance}{Accumulated\ path\ length}$$

C
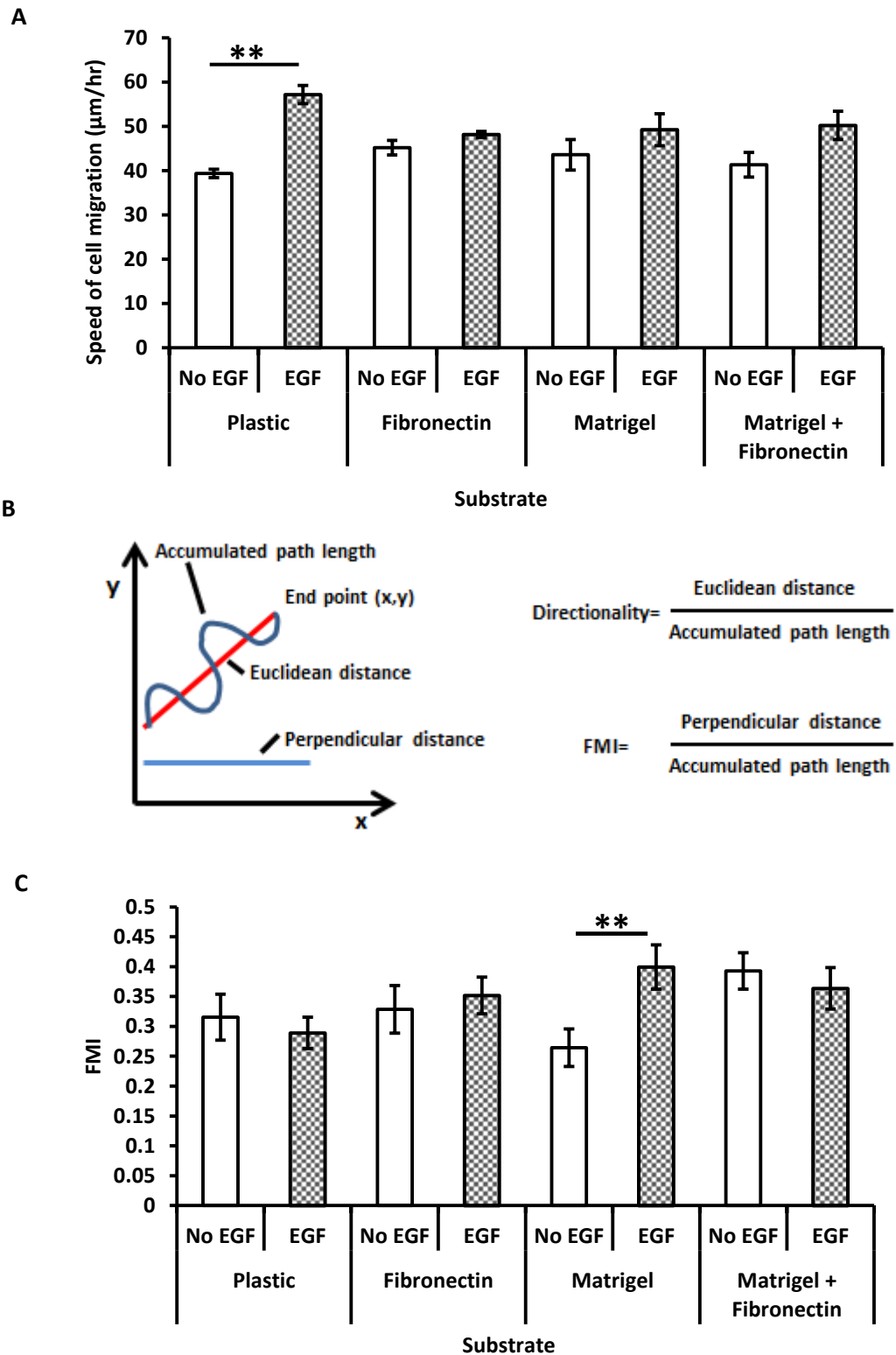
Fig 4-2. (A) The overall migration velocity of cells moving into the wound following EGF treatment. (B) Schematic diagram of cell tracking analysis. The forward migration index (FMI) is the ratio of the net distance travelled into the wound by the accumulated path length. Directionality is the Euclidean distance from the centre of mass divided by the accumulated path length. (C) Is the FMI. Values are means ± SEM of 15 cell tracks. Statistical significance was determined using the students paired t-test. This experiment was performed once.

4.2.2 MDA-MB-231 migration in response to A5

Since it was shown that the A5 isoquinolinone affected cell adhesion and that we know transient adhesive interactions between integrins the ECM mediate cellular migration, we decided to investigate whether A5 alters cell migration (VanCompernolle et al, 2003). MDA-MB-231 cells were seeded on the matrix components Fibronectin, Matrigel and type I collagen and then treated with 50μM A5 for 17 hours in which monitoring at 10 minute intervals using time-lapse microscopy was taken. Typically, baseline migration of MDA-MB-231 cells was enhanced on all matrix coated surfaces compared with plastic (Fig 4-3 and Fig 4-4). The speed of migration increased significantly in the presence of A5 by 18, 20, and 35% on Fibronectin, Matrigel, and type I collagen respectively when compared to cells migrating on the same substrates but in the presence of DMSO (Fig 4-3A and Fig 4-4A). The effect of the A5 compound was most pronounced on type I collagen which saw the most enhanced increase in migrational speed of cells when compared to Fibronectin and Matrigel. Unlike the A5 compounds effect on speed, there was no observed influence on the directionality of migrating cells on any of the substrates (Fig 4-3B and Fig 4-4B). Whilst not significant, the speed of cells migrating on all three substrates was impeded when treated with DMSO when compared to untreated migrating cells.

4.2.3 MDA-MB-231 invasion in response to chemoattractants EGF and FCS

After looking at the effect of EGF on cells migrating on 2D planar surfaces we moved on to observe its effect on cells migrating through a 3D Matrigel plug supplemented with Fibronectin (Fig 4-5). For additional comparison in this experiment we also looked at another chemoattractant FCS. In the presence of 20% FCS, EGF, and the combination of EGF and 20% FCS there was approximately a 3-fold increase in invasion through the Matrigel basement extract when compared to cells migrating towards serum devoid of any chemoattractants (Fig 4-5B). The combination of EGF and 20% FCS appeared to have no enhanced synergistic effect on invading MDA-MB-231 cells. Additionally, whilst not significant it is worth noting that the combination of chemoattratancts seemed to modestly cause less invasion when compared to cells migrating towards them individually suggesting a slight suppressive effect. Total fluorescence was greater across all conditions where a chemoattractant is present, however was only significantly different to the control in wells with a combination of both chemoattractants (Fig 4-5C).
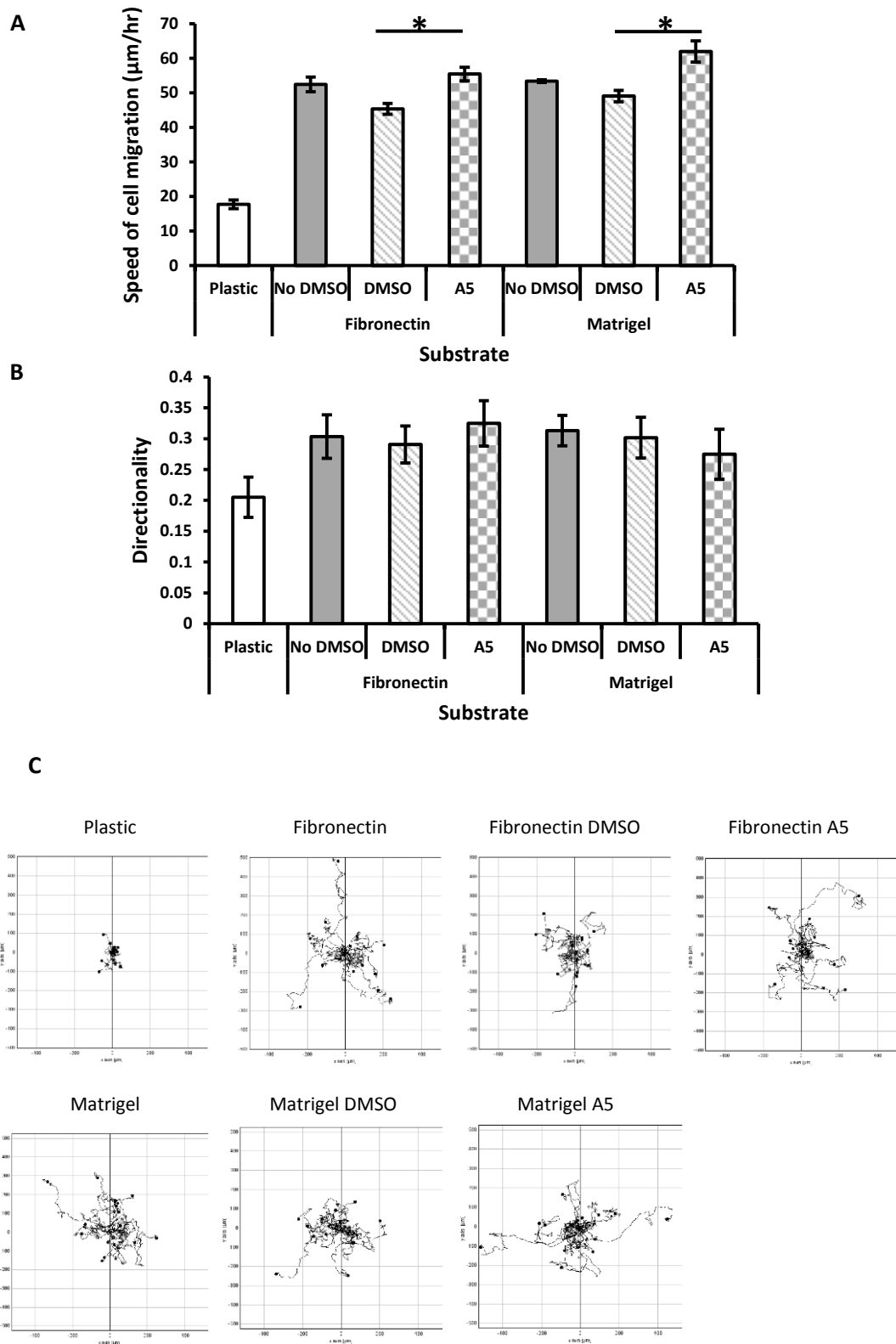
Figure 4-3. Migration of MDA-MB-231 cells on Fibronectin and Matrigel in response to A5 (50µM). The migration parameters (A) Speed and (B) directionality (a measure of how straight cells move) were calculated based on random cell migration experiment over 17 hours. (C) Migration trajectories of MDA-MB-231 cells from the same experiment. Bars represent the mean ± S.E.M of 15 cell tracks. Statistical significance was determined using the Anova *Tukey test*. This experiment was performed once.
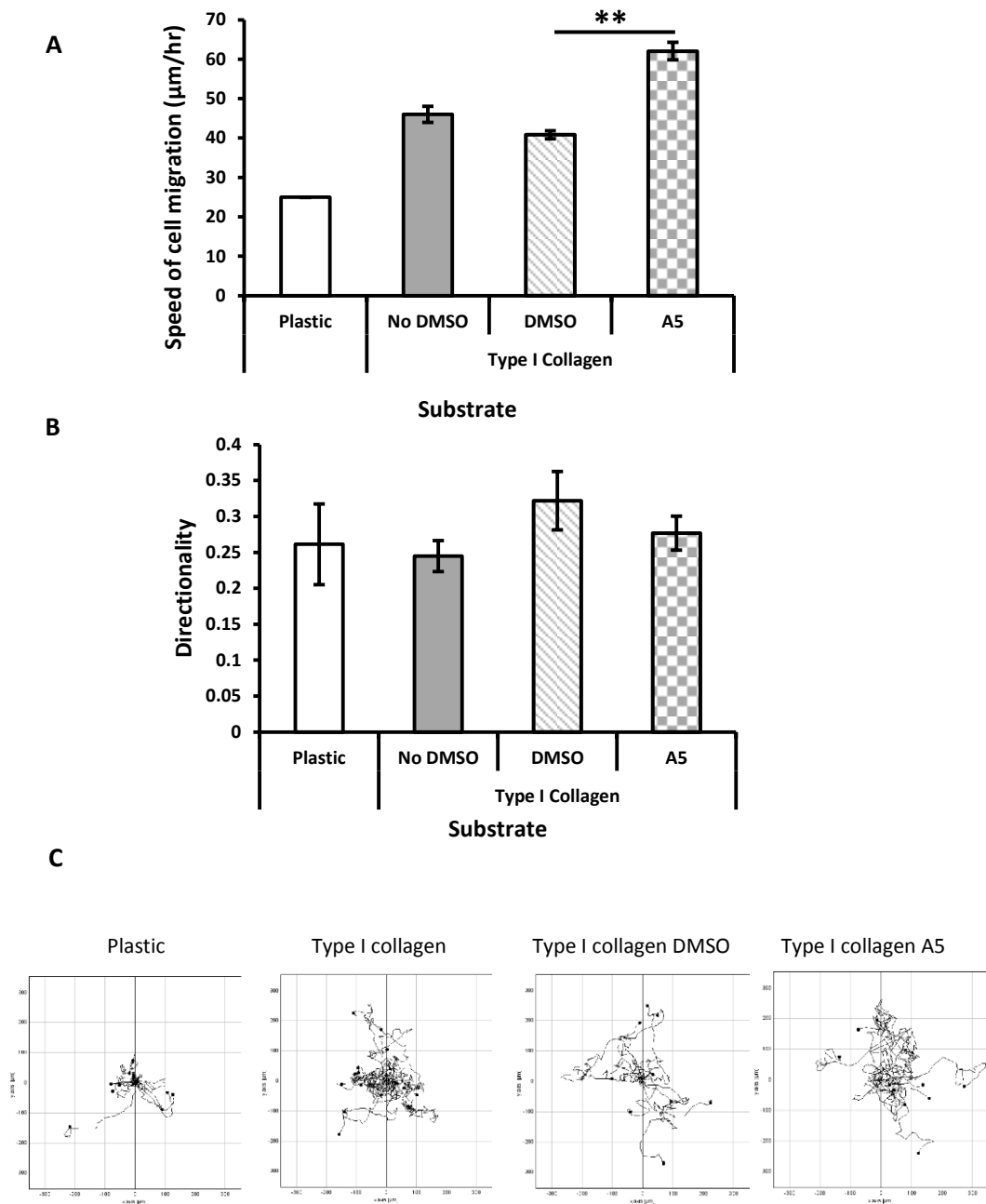
Figure 4-4. Migration of MDA-MB-231 cells on type I collagen in response to A5 (50μM). The migration parameters (A) Speed and (B) directionality (a measure of how straight cells move) were calculated based on random cell migration experiment over 17 hours. (C) Migration trajectories of MDA-MB-231 cells from the same experiment. Bars represent the mean ± S.E.M of 15 cell tracks. Statistical significance was determined using Anova *Tukey test.* This experiment was performed once.

A

0 μm ———————————————————————————→ 210 μm

Control

FCS 20%

EGF

EGF + FCS 20%

15 μm   30 μm

B

C
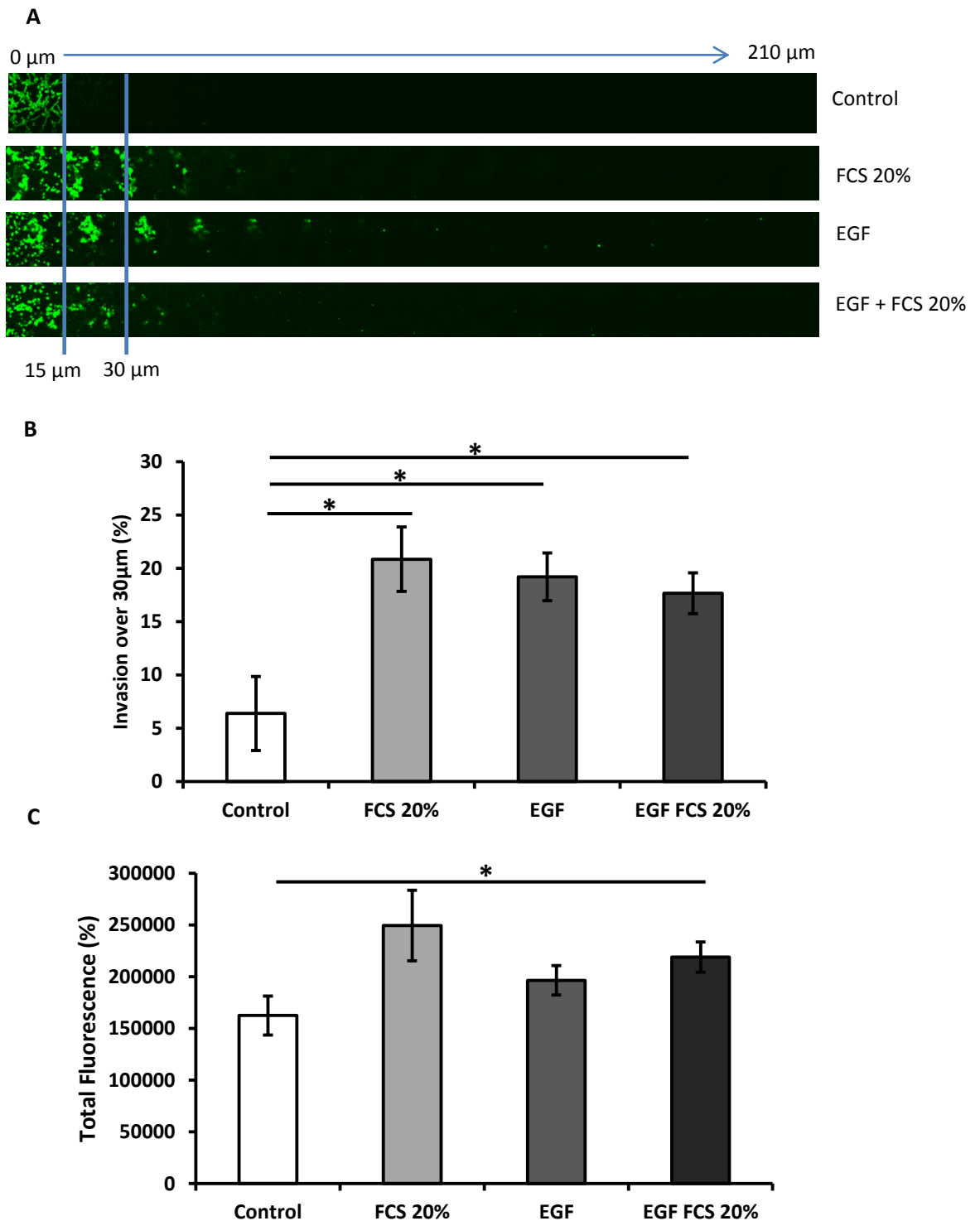
Figure 4-5: (A) Matrigel plug montages enriched with 10μg/ml fibronectin. MDA-MB-231 cells were seeded onto filter and allowed 72hours to migrate towards chemoattractants FCS and EGF. Invading cells were stained with calcein-AM and visualised using confocal microscopy taking 15μm optical sections that are collated as a sequence increasing in depth from left to right. (B) Quantification of invasion was determined through measuring the fluorescence intensity of ≥30μm. (C) The total fluorescence for each experiment was also extracted. Bars represent the mean ±S.E.M of 6 points from 2 replicates. Statistical significance of differences was determined by Anova *Tukey test.* This experiment was performed twice with one representation shown above.

4.2.4 MDA-MB-231 invasion in response to A3

Having established that isoquinolinones can reduce cell adhesion and enhance cell migration on 2D surfaces we looked to determine to what extent they may drive invasion. Just like our previous inverted invasion assay experiment, cells where tested in their ability to migrate up into, and through the Matrigel plug mixed with Fibronectin in response to media supplemented with A3 (50 µM) in both the lower and upper wells (Fig 4-6). The A3 compound significantly induced invasion by 85% and 53% compared to DMSO and control wells (Fig 4-6B). DMSO had a suppressive effect on invasion shown by a four fold decrease compared to the control. No significant difference in total fluorescence was observed between any of the conditions (Fig 4-6C).

4.2.5 MDA-MB-231 invasion in response to A5

The effect of the A5 isoquinolinone on MDA-MB-231 invasion through Matrigel mixed with Fibronectin was also determined (Fig 4-6 and Fig 4-7). Overall cell invasion into, and through the matrix was relatively low in response to A5 at 30 µm. Cells showed a greater invasive capacity in response to EGF alone, and the combination of EGF with A5 (Fig 4-7B). Additionally, there was a significant increase in invasion by 90% for cells invading in response to EGF combined with A5 compared to cells invading in response to EGF combined with DMSO. Whilst not significant, there was an 88% decrease in invasion when DMSO was combined with EGF as opposed to EGF alone suggesting a suppressive effect of DMSO (Fig 4-7B). In Figure 4-6B there is a 3-fold increase in invasion in response to A5 compared to DMSO, however overall invasion was less than that of the control wells. There was no significant difference in total fluorescence amongst the three conditions in Figure 4-7C. However it is worth noting that there is approximately 2-fold less total fluorescence for EGF compared to EGF combined with A5, and EGF combined with DMSO which had the greatest total fluorescence. In Figure 4-6C there is no significant difference in total fluorescence between conditions either.

A

0 μm ⟶ 210 μm

Control

DMSO

A5

A3

15 μm 30μm

B

C
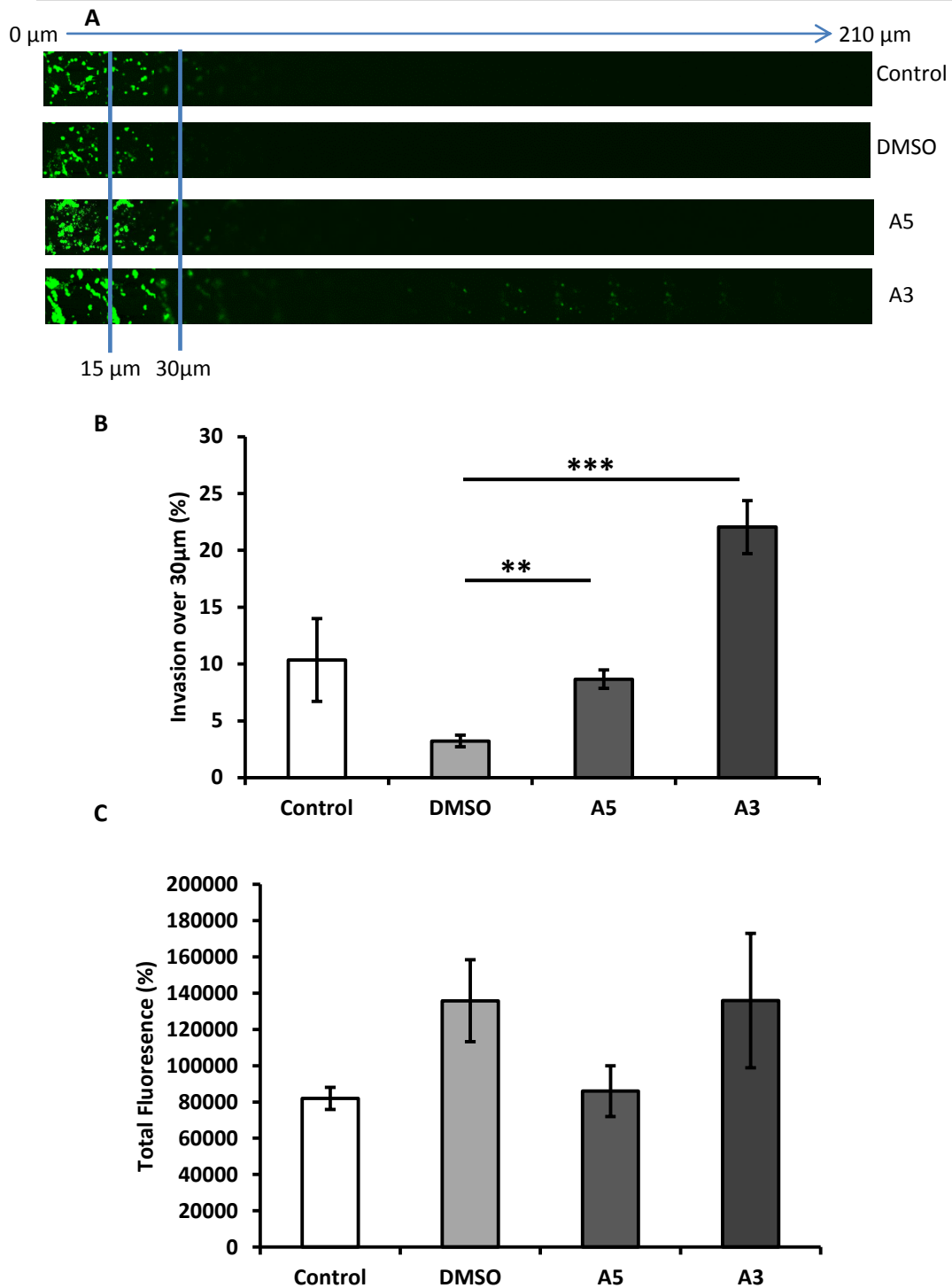
Figure 4-6: (A) Matrigel plug montages enriched with 10μg/ml fibronectin. MDA-MB-231 cells were seeded onto filter and allowed 72hours to migrate toward serum-free media, DMSO, A5, and A3. Invading cells were stained with calcein-AM and visualised using confocal microscopy taking 15μm optical sections that are collated as a sequence increasing in depth from left to right. (B) Quantification of invasion was determined through measuring the fluorescence intensity of ≥30μm. (C) The total fluorescence for each experiment was also extracted. Bars represent the mean ±S.E.M of 6 points from 2 replicates. Statistical significance of differences was determined by students Anova *Tukey test.* This experiment was performed once.

A

0 µm ———————————————————————➤ 210 µm

EGF

EGF +DMSO

EGF +A5

15 µm   30µm

B

C
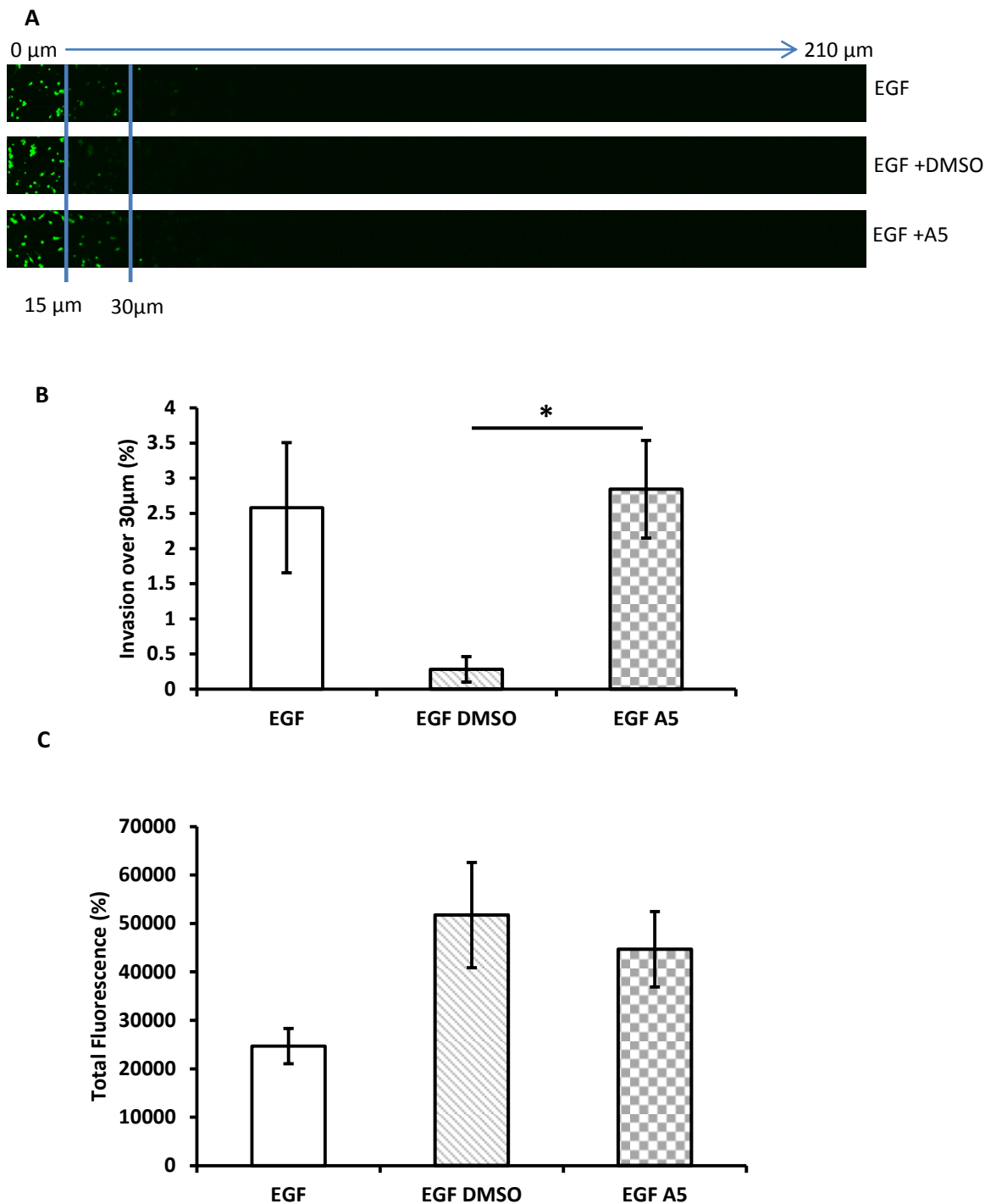
Figure 4-7. (A) Matrigel plug montages enriched with 10µg/ml fibronectin. MDA-MB-231 cells were seeded onto filter and allowed 72hours to migrate toward EGF, DMSO, and A5. Invading cells were stained with calcein-AM and visualised using confocal microscopy taking 15µm optical sections that are collated as a sequence increasing in depth from left to right. (B) Quantification of invasion was determined through measuring the fluorescence intensity of ≥30µm. (C) The total fluorescence for each experiment was also extracted. Bars represent the mean ±S.E.M of 6 points from 2 replicates. Statistical significance of differences was determined by Anova *Tukey test*. This experiment was performed twice with one representation shown above.

4.3 Discussion

In this chapter the aim was to determine what effect the isoquinolinone compounds A5 and A3 have on MDA-MB-231 cell migration and invasion using both 2D and 3D models respectively. The present data provides evidence that the A5 isoquinolinone compound induces cell migration on components of the ECM and modestly drives cell invasion whilst the A3 compound was found to induce the invasion of MDA-MB-231 significantly.

4.3.1 Involvement of EGF in wound healing

As stated previously, it is well documented that EGF acts as a chemoattractant in promoting motility of a number of human cancer cell lines. Moreover, the effect of EGF on the migration of mammary breast carcinoma cells MDA-MB-231 has been well documented. In typical *in vivo* wounds induced by trauma, cells adjacent to the site will sense the damage, proliferate, migrate, synthesize and incorporate matrix components allowing for the closure of the wound (Krishnamoorthy et al, 2012). Since its discovery, EGF has been isolated and investigated in the treatment of acute and chronic wounds in clinical practice. It is found abundantly at sites of injury and is known to be upregulated in an autocrine and paracrine fashion by migrating cells and cells of the stroma respectively (Hardwicke et al, 2008). This participation of growth factors such as EGF in enhancing cell migration make them ideal for study as we can compare them to the ability of the isoquinolinones to induce migration. In our experiment we observed a significant increase in the speed of migration on plastic, and modest, but not significant speed increases on wells coated with matrix components. Studies have shown that EGF enhances polarization and migration of MDA-MB-231 cells into artificially created wounds (Wang et al, 2013). EGF promotes MDA-MB-231 migration through the activation of a complex intracellular signalling axis which to this day has not been fully elucidated. Binding of EGF to EGFR causes activation of Arf6 GTPase through its specific GEF GEP100 which allows for the activation of Rac1 (Hu et al, 2012). Rac1 participates in the control of ROS machinery which together in concert play a central role in the activation of PI3K-AKT signalling (Yang et al, 2011). Additionally, the PI3K-AKT signalling pathway is known to cause further regulation of Rac1 and activation of Cdc42 in EGF stimulated breast cancer cells (Sturge et el, 2002). This causes subsequent activation of PAK1, a downstream effector of Rac1 and Cdc42, and major nodule of growth factor signalling. PAK1 evokes cellular ruffling, increases reorganisation of the actin cytoskeleton, induces rapid formation of lamellipodia as well as filopodia, and is associated with FAK, MEK1, and Raf phosphorylation (Yang et al, 2011: Du et al, 2010).

Typically, the motility of cells shows a biphasic behaviour with respect to adhesiveness to the substrate that leads to a well-defined spreading morphology typical on 2D surfaces (Wang et al, 2004). MDA-MB-231 cells have been shown to migrate more effectively on matrix coated surfaces in wound healing assays as opposed to control conditions (Maity et al, 2011: Yu and Machesky, 2012). This is predominately due to integrin-matrix signalling which was discussed in the last chapter. Migration on Matrigel and Fibronectin has shown to induce dynamic focal adhesion turnover and expression of MMPs through FAK and the PI3K signalling pathway (Maity et al, 2011: Yu and Machesky, 2012). In our experiment the morphology of cells migrating on plastic was not so different from those migrating on matrix-coated surfaces. This indicates an unknown degree of serum must have been present and not fully eluted in the resuspension phase. A reason why we observed a greater speed on plastic then what has been seen when compared to the matrix the ECM has been known to sequester and deposit growth factors such as EGF. This means cells migrating on the matrix may have had less exposure as opposed to those migrating on plastic (Shultz and Wysocki, 2008). Creating the wound through scraping has also been known to create barriers which could impede cell migration into the wound (Murrell et al, 2011). Observing migration over a longer may overcome these obstacles. Also, whilst many studies have used EGF at a concentration of 100ng/ml there is some debate that this is not the optimal concentration to use in wound healing assays. One study showed that whilst 100ng/ml EGF increased cell speed, the overall chemotactic and directionality response was decreased (Wang et al, 2004). This may have produced an unreliable true response of our cells migrating into the wound in our experiment.

4.3.2 EGF and FCS are inducers of breast cancer cell invasion

Our data shows that the chemoattractants EGF and FCS induce signalling that orchestrates cell migration machinery as in their absence invasion through the Matrigel basement extract supplemented with Fibronectin was significantly reduced. This coincides with what has been noted in other studies using EGF and FCS as chemoattractants (Hu et al, 2013: Abdelkarim et al, 2011). Persistent cellular movement during chemotactic experiments requires the establishment of a well-defined front to tail polarity towards the chemotactic gradient. This configuration is the result of key signalling events that allow the cell to alter its shape during migration. Many signalling pathways have been identified to operate downstream of the EGFR receptor, however the EGFR-GEP100-Arf6-AMAP1 axis has been described as a central pathway to MDA-MB-231 tumour cell invasion (Morishige et al, 2008: Sabe et al, 2009). It has been shown that inhibition of GEP100, Arf6, and AMAP1 abolishes the invasive phenotype and malignancy of breast cancer cells (Morishige et al, 2008). Moreover, knockdown of these

proteins effectively blocks EGF-induced Matrigel invasion. Arf6 and AMAP1 are essential in the formation of invadopodia at the cell front. Arf6 localises and binds Rac1 causing the activation of the PI3K signalling pathway allowing for the accumulation of PIP_3, the lipid product responsible for the maintenance of cell polarity and F-actin formation (Nakahara et al, 2003). AMAP1 binds to cortactin and is implicated in regulating the formation of invadopodia through recruitment of the Arp2/3 complex to existing actin microfilaments (Hashimoto et al, 2006). As previously mentioned, EGF also regulates FAK phosphorylation and dephosphorylation leading to increased motility through more dynamic temporal and spatial regulation of focal adhesions (Lu et al, 2001).

Whilst is has been defined that FCS can induce breast cancer cell invasion, as well as invasion of several other metastatic cancer cell lines, there is a lack of mechanistic study on how this may occur. This may be for the reason that there is a lack of consistency in the constituents that collectively make up the composition of FCS in differing batches meaning the quantification of invasion is not so easily reproducible. FCS contains a number of growth factors that can induce migration such as TGF-β, TGF-α, EGF, PDGF and insulin (Hsiao et al, 1987). Individually these are present in smaller concentration in FCS then what would be required for effective migration, however collectively they may induce a synergistic effect that allows for effective invasion (Hsiao et al, 1987). Our results showed that the combination of FCS with EGF had no synergistic effect and perhaps together was slightly suppressive. However due to only being a small decrease that was not significant we cannot fully conclude this as a suppressive effect and have to insinuate it as a result due to the nature of the experiment.

4.3.3 A5 and A3 are inducers of cell migration and invasion

Since its discovery, supporting evidence has shown that Nutlin, the molecule that our isoquinolinones are structurally based on, suppresses tumour growth and motility by preventing p53 proteasomal degradation (Moran and Maki, 2010). In this chapter we showed that the A5 isoquinolinone compound induces cell migration on ECM surfaces Fibronectin, Matrigel, and type I collagen. Furthermore, we also found that A5 modestly enhances invasion in MDA-MB-231 cells whilst A3 significantly drove cell invasion. Whilst no mechanistic function has been assigned to the isoquinolinone compounds, we can make an assumption on how they may elicit a metastatic phenotype through what is known about the functional activity of nutlin. A study by Moran and Maki showed that nutlin binds MDM2 preventing it from antagonising p53 which then performs various tumour suppressor functions such as

abrogating cancer cell growth, migration, invasion, and the proper formation of motile structures such as lamellipodia (Moran and Maki, 2010). Interestingly, they found that in sarcoma osteogenic cells nutlin actually induced cancer cell migration and invasion. The underlying explanation for this phenomenon was rationalized through the fact that sarcoma osteogenic cells have a mutant p53 as opposed to the other cell lines which had a wild-type p53(Moran and Maki, 2010). It has been identified that MDA-MB-231 breast cancer cells express high levels of mutant p53 which is characterised by both loss- and gain of function in tumourigenesis (Hui et al, 2006). Mutant p53 in cells reduces the transactivation of the lipid phosphatase PTEN leading to an increase of PIP_3 and subsequently PIP_3 –dependent GEFs which activates and regulates Rac and Cdc42. Moreover, a study has found that mutant p53 can activate Rac directly through unknown mechanisms (Yeudall et al, 2011). Alteration in p53 behaviour also leads to increased levels of GTP-bound RhoA and activation of its downstream effector ROCK which has been shown to drive rapid translocation of cells through Matrigel and collagen. In p53 mutant cells the intrinsic expression of collagens and Fibronectin is acquired allowing for more proficient interactions between the cell and the ECM matrix (Muller et al, 2011). Additionally gain of function p53 has been shown to upregulate the secretion of chemokine's involved in driving cell migration (Yeudall et al, 2011). These findings in previous studies on nutlin and mutant p53 provide a possible explanation on how our isoquionlinone compounds may work to induce cancer cell migration *in vitro*.

4.3.4 Summary

In this chapter we identified that EGF and the A5 compound can induce MDA-MB-231 breast cancer cell migration on Fibronectin, Matrigel, and type I collagen. Additionally, we also showed that EGF and FCS enhanced invasion of MDA-MB-231 through Matrigel, which was modest in response to A5 and absent in response to A3. In the next chapter we will investigate the mechanism of our isoquinolinone compounds and identify delineating pathways associated with cell migration.

Chapter 5: Isoquinolinones effect on gene expression

5.1 Introduction

In order for many cellular responses to be carried out the synthesis of new proteins is required. An environmental stimulus from a growth factor, mitogen, or motogen causes changes in protein expression which in some cases can be detected within minutes of stimulation. This alteration in expression is mediated by signal transduction pathways that regulate a multitude of transcription factors thereby allowing control of cellular processes (Parikh et al, 2010). An example of this would include the activation of several immediate early genes (IEGs) such as Egr-1 that can be activated within 30 minutes of stimulation by the MAPK-ERK signalling pathway (Sas-chen et al, 2012). As mentioned previously, there is currently no literature pertaining to the understanding of how isoquinolinones mechanistically work. Collectively studies have found that isoquinolinones have both intracellular and extracellular targets which are still being explored (Seitz et al, 2007: Rothweiler et al, 2008).

The displacement of mutant p53 from a pre-formed complex with MDM2 by intervention of the isoquinolinones is most likely to cause alterations in gene expression. Mutant p53 expression is high in many cancers and has been described to act as a transcription factor in its own right influencing promoter activity through the N-terminal transactivation domain (Muller et al, 2011). It has been shown that mutant p53 can repress MDM2 preventing Slug degradation and alleviate repression of Twist which allows for the partial induction of EMT (Wang et al, 2009, Kogan-Sakin et al, 2011). Other direct targets of mutant p53 that have been identified include guanine nucleotide dissociation inhibitor (RhoGDI), exchange factor for RhoA (Rho GEF-HI), and paxillin-β which are all implicated in Rho GTPase signalling and increased cell migration(Muller et al, 2011). There are however many more potential targets of mutant p53 still being identified that play a role in the induction of a more metastatic phenotype.

Since Isoquinolinones can also bind specific integrins, this allows for the assumption to be made that they can induce a diverse array of signalling cascades. Studies by Seitz et al showed that isoquinolinone derivatives could bind intergrin αvβ3 with high affinity (Seitz et al, 2007). Whilst these compounds had antagonist effects, it is possible that alterations in structure could lead to an agonistic effect that initiates signalling. Since this has yet to be explored it is without certainty which pathways may be activated in the presence of these compounds.

As shown in the previous two chapters, the synthetic A5 analogue reduced cell adhesion of our cells to the matrix, enhances cell migration, and to a lesser degree invasion. In this chapter some underlying mechanisms of enhanced migration and invasion are deduced. In order to accomplish this we performed an mRNA microarray screen to search for genes that are expressed in an isoquinolinone-specific manner.

5.2.2 Results

5.2.1 DNA microarray analysis identified an isoquinolinone gene expression signature

To determine whether isoquinolinone-specifc regulation of gene expression was responsible for influencing cell migration and invasion HT1080 cells were seeded onto plastic and Matrigel followed by treatment with either 0.5% DMSO or 50µM A3 compound for 24 hours by colleagues in the Gavrilovic lab. These conditions were used in the microarray because a lot of prior work had been conducted with HT1080 cells on Matrigel using A3. The time point was chosen from the HT1080 invasion assays as 24 hours is thought to be a sufficient period of time for the compound to have an effect causing gene expression changes that contribute to enhanced migration and invasion (Howe, J., Beven, D., Searcey, M. and Payerne, E. Gavrilovic, J unpublished observations). The initial analysis for the microarray was conducted by the Cambridge genomic services as explained in the materials and methods in chapter 2. The analysis of genes here was determined by an adjusted p-value of ≤0.05 and whether or not they were either up-, or down-regulated by 1.4 fold or greater. This cut-off point has been approved in many studies and it has been shown that 1.4 fold yields the most significant correlations between microarray and PCR data with higher cut-off points showing greater disparities (Morey et al, 2006). After applying our filtering criteria we obtained 70 genes on plastic, and 33 genes on Matrigel, whose genes expression was altered following A3 treatment as shown in Fig 5-1A/B and Fig 5-2A respectively. Of the 70 genes altered in cells grown on plastic, 34 were down-regulated and 36 were up-regulated in response to A3 as shown in Fig 5-1A and 5-1B respectively. For cells grown on Matrigel, out of the 33 altered genes, 27 were down-regulated and 6 were up-regulated (Fig 5-2A). These changes highlight that more genes were down-regulated in comparison to genes up-regulated in response to A3 when cells are grown on Matrigel. Whilst for cells growth on plastic there is a more even proportion of up- and down-regulated genes in response to A3. Additionally, there are more than double the

genes that are differentially regulated on plastic compared to Matrigel in A3 treated cells. Moreover,6 genes were down-regulated on both plastic and Matrigel in response to A3 (Fig 5-2B).

To further refine the list of genes whose expression was altered in the presence of the A3 compound, an additional filtering criterion was introduced. Using the DAVID (database for annotation, visualisation and integrated discovery) 6.7 web-based software all the differentially regulated genes were filtered by the functional annotation tool. This clustered the genes into high stringency functional groups which were determined by correlating those genes with individually selected literature based findings. With a focus on genes specific to cell migration the microarray surprisingly showed that several migration-associated genes such as *IL-8* and *CYR6R1* were down-regulated in response to A3. We hypothesized there may be a biphasic pattern of gene expression during the course of the 24 hour period. This has been shown in studies including one that treated HT1080 cells with trifluoperazine which increased the expression of *EGR1* up to 1 hour, and then was followed by a steady decline (Shin et al, 2001).   After analysing these genes from several studies displaying their effect and drawing up a preliminary paradigm 15 genes were carefully selected for further validation as displayed in Table 5-1.

A

B

Figure 5-1 Genes regulated in response to A3 on Plastic

HT1080 cells growth on plastic and stimulated with the A3 compound for 24 hours. RNA was extracted, labelled, and subjected to whole-genome expression profiling using Illumina expression chips. A) Genes up-regulated in response to A3 and B) Genes down-regulated in response to A3. Plotted genes are those which showed significant changes in expression p-value ≤0.05 and fold change of ≥1.4 when comparing A3 to DMSO.

Figure 5-2 Genes regulated in response to A3 on Matrigel and both plating conditions

A) Genes up-regulated and down-regulated in response to A3 and B) Genes that were regulated response to A3 on both matrix and plastic coated surfaces. Plotted genes are those which showed significant changes in expression p-value ≤0.05 and fold change of ≥1.4 when comparing A3 to DMSO.

Table 5-1- List of genes whose expression is regulated in an A3 compound dependent manner and involved in cell migration. Genes are those which showed significant changes in expression p-value ≤0.05 and fold change of ≥1.4 when comparing A3 to DMSO. "–"denotes no change.

Gene symbol	Fold change	
	Plastic	Matrigel
EGR1	↓2.4	–
CYR61	↓2.1	↓1.8
CTGF	↓1.9	↓1.6
IL-8	↓1.8	↓1.8
CCL2	↓ 1.5	–
CCL5	–	↓1.4
TGFA	↓ 1.5	–
FPR1	↓1.5	–
MYC	–	↓1.6
SAA1	–	↓ 1.6
ETS1	–	↓1.5
NFE2L3	–	↓1.4
EPHA2	–	↓ 1.4
HBEGF	–	↓1.7
RHOQ	–	↑1.4

5.2.2 Validation of A3-dependent gene expression in HT1080 cells using qRT-PCR

To verify A3-dependent gene expression signatures the HT1080 cDNA samples observed in the microarray were validated using qRT-PCR (Fig A2). All qRT-PCR experiments used the $\Delta\Delta C_t$ method to obtain the relative quantification which normalises our target genes against a reference 18s gene. Furthermore validation of a select few genes from HT1080 cells that were treated with the A3 compound for 4 hours in a separate experiment prepared by Dr Damon Bevan and Liam Pudwell was also conducted (Fig A1). Due to only a limited amount of cDNA sample remaining only seven genes were analysed from the 4 hour exposure experiment.

Out of the selected 15 genes, 10 validated by qRT-PCR correlated with the DNA microarray data at a 24 hour time point (Table 5-2). The five genes that did not correlate with the microarray data include *ETS1, FRP1, HBEGF, MYC,* and *NFE2L3* as shown by Figures A-1.G, H, I, K and L respectively. These genes failed to show significant changes in expression in response to A3; however they did show the same trend. Both *FPR1* and *HBEGF* showed a 0.06 significant down-regulation on Plastic and Matrigel respectively. Additionally, *CCL5* was found to be repressed only on Matrigel from the DNA microarray but our qRT-PCR data shows that A3 significantly repressed expression of *CCL5* on both plastic and Matrigel (Fig 5-4B and Fig 5-5A). This however is due to our cut-off point of 1.4 fold as *CCL5* was down-regulated in response to A3 on plastic in the DNA microarray but at 1.3 fold. The majority of genes were down-regulated in response to A3 by 1.5-2 fold. *EGR1* and *SAA1* showed a greater repression in mRNA by 8.5- and 4 fold respectively compared to the other genes (Fig 5-4E and Fig 5-5G).

At the 4 hour time interval we found that *CCL2* was suppressed greatly by 91 fold on plastic (Fig 5-3A). On both plastic and Matrigel A3 treated samples had high C_t values that ranged between 35 and 37 meaning either extremely low expression, or no expression of CCL2. This indicates A3 may repress expression of CCL2 completely at this time point in HT1080 cells. Whilst no other significant differences were found at 4 hours, it is worth noting that *RHOQ, CTGF, CYR61* and *IL-8* had elevated mRNA expression in response to A3 by 7.5-, 3-,2- and 9 fold respectively on plastic (Fig A-1F,B,C, and E). Moreover, *EGR1* had a non-significant increase in mRNA by 132 fold and 30 fold on plastic and Matrigel respectively in response to A3 treatment (Fig A-1D).

Table 2- HT1080 summarised gene expression response to A3 at 4- and 24 hours
Where variation in gene expression is significant when comparing A3 to DMSO treated cells the significance and fold change has been recorded. Fold change was worked out by dividing the average Log (2^-C_t) of each condition against each other. Microarray results integrated for comparison. "–" means no change. Significance was measured using the students paired *t*-test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Gene	4 hours		24 hours		*Microarray* 24 hours	
	PL	MG	PL	MG	PL	MG
CCL2	↓ 91 fold *	–	↓ 2 fold *	–	↓1.5 fold	–
CCL5	–	–	↓ 3 fold *	↓ 1.5 fold *	–	↓1.4 fold
CTGF	–	–	↓ 2 fold *	↓ 1.5 fold *	↓1.9 fold	↓1.6 fold
CYR61	–	–	↓ 2.5 fold *	↓ 1.5 fold *	↓2.1 fold	↓1.8 fold
EGR1	–	–	–	↓ 8.5 fold *	↓2.4 fold	–
EPHA2	–	–	–	↓ 1.5 fold *	–	↓1.4 fold
ETS1	–	–	–	–	–	↓1.5 fold
FRP1	–	–	–	–	↓1.5 fold	–
HBEGF	–	–	–	–	–	↓1.7 fold
IL-8	–	–	↓ 2 fold **	↓ 2 fold **	↓1.8 fold	↓1.8 fold
MYC	–	–	–	–	–	↓1.6 fold
NFE2L3	–	–	–	–	–	↓1.4 fold
RHOQ	–	–	–	↑ 1.5 fold *	–	↓1.4 fold
SAA1	–	–	–	↓ 4 fold **	–	↓1.6 fold
TGFA	–	–	↓ 1.5 fold *	–	–	↓1.4 fold

A
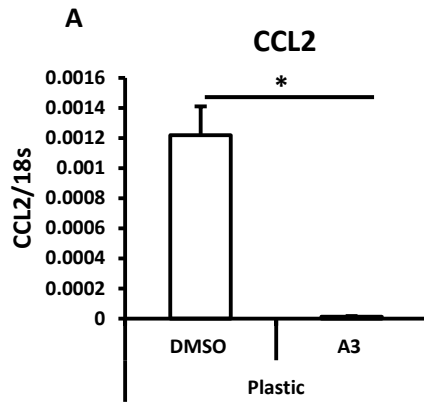
Figure 5-3 HT1080 expression of genes in response to A3 compared to DMSO at 4 hours Expression levels determined by qRT-PCR at 4 hours in HT1080 cells A) is the CCL2 gene. Each bar represents mean ± S.E.M of four samples and is displayed as the relative quantification. All mRNA expression is normalised to 18s control. Significance was measured using the students paired *t*-test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. This experiment was performed once.
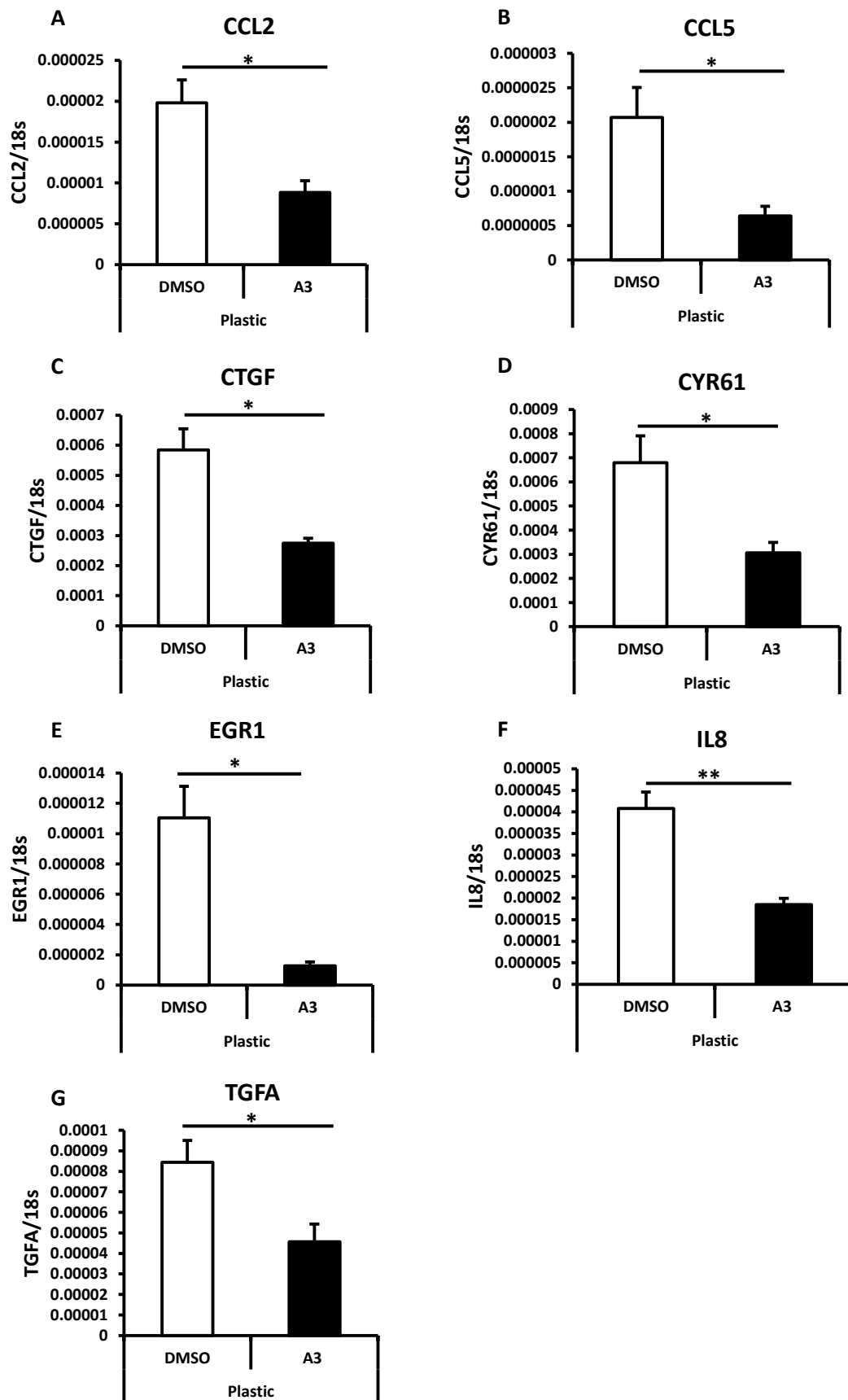
Figure 5-4 Plastic plated HT1080 cells gene expression in response to A3 compared to DMSO at 24 hours Expression levels determined by qRT-PCR at 24 hours in HT1080 cells. Genes include (A) CCL2 (B) CCL5 (C) CTGF (D)CYR61 (E)EGR1 (F)IL8 (G)TGFA. Each bar represents mean ± S.E.M of four samples and is displayed as the relative quantification. All mRNA expression is normalised to 18s control. Significance was measured using the students paired t-test, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. This experiment was performed once.

92
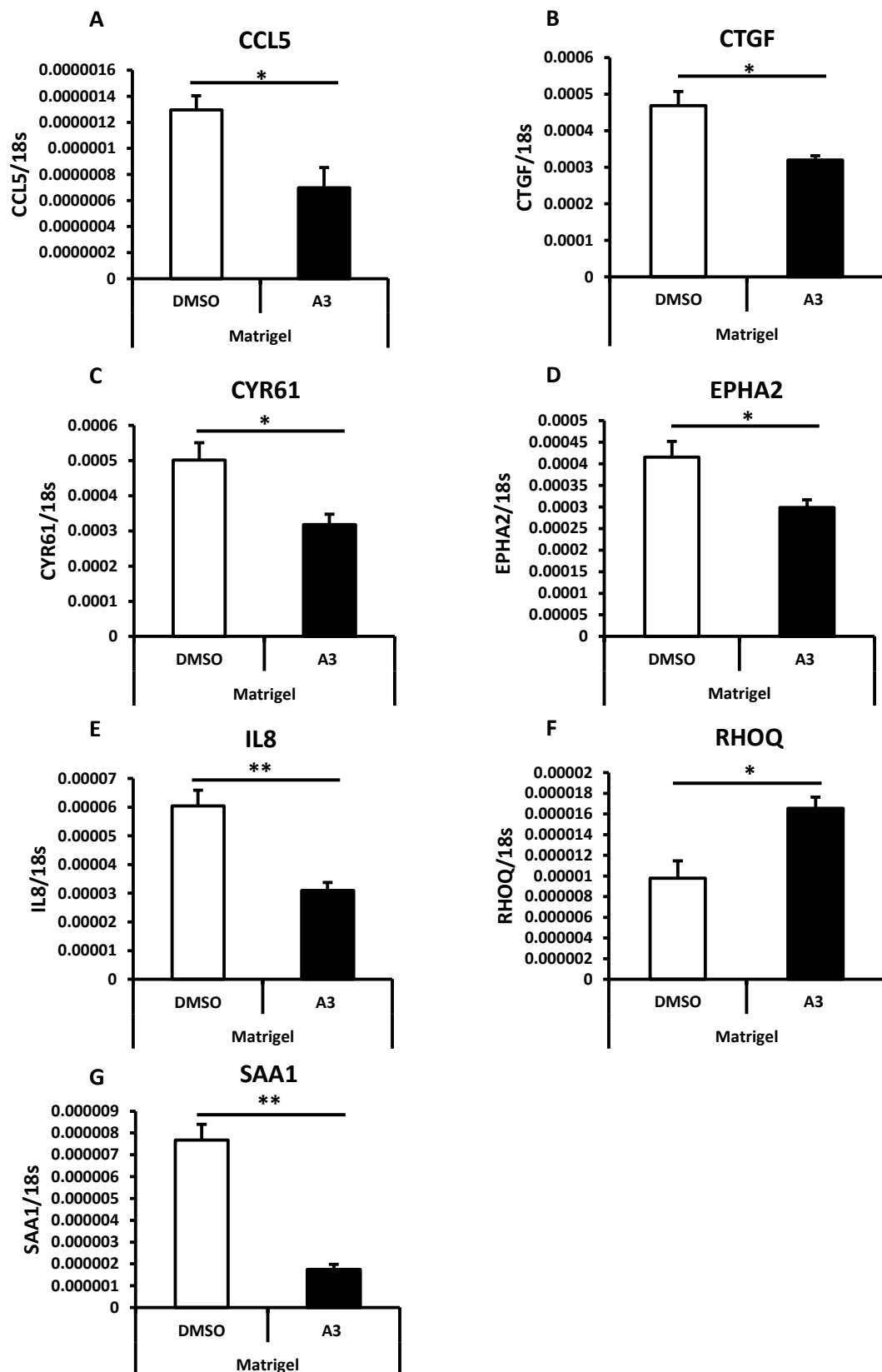
Figure 5-5 Matrigel plated HT1080 cells gene expression in response to A3 compared to DMSO at 24 hours Expression levels determined by qRT-PCR at 24 hours in HT1080 cells. Genes include (A) CCL5 (B) CTGF (C) CYR61 (D)EPHA2 (E) IL8 (F) RHOQ (G)TGFA. Each bar represents mean ± S.E.M of four samples and is displayed as the relative quantification. All mRNA expression is normalised to 18s control. Significance was measured using the students paired t-test, $*p{\leq}0.05$, $**p{\leq}0.01$, $***p{\leq}0.001$. This experiment was performed once.

5.2.3 A5-dependent gene expression in MDA-MB-231 cells

Following validation of our chosen genes in HT1080 cells in response to A3 we looked to see if these same genes may be regulated in MDA-MB-231 cells in response to the structurally identical A5 compound which also induced cell migration (Table 5-3 and Fig A-3,A-4 and A-5). At a 4 hour time point the expression of six genes, including *CYR61, EGR1, ETS1, HBEGF, MYC,* and *TGFA* were significantly up-regulated in response to A5 treatment (Fig A-3D,E,G,I,K, and O). The expression of three of those genes, including *HBEGF, MYC,* and *TGFA* was significantly up-regulated on plastic (Fig 5-6C, D and E), whilst *EGR1* was significantly up-regulated on Matrigel plus Fibronectin (Fig 5-7B). *CYR61* and *ETS1* expression was increased significantly on both plastic and Matrigel plus Fibronectin (Fig 5-6A,B and Fig 5-7A,B). Three of those genes encode secretable factors CYR61, HBEGF and TGFA. At the 4 hour time point *EGR1* had the greatest increase in expression at 154 fold on Matrigel plus Fibronectin compared to the other genes which had an increase of expression between 1.5- and 4.5 fold. Moreover, whilst not significant, a notable increase in mRNA expression on plastic was seen for *EGR1* and for *CTGF,CCL5, TGFA, HBEGF* and *MYC* on Matrigel plus Fibronectin. *IL-8* had a notable non-significant increase in expression on both plastic and Matrigel plus Fibronectin at 4 hours.

At 8 hours, A5 positively regulated the expression of four genes, including *EGR1, ETS1, HBEGF* and *IL8* (Fig 5-8B,C,E and Fig 5-9AB-E)*,* and negatively regulated four genes, including *FPR1, CCL2, TGFA* and *SAA1* (Fig 5-8A,D,F,G and Fig 5-9A,F,G). All the genes were significantly regulated on plastic and Matrigel plus Fibronectin with the exception of *HBEGF* which was regulated on just Matrigel plus Fibronectin. Two of those genes that were positively upregulated encode secretable factors HBEGF and IL8.Additionally, all genes that were regulated on both conditions at 8 hours saw a greater fold increase on Matrigel plus Fibronectin than on plastic. As with the 4 hour time point, *EGR1* had the greatest increase in expression out of all our candidate genes showing a 45 fold increase on plastic and 78 fold increase on Matrigel plus Fibronectin. At 8 hours *CCL2* had the greatest mRNA repression in response to A5 with a 17 fold and 13 fold decrease on plastic and Matrigel plus Fibronectin respectively (Fig 5-8A and Fig 5-9A). Moreover, genes that did not show a significant difference but had a noticeable increase in expression in response to A5 include *RHOQ, EPHA2* and *MYC* on Matrigel plus Fibronectin, and *HBEGF* on plastic (Fig A-4 M, F, K and I).

At the final time point of 24 hours, A5 positively regulated the expression of four genes, including *EGR1, ETS1, MYC,* and *RHOQ* (Fig 5-10C,E, Fig 5-11-1 C,E,F and Fig 5-11-2 B) and negatively regulated five genes, including *CTGF, CYR61,EPHA2, NFE2L3,* and *TGFA* (Fig 5-

10A,B,D,G,H, 5-11-1A,B,D and 5-11-2 A,C) . *EGR1* and *ETS1* expression was significantly increased on both plastic and Matrigel plus Fibronectin, whilst *MYC* and *RHOQ* was only significantly increased on Matrigel plus Fibronectin. It is worth noting though that both these genes had a notable, but not significant increase on plastic also (A-5M and K). All the genes that expression was significantly repressed by A5 occurred on both plastic and Matrigel plus Fibronectin. At 24 hours *ETS1* had the greatest A5 induced expression with a 4.5- and 3.5 fold increase on plastic and Matrigel plus Fibronectin respectively (Fig 5-10E and 5-11-1E). *NFE2L3* was the most A5 repressed gene with a 9 fold decrease on plastic and 7 fold decrease on Matrigel plus Fibronectin. There is also a greater repression in response to A5 for the same genes in cells grown on plastic compared to cells grown on Matrigel plus Fibronectin. It also appears to A5 completely suppressed the expression of *CCL2*, *FPR1* and *SAA1* even though overall expression of these genes on all conditions at 24 hours was quite low.

Overall these data indicate that A5 regulates several different genes in a unique fashion over the 24 hour time course. Some genes are regulated by A5 as an early response and then quickly repressed. An example would include *TGFA* which is up-regulated at 4 hours but then repressed at 8- and 24 hours. The expression of other genes such as *EGR1* is highly up-regulated at 4 hours, but then decreases over time. The transcription factor *ETS1* was found to be positively expressed quite consistently throughout the 24 hour period relative to its expression in cells grown in DMSO. Some genes such as *IL-8* see the greatest regulation induced by A5 at 8 hours as opposed to the other time points. Finally it appears that A5 suppresses other genes that are not very highly expressed such as *SAA1* over time to the point where the expression levels are undetectable at 24 hours. Additionally to coincide with this data we generated moving averages graph from our random migration experiments looking at cell speed in response to A5 and DMSO on Matrigel and Fibronectin. Interestingly we found the greatest spike in speed increase to occur just after 8 hours in A5 treated cells and a decrease in speed at the same time point in DMSO treated cells on Matrigel (Fig. A-6 and Fig A-7). There also appears to be a noticeable increase in speed between 3- and 4 hours on Fibronectin (Fig A-6B).

Table 5-3 MDA-MB-231 summarised gene expression response to A5 at 4- , 8- and 24 hours   Where variation in gene expression is significant when comparing A5 to DMSO treated cells the significance and fold change has been recorded. "–" means no change. Fold change was worked out by dividing the average Log (2^-C_t) of each condition against each other. Significance of A5 treated cells against DMSO treated cells was measured using the students paired *t*-test, *p≤0.05, **p≤0.01, ***p≤0.001.

Gene	4 hours		8 hours		24 hours	
	PL	MGFN	PL	MGFN	PL	MGFN
CCL2	–	–	↓17 fold *	↓13 fold ***	*No expression*	
CCL5	–	–	–	–	–	–
CTGF	–	–	–	–	↓4 fold *	↓3.5 fold **
CYR61	↑4.5 fold *	↑3.5 fold *	–	–	↓3 fold *	↓2 fold **
EGR1	–	↑154 fold **	↑45 fold *	↑78 fold *	↑2 fold *	↑2 fold *
EPHA2	–	–	–	–	↓1.5 fold **	↓1.5 fold **
ETS1	↑2 fold *	↑3 fold *	↑4 fold **	↑6 fold *	↑4.5 fold **	↑3.5 fold *
FRP1	–	–	↓2 fold *	–	↓24 fold **	*No expression*
HBEGF	↑2 fold *	–	–	↑4 fold *	–	–
IL-8	–	–	↑2.5fold **	↑4.5 fold ***	–	–
MYC	↑1.5 fold *	–	–	–	–	↑2 fold *
NFE2L3	–	–	–	–	↓9 fold **	↓7 fold **
RHOQ	–	–	–	–	–	↑2 fold *
SAA1	–	–	↓1.5fold *	↓1.5 fold *	*No expression*	
TGFA	↑2 fold *	–	↓2.5fold ***	↓2 fold **	↓6fold **	↓4 fold **

A CYR61

B ETS1

C HBEGF

D MYC

E TGFA

Figure 5-6 Plastic plated MDA-MB-231 cell gene expression in response to A5 compared to DMSO at 4 hours Expression levels determined by qRT-PCR at 4 hours in MDA-MB-231 cells. Cells were grown on plastic and treated with either DMSO or A5. Genes include (A) CYR61 (B)ETS1 (C)HBEGF D)MYC E) TGFA. Each bar represents mean ± S.E.M of four samples and is displayed as the relative quantification. All mRNA expression is normalised to 18s control. Significance was measured using the students paired *t*-test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. This experiment was performed once.
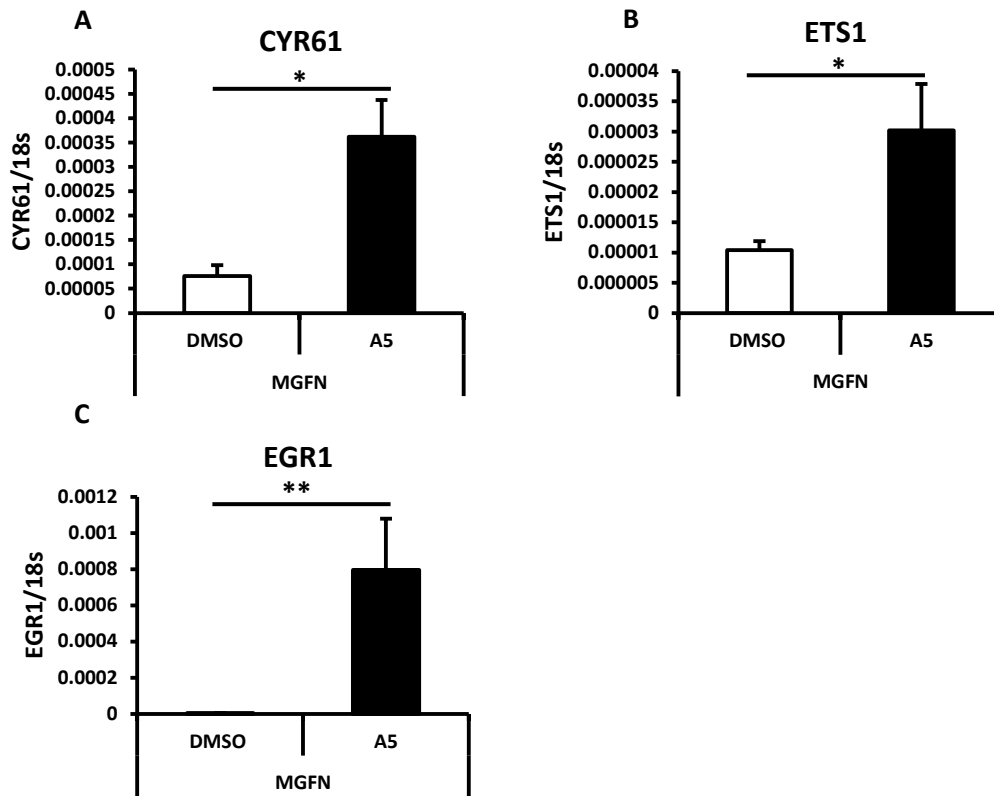
Figure 5-7 Matrigel + Fibronectin plated MDA-MB-231 cell gene expression in response to A5 compared to DMSO at 4 hours Expression levels determined by qRT-PCR at 4 hours in MDA-MB-231 cells. Cells were grown on Matrigel plus Fibronectin (MGFN) and treated with either DMSO or A5. Genes include (A) CYR61 (B)ETS1 (C) EGR1. Each bar represents mean ± S.E.M of four samples and is displayed as the relative quantification. All mRNA expression is normalised to 18s control. Significance was measured using the students paired *t*-test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. This experiment was performed once.
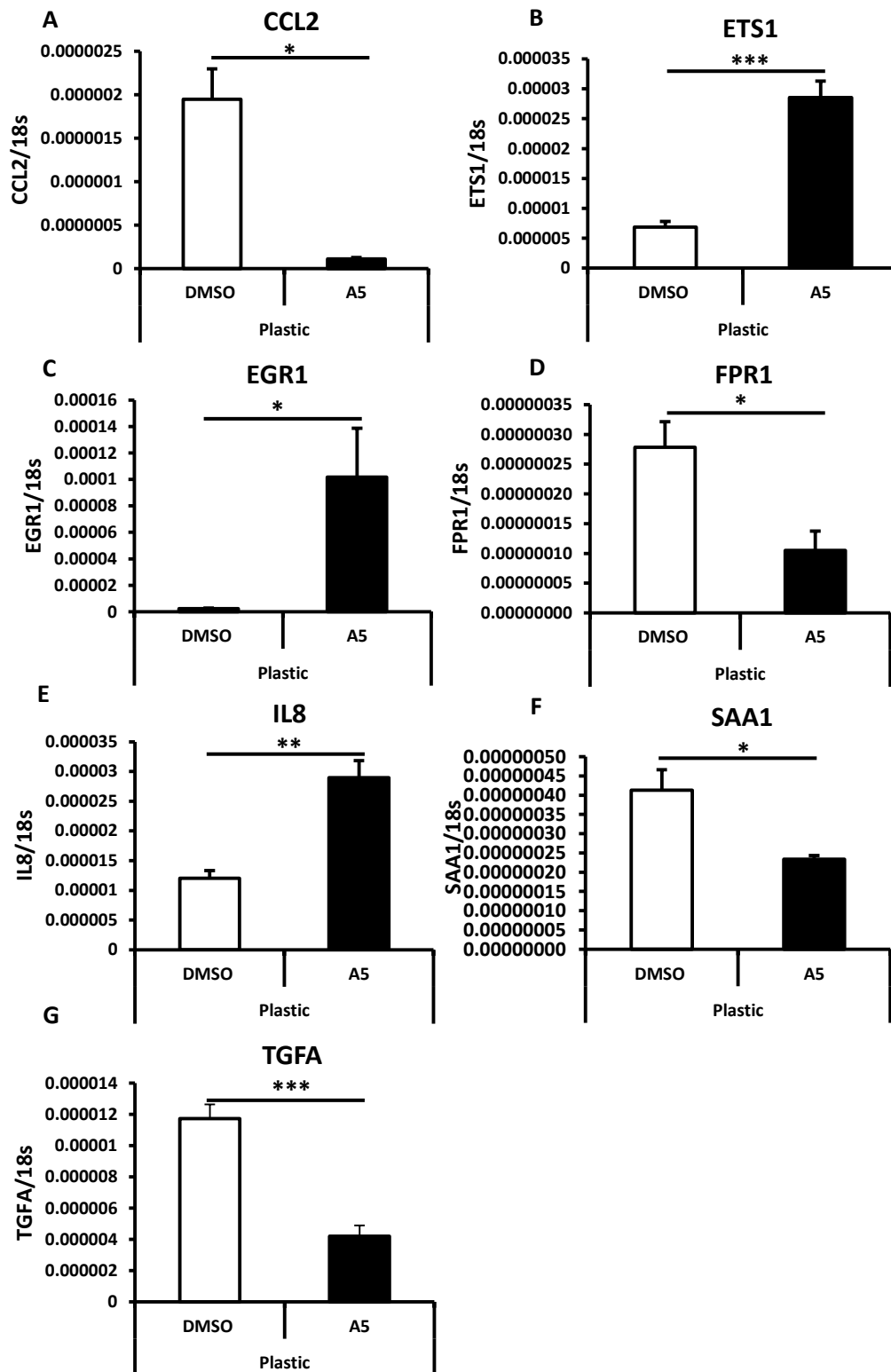
Figure 5-8 Plastic plated MDA-MB-231 cells gene expression in response to A5 compared to DMSO at 8 hours Expression levels determined by qRT-PCR at 8 hours in MDA-MB-231 cells. Cells were grown on plastic treated with either DMSO or A5. Genes include (A) CCL2 (B)ETS1 (C) EGR1 (D) FPR1 (E)IL8 (F)SAA1 (G)TGFA.Each bar represents mean ± S.E.M of four samples and is displayed as the relative quantification. All mRNA expression is normalised to 18s control. Significance was measured using the students paired t-test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. This experiment was performed once.
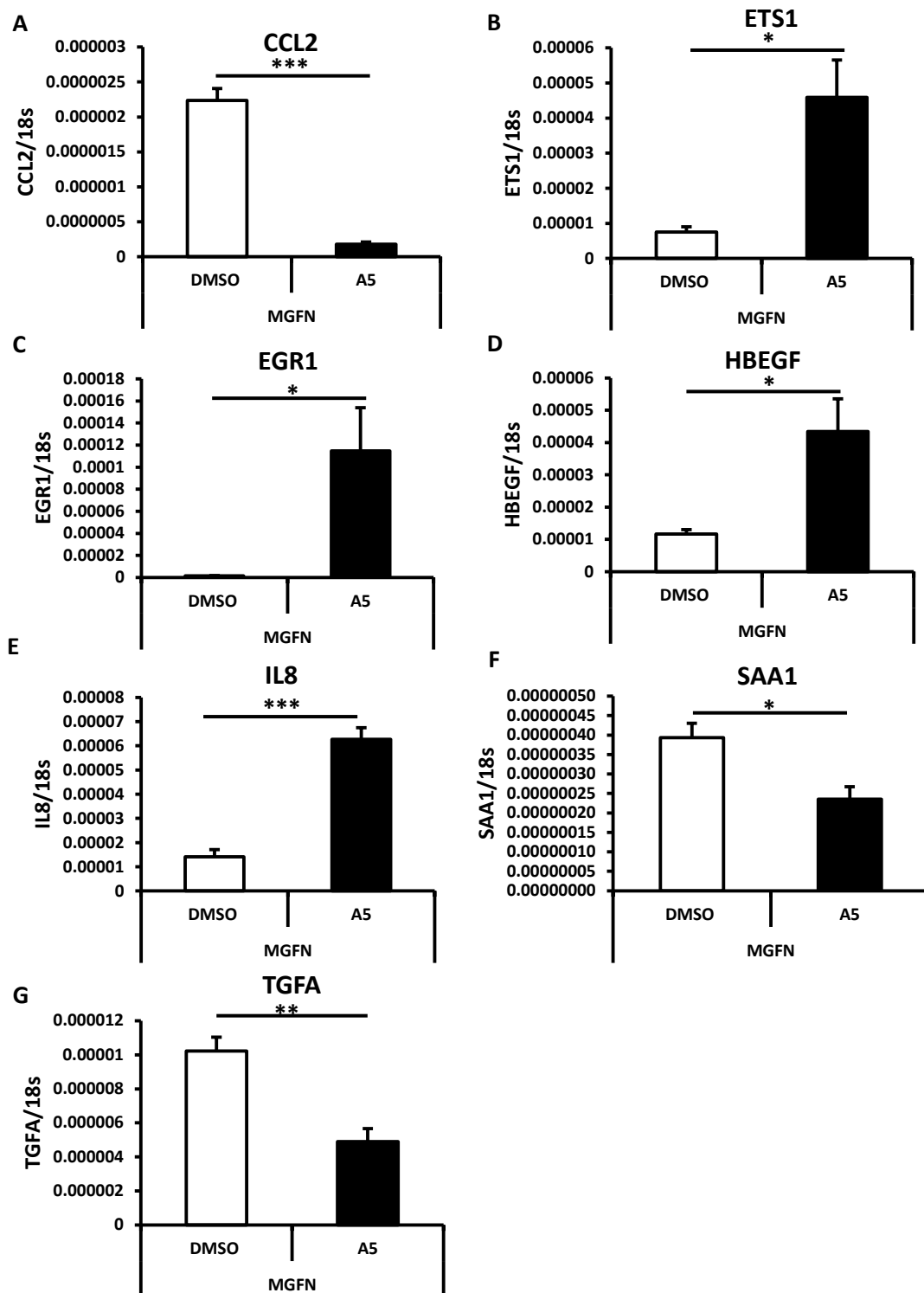
Figure 5-9 Matrigel +Fibronectin plated MDA-MB-231 cells gene expression in response to A5 compared to DMSO at 8 hours Expression levels determined by qRT-PCR at 8 hours in MDA-MB-231 cells. Cells were grown on Matrigel plus Fibronectin (MGFN) and treated with either DMSO or A5. Genes include (A) CCL2 (B)ETS1 (C) EGR1 (D)HBEGF (E)IL8 (F)SAA1 (G)TGFA.Each bar represents mean ± S.E.M of four samples and is displayed as the relative quantification. All mRNA expression is normalised to 18s control. Significance was measured using the students paired *t*-test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. This experiment was performed once.
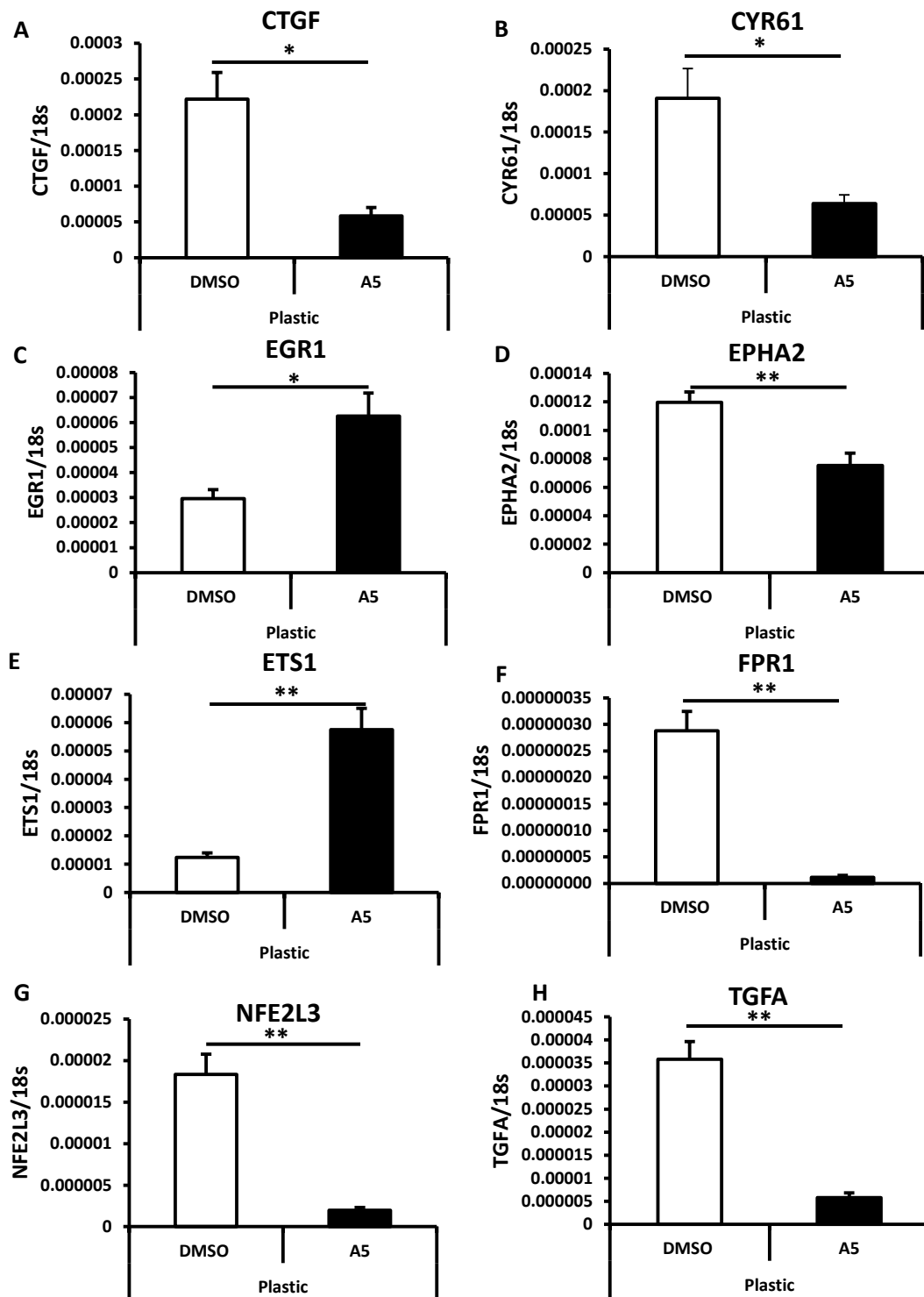
Figure 5-10 Plastic plated MDA-MB-231 cell gene expression in response to A5 compared to DMSO at 24 hours Expression levels determined by qRT-PCR at 24 hours in MDA-MB-231 cells. Cells were grown on plastic and treated with either DMSO or A5. Genes include (A) CTGF (B)CYR61 (C)EGR1 (D)EPHA2 (E)ETS1 (F)FPR1 (G)NFE2L3 (H)TGFA.Each bar represents mean ± S.E.M of four samples and is displayed as the relative quantification. All mRNA expression is normalised to 18s control. Significance was measured using the students paired *t*-test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. This experiment was performed once.
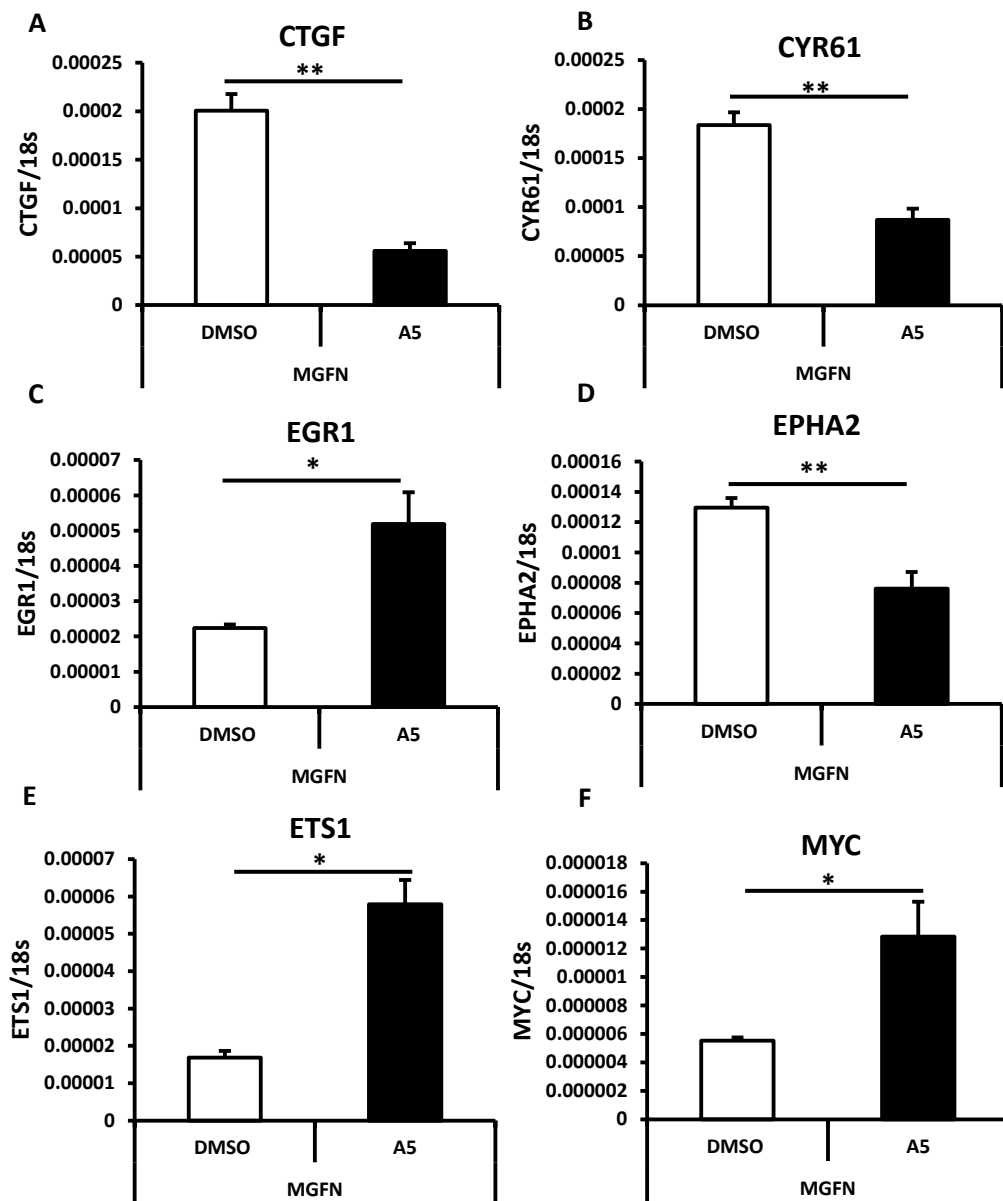
Figure 5-11-1 Matrigel + Fibronectin plated MDA-MB-231 cells gene expression in response to A5 compared to DMSO at 24 hours Expression levels determined by qRT-PCR at 24 hours in MDA-MB-231 cells. Cells were grown on Matrigel plus Fibronectin (MGFN) and treated with either DMSO or A5. Genes include (A) CTGF (B) CYR61 (C) EGR1 (D) EPHA2 (E) ETS1 (F) MYC.Each bar represents mean ± S.E.M of four samples and is displayed as the relative quantification. All mRNA expression is normalised to 18s control. Significance was measured using the students paired t-test, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. This experiment was performed once.

A

NFE2L3

B
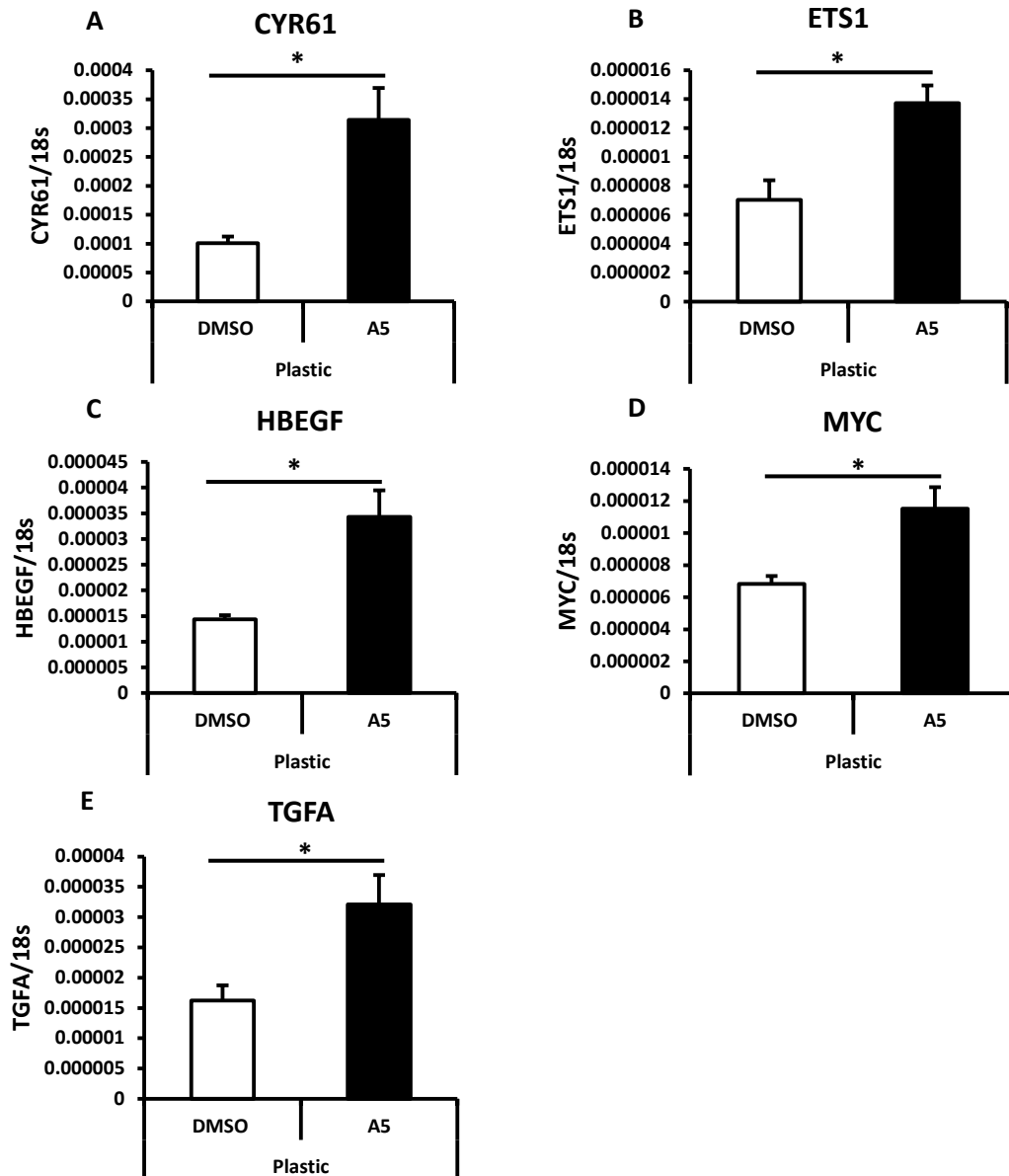
RHOQ

C

TGFA
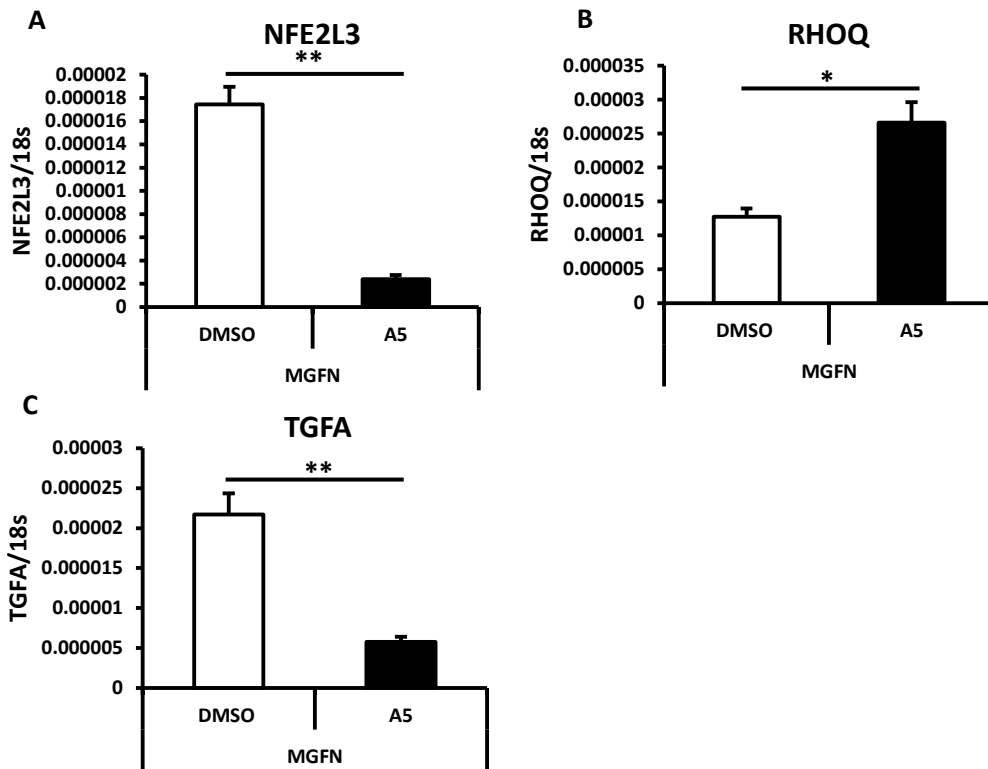
Figure 5-11-2 Matrigel + Fibronectin plated MDA-MB-231 cells gene expression in response to A5 compared to DMSO at 24 hours Expression levels determined by qRT-PCR at 24 hours in MDA-MB-231 cells. Cells were grown on Matrigel plus Fibronectin (MGFN) and treated with either DMSO or A5. Genes include (A)NFE2L3 (B) RHOQ (C) TGFA.Each bar represents mean ± S.E.M of four samples and is displayed as the relative quantification. All mRNA expression is normalised to 18s control. Significance was measured using the students paired *t*-test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. This experiment was performed once.

5.2.4 Comparison of A5 and A3-dependent gene expression in MDA-MB-231 and HT1080 cells

The comparison between the effects of isoquinolinone compound A3 and A5 on the cell lines HT1080 and MDA-MB-231 respectively should be taken cautiously due to only analysing seven genes at 4 hours and no genes at 8 hours in A3 treated HT1080 cells. Also each experiment was only performed once in HT1080 and MDA-MB-231 cells. With a large body of data unavailable this comparison serves more as a general reference of interest as opposed to an absolute conclusion of similarity between the compounds.

Our data shows that the genes *CCL2, SAA1, CTGF, CYR61* and *EPHA2* are all repressed at 24 hours in both cell lines in response to their respective compound. Both compounds appeared to up-regulate the expression of *RHOQ* at 24 hours which was the only gene to be positively regulated by A5 and A3 in both cell lines. *EGR1* and *MYC* were repressed by A3 in HT1080 cells but expressed in MDA-MB-231 in response to A5 at 24 hours. Furthermore, at 24 hours *CCL5* is repressed in HT1080 but not MDA-MB-231, and conversely the opposite is seen with *NFE2L3* which is repressed in MDA-MB-231 but not HT1080. Essentially, at 4 hours even though there was not a significant difference, there was a noticeable increase in some genes in A3 treated HT1080 that correlate with the A5 treated MDA-MB-231 cells. The genes include *EGR1, CYR61,* and *IL-8* and we speculate the reason that they may not significant is due to some variation in expression in the 18s samples.

In both cell lines we noticed that there seems to be a difference in gene expression between cells grown on plastic, Matrigel, or Matrigel plus Fibronectin coated surfaces. In A5-treated MDA-MB-231 cells there was an increase in *FPR1, CCL2* and *RHOQ* expression on Matrigel plus Fibronectin compared to plastic at 4 hours (Fig 5-12A,E,F,G,H) and visa versa was seen for *CTGF* at 4 hours (Fig 5-12C) . In A3-treated HT1080 cells there was repression in *CCL2* and *FPR1* expression at 24 hours on Matigel compared to plastic (Fig 5-12B and D).
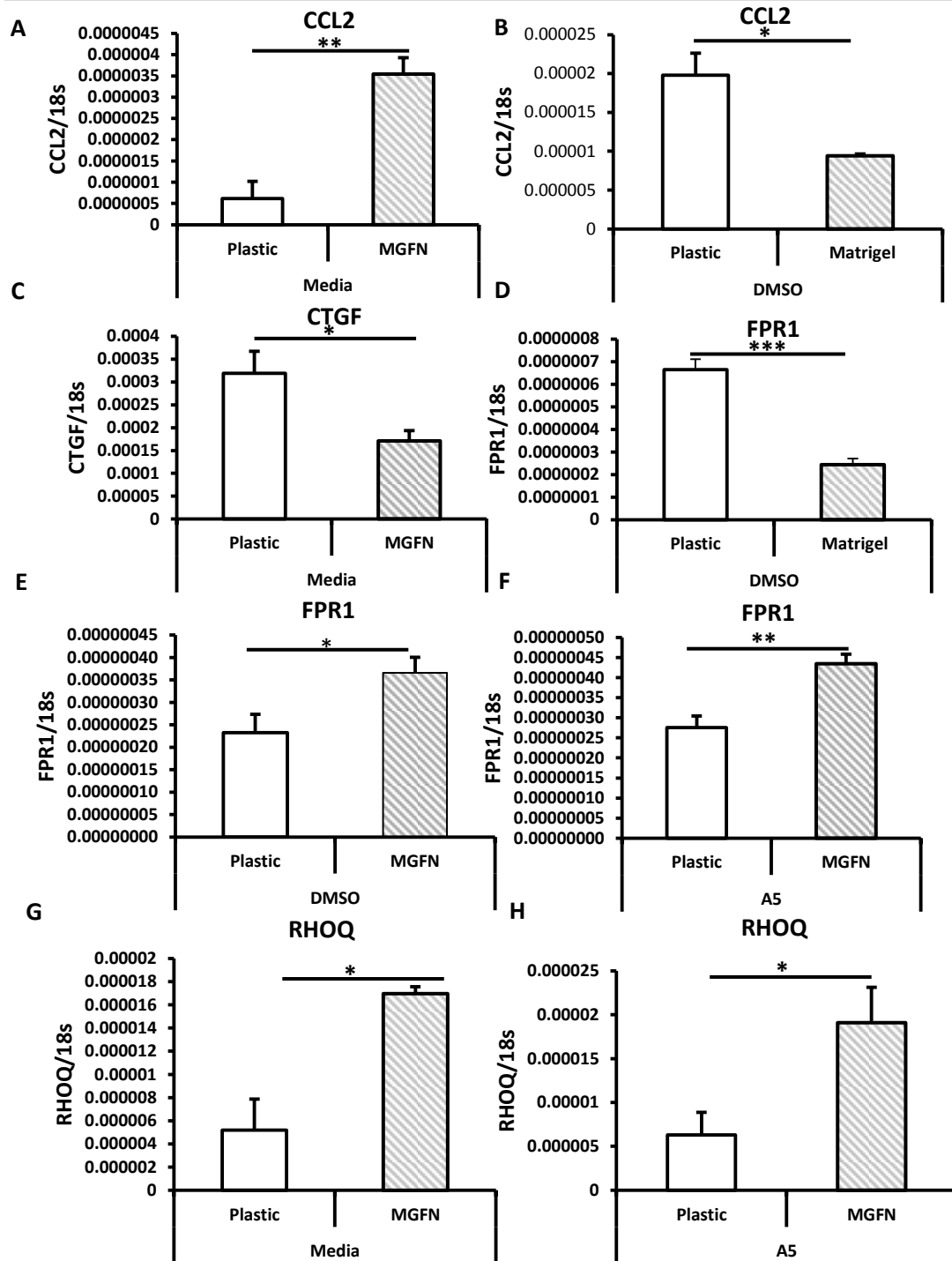
Figure 5-12 Differences in gene expression between cells grown on plastic and matrix coated surfaces Expression levels determined by qRT-PCR. Cells were grown on either plastic, Matrigel, or Matrigel plus Fibronectin (MGFN). Differences in expression were identified in some genes depending on the surface the cells were grown on in A5/A3 experiments. Genes include A) **CCL2**-*MDA-M*B-231 4hr B) **CCL2**-*HT1080 24hr* C) **CTGF**-*MDA-M*B-231 4hr D) **FPR1**-*HT1080 24hr* E,F) **FPR1**-*MDA-MB-231 4hr* G,H) **RHOQ**-*MDA-M*B-231 4hr.Each bar represents mean ± S.E.M of four samples and is displayed as the relative quantification. All mRNA expression is normalised to 18s control. Significance was measured using the students paired *t*-test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. This experiment was performed once.

5.2.5 Genomatrix generated paradigm

All genes identified to be regulated by the isoquinolinones in this study were input into the Genomatrix software suite. This software conducts a scientific analysis based on comprehensive literature searches which allows for the generation of networks and pathways (Fig 5-13). Here it shows that the transcription factor Ets1 can bind and modulate CTGF,Egr1 and IL8. Egr1 is shown to modulate the activity of Ets1, NFE2L3, HBEGF and RhoQ. Myc, another transcription factor, can activate, and be activated by TGF-α. Myc has been found to inhibit FPR1, EphA2, and CTGF but modulate the activity of Cyr61. IL8 interacts with HBEGF and SAA1, and has a known binding site for activation by chemokines CCL5 and CCL2.
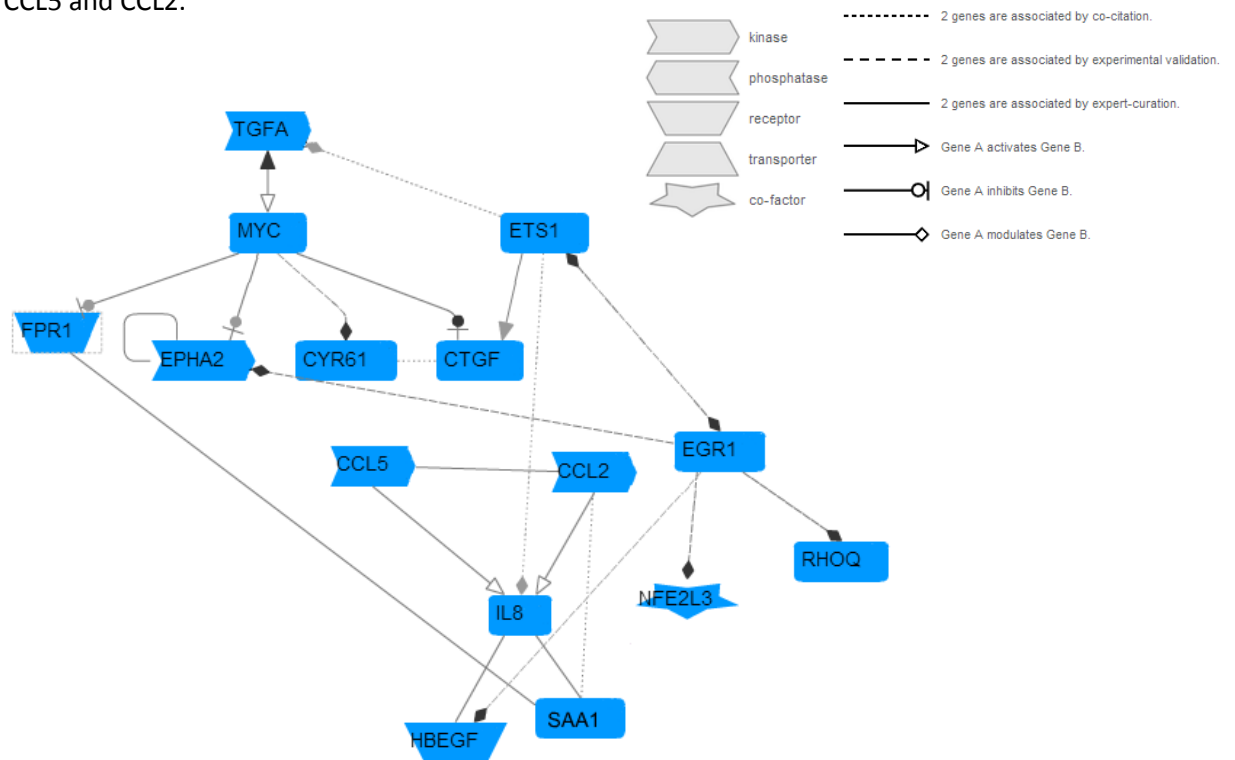
Figure 5-13 Schematic diagram depicting paradigm of isoquinolinone induced gene expression Generated using Genomatrix software. Our fifteen candidate genes are linked through what has been established in published literature to construct a plausible paradigm.

5.2.6 Chemokines CCL2 and CCL5 induce cancer cell migration

To determine a role for chemokine signalling in mammary breast carcinoma cells and fibrosarcoma cells both MDA-MB-231 and HT1080 cells were seeded onto 2D planar ECM coated surfaces and treated with 20ng/ml recombinant CCL2 and CCL5. The concentration of the chemokines used in these experiments was based on previous studies predominantly using 20ng/ml as the optimal concentration to induce cell migration. Migration tracks can be seen in Figures 5-15, 5-17, 5-19 and 5-21. During these experiments changes for cell speed, proliferation, and directionality were evaluated over a 17 hour time period. Taken together, both CCL2 and CCL5 did not induce a significant effect in cell proliferation of MDA-MB-231 or HT1080 cells during the 17 hour stimulation (Fig 5-14B, Fig 5-16B, 5-18B and 5-20B). Furthermore, there is no indication between the two chemokines of a pattern suggesting they may induce cell proliferation as the results show highly variable rates of division on the same ECM substrates between experiments. CCL2 and CCL5 treatment significantly enhanced migration of both MDA-MB-231 and HT1080 cells. The addition of CCL2 increased the speed of migration by 34% and 22% on Matrigel in MDA-MB-231 and HT1080 cells respectively (Fig5-14A and 5-18A). CCL2 also significantly increased the speed of HT1080 cells migration on Fibronectin by 37% and whilst not significant, a notable increase in speed by 21% was observed in MDA-MB-231 cells. The chemokine CCL5 enhanced cell migration on Fibronectin by 25% and 18% in MDA-MB-231 and HT1080 cells respectively (Fig 5-16A and Fig 5-20A). Additionally, CCL5 also increased the speed of MDA-MB-231 cells by 18% on Matrigel but had no distinguished effect on HT1080 cells. Interestingly, in both cell lines migrating in response to CCL2 and CCL5 treatment, there was no notable effect on type I collagen which consistently had the greatest overall migration speed of cells in both the presence and absence of the chemokines. Both cell lines used for these experiments are highly metastatic which may account for the similar migration speeds observed between the two cell lines across all ECM surface conditions. The addition of the chemokines did not have any effect on the directionality of the cells on any of the substrates and so therefore did not enhance their ability to move in straight lines for more efficient migration (Fig 5-14C, Fig 5-16C, 5-18C and 5-20C).
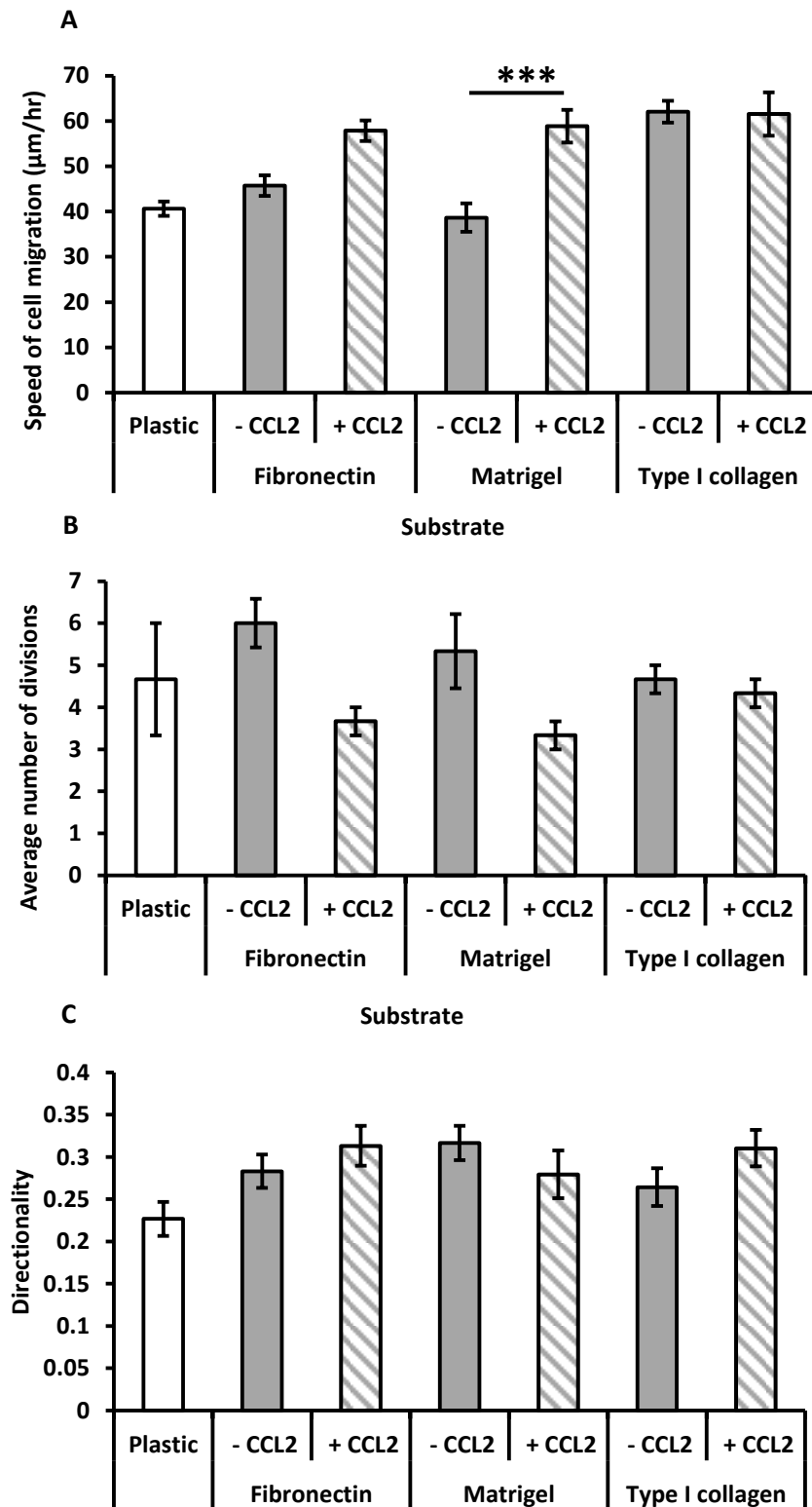
Figure 5-14 MDA-MB-231 migration in response to chemokine MCP-1/CCL2
MDA-MB-231 cells were seeded on either 10μg/ml Matrigel, Fibronection or type I
collagen and treated with 20ng/ml CCL2. Using time-lapse microscopy an image was
taken every 10 minutes for a total period of 17 hours. A) The speed of cell migration, B)
Number of divisions and C) directionality were pulled from this data. Each bar represents
the mean ± S.E.M of 15 tracks from 3 separate wells. Significance was measured using
the students paired *t*-test. This experiment was performed once.
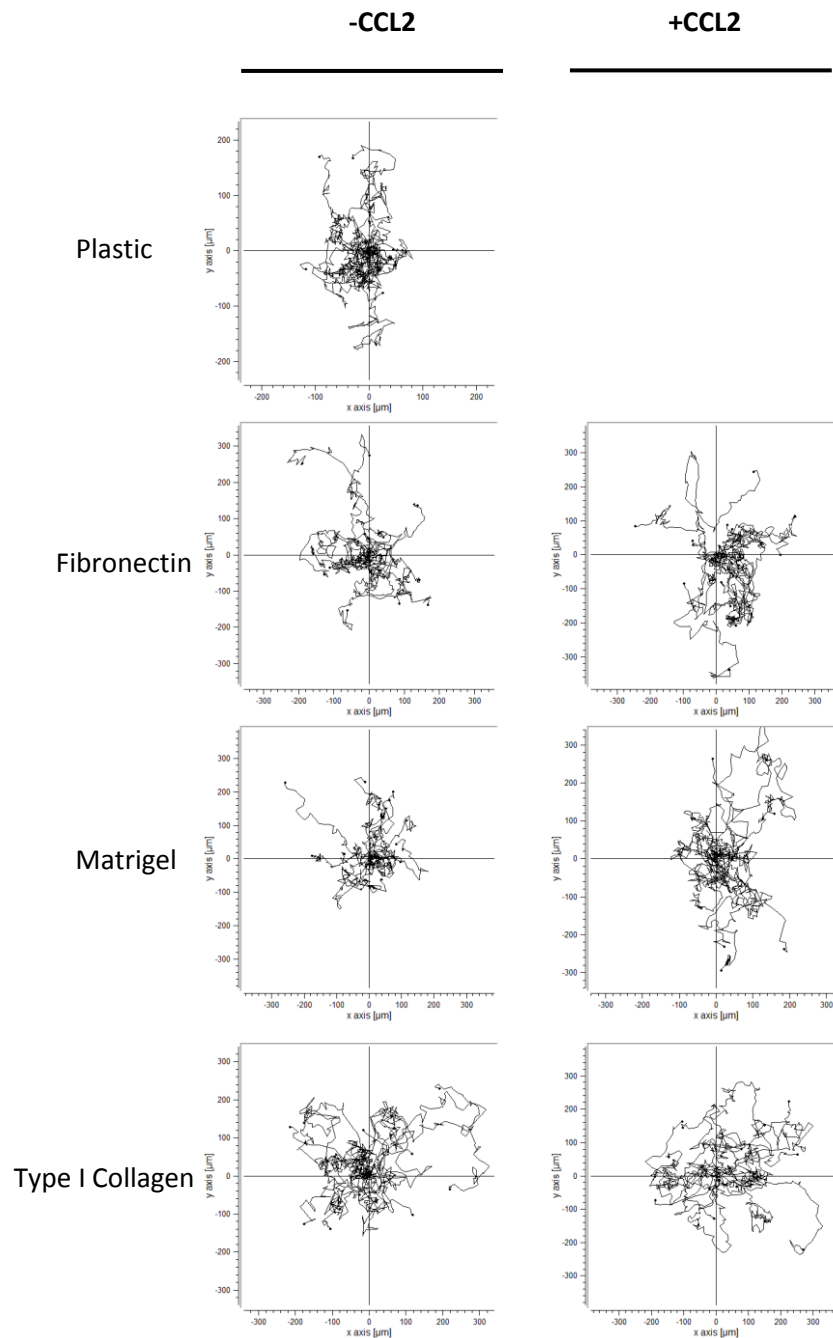
Figure 5-15 MDA-MB-231 migration in response to chemokine MCP-1/CCL2
Migration plots illustrating the motility and movement of MDA-MB-231 cells on either 10μg/ml Matrigel, Fibronection or type I collagen with or without 20ng/ml CCL2 . This experiment was performe once.
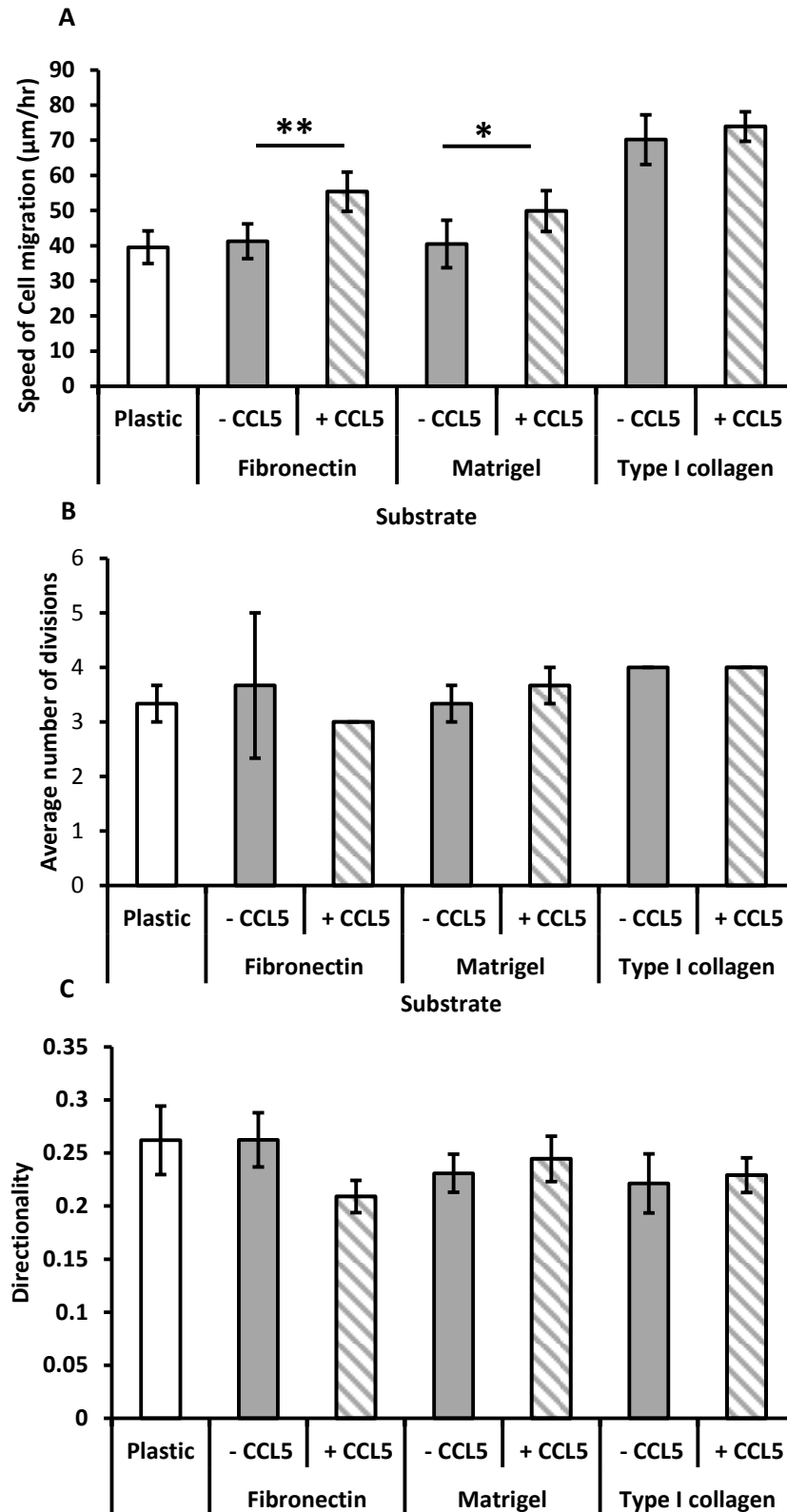
Figure 5-16 MDA-MB-231 migration in response to chemokine RANTES/CCL5
MDA-MB-231 cells were seeded on either 10µg/ml Matrigel, Fibronection or type I collagen and treated with 20ng/ml CCL5. Using time-lapse microscopy an image was taken every 10 minutes for a total period of 17 hours. A) The speed of cell migration, B) Number of divisions, and C) directionality were pulled from this data. Each bar represents the mean ± S.E.M of 15 tracks from 3 separate wells. Significance was measured using the students paired *t*-test. This experiment was performed once.
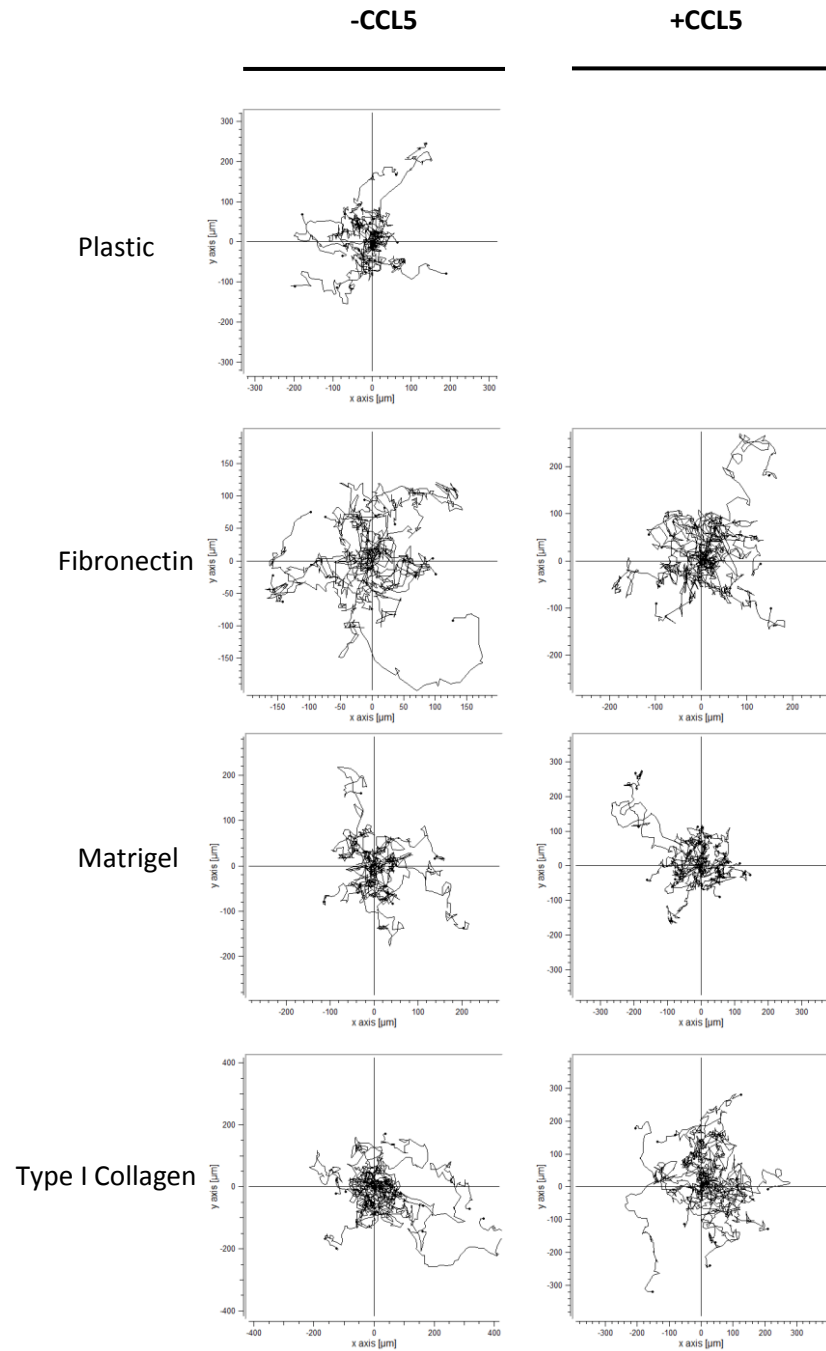
Figure 5-17 MDA-MB-231 migration plots in response to chemokine RANTES/CCL5
Migration plots illustrating the motility and movement of MDA-MB-231 cells on either 10µg/ml Matrigel, Fibronection or type I collagen with or without 20ng/ml CCL5 . This experiment was performed once
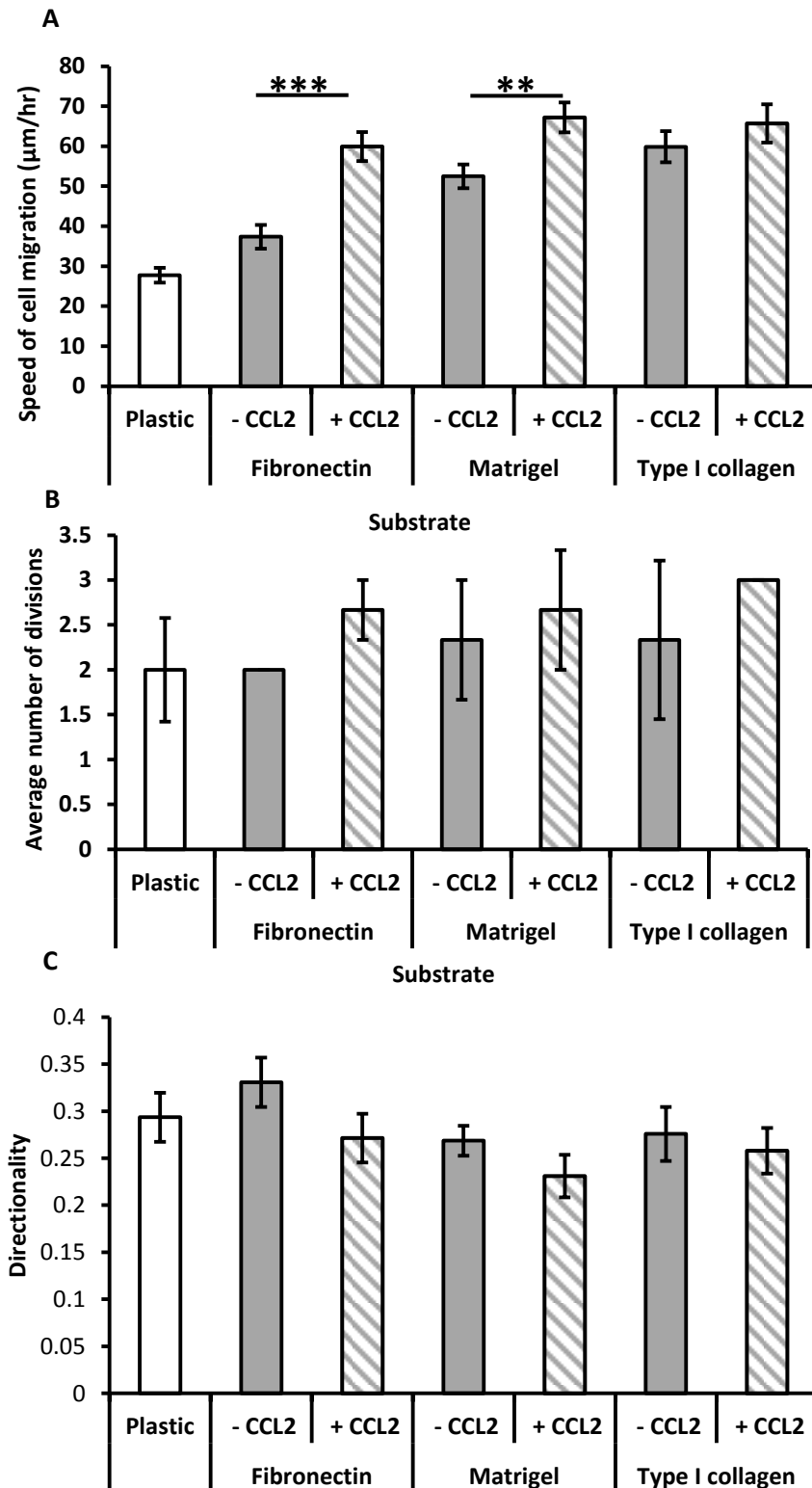
Figure 5-18 HT1080 migration in response to chemokine MCP-1/CCL2

HT1080 cells were seeded on either 10μg/ml Matrigel, Fibronection or type I collagen and treated with 20ng/ml CCL2. Using time-lapse microscopy an image was taken every 10 minutes for a total period of 17 hours. A) The speed of cell migration, B) Number of divisions, and C) directionality were pulled from this data. Each bar represents the mean ± S.E.M of 15 tracks from 3 separate wells. Significance was measured using the students paired *t*-test. This experiment was performed once.
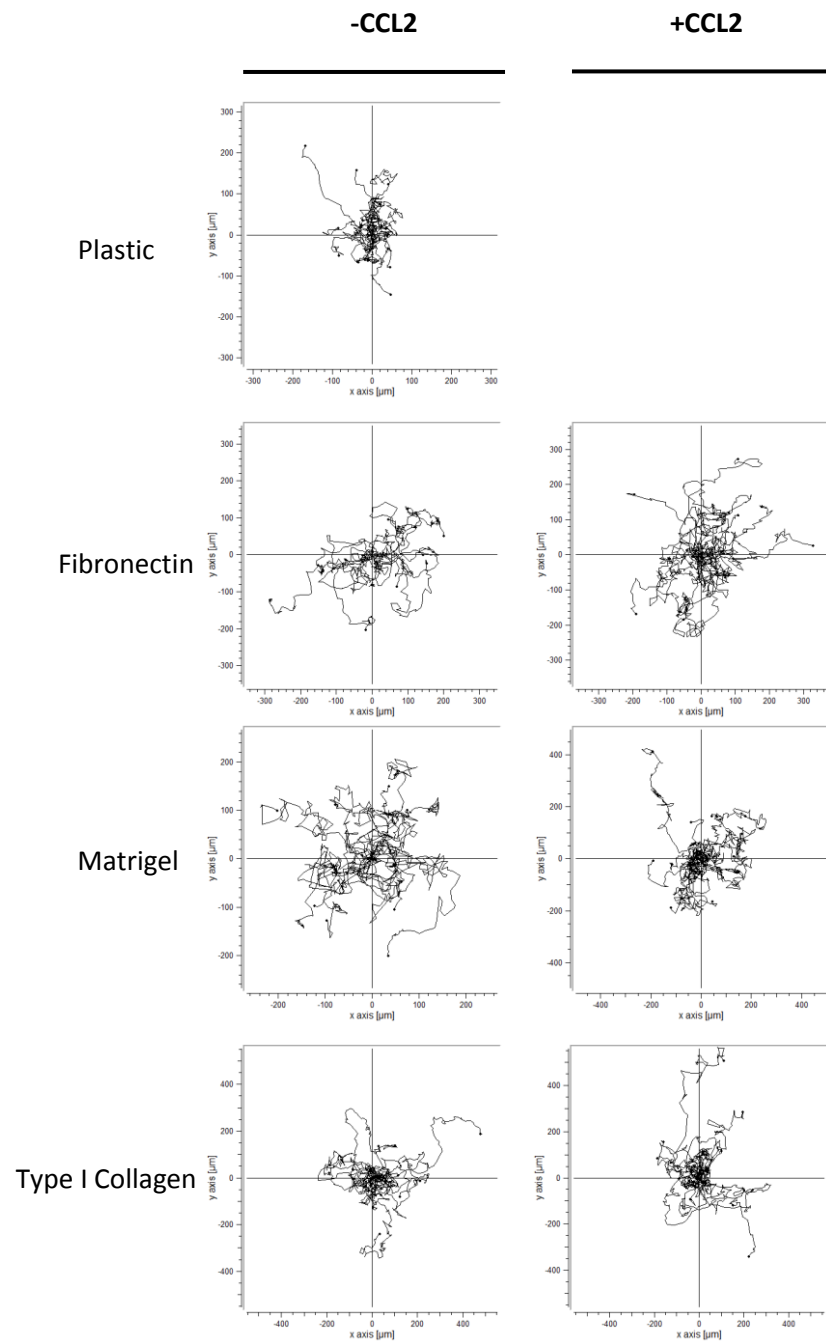
Figure 5-19 HT1080 migration plots in response to chemokine MCP-1/CCL2
Migration plots illustrating the motility and movement of HT1080 cells on either 10µg/ml Matrigel, Fibronection or type I collagen with or without 20ng/ml CCL2 . This experiment was performed once.
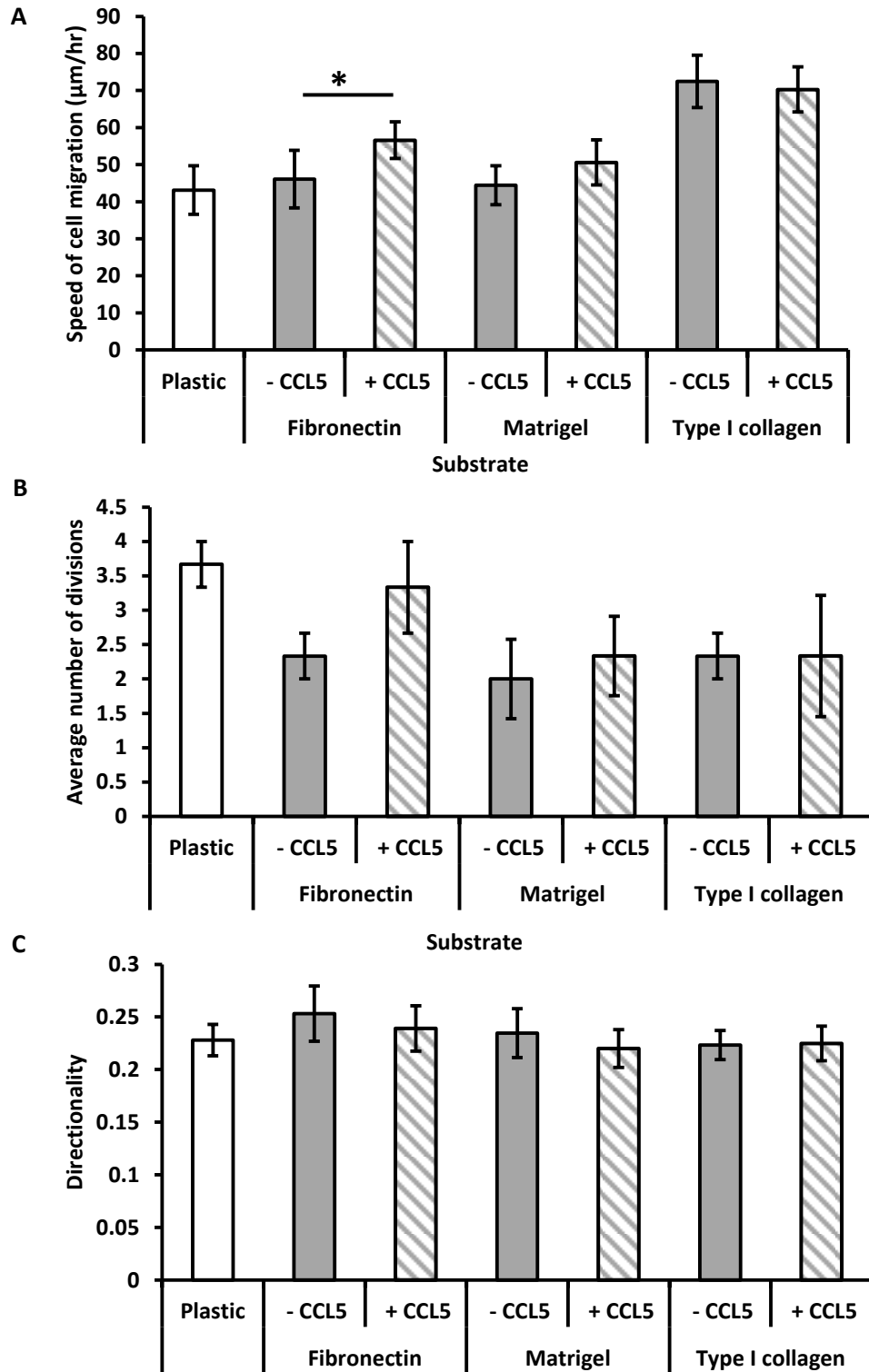
Figure 5-20 HT1080 migration in response to chemokine RANTES/CCL5

HT1080 cells were seeded on either 10μg/ml Matrigel, Fibronection or type I collagen and treated with 20ng/ml CCL5. Using time-lapse microscopy an image was taken every 10 minutes for a total period of 17 hours. A) The speed of cell migration, B) Number of divisions, and C) directionality were pulled from this data. Each bar represents the mean ± S.E.M of 15 tracks from 3 separate wells. Significance was measured using the students paired *t*-test. This experiment was performed once.
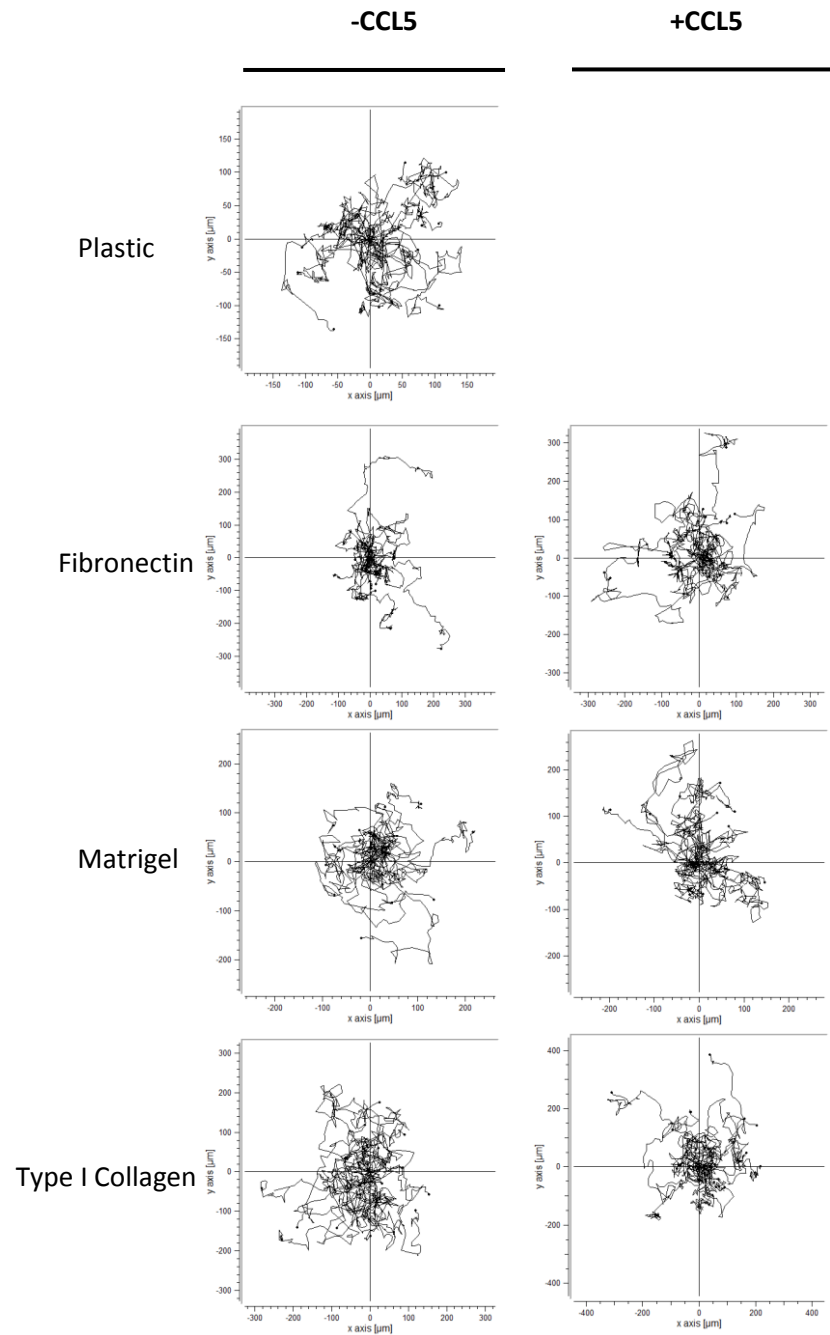
Figure 5-21 HT1080 migration plots in response to chemokine RANTES/CCL5
Migration plots illustrating the motility and movement of HT1080 cells on either 10µg/ml Matrigel, Fibronection or type I collagen with or without 20ng/ml CCL5 . This experiment was performed once.

5.2.7 MDA-MB-231 invasion in response to chemokine's CCL2 and CCL5

After assessing the ability of CCL2 and CCL5 to induce migration in 2D we next looked to see if they could stimulate invasion of our breast carcinoma cells through a 3D matrix composed of Matrigel mixed with Fibronectin as in chapter 4. Results showed that there was no significant difference in the invasive capacity of MDA-MB-231 cells to move through the matrix in response to 100 ng/ml CCL2 and CCL5 (Fig 5-22A). The invasive response of cells to EGF was also explored for a direct comparison of the effect between chemoattractants. Here the data shows that 10 ng/ml EGF causes a significant increase in invasion of MDA-MB-231 cells which is consistent with our prior invasion assay performed in response to EGF (see chapter 4). Even though a significant increase in invasion was not observed, there is an increasing trend in invasion by 20% and 32% in response to CCL2 and CCL5 respectively. Interestingly, the total fluorescence was lower in all conditions when compared to the control with it being significantly lower by 46% and 30% in CCL5 and EGF respectively (Fig 5-22C).

A 0µm ⟶ 210µm

Control

CCL2

CCL5

EGF

15 µm   30 µm

B

C
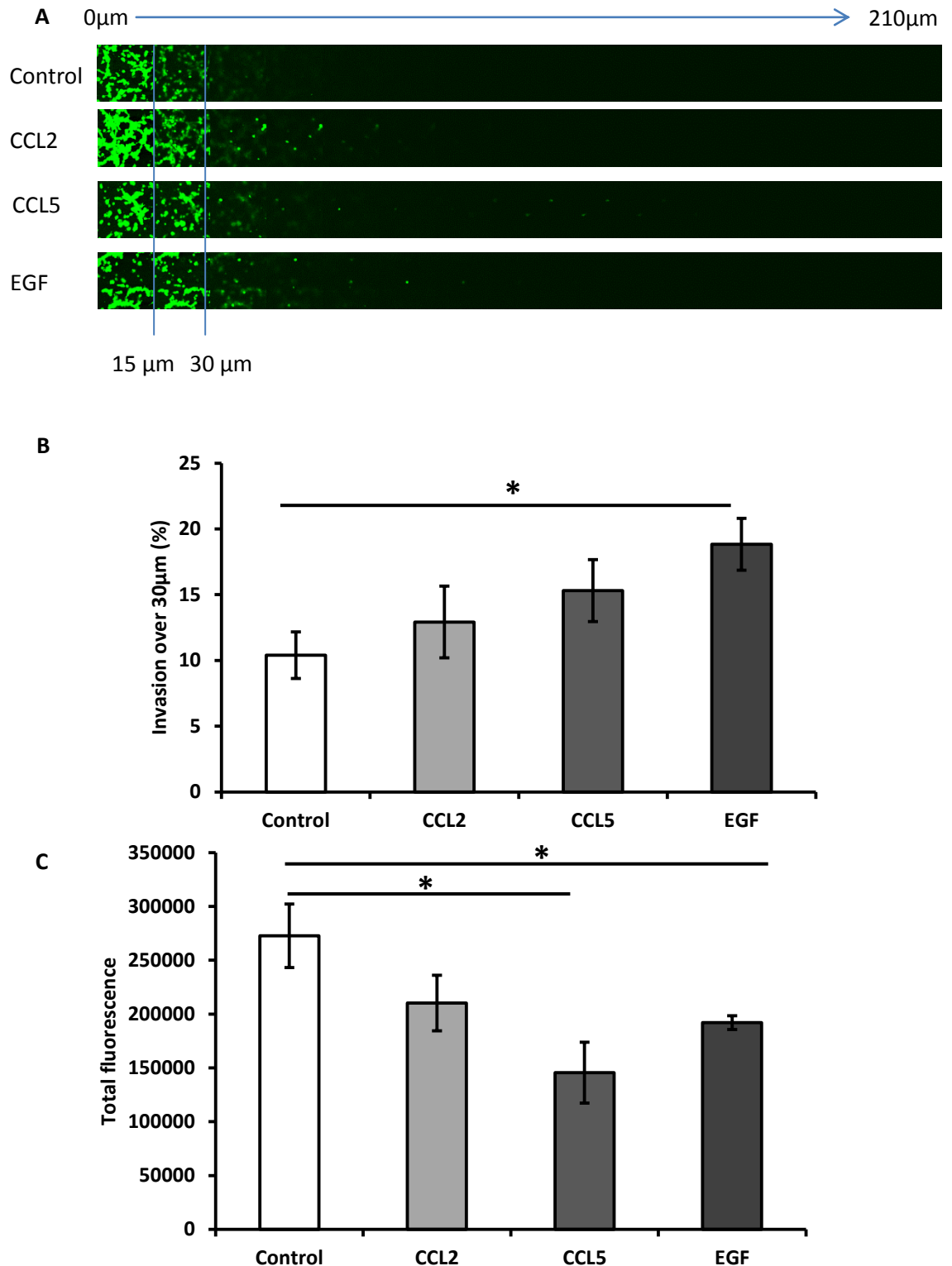
Figure 5-22 MDA-MB-231 invasion in response to chemokines CCL2 and CCL5
MDA-MB-231 cells were allowed 72 hours to migrate through Matrigel plugs
supplimented with 10µg/ml Fibronectin in response to CCL2, CCL5 and EGF. A) Invading
cells stained with calcien-AM and visualised using confocal microscopy taking 15µm
optical sections that are collated as a sequence increasing in depth from left to right. B)
True invasion into the Matrigel plug was determined through measuring fluorescence
intensity of ≥30µm. C) The total fluorescence for each experiment was also extracted.
Values are the means ±SEM of 6 points from 2 replicates. Statistical significance of
differences was determined by students paired *t*-test. Six fields of view were assessed in
duplicate wells for each condition.

5.3 Discussion

The aim of this chapter was to identify what genes are regulated in response to cell stimulation with the isoquinolinone compounds, construct a sensible paradigm from these genes that leads to a migratory phenotype, and test whether these genes do in fact effect the migration of cancer cells. The presented evidence confirms that the A3 and A5 compound do regulate the expression of genes that are involved in migration and invasion of cancer cells.

5.3.1 Isoquinolinone compounds regulate gene expression in HT1080 and MDA-MB-231 cells

Together, the illumina microarray with our qRT-PCR data in this chapter provides clear evidence for an isoquionlinone compound-dependent gene expression signature that details a potentially novel mechanism for inducing migration and invasion in MDA-MB-231 and HT1080 cells (Fig 5-13). As mentioned earlier, although we were unable to attain complete data for all our candidate genes at 4- and 8 hours in HT1080 cells, we would still like to discuss plausible tiers of gene expression that might be influenced by A3 at these time point based on what we found in our A5 treated breast cancer cells.

As we discussed in our previous chapter, the isoquinolinones are analogs of the *cis*-imidazoline Nutlin which functions to inhibit the interaction between MDM2 and p53. We theorise that both A3 and A5 compounds may target this complex and subsequently allow p53 transcriptional activity to occur. Active p53 has been shown to target Ets-1, a member of the ETS family of transcription factors that can act as activators or repressors of numerous genes in many cellular processes (Dwyer et al, 2007). In MDA-MB-231 cells we showed that Ets-1 expression is increased in response to A5 on plastic and Matrigel plus Fibronectin conditions throughout the 24 hour stimulation. In HT1080 cells, Ets-1 was shown to be repressed by A3 at 24 hours in the microarray. This however did not match the qRT-PCR validation which instead showed that A3 had no altered effect on Ets-1 expression at 24 hours. Until further validation we speculate that Ets-1 could be up-regulated at earlier time points in response to A3 treated HT1080 cells. The expression of Ets-1 can become augmented by p53 in a variety of different cancers giving it pro-oncogenic activity (Freed-pastor and Prives, 2012). Interestingly the p53 promoter contains binding sites for Ets-1 and these binding elements are important for increasing p53 expression. This creates a positive feedback-loop in which p53 is continually expressed enhancing metastatic activity (Agarwal et al, 2001). In MDA-MB-231 cells Ets-1 has been shown to increase MMP1 and MMP9 expression, whilst in HT1080 cells it regulates the expression of integrin subunits and induces the expression of cancerous inhibitor of protein phosphatase 2A through the ERK pathway to induce malignant growth (Dittmer et al, 2004:

Kamoshida et al, 2012: Kanna et al, 2011). Preliminary experiments conducted in A3-treated HT1080 cells by Dr Damon Bevon and Liam Pudwell showed that MMP9 was upregulated upon treatment (unpublished observations by Howe, J.D., Payerne, E.,Woon, E.C.Y., Searcey, M., and Gavrilovic, J).

Ets-1 can regulate the transcription of Egr-1, a zinc finger transcription factor, which in turn has also been identified as transcriptional activator of Ets-1 (Robinson et al, 1997: Kawai-kowase et al, 1999).Egr-1 was the most highly up-regulated genes in A5 treated MDA-MB-231 cells, and whilst suppressed at 24 hours in A3 treated HT1080 cells, there was a noticeable increase at 4 hours. Egr-1 protein typically has a short half-life of 90 minutes however its expression can be extended by additional stimulation. Egr-1 has been described in many studies as an important regulator of cell proliferation and tumour formation. A study by Mitchell et al showed that the use of catalytic antisense DNA which targets Egr-1 inhibited MDA-MB-231 breast cancer proliferation, migration, chemoinvasion and tumour growth (Mitchell et al, 2004). The mechanism by which Egr-1 induces migration and invasion is still not fully understood; it has been shown to co-operate with the ERK signalling pathway by suppressing the MAPK-specific phosphatase DUSP4 and regulate PLAUR, the urokinase receptor that mediates proteolysis (Tarcic et al, 2012). A recent study by Wang et el has also showed that Egr-1 interplays with PAK5, a member of the PAK family that is involved in stimulating cytoskeletal reorganisation, as well as mediating MMP2 expression (Wang et al, 2012). Egr-1 has been noted to interchangeably switch from a tumour inducer to tumour suppressor in many cell lines. A study by Liu et al has shown that HT0180 cells transfected to overexpress Egr-1 had suppressed growth and tumorigenicity through the up-regulation go TGF-β1, Fibronectin and plasminogen activator inhibitor-1. This prevented anchorage independent growth, degradation, and cell cycle progression (Liu et al, 1999).

Egr-1 has been shown to target several genes on our list that were regulated in response to the compounds. One of those genes is the cap 'n' collar basic-region leucine zipper family of transcription factors NFE2L3. In our data NFE2L3 expression was repressed in the microarray and at 24 hours in A5-treated MDA-MB-231 cells but was unaffected in A3-treated HT1080s. NFE2L3 has been shown to be expressed in MDA-MB-231 cells and have a protective role in carcinogen-induced lymphomas but there is no evidence for its involvement in driving cell migration or invasion (Chevillard and Blank, 2011).

HBEGF, another target of Egr-1 had increased expression at 4 and 8 hour in MDA-MB-231 cells. HT1080 and MDA-MB-231 cells have been shown to express HBEGF which remains tethered to

the plasma membrane by a transmembrane domain requiring proteolytic cleavage by ADAM17 before being able to act on surrounding receptors (Kveiborg et al, 2011: Nie et al, 2012). The induction of migration and invasion through HBEGF in breast cancer is similar to EGF, which we discussed in the last chapter, primarily activating signalling pathways such as PI3K, GEP100-Arf-6-AMAP100, and MAPK (Nie et al, 2012). Pubmed research did not reveal any studies where the migratory potential of HBEGF on HT1080 has been explored. If HBEGF is verified to be up-regulated at earlier time points like we speculate, this may provide a novel inducer of migration in this cell line.

Binding sites for Egr-1 in the promoter of EphA2 have been identified suggesting it may regulate EphA2 activity (Encode, 2013). However, EphA2 can autophosphorylate itself leading to post-translational modifications that cause an abundant accumulation of EphA2in cancer cells (Walker-daniels et al, 2002). In concert with the microarray, EphA2 expression was downregulated in response to A3- and A5-treated HT1080 and MDA-MB-231 cells respectively at 24 hours. A slight increase at 4 and 8 hours was seen in MDA-MB-231 cells however which may have contributed to compound-enhanced motility. EphA2 overexpression is associated with an aggressive and metastatic and cellular phenotype. In breast cancer cells EphA2 binds Ephexin4 to mediate the activation of RhoG which then binds ELMO2. ELMO2 recruits Dock4 which promotes a complex with EphA2 and induces the activation of Rac. This complex further co-localises with cortactin and induces cancer cell motility and invasion (Hiramoto-Yamaki et al. 2010).

RhoQ expression was significantly upregulated in response to compound treated HT1080 and MDA-MB-231 cells. RhoQ remains one of the lesser well studied Rho GTPases but is described to be structurally similar to Cdc42 and Rac1 (Neudauer et al, 1998). RhoQ induces the formation of filopodia through binding and activating N-WASP which subsequently induces Arp2/3 complex mediated F-actin polymerisation in the perinuclear region (Murphy et al, 1999: Abe et al, 2003). Currently there are no studies looking at the effect of RhoQ on cell migration, however RhoQ has been shown to associate with Cdc42 and Rac with its knockdown abolishing filopodial formation. RhoQs ability to interact with proteins such as N-WASP to induce F-actin polymerisation indicates it most likely plays an important role in cell migration and invasion. Being structurally similar to Cdc42, it would be interesting to further clarify how these proteins interact with each other and if any redundant roles overlap between the two. We believe RhoQ may have an important role in isoquinolinone induced cell migration and invasion.

A study by Holterman et al showed that Ets-1 regulates the transcription of TGF-α, a gene that we found to be upregulated in A5 treated MDA-MB-231 cells at 4 hours (Holterman et al, 2010). TGF-α induces MDA-MB-231 invasion through the activation of the downstream effector Cdc42 with its silencing suppressing TGF-α induced protrusion formation and cell invasion (Kituchi et al, 2011). TGF-α can stimulate expression of oncogene myc, and conversely myc has been shown to regulate the expression of TGF-α (Yoshida et al, 1990: Christensen et al 1999).

Interestingly myc can also be transactivated by mutant p53 with a study by Frazier et al showing that myc is an important target of mutant p53 tumourigencity (Frazier et al, 1998). Due to TGF-α and myc being repressed or unaffected by A3 in the microarray and qRT-PCR data is unclear or doubtful these genes work in the same way in HT1080 cells. Additionally HT1080 express wild-type p53 as opposed to mutant which does not increase myc transcription. Only future validation at 4 and 8 hours may suggest otherwise but as the current data stands this part of the mechanism differs in breast and fibrosarcoma cell lines. Myc has been shown two differential effects on cell migration with one study shown that its knockdown in MDA-MB-231 cells impairs cell migration and another showing its overexpression can impede breast cancer cell migration (Cappellen et al, 2007: Liu et al, 2012). This provides an unclear idea as to how A5-induced and A3-repressed myc expression in MDA-MB-231 and HT1080 cells respectively affects their migratory properties.

Myc itself has been identified to interact with multiple genes on our list. Two of these genes include the CCN family members CYR61 and CTGF. CTGF is typically strongly expressed in both cells lines and has been found in studies to be inhibited by myc and regulated by Ets-1 (Shimo et al 2001 :Dews et al, 2006: Encode, 2013). Whilst not significant, there was a noticeable increase in CTGF expression at 4 hours in both compound treated cell lines. We theorise that Ets-1 may induce early expression of CTGF, which then over time becomes inhibited with increasing myc expression. A study by chen showed that CTGF enhances migration of MDA-MB-231 cells through binding of the αvβ3 integrin which activates the ERK signalling pathway and leads to the increased expression of S100A4 upregulation (Chen et al, 2007). The precise function of S100A4 remains unclear but its ability to interact with cytoskeletal moieties results in reorganisation of cytoskeletal components and its knockdown abolishes CTGF induced migration (Chen et al, 2007). A more recent study has revealed that tissue transglutaminase 2 regulated S100A4 migration involves the activation of sydecan-4 and α5β1 integrin co-signalling pathway linked by PKCα (Wang and Griggin, 2013). Interestingly S100A4 was

significantly downregulated in the microarray at 1.3 fold coinciding with a downregulation of CTGF indicating this mechanism could be applicable in HT1080 cells as well as MDA-MB-231.

As with CTGF, there are no studies on the migratory effect of Cyr61 on HT1080 and few in MDA-MB-231. Our data showed a significant increase in Cyr61 expression in A5-treated MDA-MB-231 cells and notable increase in A3-treated HT1080 cells at 4 hours. A study by Nguyen shows that breast cancer cells secrete Cyr61 which induces stromal fibroblasts to express matrix remodelling MMP1 which in turn stimulates the effector molecule PAR-1 receptor that induces cell motility (Nguyen et al, 2006). Whilst not fully understood, it is believed PAR-1-medated cell migration occurs through activation of RhoA which enhances stress fibre formation, and increased FAK phosphorylation to elevate focal adhesion turnover (Diaz et al, 2012).

Interestingly, Cyr61 has been shown to induce the expression of chemokine G-coupled receptors CXCR1 and CXCR2 (Lin et al, 2007). These are the main receptors that IL8, a proinflammatory chemokine, mediates its biological effects through (Waugh and Wilson, 2008). We found IL8 to be significantly expressed at 8 hours in A5-treated MDA-MB-231 cells with a notable increase in expression at 4 hours which was also seen in A3-treated HT1080 cells. IL8 is expressed in response to Ets-1 and could be Ets-1 dependent in isoquinolinone stimulated cells (Qiao et al, 2007). Its expression has also been found to be indirectly increased by HBEGF (Mehta and Besner, 2003). IL8 is a potent inducer of cell migration and invasion in many cancer cell lines such as breast, gastric and ovarian. Studies show IL8 coordinates these events through activation of the PI3K, MAPK, and Src-FAK signalling pathways which induce the expression of MMPs and activate members of the Rho GTPases Rac,Cdc42, and RhoA to regulate cytoskeletal architecture (Waugh and Wilson, 2008). A study by Hjortoe et al found that IL8 suppression inhibited breast cancer migration and invasion indicating its importance in these processes and potentially A5-treated MDA-MB-231 (Hjortoe et al, 2004). Whilst no studies have looked at the effect of IL8 on HT1080 migration and invasion, IL8 has been shown to be expressed in this cell line indicating it may induce a migratory phenotype through similar means (Wyzgol, et al, 2009).

Myc has also been shown to inhibit FPR1, a seven transmembrane G protein-coupled receptor that is well known as a powerful leukocyte promoting chemoattractant (Kanayasu-Toyoda et al, 2003: VanCompernolle et al, 2003). Our data showed repression and overall low expression of this gene during the 24 hour stimulation in response to compound treated cells. Regardless of this we found some studies that provide an interesting mechanistic insight to how this gene

functions. A study by VanCompernolle showed that FPR1 triggers dose-dependent migration of HT1080 cells through PKC and the PI3K signalling pathway which lead to a Rac-dependent increase in F-actin and redistribution of the cytoskeleton (VanCompernolle et al, 2003). There is no apparent literature asserting the migratory effects on FPR1 on MDA-MB-231 cells.

FPR1 has also been shown to interact with SAA1, an acute-phase protein with cytokine-like properties (Lee et al, 2008). In our data, SAA1 was also found to be not very highly expressed and repressed in response to compound treated cancer cells. There are very few studies looking at the effects of SAA1 on cell migration. One by Knebel showed that SAA1 actually had dual effects on invasion and migration in two different glioma cell lines. In one cell line, migration and invasion was induced in a dose-dependent manner through the upregulation of IL8, MMP2, MMP9, ROS, and NO. Whilst in the other cell line there was an inverse correlation seen coinciding with a decrease in expression of these proteins (Knebal et al, 2013). SAA1 is an endogenous ligand of FPR1 and has been shown to stimulate the production of CCL2 by activating the ERK and NF-κB signalling pathways in monocytes (Lee et al, 2008). Yet again however, in our data overall expression of CCL2 was low and significantly repressed in compound treated cell lines. Altogether this suggests that the mechanism by which FRP1 and SAA1 act together, or individually is most likely not driving isoquinolinone-dependent migration or invasion in MDA-MB-231 or HT1080 cells.

The effects that the chemokines CCL2 and CCL5 have on cancer cell migration are discussed in detail later. Overall these chemokines were either markedly repressed during the 24 hour compound treatment with only a modest increase in CCL5 expression in MDA-MB-231 cells at 4 hours on Matrigel plus Fibronectin. Both chemokines have been shown to induce the expression of IL8; however our data suggests that Ets-1 is more likely to be the mediator of isoquinolinone-dependent IL8 expression (Nanki et al, 2001). Moreover, our current findings imply that the chemokines may not be involved in isoquinolinone A3- or A5-induced migration and invasion.

Moving averages data was extracted from our compound induced migration assays (Fig A-6 and A-7). We looked at this data to see if there were any notable patterns in migrational speed and gene expression. However, the findings appear to be to variable to draw conclusions from and without knowing the timing of protein expression of these genes we are unable to say if a true coinciding effect was apparent.

For some of our genes we observed differences in expression between cells grown on Plastic, Matrigel, and Matrigel plus Fibronectin. An explanation for higher expression of Matrix coated surfaces could be due to integrin mediated upregulation of those genes. A study by Wantanabe et al showed that components Fibronection, laminin, and type I collagen induced FAK phosphorylation which subsequently lead to a substantial increase in CCL2 expression. Matrigel in rich in Laminin and undoubtedly there would have been a higher concentration of fibronectin present due to being added compared to plastic. A similar explanation for RhoQ may also hold true as matrix coated surfaces would initially provide more integrin binding site with which Rho kinases regulate the relaying signals and so would be up-regulated themselves in this response (Clark et al, 1998). It is less clear why some genes may be more upregulated on plastic. In the case of CTGF, it is possible through sensing on matrix coated surfaces that it is naturally repressed as over-expression of CTGF leads to enhanced ECM production and could impair cell motility through increased binding to the matrix (Abraham, 2008).

5.3.2 Chemokines enhanced cell migration In HT1080 and MDA-MB-231 cells

We investigated whether two well-studied chemokines, CCL2 and CCL5, induced cell migration and invasion in our cell lines. qRT-PCR data indicated that the isoquinolinone compounds suppressed CCL2 and CCL5 in both our cell lines, however we decided to pursue these experiments because they are known to induce migratory properties in cancer cells. Additionally, there was some form of positive regulation of CCL5 at 4 hours in MDA-MB-231 cells. The data shows that both these chemokines induce enhanced migration in MDA-MB-231 and HT1080 cells on Matrigel and Fibronectin coated surfaces and modest invasion of MDA-MB-231 through a Fibronectin supplemented Matrigel plug.

Several studies show that these chemokines act directly on breast cancer cells increasing their migratory and invasion-related properties (Soria and Ben-Baruch, 2008). In the case of the fibrosarcoma cell line, HT1080 cells have been shown to express both CCL2 and CCL5, however there are no current studies pertaining to their ability to induce enhanced migration or invasion in this cell line (Germano et al, 2010). This therefore means we have defined CCL2 and CCL5 as novel inducers of migration in HT1080 fibrosarcoma cells in our study.

In our studies we found that CCL2 and CCL5 did not promote proliferation in either MDA-MB-231 or HT1080 cells on any matrix coated surface which is consistent with several *in vitro* studies in MDA-MB-231 cells (Potter et al, 2012: Kuang et al, 2011). We also found in our experiments that directionality was unaffected in both cell lines in response to CCL2 and CCL5. Studies have shown that several chemokines, including CCL2 and CCL5, can influence directed

cell migration in a number of cell lines such as leukocytes and macrophages (Kulbe et al, 2004). However, no studies have shown if these chemokines induce directed movement in 2D experiments for MDA-MB-231 or HT1080 cells.

Both CCL2 and CCL5 induce cell migration in cancer cells, however the mechanisms in which they do this differ. A study by Fang et al showed that CCL2 works by binding the overexpressed G protein-coupled receptor CCR2 on MDA-MB-231 breast cancer cells to stimulate smad3, a mediator of the TGF-β signalling pathway, in concert with the p42/44 MAPK pathway which together coordinate survival and motility (Fang et al, 2012). CCL2 induced phosphorylation of Smad3 and p42/44 MAPK proteins causes cross-talk between these two independent signalling pathways and enhances expression and activity of RhoA to regulate breast cancer cell motility (Fang et al, 2012). The exact mechanism of how Smad3 co-operates with the MEK-MAPK pathways is still to be explored but it is speculated to be through either Smad3 phosphorylation of MAPK, or Smad3 suppression of phosphatases that target p42/44 MAPK such as PP2A. Since this study was performed in several cell lines including MDA-MB-231, we believe this to be the mechanism by which CCL2 induced migration in our experiments.

Current studies have revealed less about the mechanism by which CCL5 induces migration of cancer cells. A study by Li et al showed that a 20 ng/ml CCL5 stimulation of breast cancer cells initiated a redistribution of the cytoskeletal F-actin towards the periphery of the cell in a polarised manner (Li et al, 2010). They went on further to define a mechanism by which CCL5 stimulates cytoskeletal reorganisation to induce motility and identified a novel downstream effector called ezrin. Ezrin is a member of ERM (ezrin-radixin-moesin) cytoskeletal-associated protein family and acts as a linker of the cell membrane to the cytoskeleton. Studies using osteosarcoma cell lines have shown that connection of the cell membrane and actin cytoskeletin through ezrin caused activation of the MAPK signalling pathway which was associated with an increased metastatic phenotype (Kanna et al, 2004). Together these studies provide a plausible mechanism by which the speed of migration in our cells was increased in response to CCL5.

In our studies we noticed that MDA-MB-231 and HT1080 cells plated on type I collagen migrated very rapidly in all experiments at around 60-70 μm/hr with or without CCL5. We speculate that the cells may reach a maximal migration speed on this substrate that alleviates any effect by the chemokines. Studies have shown that cells plated on type I collagen, but not other substrates undergo EMT through the activation of several integrin mediated pathways such as Src-FAK , PI3K , TGF-β, and JNK which lead to an up-regulation of key motility

associated proteins such as Rac1 (Shintani et al, 2008). This may provide a logical explanation why we saw no migration inducing effect of CCL2 of CCL5 for MDA-MB-231 and HT1080 cells in any of our experiments.

In our studies we failed to show significant invasion of MDA-MB-231 cells in response to both chemokines. However, there was a noticeable increase in invasion in response to CCL2 and CCL5. Several studies have showed that these chemokine do induce MDA-MBP-231 invasion through Matrigel basement membrane extract via the mechanisms already described (Karnoub et al, 2007: Nam et al, 2006). We did find that there was significantly lower total fluorescence in CCL5 chambers, and whilst not significant a visible decrease also in CCL2 chambers. As fluorescence correlates with the amount of cells present, this may provide a reason why we did not see a significant difference in invasion in conditions where chemokines were present. It is reasonable to rule out difference in cell survival between conditions as cells survive in 2D conditions in response to these factors. The reduced overall fluorescence is difficult to justify, and may be simply due to the nature of the experiment as all conditions prior to chemoattractant addition were set up identically.

5.3.3 Summary

In this chapter the isoquinolinone compounds were shown to differentially regulate the expression of many genes, many of which were shown to be positively regulated and involved in cell migration. We also believe that some genes such as RhoQ, which was positively regulated, may be a driver of isoquinolinone dependent-cell migration, even though to date it has not yet been studied for this function. Other genes such as CCL2 and CCL5 were found to be largely repressed, indicating they may not play a role in Isoquinolinone dependent motility.

Chapter 6: General discussion and future directions

6.1 Discussion

In summary this thesis details the role Isoquinolinone compounds play in cell migration and invasion, and begins to identify a potential mechanism for an alteration in effect. The evidence in Chapter 3 shows that compounds A3 and A5 significantly reduce adhesion of the mammary breast cancer cell line MDA-MB-231 and the human fibrosarcoma cell line HT1080 to matrix substrates that are representative of an *in Vivo* ECM. The results in Chapter 4 characterise the isoquinolinone compounds as inducers of cell migration and invasion in MDA-MB-231 cells in *Vitro*. And finally in Chapter 5 attempts have been made to identify and outline a novel mechanism for isoquinolinone-dependent migration.

As mentioned before in this thesis, the Isoquinolinones appear to have several binding targets both extracellularly and intracellularly. They can bind integrins and have been shown to have an antagonistic effect (Seitz et al, 2007). There are only very few studies looking at the relationship between Isoquinolinones and Integrins. We showed from our adhesion assays that the compounds A5 and A3 are probably having an effect on integrin-ECM binding. Isoquinolinones comprise a broad family of structural isomers and so the binding to specific integrins, affinity of that binding, and effect could vary greatly between compounds (Seitz et al, 2007). In this study it is undetermined how the compounds A3 and A5 interact with integrins. It could be that they bind integrins which induces more dynamic turnover of focal adhesions through regulation of FAK and Src tyrosine kinases thereby decreasing the number of peripheral adhesions (Huttenlocher and Horwitz, 2011). Antagonism or decreased expression of integrins, as shown by berberine treatment in chondrosarcoma, may also be likely, however fit less well with providing an explanation for enhanced migration in relation to this effect (Wu et al, 2013). Further studies, which will be discussed later, would need to be carried out to determine how compounds A3 and A5 interact with integrins and whether or not they mediate signalling through them to drive cell motility.

There are several other cell surface receptors that have been identified as binding targets of Isoquinolinones, but just like with integrins are also not very well studied. An example would be that Isoquinolinone-derivatives have been shown to bind to the G protein coupled opioid receptors which are classified into three main types designated μ (MOR), δ (DOR), and κ (KOR), (Freissmuth et al, 1993: Gach et al, 2011). With a main focus on nociceptive processing these receptors have received little attention in cancer studies but have been shown to be expressed

in MDA-MB-231 and HT1080 cells (Gach et al, 2011: Zagon, and McLaughlin, 1990). It has been noted that stimulation of the opioid receptor can transactivate EGFR leading to receptor dimerization, phosphorylation and increased tyrosine kinase activity (Farooqui et al, 2006). A study by Charbaji et al in oral epithelial cells showed that opioid induced EGFR activation led to subsequent MAPK-ERK1/2 and p38 phosphorylation which were involved in driving cell migration during would healing (Charbaji et al, 2012).

Isoquinolinones have also been shown to target the 5-HT$_3$ receptor which is a ligated-gated ion channel that induces emesis (Gant, 2010). It has not yet been established whether or not this receptor is expressed in MDA-MB-231 or HT1080 cells. However, it has been demonstrated in smooth muscle cells and helper T-cells that 5-HT$_3$ induces cell migration through concomitant activation of the PI3K, MAPK/ERK and Rho kinase (ROCK)-signalling pathways along with the secretion of chemotactic chemokines such as CXCL12 (Liu and Fanburg, 2006: Magrini et al, 2011). Current evidence suggests this receptor is expressed in cells involved in inflammation. It is well known that inflammation is a critical component of tumour progression with cancer cells co-opting signalling molecules of the innate immune system such as chemokines (Coussens and Werb, 2002). One study even showed that stimulation of the 5-HT$_3$ receptor led to the production of the chemokines CCL5, and IL8, both of which we found to be regulated by isoquinolinones in our data (Bayer et al, 2007). Since this receptor has not been identified as expressed in our cell lines it is uncertain whether this may be a potential cause of Isoquinolinone induced cell migration.

As we know, the isoquinolinone compounds were designed to transverse the tumour cell membrane through passive diffusion or by parasitizing a transporter in order to dissociate the MDM2-p53 complex (Rotheweiler et al, 2008: Goldman, 2002). There are studies showing that Isoquinolinones can also interact with PARP-1, a DNA repair enzyme, however in truth it is unknown how many intracellular targets the compounds interact with (Chiarugu et al, 2003). In chapter 5, analysis of our microarray and qRT-PCR data in regard to compound treated cells was carried out as well as an overview of how those genes regulated by the compounds have been shown to induce cancer cell migration and invasion. We speculate that the isoquinolinone compounds infiltrate the cell bypassing the membrane and dissociate the MDM2-p53 complex allowing p53 to activate transcription factor Ets-1. However, taking into consideration of other binding targets of these compounds there could by other means of activation for Ets-1. For instance, if compounds A5 and A3 do bind the EGFR then they could activate the MARK-ERK signalling pathway which has been shown to induce the expression of

Ets-1 and Egr-1 in vascular smooth muscle cells (Goetze et al, 2001). We also discussed in Chapter 5 how mutant p53 can activate myc which stands as a plausible link in MDA-MB-231 cells; however since HT1080 cells contain wild-type p53, this same activation is not possible. This creates a slight alteration in our paradigm (Fig 6-1) in which myc may be activated still in an isoquinolinone dependent manner, but by a different means in HT1080 cells. In one study, a MEK-ERK inhibitor caused a marked reduction in myc expression in rhabdomyosarcoma cells. The ERK proteins lie upstream of myc and are important also for the stability of myc through phosphorylation (Marampon et al, 2006). Thus, myc expression could be up-regulated in compound treated HT1080 cells through binding of EGFR to induce MAPK-ERK signalling. But obviously since this Erk-mediated myc stability has been described as vital for its activity this could also occur in MDA-MB-231 cells also. It remains unclear how myc and TGF-α regulate each other's expression, however studies have shown that these proteins interact in a strong, synergistic fashion to impair apoptosis and induce proliferative and survival signals (Amundadottir et al, 1996).

There were five genes that encode soluble secreted factors which were positively upregulated in response to isoquinolinone treatment. The proteins encoded by these genes include TGF- α, CTGF, Cyr61, IL8, and HBEGF and are all involved in inducing cell migration. CTGF is expressed in response to Ets-1 activation and as we discussed in chapter 5 has been shown to induce MDA-MB-231 migration through cytoskeletal rearrangement (Nakerakanti et al, 2006: Chen et al, 2007). As such there is confidence in assuming that the isoquinolinone compounds induce Ets-1 expression which subsequently activates CTGF. CTGF is then secreted to act back on the cell through an autocrine outside-in signalling manner through the binding of integrin $\alpha v\beta 3$. This causes phosphorylation of the ERK proteins ERK1/2 which regulate S100A4 activity. As mentioned previously, it is not exactly clear how S100A4 functions in cytoskeletal rearrangement; however it is known to interact with F-actin, tropmyosin, and heavy chain of nonmuscle myosin II (Mishra et al, 2012). Additionally it has been shown that S100A4 activates transcription of MMP11, MMP13, and MMP14 which leads to increased metastasis (Chen et al, 2007). We believe this mechanism also occurs in HT1080 cells as S100A4 was significantly downregulated in the microarray at 24 hours which coincided with the downregulation of CTGF at 24 hours in the microarray and qRT-PCR. Since S100A4 is a downstream effector of CTGF with studies showing the knockdown of CTGF causing suppression of S100A4 expression there is no reason to suggest this mechanism is not also applicable in HT1080 cells (Chen et al, 2007).

The other CCN family member Cyr61 may also contribute to isoquinolinone induced cell migration and invasion by working in a similar manner to CTGF. As explained in chapter 6 Cyr61 has been shown to be secreted by breast cancer cells to induce migration via the expression of MMP1 and PAR-1 mediated activation of RhoA (Diaz et al, 2012). Studies in prostate cancer cell lines have shown that *de novo* Cyr61 is secreted to act back on the cell. Cyr61 can bind several integrins including $\alpha v\beta 3$ and $\alpha 6\beta 1$ and elicit intracellular signalling pathways to regulate cell migration (Sun et al 2008). It seems that Cyr61 accomplishes this by regulating the Src-FAK signalling pathway as knockdown of Cyr61 led to excessive focal adhesion assembly and loss of normal focal adhesion regulation by integrins (Sun et al, 2008). This in part may also be an explanation for the decrease in cell adhesion we observed in response to compound treated cells. Additionally, Cyr61 binding to integrins activates Src which phosphorylates and activates the p130^{cas}-CrkII-DOCK180 pathway that regulates Rac activation leading to the formation of motile structures (Gu et al, 2001).

The chemokine IL8 has been shown to play an important role in inducing cancer cell migration and invasion. As mentioned in Chapter 5, we speculate that Cyr61 upregulates IL8 receptors CXCR1 and CXCR2 as it has been shown to in gastric adenocarcinoma cells. Then isoquinolinone induced activation of Ets-1 leads to IL8 expression which is secreted to act back on the CXCR1/2 receptors leading to enhanced migration and invasion in our cell lines. IL8 activates many signalling pathways through the CXCR1/2 receptors which are involved in cell motility. As stated in the last chapter, IL8 can regulate the Rho GTPases Rac, Cdc42 and RhoA as well as induce the FAK-Src-cortactin paradigm (Waugh and Wilson, 2008). Studies have shown that HT1080 and MDA-MB-231 cells endogenously express IL8 and that IL8 is important in MDA-MB-231 cell invasion with IL8 knockdown impairing the cells ability to invade through Matrigel (Yao et al, 2007: Kreuz et al, 2004). However, the exact mechanism in these two cell lines has not been defined and so whilst we can be confident that IL8 is driving isoquinolinone-induced cell migration and invasion we can't elaborate on exactly how it does so.

Both TGF-α and HBEGF were only found to be positively regulated in A5-treated MDA-MB-231 cells at 4 hours. Since there was not enough convincing evidence we excluded them from the A3-treated HT1080 paradigm (Fig 6-1) and for now are assuming that A3 does not positively regulate the expression of these two genes in HT1080 cells. In MDA-MB-231 cells isoquinolinone activation of Ets1 and Egr-1 could lead to the expression of TGF-α and HBEGF which are then secreted. TGF-α acts back on cell to induce cell spreading and invasion through Cdc42 activation (Kituchi et al, 2011). The precise mechanism of how TGF-α activates Cdc42 is

not very well established. There is some evidence shown in a study using human gastric epithelial cells to suggest TGF-α acts binds integrins $\alpha3\beta1/\alpha2\beta1$ to activate the PI3K signalling pathway which targets the RhoGTPases including Cdc42 (Tetreault et al, 2006). After synthesis and cleavage HBEGF can act on many cell surface receptors such as EGFR or integrin $\alpha3\beta1$ to carry out isoquinolinone-induced cell migration. As mentioned previously, HBEGF can activate many signalling pathways such as PI3K and MAPK-ERK involved in migration. Through these pathways HBEGF has been shown to activate RhoA in corneal epithelial cells which enhanced cell migration (Yin et al, 2009). It is likely that a similar mechanism could occur in A5-treated MDA-MB-231 cells.

RhoQ was upregulated in response to isoquinolinone treatment in both cell lines indicating it is important in A5 and A3 induced cell migration and invasion. As mentioned in chapter 5, it has been shown that Egr-1 can induce expression of RhoQ, however there is little more known about what other factors can induce RhoQ expression and what RhoQ itself can target. Studies have mainly focused on the role of RhoQ in the translocation of glucose transporter 4 (GLUT4) upon insulin stimulation, nerve regeneration and vesicle formation (Tong et al, 2007). Since RhoQ has been shown to associate with Cdc42 and Rac and induce cytoskeletal rearrangement it is surprising that more attention hasn't been focused on looking at its ability to enhance cell migration and invasion (Murphy et al, 1999). However, we speculate since RhoQ shares high homology with Cdc42 and its knockdown abolishes cellular ruffling that it may be vital to isoquinolinone induced cell migration.

It is curious as to why some genes were downregulated in response to isoquinolinone treatment. The chemokines CCL2 and CCL5 are well known to be strong promoters of cell migration and invasion in many cell lines including breast cancer (Fang et al, 2012:Karnoub et al, 2007:Nam et al, 2006). There is also no evidence to suggest that these chemokines could have a negative effect on cell motility. One possibility could be that in some cell lines, including pancreatitis acinar cells, the expression of chemokines such as CCL2 play a role in early activation of the inflammatory response (Grady et al, 1997). We saw a modest increase in CCL5 expression in MDA-MB-231 cells at 4 hours. This could mean that the isoquinolinone compounds could induce early expression of chemokines within the first couple of hours for an initial response. In the case of SAA1, as we stated in chapter 5, it has been shown to have dual effects in migration and invasion of glioma cells (Knebal et al, 2013). Since the effect of SAA1 has never been studied in either of our cell lines for now we could propose the idea that SAA1 may negatively regulate the migration and invasion of both MDA-MB-231 and HT1080 cells

and so was the reason for being targeted for suppression by the isoquinolinones. Obviously further study to clarify this claim of effect would need to be carried out. Both EphA2 and FPR1 are involved in positively regulating cell motility and it is hard to justify why they are suppressed by the compound treatment especially as FPR1 is a strong promoter of HT1080 migration. EphA2 is associated with inducing amoeboid motility and it is possible that since the cells were grown on a 2D surface as opposed to a 3D environment that this phenotype of using mechanical shaping independent of proteases to squeeze through holes in the matrix was not necessary or the most effective mode of migration (Taddai et al, 2011).

In conclusion it appears we may have not identified a novel signalling pathway, but multiple novel targets that may be a cause of enhanced breast cancer and fibrosarcoma migration which have not yet been studied. This statement is particularly true for HT1080 cells were virtually all the genes regulated by the compound, which have been shown in other cell lines to induce migration and invasion, have not been studied in depth in this cell line. We propose that all the genes discussed above contribute to isoquinolinone-dependent migration and invasion and prompt further study, especially genes such as RhoQ which are quite poorly studied but are a promising new potential therapeutic target.
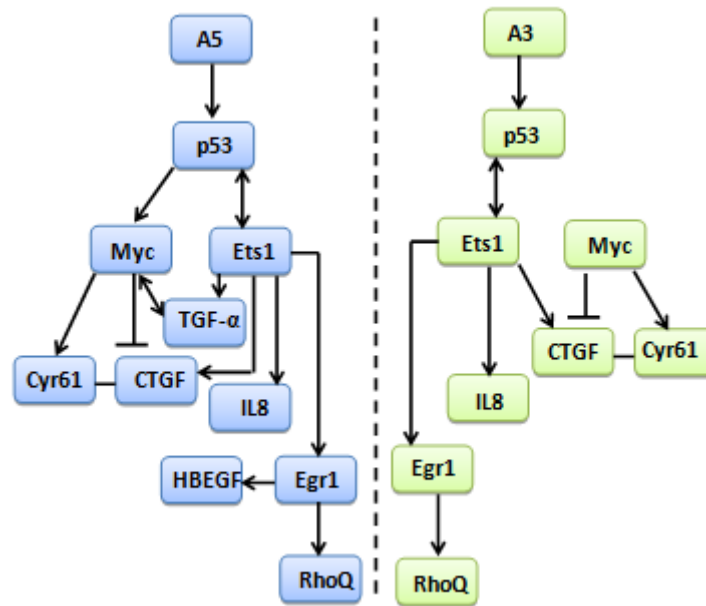
Figure 6-1 Compound A5 and A3 induced gene expression in MDA-MB-231 and HT1080 Diagram depicts conclusion of how gene may be regulated by A3 (green) in HT1080 cells and by A5 (Blue) in MDA-MB-231 cells.

6.2 Future directions

The identification between differences in gene regulation by A3-treated HT1080 cells and A5-treated MDA-MB-231 cells still remains to be elucidated. Here, complete data at three time points was collected for A5-treated MDA-MB-231 cells however in A3-treated HT1080 cells we managed to only collect full data at 24 hours, partial at 4 hours and none at 8 hours. For a better comparison between the effects of the isoquinolinone compounds on these two cell lines complete data at 4- and 8 hours in HT1080 needs to be collected. This then would then instil a greater confidence in some of the assumptions we have made about the differences and similarities between HT1080 and MDA-MB-231 cells treated with the compounds.

Unpublished observations in the Gavrilovic Lab showed that HT1080 cells migrate and invade in response to the A3 compound (Howe,J.D., Payerne, E., Woon, E.C.Y., Searcey, M., and Gavrilovic, J). It would be good to repeat these experiments with A3 and also the A5 compound which was primarily used in this study to identify if either compound induces a stronger effect. This also applies to testing the effect of newly synthesised compound A3 on MDA-MB-231 cell migration. Additionally, looking into how the compounds induce invasion of HT1080 and MDA-MB-231 cells through a type I collagen plug would give more physiologically relevant data of the compounds motility inducing properties (Sabeh et al, 2009). Since collagen is the most abundant component of the ECM and requires proteolytic activity to navigate unlike Matrigel in most cell types, this testing would be more indicative of what one might expect to find *in vivo*.

Naturally testing of some of these genes we found to positively upregulated in response to isoquinolinone treatment would be a next step in determining how much they do actually contribute to compound induced cell migration and invasion. One approach, for secreted proteins including IL8, TGF-β and HBEGF, would be direct addition of the proteins to the cells as we did with the chemokines. Or we could transfect the cells with vectors that are overexpressing the protein in question. As we discussed, IL8, TGF-α, HBEGF, CTGF and Cyr61 have all been implicated in inducing a more motile phenotype in cancer cells however have not been extensively studied in our cell lines. Therefore we believe they need more focus in future studies.

The use of siRNAs would also be vital in further studies as it would determine the extent in which these upregulated proteins hinder isoquinolinone-induced migration and invasion. We already know in other cell lines that knockdown of some of these genes such as RhoQ, TGF-α,

and CTGF abolishes the formation of migratory and invasive structures impairing cell migration (Murphy et al, 1999: Kituchi er al, 2011: Chen et al, 2007). It would also be interesting to knockdown the expression of transcription factors p53, myc, Ets-1 and Egr-1 to identify what effects they have on the genes they supposedly regulate.

We discussed a lot of signalling pathways that were speculated to be involved in carrying out the migration and invasion inducing properties of many isoquinolinone regulated genes. Unfortunately due to time constraints no signalling pathways that may be involved were identified. In further studies it would be important in implicating whether any of the signalling pathways discussed including PI3K, MAPK, EGFR-GEP100-Ar6-AMAP and Src-FAK are involved in compound induced motility. This could be done through western blotting or by transfecting cells with pathway-specific response element luciferase vectors. The identification of pathways involved is essential for therapeutic value allowing us to target them with specific inhibitors.

A few potential binding targets of the isoquinolinone compounds were discussed in this thesis, however as the current data stands none have been confirmed. Identifying targets of the compound is essential in clarifying what pathways may be triggered or suppressed. Some preliminary work was conducted in the Gavrilovic lab fluorescently tagging the isoquinolinones to observe their localisation and binging partners. However this method has currently proved unsuccessful as the fluorescent molecules appeared to cause cell death (Howe,J.D., Payerne, E., Woon, E.C.Y., Searcey, M., and Gavrilovic, J). There are other ways of identifying binding partners with an example being a method known as stable isotope labelling of amino acids in cell culture (SILAC) which determines the binding potencies of compounds to affinity-captured proteins from cell extracts (Ong et al, 2012).

In this thesis the regulation of gene expression in response to isoquinolinone treatment was performed on cells grown on a 2D culture. Primary cancer cells are embedded in complex three dimensional structures and when grown on 2D substrates have been shown to lose many hallmarks associated with their *in vivo* counterparts (Luca et al, 2013). A study by Kenny et al looking at breast cancer cells grown in 2D and 3D showed that cells had different growth kinetics, morphology and gene expression patterns between these two conditions. They also provided evidence that signalling events of cells grown in 3D conditions are significantly closer to the cognate events *in vivo* (Kenny et al, 2007). Thus, it is important in further studies to observe how gene expression is differentially regulated by the compounds in response to being grown in a 3D environment.

Moreover, it may be useful to explore the use of these compounds in different applications. For example, as we have shown they induce migration, they could potentially be used in impaired wound healing of keratinocytes which has been seen in type II diabtetes as they are of epithelial origin like MDA-MB-231 cells (Yoshioka et al, 2010). Growth factors such as HBEGF are quite expensive to produce in mass quantities, however have been shown to enhance impaired wound healing. Thus, if the isoquinolinone compounds can upregulate the expression of this gene natively, then it may provide a cost effective solution.

The main focus of this research was looking at how the compounds affected the migration and invasion of cancer cells. However there are many more important factors associated with the metastasis of cancer cells. In further studies it would be good to identify how the compounds effect cell survival, growth, proliferation, stress, and angiogenesis in both 2D and 3D conditions. This would then allow for an overall comprehensive analysis of the metastatic inducing potential of the isoquinolinone compounds. Moreover the treatment of cancer is frequently described as a two-pronged attack and so combating these other factors may prove necessary for therapeutic intervention.

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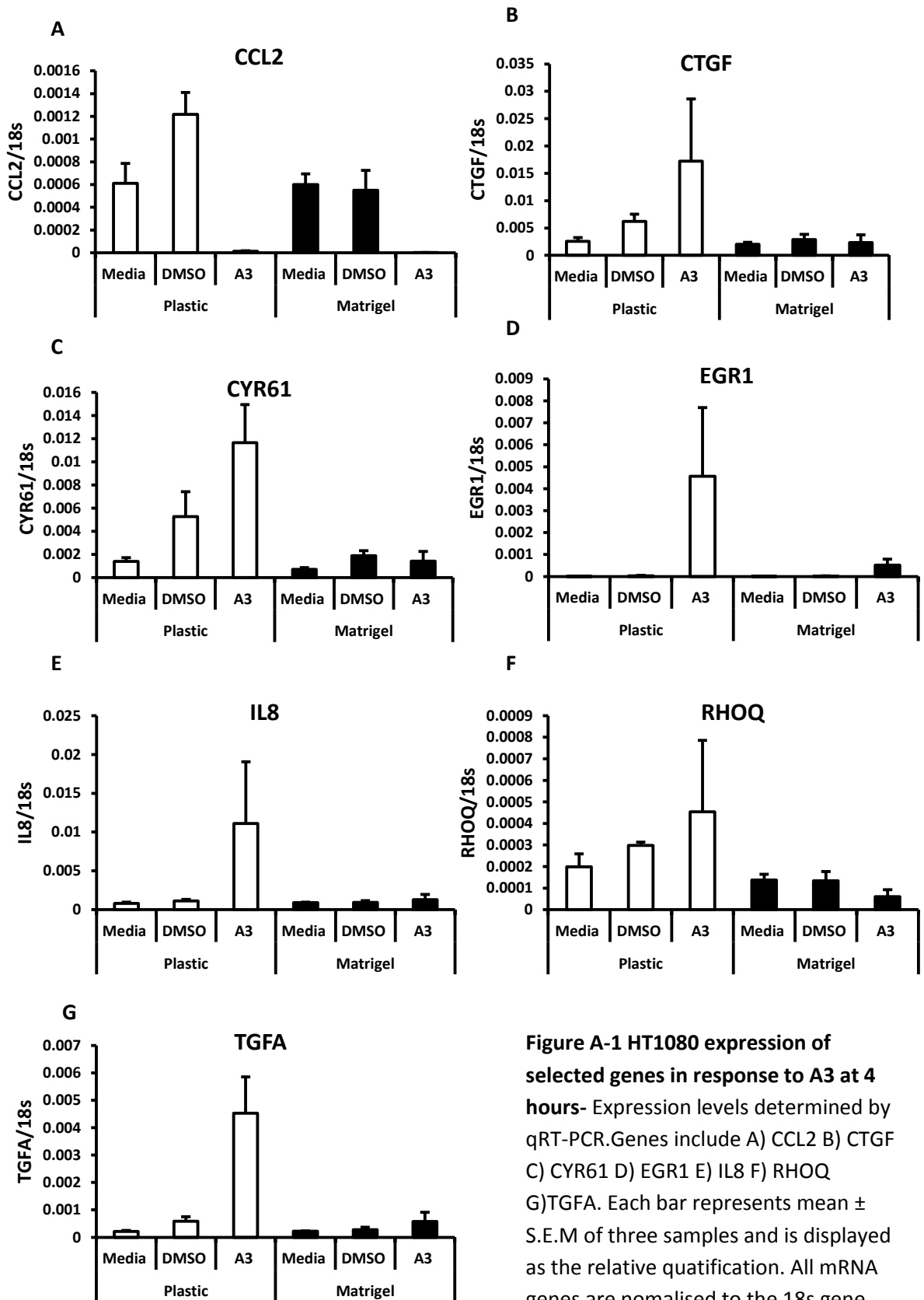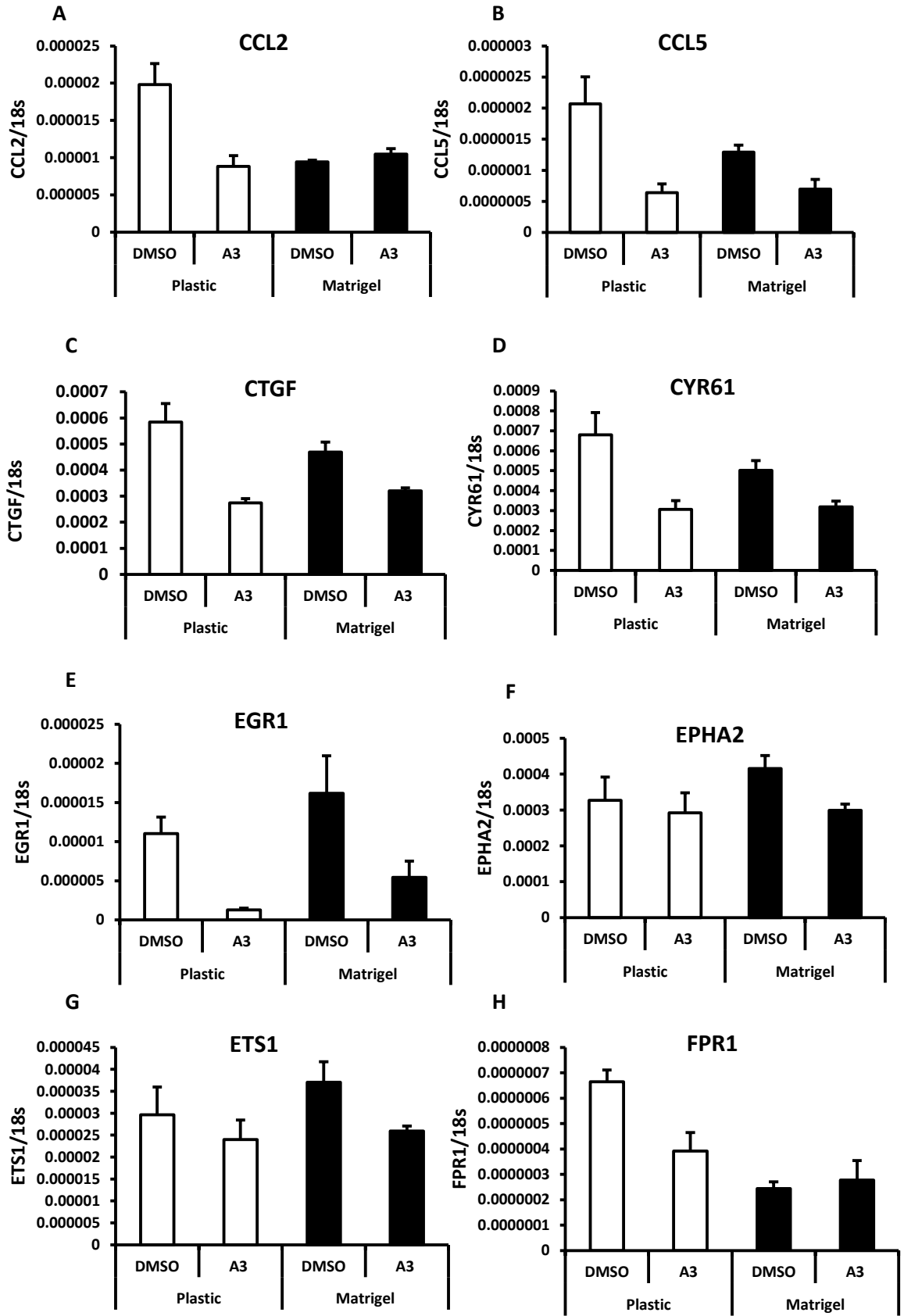
Appendix

Figure A-1 HT1080 expression of selected genes in response to A3 at 4 hours- Expression levels determined by qRT-PCR.Genes include A) CCL2 B) CTGF C) CYR61 D) EGR1 E) IL8 F) RHOQ G)TGFA. Each bar represents mean ± S.E.M of three samples and is displayed as the relative quatification. All mRNA genes are nomalised to the 18s gene.
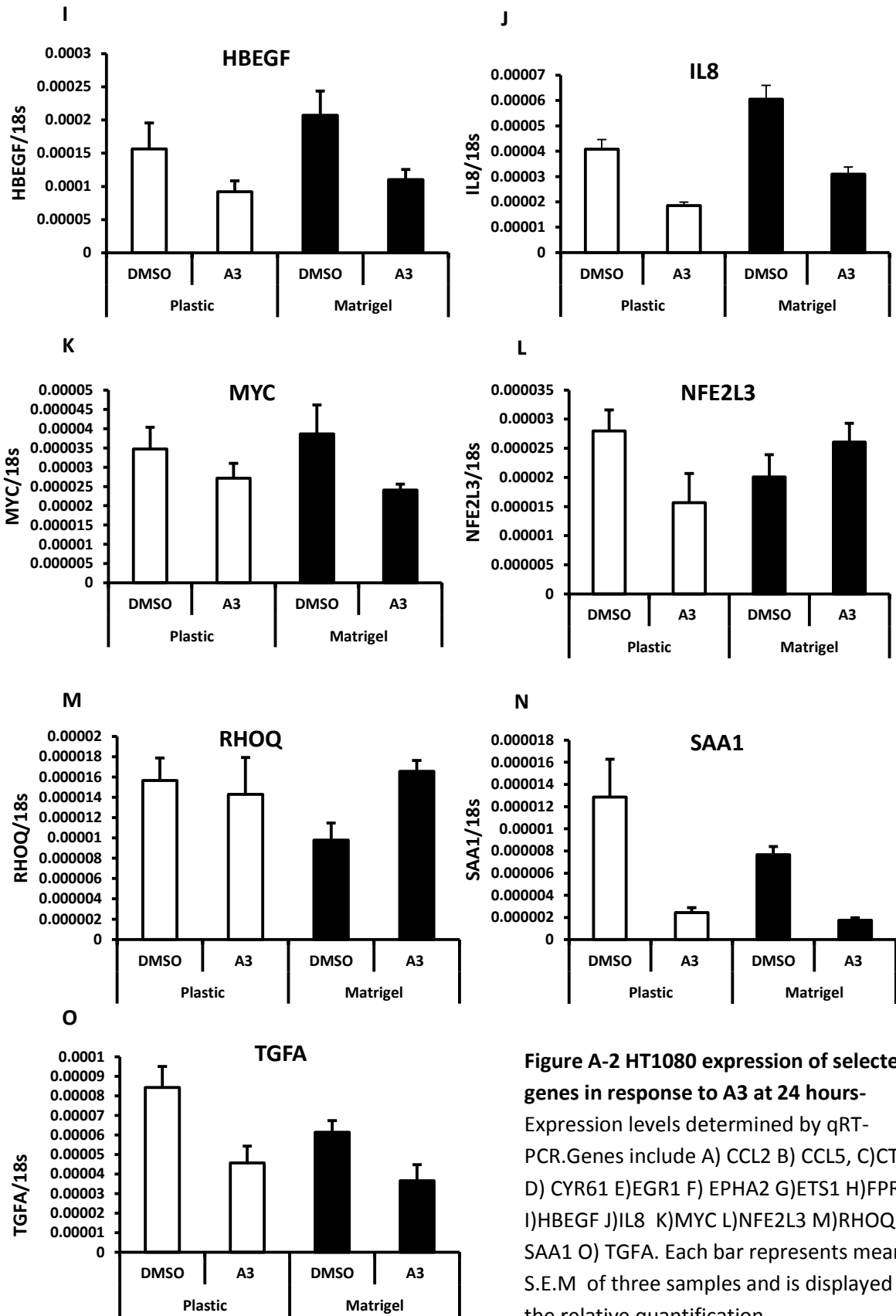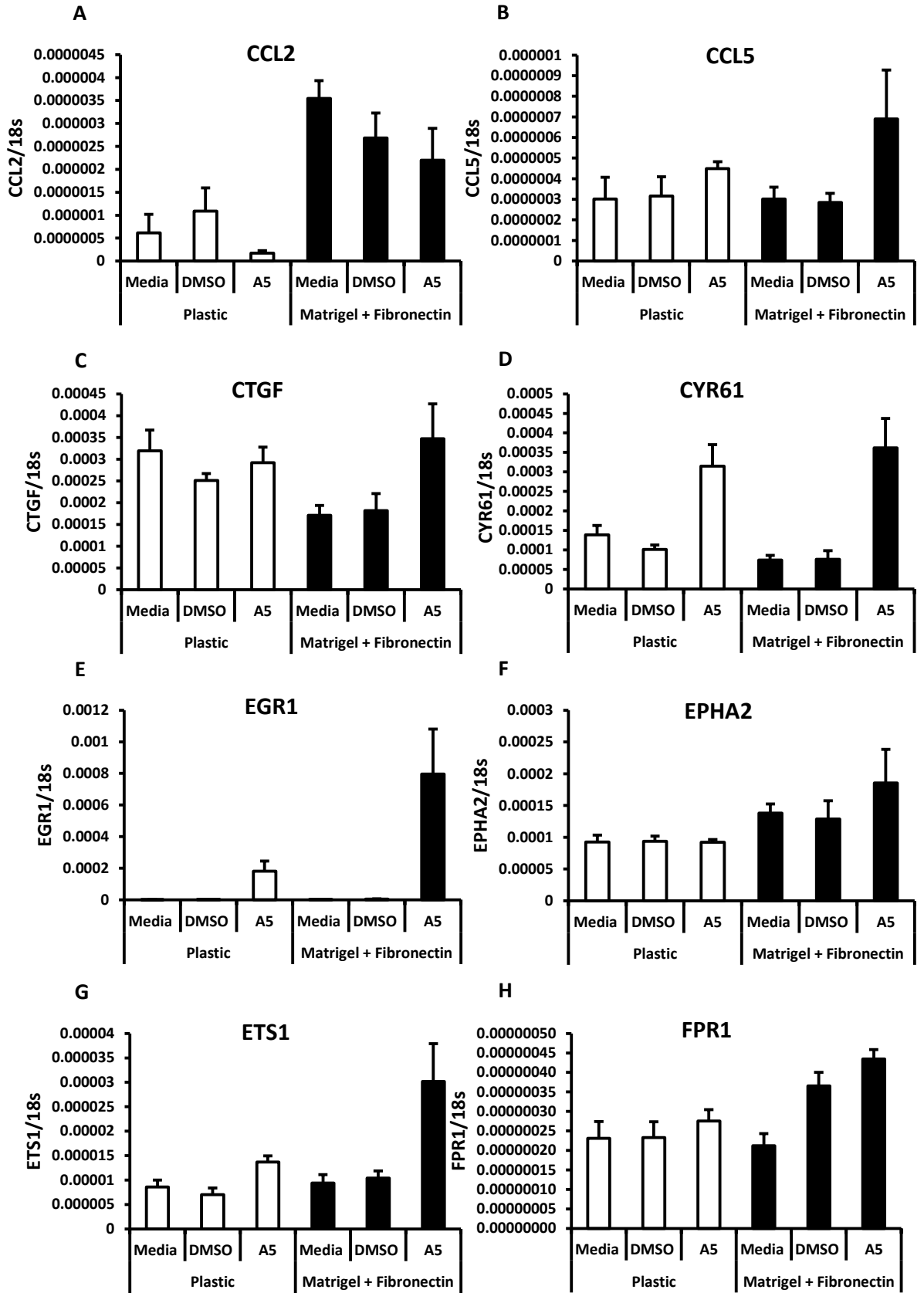
A CCL2

B CCL5

C CTGF

D CYR61

E EGR1

F EPHA2

G ETS1

H FPR1

Figure A-2 HT1080 expression of selected genes in response to A3 at 24 hours- Expression levels determined by qRT-PCR.Genes include A) CCL2 B) CCL5, C)CTGF D) CYR61 E)EGR1 F) EPHA2 G)ETS1 H)FPR1 I)HBEGF J)IL8 K)MYC L)NFE2L3 M)RHOQ N) SAA1 O) TGFA. Each bar represents mean ± S.E.M of three samples and is displayed as the relative quantification.
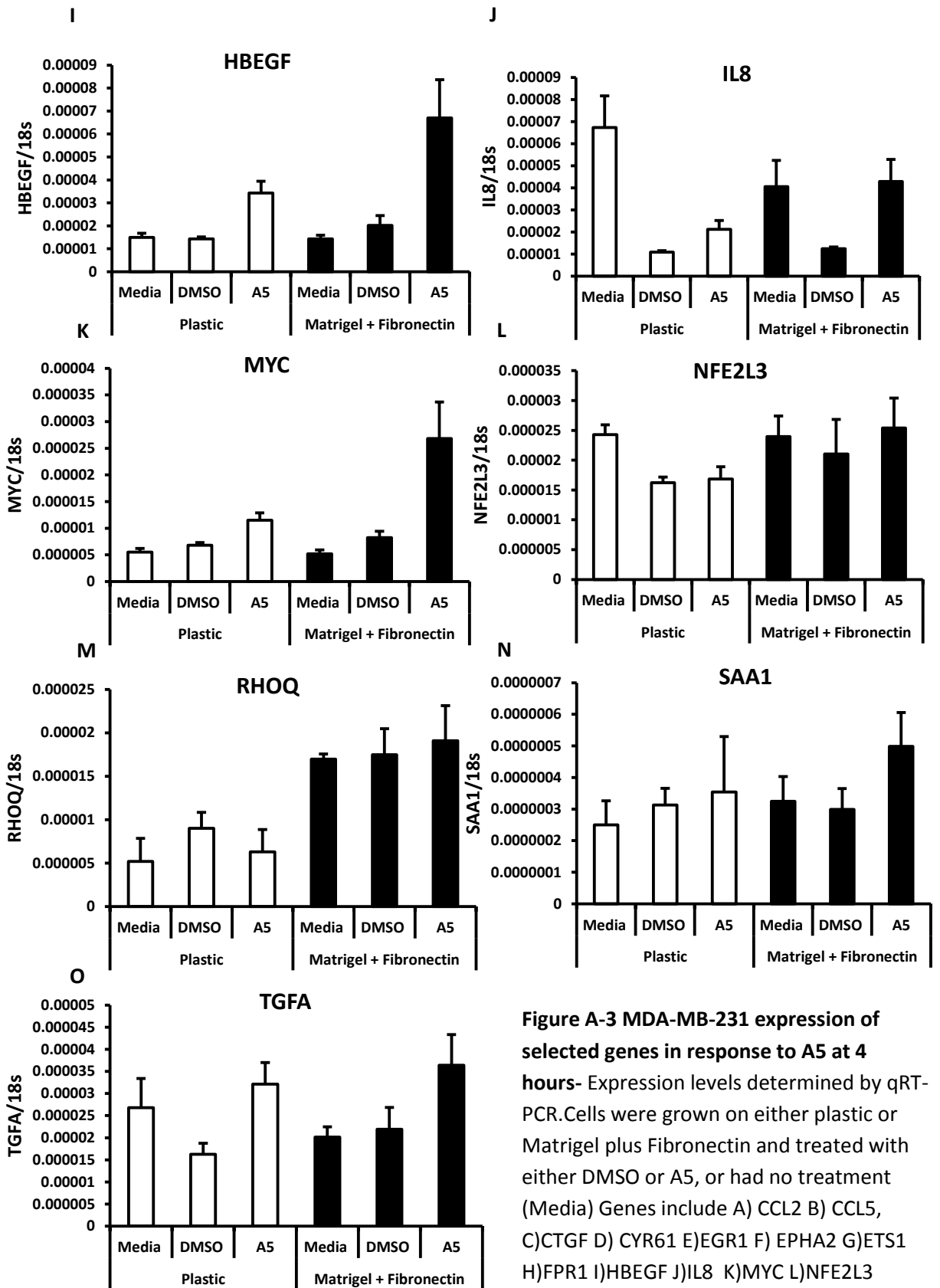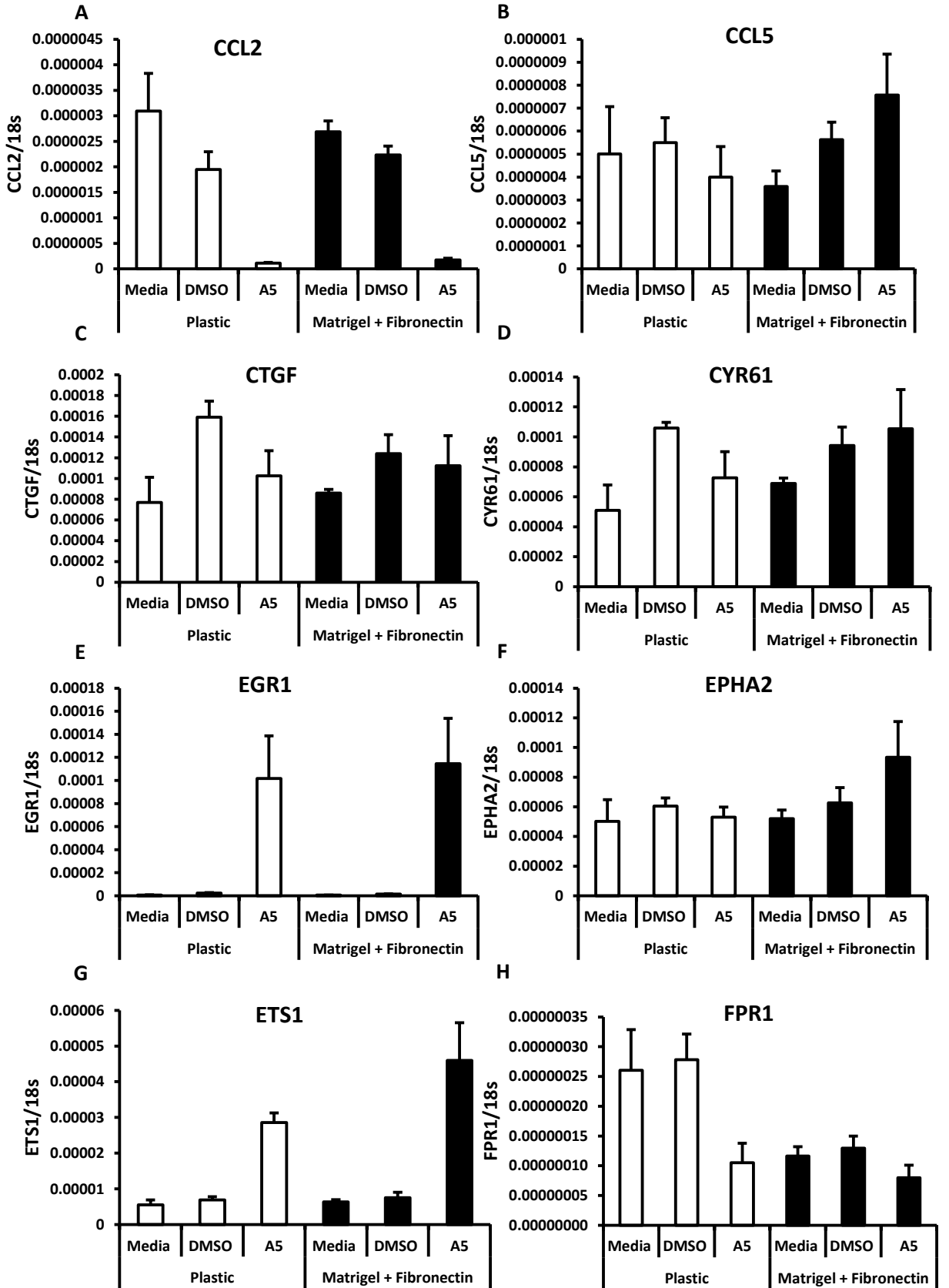
A CCL2

B CCL5

C CTGF

D CYR61

E EGR1

F EPHA2

G ETS1

H FPR1

Figure A-3 MDA-MB-231 expression of selected genes in response to A5 at 4 hours- Expression levels determined by qRT-PCR.Cells were grown on either plastic or Matrigel plus Fibronectin and treated with either DMSO or A5, or had no treatment (Media) Genes include A) CCL2 B) CCL5, C)CTGF D) CYR61 E)EGR1 F) EPHA2 G)ETS1 H)FPR1 I)HBEGF J)IL8 K)MYC L)NFE2L3 M)RHOQ N) SAA1 O) TGFA. Each bar represents mean ± S.E.M of three samples and is displayed as the relative quantification. All mRNA expression is normalised to 18s control.
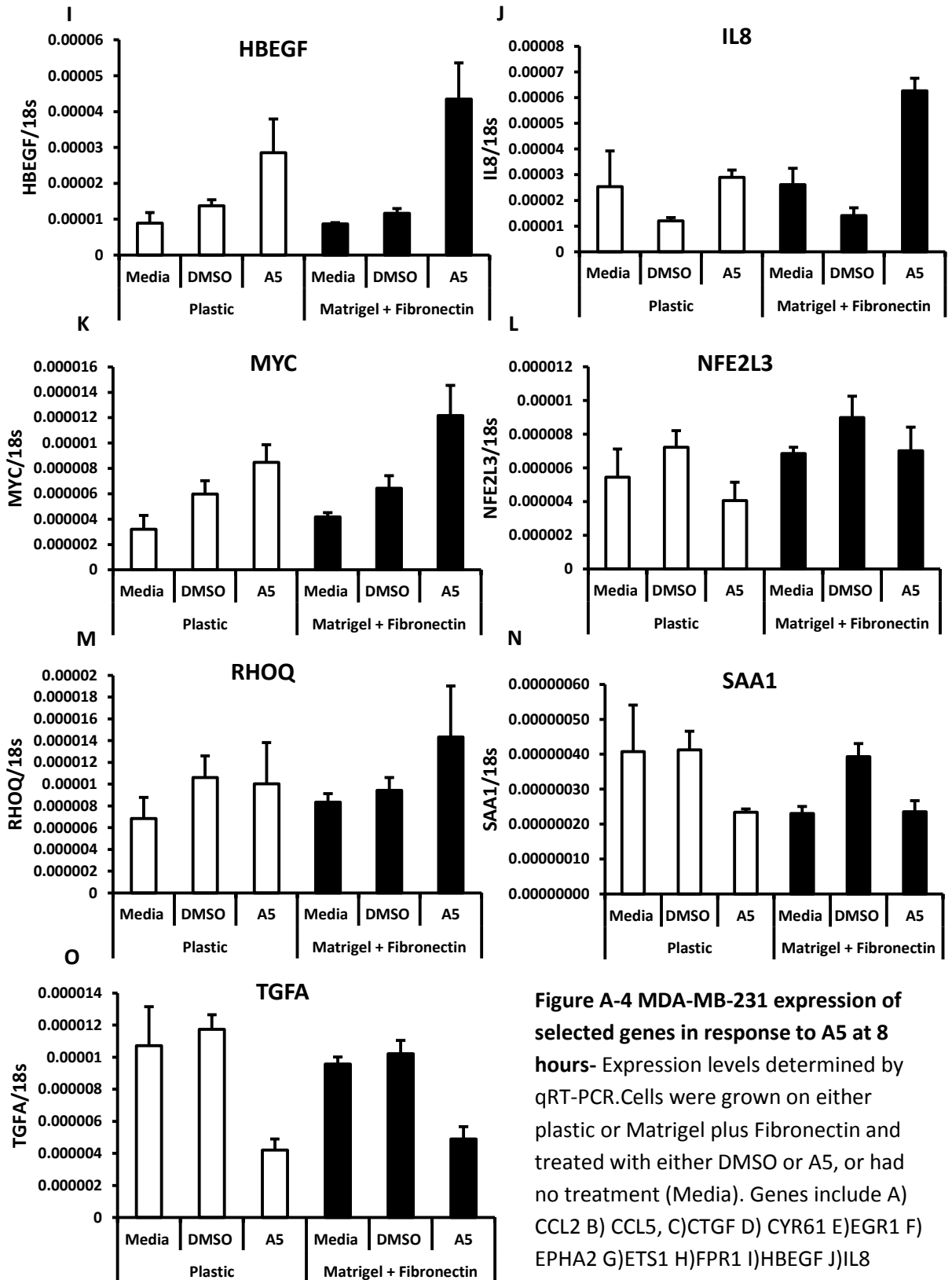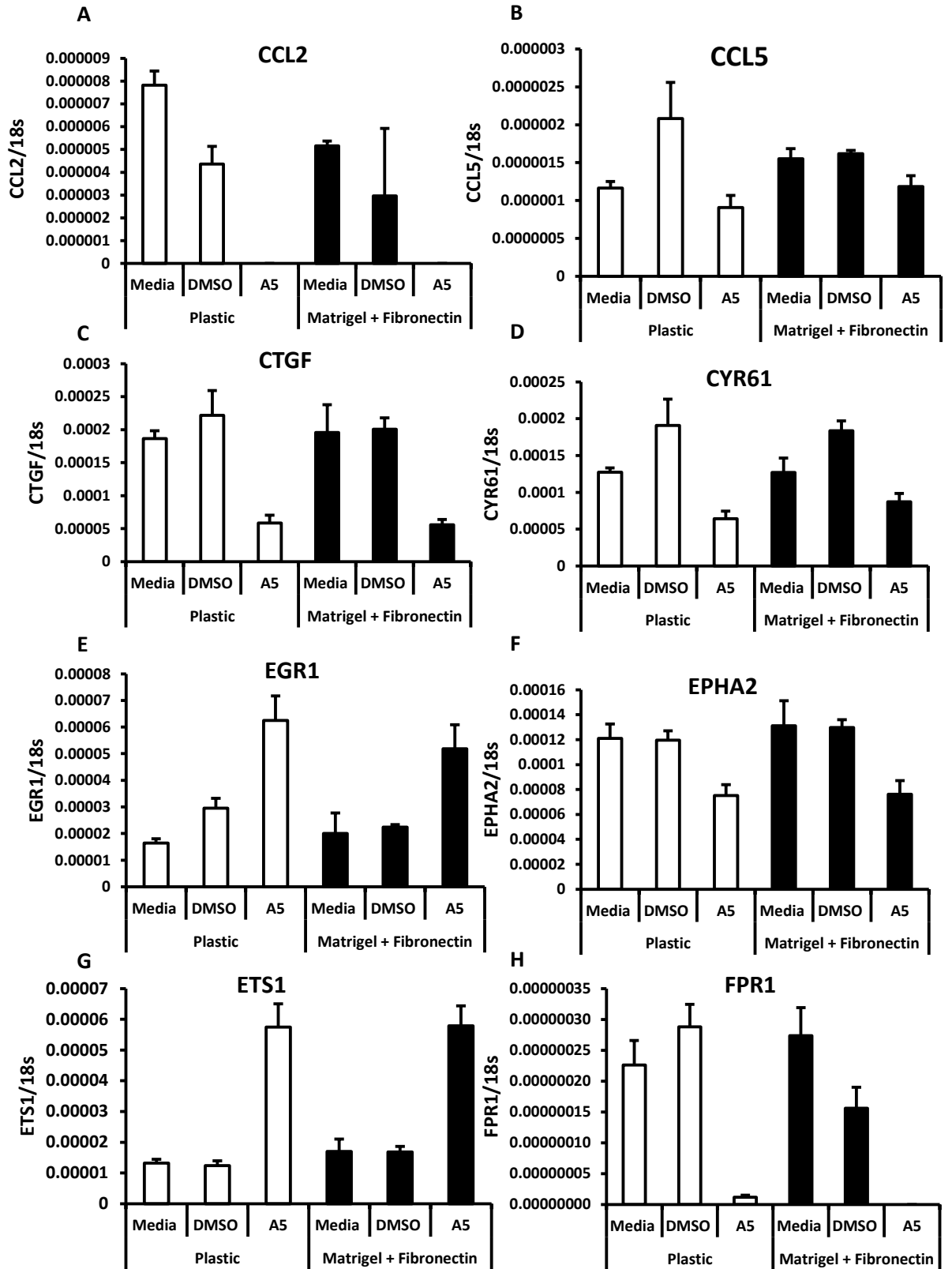
Figure A-4 MDA-MB-231 expression of selected genes in response to A5 at 8 hours- Expression levels determined by qRT-PCR.Cells were grown on either plastic or Matrigel plus Fibronectin and treated with either DMSO or A5, or had no treatment (Media). Genes include A) CCL2 B) CCL5, C)CTGF D) CYR61 E)EGR1 F) EPHA2 G)ETS1 H)FPR1 I)HBEGF J)IL8 K)MYC L)NFE2L3 M)RHOQ N) SAA1 O) TGFA. Each bar represents mean ± S.E.M of three samples and is displayed as the relative quantification. All mRNA expression is normalised to 18s control.

I

J

K

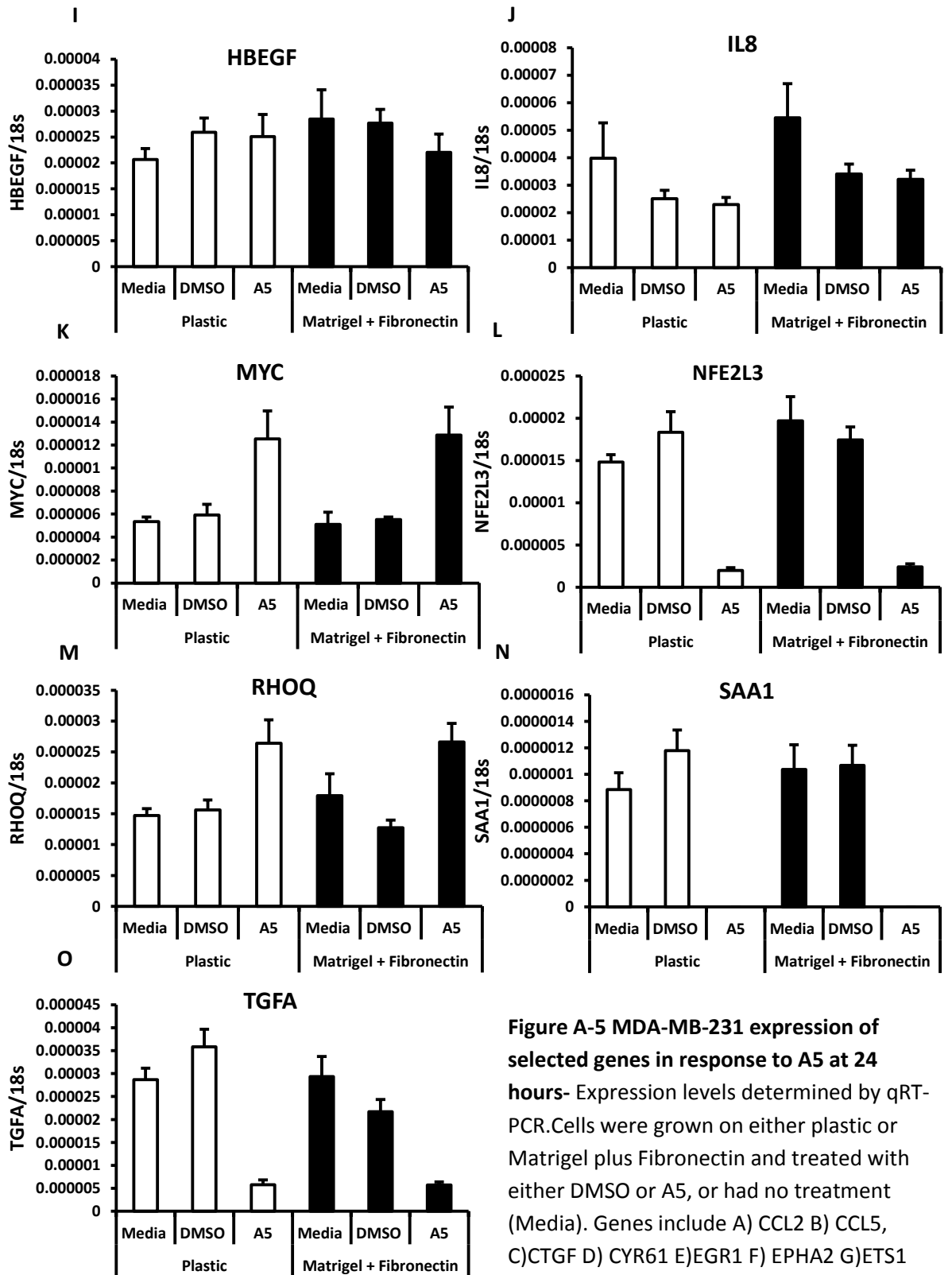**L**

M

N

O

Figure A-5 MDA-MB-231 expression of selected genes in response to A5 at 24 hours- Expression levels determined by qRT-PCR.Cells were grown on either plastic or Matrigel plus Fibronectin and treated with either DMSO or A5, or had no treatment (Media). Genes include A) CCL2 B) CCL5, C)CTGF D) CYR61 E)EGR1 F) EPHA2 G)ETS1 H)FPR1 I)HBEGF J)IL8 K)MYC L)NFE2L3 M)RHOQ N) SAA1 O) TGFA. Each bar represents mean ± S.E.M of three samples and is displayed as the relative quantification. All mRNA expression is normalised to 18s control.

Reagents

1:1 Ethanol: 0.1m Hydrochloric acid releasing agent

A 50ml tube was filled with 25ml distilled water (dH$_2$O) and then 208μl of hydrochloric acid (HCL). The final concentration was made up to 50ml using absolute ethanol (EtOH).

1% Bovine serum albumin

Exactly 2.5g of Albumin, bovine serum (99% protease free, essentially γ-globulin free) was placed in storage flask containing 250ml Phosphate Buffer Saline (PBS) and dissolved using a magnetic stirrer. Storage flask was then placed in 56˚C water bath for 10 minutes to heat inactivate contents.

1% Methylene blue

Added 0.762g sodium tetraborate to 180 ml dH$_2$O and adjusted the pH to 8.5 using HCL and sodium hydroxide. Fill container to 200 ml and then add 2 g of methylene blue powder (Sigma). Shake gently and leave to dissolve.

Freezing and thawing cells

After spinning cells down and dispensing the supernatant, cell pellets are resuspended in 3 ml DMEM (10% FCS) plus 10% DMSO. Cryo tubes (thermo scientific) are then filled with 1 ml aliquots of the resuspension and placed in a 'Mr Frosty' containing isopropyl alcohol. Mr Frosty is placed in the -80°C freezer overnight to achieve repeatable -1°C per minute cooling and then the next day placed in liquid nitrogen stores.
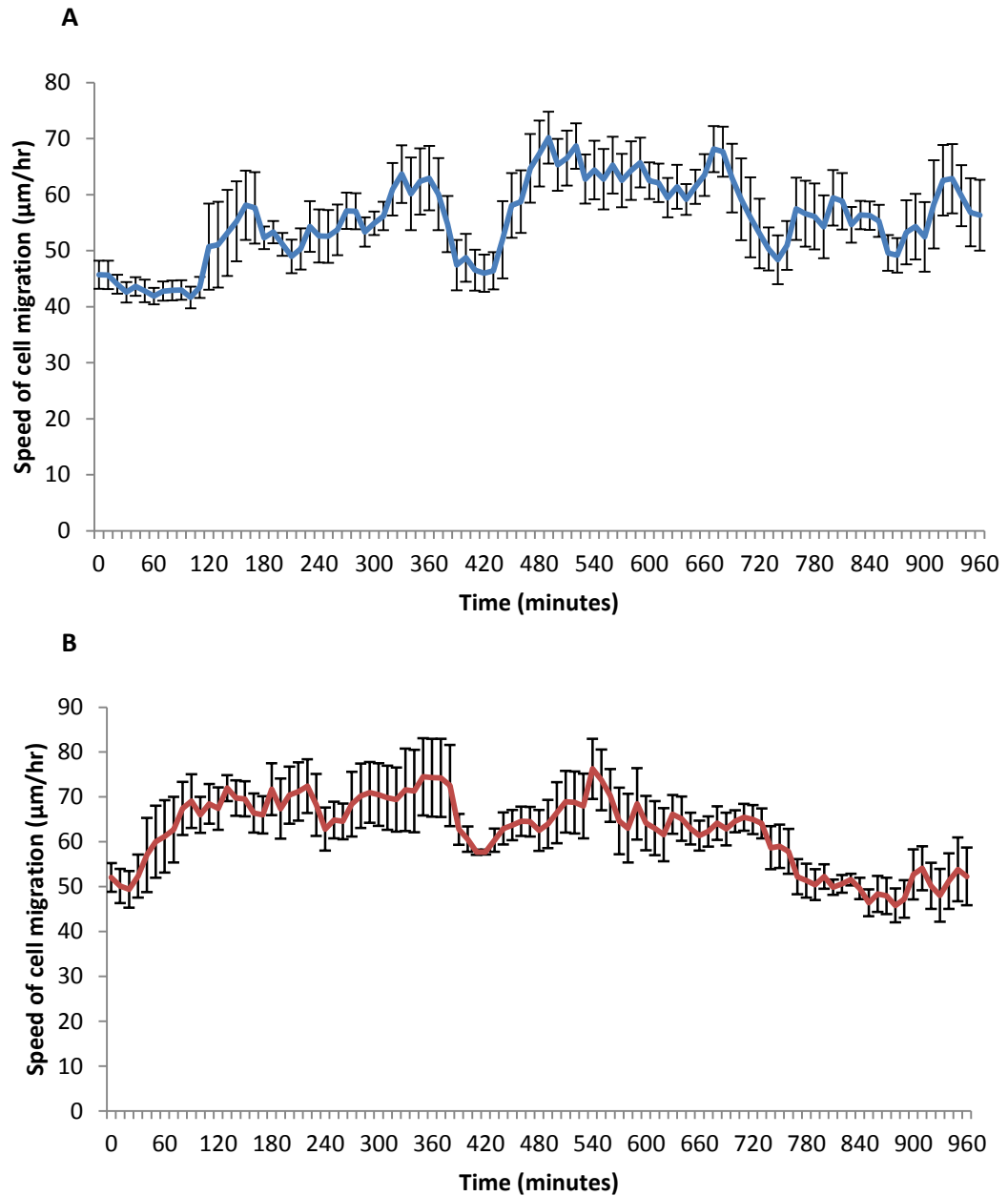
A

B

Figure A-6 MDA-MB-231 moving averages charts from migration assays in response to A5 MDA-MB-231 cells were seeded on to A) Matrigel (red) and B) Fibronectin (blue) which was then followed by stimulation with 50µM A5. Cells were monitored over 17 hours with an image being taken every ten minutes with time-lapse microscopy. Graphs represent the moving average every ten minutes collated over the 17 hour period. This experiment was performed once.
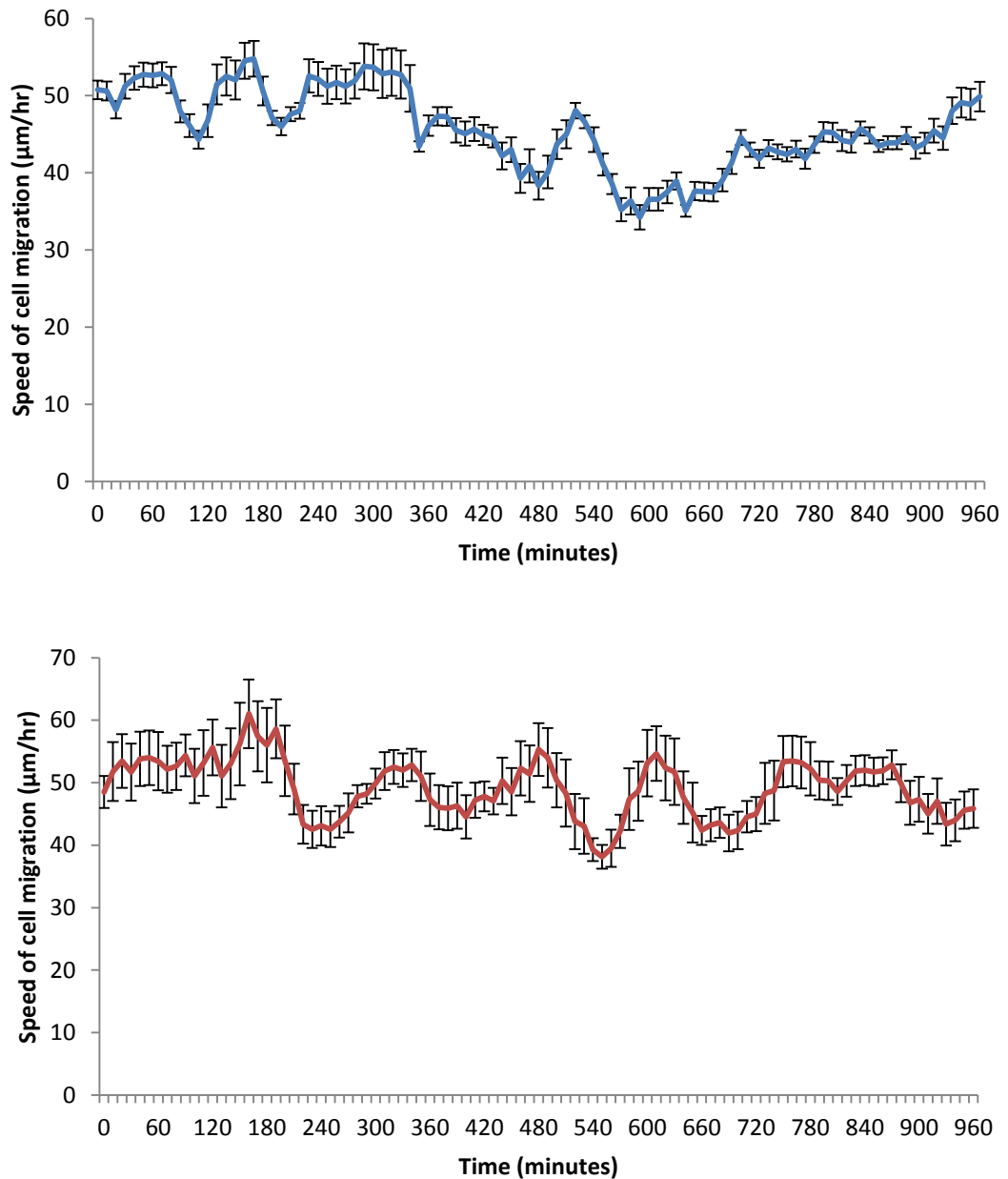
Figure A-7 MDA-MB-231 moving averages charts from migration assays in response to DMSO MDA-MB-231 cells were seeded on to A) Matrigel (red) and B) Fibronectin (blue) which was then followed by stimulation with 0.5 % DMSO. Cells were monitored over 17 hours with an image being taken every ten minutes with time-lapse microscopy. Graphs represent the moving average every ten minutes collated over the 17 hour period. This experiment was performed once.