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Abbreviations

2D – 2-Dimensional

3D – 3-Dimensional

AMT – Amoeboid to Mesenchymal Transition

CAF – Cancer-Associated Fibroblast

CSPG4 – Chondroitin Sulphate Proteoglycan 4

CTLA4 – Cytotoxic T-Lymphocyte Antigen-4

E-Cadherin – Epithelial Cadherin

ECM – Extracellular Matrix

EMT – Epithelial to Mesenchymal Transition

FAK – Focal Adhesion Kinase

GAP – GTPase-activating proteins

GDI – Guanine Nucleotide Dissociation Inhibitor

GEF – Guanine Nucleotide Exchange Factor

Gp130 – Glycoprotein 130

HMW-MAA – High Molecular Weight-Melanoma-Associated Antigen

IL-6 – Interleukin-6

IL-8 – Interleukin-8

JAK – Janus-Activated Kinase

MAT – Mesenchymal to Amoeboid Transition

MCSP – Melanoma Chondroitin Sulphate Proteoglycan

MET – Mesenchymal to Epithelial Transition

MF – Myofibroblast

MLC – Myosin Light Chain

N-Cadherin – Neuronal Cadherin

NG2 – Neuron Glia Antigen-2

PI3K – Phosphoinositol-3 Kinase

PKCα – Protein Kinase C α

STAT – Signal Transducers and Activators of Transcription

TGF- β – Transforming Growth Factor-β

α-SMA – α-Smooth Muscle Actin

Abstract

Cancer cell invasion and metastasis are hallmarks of cancer. It is becoming apparent that the interaction between cancer cells and the surrounding microenvironment are involved in their ability to invade and metastasise. In general, cancer cells can either migrate individually, in an amoeboid or mesenchymal manner, or collectively. The first aim of this thesis was to analyse the role of NG2 in amoeboid to mesenchymal transition (AMT) and Rho/ROCK signalling. We found that NG2 promotes an amoeboid morphology, and increased invasiveness, in a Rho-dependent manner. Secondly, we analysed the role of the major tumour microenvironment (TME) component, cancer-associated fibroblasts (CAFs), on melanoma cell invasiveness. We found the CAF interaction with melanoma cells leads to increased levels of interleukin-6 (IL-6) and IL-8, and this leads to increased invasiveness. Simultaneous blocking of IL-6 and IL-8, using neutralising antibodies, inhibits CAF-dependent invasion. Further analysis of another major component in the melanoma TME, keratinocytes, has highlighted the importance of the tumour cell niche in invasion. Our results indicate that cancer cells have the ability to change morphology, and that the TME plays an important role in melanoma cell invasiveness. Metastatic melanoma treatment has proven difficult over the years, and the use of natural compounds may be desirable. We analysed the effect of natural curcumin, purified from turmeric, on melanoma invasion and found that curcumin inhibits invasion of melanoma cells, in 3D. These results demonstrate the importance of the tumour cell-TME interaction.

Keywords: Melanoma, invasion, fibroblasts, microenvironment, interleukins

Abstrakt

Invazivita a metastázování patří mezi určující vlastnosti buněk zhoubných nádorů. Je nyní již zjevné, že v invazivitě a metastázování nádorových buněk má důležitou roli nádorové mikroprostředí. Nádorové buňky mohou invadovat buď individuálně, s využitím améboidního nebo mezenchymálního způsobu invazivity, nebo kolektivně. Prvním cílem této disertační práce bylo analyzovat roli NG2 v améboidně-mezenchymálním přechodu (AMT) a signalizaci přes dráhu Rho/ROCK. Zjistili jsme, že NG2 podporuje améboidní morfologii nádorových buněk a zvyšuje invazivitu prostřednictvím Rho/ROCK dráhy. V druhé části disertační práce jsme analyzovali úlohu jedné z hlavních složek nádorového mikroprostředí – s nádory asociovaných fibroblastů (CAFs) na invazivitu melanomových buněk. Zjistili jsme, že interakce CAFs a melanomovými buňkami vede ke zvýšení hladin interleukinů IL-6 a IL-8 a následně ke zvýšené invazivitě melanomových buněk. Současné blokování signalizace přes IL-6 a IL-8 s pomocí specifických neutralizačních protilátek inhibuje zvýšení invazivity melanomových buněk indukované pomocí CAFs. Následná analýza další důležité složky nádorového mikroprostředí u melanomů – keratinocytů – ukázala důležitost nádorového niche pro invazivitu. Naše výsledky ukazují, že nádorové buňky jsou vysoce plastické a nádorové mikroprostředí hraje důležitou roli v invazivitě melanomů. Léčba metastazujících melanomů je velmi komplikovaná a je zvažováno i testování přírodních produktů v terapii. V rámci disertační práce byl analyzován efekt přírodního kurkuminu, purifikovaného z indického šafránu, na invazivitu melanomových buněk a zjistili jsme, že kurkumin invazivitu ve 3D prostředí inhibuje. Celkově výsledky potvrzují zásadní roli nádorového mikroprostředí v invazivitě melanomových buněk.

Klíčová slova: Melanom, invazivita, fibroblasty, mikroprostředí, interleukiny

1 Introduction

1.1 Introduction to Cancer

Cells that are embedded in an *in vivo* tissue constantly interact with the biochemical and mechanical nature of their surroundings, converting extracellular signals into intracellular signals and responding accordingly. At a cellular level, responses may include changes in gene expression, proliferation, migration and/or apoptosis. Retaining tissue homeostasis requires overall integration of, and response to, signals from within as well as outside of the tissue and its environment. Once this balance and maintenance is disrupted, neoplastic growth may result. This involves the abnormal, uncontrolled growth of tissue, resulting in what is termed a tumour. Tumours can be benign or malignant, and malignancy takes place when abnormally proliferating cells invade nearby tissue, and spread to other parts of the body, using the blood and lymph systems. In the year 2000, Hanahan and Weinberg produced a list of six characteristics of cancer cells that facilitates their transition from being normal cells to becoming tumourigenic, and eventually malignant (Hanahan & Weinberg, 2000). These traits are, sustenance of proliferative growth, avoiding and resisting apoptosis, escaping methods that negatively regulate cell proliferation, constant replication, induction of angiogenesis, and finally, stimulation of invasion and metastasis. An essential trait of cancer cells is their ability to progress from their initial location.

Until recently, the exact mechanisms that result in invasion and metastasis were unclear. There was knowledge of tumour progression once malignancy reached greater pathological grades, which was demonstrated by resident invasion and distant metastasis. Tumour cells involved in invasion showed modifications in their

morphology in addition to adhesion to other cells and the extracellular matrix (ECM). In certain highly aggressive tumours, alterations in genes that encode cell-cell and cell-ECM adhesion are frequently observed. Genes that aid cytostasis are usually downregulated, conversely, adhesion molecules that promote migration, usually during embryogenesis and inflammation, are frequently upregulated.

Metastasis is a multi-step process, termed the invasion-metastasis cascade, which involves (I) penetration of cancer cells into the adjacent tissue, (II) intravasation, which is the migration of cells into blood and lymphatic vessels, (III) persistence in the circulatory system, (IV) extravasation, which is the migration of cancer cells outside of the vessels, followed by (V) the proliferation of tumour cells into micrometastases in compatible organs, resulting in colonisation (Eger & Mikulits, 2005). Recently, it is becoming apparent that the crosstalk between cancer cells and the cells in the reactive stroma are involved in the developed ability to invade and metastasise. These heterotypic interactions are able to stimulate the expression of malignant cell phenotypes. Approximately 90% of cancer deaths are due to metastasis, thus the prevention of cancer cell dissemination and consequent secondary tumour formation is a primary goal of cancer therapies.

1.2 Cancer Cell Invasion and Migration

Cell migration is a complex and heterogeneous process that is undergone by all nucleated cells at a particular time in their development. Majority of cell types, such as epithelial, stromal, and neuronal cells, the phases of migration are limited to morphogenesis, and migration ceases when cells are differentiated (Friedl & Wolf, 2010). Migration is only reinitiated during tissue regeneration or cancerous processes. In other cell types, such as leukocytes, migration persists throughout the life cycle of the cell, as it is essential for their function (Friedl & Weigelin, 2008). Some cells

migrate only within a restricted substrate, for example epithelial cells, which move along a basement membrane but not via interstitial tissues (Friedl et al., 2004). On the other hand, cell types such as leukocytes adapt to the particular substrate available, and are able to interact and migrate accordingly (Friedl & Weigelin, 2008). Therefore, even though the cells undergo the same basic process of migration, each cell executes migration in varying contexts, using distinct molecular mechanisms and extracellular support signals.

Generally, cell migration takes place in tissues via a series of interdependent steps (Friedl & Brocker, 2000; Friedl and Wolf, 2003): (I) the migrating cell becomes polarised and elongated, (II) this is followed by pseudopod production by extension of the cell's leading edge, which then adheres to the extracellular matrix substrate, (III) then, areas of the cell's leading edge or the whole cell body contract, (IV) this then produces a traction force that leads to subsequent forward gliding of the cell body and the rear edge.

The invasion and metastasis of cancer is not a cell autonomous process but includes a multifaceted network of interactions with other cells and non-cellular components of the tumour stroma. Cancer invasion is a cyclic process, which requires a change in cell morphology, driven by the actomyosin cytoskeleton, thereby leading to the translocation of the cell body. Based on the cell type and the surrounding environment, cells can migrate in two main modes: individually, where cell-cell junctions are absent, or collectively as multicellular groups containing cell-cell adhesions. The primary mechanism in both modes of migration is the dynamics of the cytoskeleton coupling with the cell surface receptors that associate with the tissue environment. Hence, the cell cytoskeleton acts as the cell's primary machinery, and the cell surface receptors act as transmitters.

1.2.1 The Various Modes of Cancer Cell Migration

Initially, morphology was used to describe and classify migration modes. The nomenclature then included certain molecular parameters, which include the organisation of the cytoskeleton, the manner in which the cell and the surrounding matrix interact and the force generated during this interaction, as well as the effect of the migrating cell on the surrounding tissue structure (Friedl & Wolf, 2009). In general, cells can either migrate individually, in an amoeboid or mesenchymal manner, or collectively, consisting of an organized multicellular network (Pankova et al, 2010). Even though the nomenclature is arbitrary and the differences between the molecular machineries are not fully elucidated, they aid in the simplification and categorisation of an otherwise complex literature. In addition, they also permit the analysis of the various molecular mechanisms, which underlie each mode of migration. In recent years, the ideology that each cell type must be assigned a particular mode of migration has disappeared, as there is increasing evidence that there is plasticity in cell motility, and cells can switch from one migration mode to another. The function of environmental properties, which include adhesion, confinement and substrate stiffness, in controlling cell polarity and cell migration has been emphasised. Furthermore, features that are common in different modes of migration, such as actin flows, which was primarily associated with mesenchymal mode of migration, have been recognised in amoeboid migration as well.

Cancer cells have the ability to invade other tissues by either migrating collectively as epithelial layers or detached clusters, or as individual mesenchymal or amoeboid cells. During the process of tumour progression, various cancer cells exhibit modifications in their plasticity via morphological and phenotypical transitions, such

as epithelial to mesenchymal transition (EMT), collective to amoeboid transition, and mesenchymal to amoeboid transition (MAT).

1.2.1.1 *Epithelial-Mesenchymal Transition*

EMT represents the lack of epithelial characteristics and the parallel gain of a mesenchymal gene expression mechanism. It is a highly conserved process that is observed in embryogenesis, chronic inflammation, and during cancer progression (Thierry, 2002). The highest grade of EMT denotes a trait in the malignant tumour progression to an aggressive cancer, and takes place in a number of carcinomas, which include breast (Vincent-Salomon & Thiery, 2003), lung (Tauler et al., 2010), prostate (Giannoni et al., 2010), and colorectal cancers (Brabletz et al., 2001) (Tester et al., 2000; Klein et al., 1991; Giannelli et al., 2005). One important characteristic of EMT is the loss of epithelial (E) cadherin, a primary component that mediates the adhesion in cell-cell contact (Friedl & Wolf, 2003). Concomitantly, there is subsequent increase in mesenchymal markers, including vimentin and neuronal (N) cadherin (Kalluri & Weinberg, 2009; Lehembre et al., 2008; Yilmaz & Chrisofori, 2010). Upregulation of N-cadherin is associated with the cytoskeletal modification by controlling Rho GTPases (Harris & Tepass, 2010). The transformation from E- to N-cadherin expression, also known as the cadherin-conversion, results in increased motility of EMT-transformed cells. Hence, cells that have gone through EMT lack epithelial organisation and demonstrate an increased ability to separate from epithelial cell clusters so as to migrate as individual cells in a mesenchymal manner. In addition to increased motility, EMT also leads to prevention of apoptosis and senescence by obtaining stem cell properties (Thiery et al., 2009). The reversal of EMT is mesenchymal to epithelial transition (MET), which is represented by epithelial

reorganisation. During MET, mesenchymal cells redeem epithelial cell-cell adhesion in other to colonise distal sites in metastasis (Kalluri & Weinberg, 2009)

1.2.1.2 *Amoeboid and Mesenchymal Cell Invasion*

Mesenchymal to amoeboid transition (MAT) and amoeboid to mesenchymal transition (AMT) refers to the transition of mesenchymal cells to amoeboid cells, and vice versa. It has been detected in breast cancer and melanoma, amongst other cancers (Friedl et al., 2012). These transitions are dependent on Rho GTPase signalling, and are independent of protease activity, including matrix metalloproteinases (MMPs) (Ridley, 2015). It was shown that blocking extracellular proteolysis stimulates MAT (Wolf, 2003).

1.2.1.2.1 Amoeboid Mode of Migration

Amoeboid mode of migration generally refers to the movement of cells with a rounded or ellipsoid morphology, lacking in mature focal adhesions and stress fibres (Ridley, 2015). Amoeboid cells adhere loosely to the ECM, demonstrating an apparent lack of cell polarity and the ability of chemotaxis (Sahai & Marshall, 2003). This form of migration is often observed in numerous cancers, such as breast, lymphoma, prostate, as well as melanoma and sarcoma (Narumiya et al., 2009). There are two main subtypes of amoeboid migration; one being the migration of rounded, blebbing cells, which do not adhere or pull on the substrate, but instead utilise a propelling and pushing mode of movement (Lammermann & Sixt, 2009). The other type of amoeboid migration is seen in amoeboid cells that have a more elongated morphology, which produce actin-rich filopodia at the leading edge that are involved in weak, poorly defined interactions with the substrate (Lorentzen et al., 2011). In a unique case of amoeboid migration, dendritic cells that are terminally matured and

nonadhesive, create actin-rich filopodia instead of producing blebs at their leading edge (Lammermann & Sixt, 2009). This causes the cell to become entrapped within the extracellular matrix substrate during migration. Amoeboid tumour cells do not exhibit any production of stress fibres, are not involved in ECM remodelling and are lacking in focalised integrins (Sahai & Marshall, 2003). Amoeboid cell migration is considered the fastest migratory morphology as cells can reach a velocity of 20 micrometres per minute, as compared to more elongated mesenchymal cells, which demonstrate a velocity of up to 1 micrometre per minute (Friedl & Wolf, 2003). Amoeboid migration is independent of proteases, as cells generally recruit actomyosin-mediated forces that shift matrix fibrils instead of degrading them. I contrast to the migration process explained above, in amoeboid migration, the focal contacts and focalisation of proteolysis are thus abolished, whereas immediate and non-focalised receptor assemblies at the cell-matrix contacts persist. Specifically, it is suggested that the amoeboid mode of invasion, independent of proteases, takes place when structural pores present in collagen networks do not display stabilising covalent crosslinks that regulate ECM stiffness (Lammerman et al., 2008). The migration of amoeboid cells may be inhibited due to structural limitations as cells that contain a nucleus larger than the collagen pores cannot be negotiated.

1.2.1.2.2 Mesenchymal Mode of Migration

Conversely, individual cells with high adhesive potential and low cytoskeletal contractility acquire a mesenchymal mode of migration, which intensifies cell-matrix interactions and migration in a manner that resembles fibroblasts (Sanz-Moreno et al., 2008). Generally, mesenchymal cells utilise a five-step sequence of migration, which includes pseudopod protrusions, production of focal contacts, focalised proteolysis, actomyosin contractility, and lastly, the disassociation of the rear end (Wolf et al.,

2003). Invasion of individual migrating mesenchymal cells is observed in melanoma, glioblastoma, breast and lung carcinomas, in addition to other cancers (Paulus et al., 1996). Interestingly, a characteristic of some cancers is the movement of individual cells that form and resolve temporary cell-cell adhesion, all the while migrating along the same track, which is known as chained migration or cell streaming (Tester et al., 2000). These mesenchymal cells typically lose E-cadherin expression and demonstrate numerous traits of active invasion, such as the expression of integrins and surface proteases (Tester et al., 2000).

Lastly, cells that maintain rigid cell-cell contact partially or completely silence the movement of cells inside a group, nevertheless the cytoskeletal activity of cell in the leading and outward edges or points of cell-substrate contact is supported. As a result, collective migration occurs, with an appearance that resembles irregularly shaped sheets or masses of a multitude of cells.

1.2.2 Rho GTPase Signalling in Cell Migration

It is becoming clearer that cells are not rigid in their migration, and can interchange between various modes of migration (Friedl et al., 2012). As described above, they can migrate on their own, as individuals, or collectively, as a group. They are able to change between a lamellipodium-based manner and motility based on the formation of blebs (Paluch & Raz, 2013; Wolf et al., 2003). This change is dependent on the confinement and stiffness, as well as the composition of their current environment, such as the ECM components, and cells in the surrounding (Zhang et al., 2008; Charas & Sahai, 2014). The interaction between cells greatly affects the manner in which the cells move and what regulatory pathways are involved in their motility. When cells are in contact with each other, migration is often halted due to a process called contact inhibition, and they either create cell-cell adhesions or alter their

direction of travel, the latter leading to the dispersal of cells *in vivo* (Batson et al., 2013). Cells may undergo directional migration, where they are guided towards a specific area by soluble or matrix-associated stimulations, or may randomly migrate with frequent directional changes (Graziano & Weiner, 2014). A mutual factor in all these migration modes is the role of Rho GTPases.

The role of Rho GTPases in migration was first found about two decades ago (Ridley et al., 1995). The Rho GTPases form a subgroup of the Ras superfamily of 20- to 30kD GTP binding proteins that have been shown to regulate a number of cellular functions (Boureux et al., 2007). Recently, the use of 3-dimensional (3D) models of migration *in vitro* and work on animals *in vivo* has greatly increased our understanding of the various functions of Rho GTPases in cell migration in tissues, as well as in environments that resemble tissues.

Currently, there are approximately twenty known Rho GTPase genes in humans. Like all members of the Ras superfamily, the majority of Rho GTPases are active and are involved in the stimulation of downstream targets when bound to GTP, and inactive when bound to GDP. The activity of the two forms is controlled by guanine nucleotide exchange factors (GEFs), which enhance the exchange of bound GDP for GTP, and the GTPase-activating proteins (GAPs), which increase the hydrolysis of GTP to GDP on Rho proteins (Boureux et al., 2007). There are more than eighty GEFs and over seventy GAPs, which suggests that the Rho GTPase family is multifaceted and that their activity can be regulated by numerous signalling pathways subject to the spatiotemporal context (Boureux et al., 2007). Furthermore, Rho-like GTPases are also controlled by guanine nucleotide dissociation inhibitors (GDIs), which are able to inhibit the exchange of GTP, as well as the hydrolysis of bound

GTP (Boureux et al., 2007). The most studied Rho GTPases are Rho, Cdc42 and Rac, which are greatly conserved members of the Rho family members in eukaryotes, as they are found in animals, fungi, as well as plants (Hall, 2012). The analysis of the contribution of the Rho GTPases in cell migration has been complicated, due to the similarities between the Rho and Rac genes, as well as the splice variants of Rac1 and Cdc42. Moreover, there are at least thirteen other Rho family members in mammals, which possess diverse and less characterised functions in cell migration (Lawson & Burridge, 2014).

1.2.2.1 *Rho, Rac, and Cdc42 in 2D environments*

Hall and colleagues first found that Rho GTPases were involved in the remodelling of cellular architecture during cell movement in 2D (Ridley et al., 1992). They showed that the plasma membrane extension in lamellipodia is primarily driven by Rac-dependent actin polymerisation, whereas RhoA promotes the formation contractile actomyosin fibres, also known as stress fibres (Ridley et al., 1992). Later, it was shown that Cdc42 is involved in the stimulation of filopodia formation, as well as the activation of Rac (Nobes & Hall, 1995).

During cell migration, Rho GTPases play a role in actin polymerisation, in addition to force generation, by interacting with, and activating, the kinase ROCK (Machacek et al., 2009). In 2D, it was assumed that Rac and Cdc42 are active at the leading edge, in order to stimulate the formation of protrusions, whereas Rho is only active in the cell body and the rear of the cell, thereby providing the actomyosin-dependent force required for the retraction of the rear-end and facilitate forward movement (Machacek et al., 2009). Further analysis of the Rho GTPase activity has shown this model to be inadequate, as it was then shown that Rho is also active at the leading edge, and its activation takes place before that of Rac and Cdc42 (Raftopoulou & Hall, 2004).

These findings emphasise the complex nature of the role of the Rho GTPases during cell migration, as well as the disparities seen in the use of 2D environments in order to analyse migration.

1.2.2.2 *Rac, Rho and Cdc42 GTPases in 3-Dimensional Migration*

The use of 3D environments to study the modes of migration has revealed that Rho GTPases are regulators of different modes of migration. Cells moving through collagen-rich connective tissue demonstrate different physical and chemical properties. As previously mentioned, the migration of single tumour cells that are in these 3D environments either exhibit a rounded, highly contractile morphology, which is mediated by Rho, or an elongated, lower contractility morphology that is dependent on Rac (Friedl & Wolf, 2010). These two modes of migration are interchangeable, as, in a permissive environment, blocking of particular signalling pathway components that stimulates a certain mode of migration alters the cell's migration to the other mode. It is important to note that both modes of migration depend on actomyosin contractility in order to produce the force required for movement, however, they vary in the amounts of contractility that is needed (Sahai, 2005). The canonical Rho/ROCK signalling pathway mediates actomyosin contractility (Fig. 1), where the kinase ROCK, activated by Rho, phosphorylates and inactivates the myosin light chain (MLC) phosphatase (Sanz-Moreno et al., 2008). This leads to the activation of myosin-II (Sanz-Moreno et al., 2008; Kimura et al., 1996). It was previously demonstrated that Rho and Cdc42 interact and produce the actomyosin contractility that is required for the elongated mode of migration (Wilkinson et al., 2005). It was shown that most of the actomyosin contractility required for the rounded mode of movement is produced downstream of the canonical Rho/ROCK signalling pathway (Wilkinson et al., 2005). On the other hand, activation

of Cdc42 downstream of DOCK10 stimulates actomyosin contractility via the activation of kinases, which directly phosphorylate the MLC, thereby leading to Myosin-II activation (Gadea et al., 2008). The use of co-culture systems to analyse migration has shown, using squamous cell carcinoma and stromal fibroblasts, that cancer associated fibroblasts (CAFs) have the ability to remodel the extracellular matrix by traction forces driven by actomyosin contractility. This leads to the production of tracks that are followed by carcinoma cells (Gaggiolo et al., 2007). A novel characteristic of migration was emphasised, that Rho and Cdc42 interact with each other to regulate actomyosin contractility. This was shown by carcinoma cell use of Cdc42 to drive actomyosin contractility, in order to follow the tracks created by the CAFs, which utilised the Rho/ROCK signalling pathway to mediate actomyosin contractility (Gaggiolo et al., 2007).

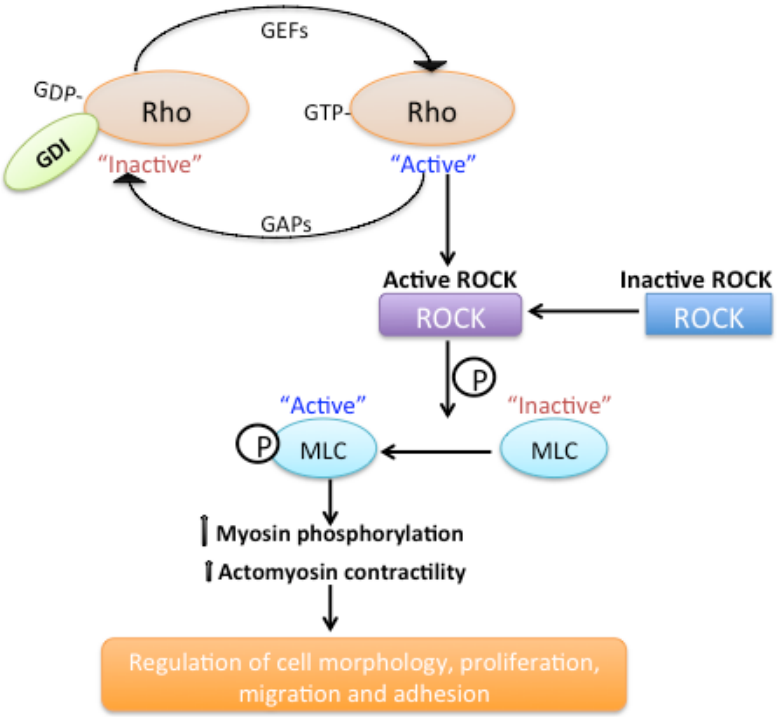


Figure 1. Components of the Rho/ROCK signalling pathway. A number of extracellular stimuli, such as growth factors and hormones, bind to cell membrane receptors, which act upon GEFs and GAPs to mediate the activation of Rho GTPases. In its GTP-bound active state, Rho GTPases binds to ROCK to stimulate major downstream effector proteins such as MLC, which, when phosphorylated, promotes the phosphorylation of myosin and enhances actomyosin contraction.

1.2.3 The NG2 Proteoglycan Plays An Essential Role in Cancer Invasion

Neuron glia antigen-2 (NG2) / CSPG4 (chondroitin sulphate proteoglycan 4), originally referred to as high molecular weight-melanoma-associated antigen (HMW-MAA) or melanoma chondroitin sulphate proteoglycan (MCSP), hence called NG2, is a unique, transmembrane proteoglycan. It is responsible for a number of interactions that take place between cancer cells and their environment, in order to facilitate a number of cellular events, which lead to tumour growth and invasion (Stallcup, 2002). NG2 is predominantly found in tissue specific progenitor cells during development (Stallcup, 2002). The *CSPG4* gene that encodes NG2 is switched off once differentiation terminates, however it is abnormally re-expressed in numerous malignancies (Burg et al., 1998). Initially, it was believed that NG2, which is composed of three functionally distinct subunits, was distributed in a fashion restricted to pericytes (PCs) and melanocytes. In recent studies, it is apparent that NG2 is distributed in a wide range of progenitor cells, such as pericytes, smooth muscle cells, mesenchymcal stem cells, chondrocytes and oligodendrocyte precursor cells (Campoli et al., 2010). Notably, even though it is difficult to observe during development, it was shown that the downregulation of NG2 is associated with deficiencies in progenitor pools, as well as defective vasculature (Huang et al., 2010). This suggests that NG2 has a crucial role in development, in particular, tissue homeostasis and repair, and angiogenesis. Certainly, NG2 is a known modulator of oligodendrocyte precursor cell migration, and its expression is also associated with the invasiveness of melanoma and glioma cancers (Campoli et al., 2010).

NG2 is of great interest in the context of tumourigenesis, as it is not only expressed by cancer cells but also particular cells in the tumour microenvironment. Increased NG2 expression is frequently observed in glioma, melanoma and sarcoma cells, and it is thought that it contributes to aggressive tumour growth, as well as poor patient outcome (Price et al., 2011).

1.2.3.1 *The NG2 Protein*

NG2 was first reported in malignant melanoma, more than three decades ago. It was identified as highly glycosylated integral proteoglycan present in two forms that are expressed on the cell membrane, an N-linked 280-kDa core protein, and a highly glycosylated 450-kDa protein (Price et al, 2011). As the two consist of the same core protein but are independently expressed, a great challenge is determining to what extent NG2 is targeted to the extracellular matrix in cancer, and how this promotes carcinogenesis. A greater task is the use of NG2 in targeted therapy, and the development of drugs that are aimed at NG2-expressing tumour cells, and the surrounding environment.

The NG2 proteoglycan consists of a large 2,225 amino acid extracellular domain and a smaller, 76-amino acid cytoplasmic domain (Campoli et al, 2010) (Fig.2). The extracellular domain allows anchorage to glycosaminoglycan chains, and is involved in adhesion and growth factor signalling as a co-receptor to ECM components (Campoli et al, 2010). Even though the cytoplasmic domain of NG2 is not essential for co-receptor function, it is necessary for its role in adhesion and migration, which suggests that NG2 directly stimulates intracellular signalling (Campoli et al, 2010). In the cytoplasmic domain, it was shown that certain threonines are phosphorylated by various stimuli, which include protein kinase C α (PKCα) and extracellular signal-regulated kinase (ERK) (Cooney et al., 2011). The C-terminal PDZ-binding domain

interacts with PDZ proteins GRIP, syntenin and MUPP1 (Barritt et al., 2000). Furthermore, NG2 has been linked to the regulation of the Rho GTPases Cdc42 and Rac, which, as mentioned above, are crucial mediators of cell polarity and migration. NG2 phosphorylation results in the stimulation of Rac activity via the polarity complex protein PATJ, as well as the Rho GEF Tiam1 (Biname et al., 2013). In addition, the NG2 proteoglycan also contains chondroitin sulphate GAG chains, facilitating its involvement in cell migration (Campoli et al., 2010). Our recent work, as well as others', has highlighted the role of NG2 in Rho stimulation. Taking into consideration NG2's role in Rho GTPase regulation, adhesion, and association with growth factors, it is clear that NG2 is an important mediator of cell polarity and migration.

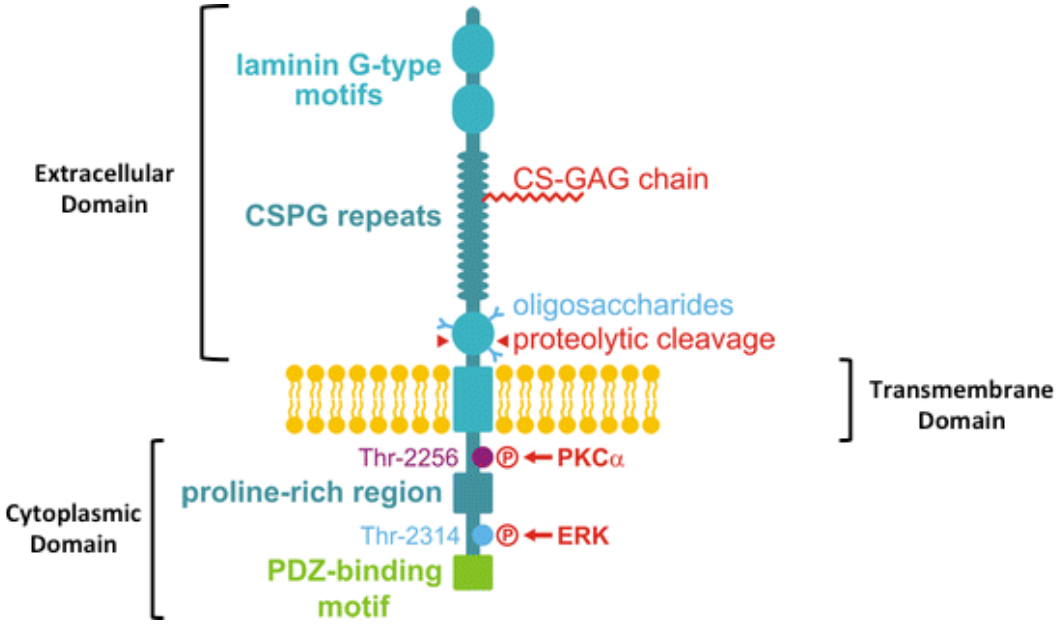


Figure 2. The structure of the NG2 proteoglycan. The large extracellular domain consists of 2,225 amino acids, with an N-terminal domain that contains two laminin G-type motifs that are stabilised by disulphide bonds, as well as a CSPG repeat domain. The small transmembrane domain contains 25 amino acids, and the cytoplasmic domain, which consists of 76 amino acids, contains a C-terminal PDZ-binding motif as well as threonines phosphorylated by distinct stimuli. *Adapted from Biname, 2014*.

1.2.3.2 *NG2 Interactions and Adhesion*

The most significant concept of the importance and role of NG2 was derived from a study using rat orthologs. Subsequently, numerous studies have followed this unique protein in wide range of fields under physiological and pathological conditions.

Generally, cells need to attach to the substratum in order to produce forces required for migration. In tissues, cells are in contact with the extracellular matrix, which consists of other cells and a complex network of macromolecules. Attachment of cells to the surrounding extracellular matrix is facilitated by integrins, membrane receptors and cell surface proteoglycans. Integrins are heterodimeric receptors that contain a large extracellular domain, which binds the extracellular matrix. This binding links the extracellular matrix to the cytoskeleton via a short cytoplasmic domain. The specificity of integrins is determined by their α and β subunits.

The binding of integrins to certain extracellular substrates stimulates integrin assembling and the production of adhesion complexes whereby adaptor proteins connect the integrins to the actin cytoskeleton. The adhesions that are formed at the edge of the cell mature to form focal complexes, which then develop into focal adhesions due to the mechanical stress from inside and outside of the cell. The external mechanical forces are due to substrate stiffness, and internal forces come from actomyosin contractility, which is controlled by Rho GTPases. During this process, focal adhesion kinase (FAK) is recruited to the focal adhesions, which then leads to the activation of PI3K, Rac and Cdc42, thereby mediating actin polymerisation (Yang et al., 2004). In addition, integrins also recruit cell surface matrix metalloproteinases (MMPs) that are involved in the degradation of the extracellular matrix in order to create space for the cells to move in 3D environments (Chekenya et al., 2008). It is important to note that directional movement involves

ROCK-mediated disassembly of the integrin-dependent adhesion and cytoskeleton contraction at the rear of the cell, which allows the cell to engage in polarised movement (Chekenya et al., 2008).

The extracellular matrix (ECM) is not a static structure, and is activated during tumourigenesis. In addition to integrins, cancer cells interact with the ECM via various receptors, including NG2. Even though NG2 itself is not known to be a predominant regulator of transmembrane signalling, it is able to mediate the proliferation, migration, and resistance of apoptosis of cancer cells by interacting with integrins, growth factor receptors, and extracellular matrix components. Analysis of NG2 in surgically removed cancer lesions has shown that NG2 is deposited in the cancer ECM during the tumour reaction, and is involved in the re-organisation of the ECM in order to promote cancer cell migration and metastasis (Burg et al., 1996). Furthermore, more in depth hispathological studies have revealed that NG2 levels do not differ between primary and metastatic lesions, during early stages of cancer. However, at later stages, there is significantly higher expression of NG2 in metastatic lesions compared to the primary tumour (Higgins et al., 2014). This suggests that NG2 is involved in the migratory potential of tumour cells, which has positive correlation with poor prognosis.

NG2 is highly conserved in mammals and other organisms including chicken, zebrafish and *Drosophila* with the maintenance of domains, which include laminin G-type motifs, CSPG repeats, and the PDZ-binding domain (Price et al., 2011). NG2 binds to a number of ECM components, such as collagen II, collagen V, tenascin, laminin, and its popular, highly characterised interaction with collagen VI (Burg et al., 1996). The central non-globular domain of NG2 binds to collagen VI and mediates *in vitro* glioma migration towards gradients on soluble collagen VI (Burg et

al., 1996). Due to the high affinity NG2 has for collagen VI, cells expressing NG2 can keep the glycoprotein at the cell surface and could as a result alter the assembly of the pericellular matrix (Burg et al., 1996). NG2 also interacts with integrins, which include α3β1 and α4β1, to regulate adhesion. For example, activation of both NG2 and α4β1 integrin promotes integrin-dependent cell spreading. NG2 and α4β1-mediated adhesion leads to FAK phosphorylation and syntenin binding (Fukushi et al., 2004). NG2 is also involved in the proteolysis of collagen I by interacting with the metalloproteinase, MT3-MMP, through its GAG chains leading to invasion through collagen I (Price et al., 2011).

Previous reports have shown that in a number of cell types, NG2 contributes to the activity of Rho GTPases (Biname et al., 2013; Barritt et al., 2000; Pankova et al., 2012; Sivasankaran et al., 2004). In glioma cell lines, the most popular cell lines used in NG2 research, transfection with NG2 and cell subsequent cell spreading on surfaces coated with NG2 antibodies revealed specific membrane protrusions, such as filopodia and lamellipodia. In melanoma cells, NG2 clustering was shown to recruit and activate Cdc42, which may explain the role of NG2 in filopodia formation as this process is regulated by Cdc42, while it was shown that lamellipodia induction was controlled by Rac. Recently, it was revealed that PKCα phosphorylation of NG2, in the cytoplasmic tail, leads to lamellipodia formation and migration, in association with integrins.

1.3 Tumour Microenvironment and invasion/metastasis

1.3.1 The Tumour Microenvironment and Cancer Progression

Over a century ago, Paget proposed the "seed and soil" theory (Paget, 1889). The analysis of the molecular features of the "seed", i.e. cancer cells, has been extensive. Numerous oncogenes, as well as tumour suppressor genes, of cancer cells have been identified and characterised. This has led to the general consensus that cancer cells are genetically and/or epigenetically modified. On the other hand, the "soil", which is created by both cancer cells and the host stromal cells, is not fully characterised, possibly due to the complex nature of its structure and function. Recently, particular developments in molecular technology have highlighted the biomedical importance of the "soil" (Marsh et al., 2012).

Tissues contain a plethora of cells that work together to effect normal physiology. These cells also have positional identity so that their location is distinct and their number limited. Cancers lose these restrictions via mutations in oncogenes and tumour suppressor genes. However, these tumour cells do not lose all their interactions with the surrounding non-cancerous cells or with the extracellular architecture.

Homeostasis in normal tissues involves strict control of the balance between cell proliferation and death, which is attained and sustained through intercellular communication. A crucial regulator of normal cell behaviour and tissue homeostasis is the surrounding extracellular matrix (ECM) (Duda et al., 2010). The ECM has a number of functions, such as acting as a physical scaffold, mediating interactions between different cell types, and providing survival and differentiation of signals. Preserving organ homeostasis can inhibit neoplastic transformation in normal tissues

by ensuring stable tissue structure (Fukushi et al 2004). It is this mutual interchange of information between cells and their surroundings that permits the functional organisation of the cells into tissues and guides organogenesis during development. Communication between cells and their microenvironment occurs through a complex network of signals generated by cell-ECM and cell-cell adhesion and junctional molecules, as well as by collaboration between the epithelial, stromal and other organ-specific cell types (Hynes, 2009). These ECM-molecules, in addition to the enzyme molecules that remodel them, not only organise and form tissues, but also directly signal the cells (Hynes, 2009). If the microenvironment were not dominant, each cell would have its own way and the end results would be either uniformity in similar fate or absolute chaos. Studies in this field have given considerable insight into the supremacy of the microenvironment over epithelial cell behaviour. Mintz et al demonstrated that the microenvironment of a mouse blastocyst in addition to supressing tumourigenicity of teratocarcinoma cells, also stably reprogrammed these cells, which resulted in normal chimeric mice (Mintz et al., 1975). Studies that followed also showed that the embryonic microenvironment is potent in its ability to reprogram various cancer cells, which include metastatic cells, to a less aggressive phenotype (llmensee & Mintz, 1976). During early tumour development, the protective constraints of the microenvironment are overridden by conditions such as chronic inflammation, and the local tissues microenvironment becomes a growth-promoting site (Bissell & Hines, 2011).

The existence of the reactive stroma has been known for a long time, although it is now known whether the sequence of events does not always begin with the tumour (Bissell & Hines, 2011). However, upon tumour formation, irrespective of the

mechanism, in addition to drastic modification of the stroma, an inflammatory reaction is initiated, as well as a complex immune response (Balkwill et al., 2005). The inflammatory reaction either already exists prior to tumour formation, and may be partially responsible for tumour progression, or the inflammatory cells are mobilised in response to signals stemming from the tumour microenvironment.

1.3.2 Cancer-Associated Fibroblasts

Cancer-associated fibroblasts (CAFs) are a significant component of the tumour microenvironment. They are a key population of cells in the tumour stroma that, in a number of cases, dictate the outcome of the cancer by promoting proliferation, inflammation, angiogenesis and metastasis. CAFs generally originate from mesenchymal cells, which are found in a number of healthy tissues (Madar et al., 2013). Normal fibroblasts are elongated cells that reside in connective tissues and mediate the formation and turnover of the ECM (De Wever et al., 2008). Fibroblasts regulate and maintain normal tissue homeostasis and are involved in a wide range of biological processes, including wound healing and senescence. Quiescent fibroblasts are activated into myofibroblasts (MFs) during tissue remodelling (De Wever et al., 2008). In wound healing, for example, MFs gain actin contractility by expressing α-smooth muscle actin (α-SMA) and form cell-cell adhesions via gap junctions (Chung et al., 2005). Once the wound healing process terminates, MFs undergo programmed cell death and are eventually removed from the tissue (Olumi et al., 1999).

1.3.2.1 *Cancer-Associated Fibroblasts and the ECM*

As tumours are referred to as “wounds that do not heal” (Dvorak, 1986), it is logical that CAFs and MFs have comparable properties, such as the expression of SMA.

However, CAFs persist in the tumour stroma and do not undergo apoptosis, resulting in accumulation of redundant ECM and the production of a number of soluble factors (Kalluri & Zeisberg, 2006; Joyce & Pollard, 2009; Gaggioli C et al, 2007; Calvo F et al., 2013; Lu et al., 2012). As a major component of the tumour microenvironment, the ECM is known to mediate a number of biochemical and mechanical functions via its complex composition consisting of proteins, proteoglycans, glycoproteins and other macromolecules (Lu et al., 2012). ECM components such as collagen and fibronectin are known to interact with adhesion molecules, including integrins (Lammerman et al., 2008). Furthermore, ECM proteins are able to interact with, and regulate, particular growth factors, resulting in their distribution and subsequent activation.

In order to ensure tissue integrity in normal tissue, the ECM maintains force distribution and balance. The biophysical controls of the ECM tightly regulate the cell integration into epithelial monolayers. In cancer, ECM stiffness may lead to cell confinement, as it was suggested that the compressive stress from the ECM limits tumour progression by reducing cell proliferation (Lu et al., 2012). However, this may have a negative effect by promoting tumour cell invasion and metastasis. It is clear that the biophysical traits of the ECM in the tumour microenvironment are important regulators of the proliferative and invasive potential of cancer, even at the initial stages of the cancer development (Lu P et al., 2012).

CAFs form a heterogeneous cell population with varying biological properties. Presently, there is uncertainty in the exact manner in which CAFs and its role in ECM modelling promotes cancer cell invasion. It was previously shown that cancer cells invade in a CAF-dependent manner, due to the ability of CAFs to remodel the ECM (Gaggioli et al. 2007; Neri et al. 2015; Satoyoshi et al. 2015). It was also reported that

CAF-dependent remodelling leaves residues of ECM components, facilitating cancer cell adhesion (Gaggioli et al. 2007). Recently, Neri *et al* showed that ECM remodelling by CAFs is a rate-limiting factor in cancer cell invasion as there was positive correlation between cancer cell invasion and CAF-dependent ECM remodelling (Neri et al, 2016). Taken together, these results highlight the biomechanical and biochemical importance of ECM remodelling in cancer cell invasion, and further evidence that more studies on CAFs are required to help explain their exact role in local and distant metastasis of heterogeneous cancer forms.

1.3.2.2 *Cancer-Associated Fibroblasts and Melanoma Progression*

CAFs are present in great numbers in various cancers, such as breast and prostate carcinomas, as well as melanoma (Kalluri & Zeisberg, 2006; Pietras & Ostman, 2010). Melanoma is an aggressive form of cutaneous neoplasia that plays a significant role in the number of skin cancer deaths. Data shows that melanoma accounts for approximately 2% of skin cancer cases, however, it is responsible for almost 80% of deaths in skin cancer.

1.3.3 Interleukin-6 and -8 Play a Significant Role in Malignant Melanoma

Tumour cells stimulate a number of molecular changes, which are known to promote cancer progression, and subsequent metastasis. These modifications include autocrine expression of cytokines and growth factors, which have been implicated in tumour growth and metastasis. Melanoma cells are known to secrete a number of growth factors and cytokines, either constitutively or due to stimulation. These primarily include interleukin-6 (IL-6) and interleukin-8 (IL-8).

1.3.3.1 *Interleukin*-6 (IL-6)

Several serum cytokines, which include IL-6, play an important role in the initiation and progression of melanoma; however, the precise biological roles that IL-6 plays in melanoma progression are unclear (Sullivan et al., 2009). Interluekin-6 (IL-6) is a multifunctional cytokine that plays an important role in a number of processes, which include the regulation of inflammation and immune responses (Barton et al., 1997). It is produced by a number of cells, which include the monocytes, fibroblasts, keratinocytes, macrophages and various cancer cell types, and it binds to the IL-6 receptor, IL6R (Colomiere et al., 2009). Inflammation plays a significant role in tumourigenesis. The IL-6/IL6R receptor complex stimulates glycoprotein 130 (gp130), which then induces the signalling of Janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT) (Colomiere et al., 2009).

A number of studies have demonstrated increased serum levels of IL-6 in cancer patients, which are associated with disease severity and outcome (Conze et al., 2001). IL-6 has been implicated in the regulation of growth and differentiation in numerous cancers, and is correlated with poor prognosis in several cancers, including melanoma (Mouawad et al., 1996). IL-6 is an important mediator of cell survival as it aids cells in evading programmed cell death. Furthermore, IL-6 has been shown to enhance tumour progression by promoting cancer cell proliferation, migration, and subsequent metastasis (Jee et al., 2004).

In melanoma initiation, studies have shown that IL-6 inhibits the growth of melanocytes *in vitro* (Hoejberg et al., 2012). In addition, IL-6 also inhibits the growth of cancer cells derived from nonmetastatic melanoma. However, IL-6 promotes the proliferation of tumour cells derived from metastatic melanoma, which suggests there is a change from paracrine to autocrine growth stimulation (Hoejberg et al., 2012). It

was also shown that cells derived from metastatic melanoma have increased expression of both IL-6 and IL6R (von Felbert et al., 2005). von Felbert and colleagues showed, using transgenic mice predisposed to develop melanoma, that mice that are unable to produce IL-6 demonstrate fewer and smaller melanomas, in comparison to IL-6 producing mice (von Felbert et al., 2005). This suggests that lack of IL-6 decreases melanoma incidence and size, indicating that IL-6 promotes the development and progression of melanoma. IL-6 may be produced as a paracrine inducer by cells in the tumour microenvironment (Whipple & Brinckerhoff, 2014).

1.3.3.2 *Interleukin* – 8 (IL-8)

CXCL8 or interleukin-8 (IL-8), hence referred to as IL-8, is a member of the C-X-C family of chemokines, which are a group of cytokines involved in inflammatory, chemotactic and growth-stimulating processes (Varney et al., 2006). IL-8 is an immunoregulatory molecule that has gained growing interest in melanoma research. It is a chemokine produced by a plethora of cells, including macrophages, epithelial cells, fibroblasts and endothelial cells (Wang et al., 1990). IL-8 can bind to a number of receptors, although the most common are the G-protein coupled receptors CXCR1 and CXCR2 (Singh et al., 2009). IL-8 is an important regulator of the innate immune response, and can induce neutrophils, as part of the immune response, to the site of infection (Wang et al., 1990). Furthermore, IL-8 plays an important role in a number of biological functions, including angiogenesis and migration, and studies have shown that it may play an important role in tumour growth and metastasis.

IL-8 and its receptors have been associated with melanoma progression, by stimulating a number of processes such as tumour cell proliferation and migration (Singh et al., 2009). Previously, it was shown that IL-8 expression was correlated with human melanoma metastasis, and IL-8 was not expressed in non-tumourigenic and

nonmetastatic cases (Singh et al., 1999). More than 20 years ago, IL-8 was shown to promote angiogenesis, and melanoma cell migration, and therefore its expression by tumour cells was postulated to be indicative of a metastatic phenotype (Singh et al., 1994). IL-8 levels were shown to positively correlate with a metastatic phenotype in human melanoma cell xenografts in nude mice (Ugurel et al., 2001). Furthermore, UV-B radiation on nude mice promoted IL-8 expression at both mRNA and protein levels, subsequently leading to enhanced melanoma tumour cell progression and metastasis (Gebhardt et al., 2007). In tumour samples from varying stages of melanoma, IL-8 was differentially expressed in the radial and vertical growth phases (Varney et al., 2003). While IL-8 expression was not observed in radial growth phase tumours, more than half of the vertical growth phase tumours heterogeneously expressed IL-8 (Varney et al., 2003). Comparable data was also reported in another study, which showed correlation between IL-8 expression and the depth of the tumour (Singh et al., 2009). Furthermore, it was recently demonstrated that in melanoma patients, plasma IL-8 levels significantly increases in the third and fourth Breslow score stages, which is based on the measurements of the thickness of the melanoma (Kucera et al, 2015). These results indicate that IL-8 expression is associated with melanoma metastasis, and abnormal expression of IL-8 may be a general feature of metastatic melanoma. IL-8 may be involved in melanoma tumour development and their ability to metastasise.

1.3.4 CAFs, Melanoma and IL-8 and IL-6

Metastasis is a vital step in the cancer progression and continues to be a significant setback in cancer therapy (Li et al, 2009). There is increasing evidence that the tumour microenvironment plays a crucial role in metastasis. The interaction between cells in the tumour stroma and cancer cells is considered bidirectional, as they all play

a part in the process of metastasis. One hypothesis is that tumour cells may modify gene expression in cells residing in the tumour microenvironment in order to produce a favourable environment for tumour cell invasion (Joyce & Pollard, 2009). Similarly, cells in the tumour stroma may stimulate genetic modification, as well as clonal selection in cancer cells, which leads to the production of tumour cells that are able to invade into the surrounding tissue (Joyce & Pollard, 2009). Recently, a number of studies have supported the former hypothesis; one demonstrated that macrophages in the lung tumour stroma showed distinct expression of genes that represent a role in the promotion of tumour metastasis (Hedbrant et al., 2015). Others showed that the tumour stroma displayed significantly upregulated gene transcripts in comparison to normal stroma (Crnogorac-Jurcevic et al., 2002; Okochi et al., 2012). Gallagher *et al* also demonstrated, using gene profiling of the melanoma and stromal fibroblast crosstalk, that human fibroblasts only slightly affected the gene expression of melanoma cells, on the other hand, the gene expression of the fibroblasts were significantly altered by the melanoma cells (Gallagher et al, 2005). Recent studies have also showed that CAFs in the tumour stroma assist melanoma tumour chemoresistance by inducing the secretion of cytokines and chemokines, which favour tumour cell growth and stimulate the production of proteins that aid the cells to evade programmed cell death. Tiago *et al* showed that melanoma cells grown with CAFs significantly increased the production of IL-6 and IL-8, compared to cells grown without (Tiago et al., 2014). These studies suggest that the interaction between melanoma cells and the fibroblasts in the tumour stroma, which are known to inhibit tumour progression, may lead to the secretion of products by melanoma cells, which affect gene expression in normal fibroblasts. This causes them to possess a CAF-like phenotype (Eck et al., 2009) (Fig.3). These studies also imply that there is direct

interaction between melanoma cells and stromal fibroblasts, which is required to increase the production of these pro-invasive cytokines (Fig.3). This provides further evidence that the tumour microenvironment in melanoma has the ability to promote melanoma tumour progression.

IL-6 and IL-8, as well as CAFs have key roles in tumour progression. The relationships between IL-6 and -8 secretion and the activities of melanoma cells and CAFs have not been fully examined. However, there is increasing evidence that upregulation of key chemokines in CAFs in the melanoma stroma supports immune cell infiltration, which is pro-invasive and pro-metastatic (Cheng et al., 2016). With regard to tumour progression, interleukins 6 and 8, and fibroblasts are considered important players. Several studies have demonstrated that IL-6, IL-8 and CAFs represent potential therapeutic targets in a number of cancers (Li et al., 2009, Na et al., 2013; Kolar et al., 2012).

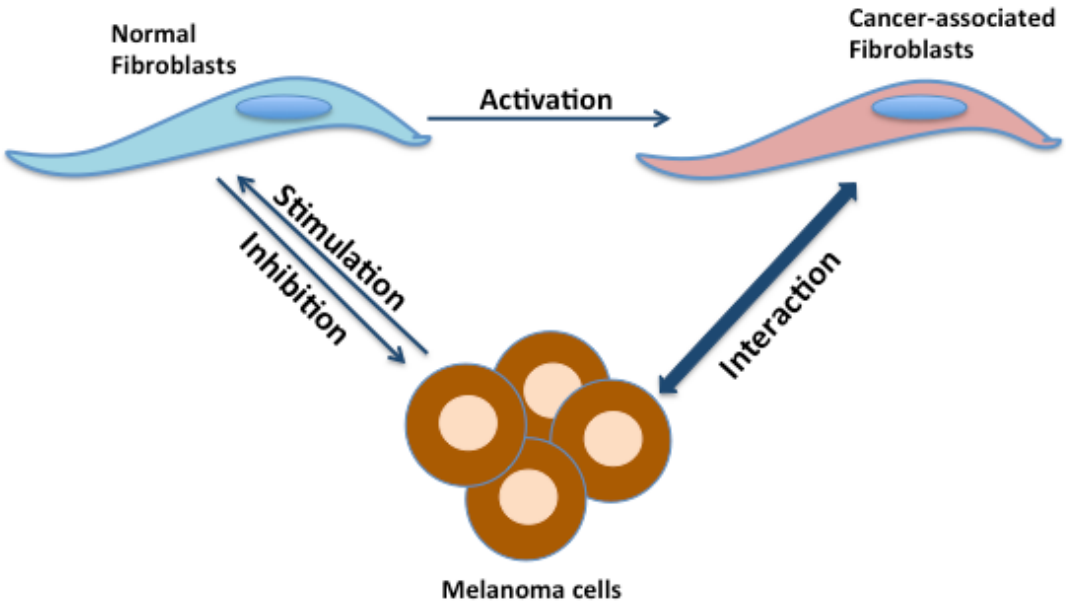


Figure 3. The interaction between melanoma cells and fibroblasts in the stroma. Melaoma cell interaction with normal fibroblasts may stimulate gene expression of factors that lead to fibroblast activation into CAFs. CAF interaction with melanoma cells also leads to the production of factors which promote melanoma progression.

1.3.5 CAFs and Melanoma Therapy Resistance

Malignant melanoma is recognised as a treatment resistant form of cancer. CAFs, in addition to stimulating proliferation, angiogenesis and inflammation, also contribute to tumour resistance against therapies. It was previously shown that an increased level of CAFs in the tumour stroma is positively correlated with a greater risk of metastasis and poor clinical outcome in breast, lung and pancreatic cancers (Hwang et al., 2008; Liao et al., 2009; Bremnes et al., 2011). The use of co-culture experiments *in vitro* have shown that CAFs promote tumour progression in melanoma and other cancers, such as pancreatic and prostate cancer (Li et al, 2009). In addition, *in vivo* studies have also demonstrated that CAFs enhance melanoma tumour growth, and were then used as a target to suppress tumour progression (Gascard & Tlsty, 2016).

Recently, more studies are showing that the tumour stroma promotes melanoma therapy resistance (Seip et al., 2016). The protective role of the tumour microenvironment may have significant implications for a number of targeted therapies, such as MAPK, as it can result in the production of non-responding melanoma cell subpopulations, in an otherwise responsive tumour. Seip et al demonstrated that CAFs decreased melanoma tumour sensitivity to BRAF inhibitors *via* stromal contact (Seip et al., 2016). The tumour microenvironment may also induce melanoma cancer cell plasticity as a protective mechanism. Fibroblasts have been demonstrated to induce a de-differentiated mesenchymal-like morphology in melanoma cells, which has been associated with BRAF inhibitor resistance in a number of studies (Konieczkowski et al., 2014; Muller et al., 2014; Ramsdale et al., 2015). Other studies also showed that CAFs in the tumour stroma could deposit ECM to which melanoma cells could adhere to, thus gaining a survival advantage (Hirata et al., 2015; Fedorenko et al; 2015). Further research on the interaction between CAF-

dependent cancer cell plasticity and modifications in signalling should improve therapeutic targeting. Combination therapies could be developed to target both CAF-dependent and independent mechanisms in metastases.

In addition to their role in melanoma tumour progression, CAFs are more genetically stable, compared to cancer cells, thus they have shown promise as an ideal therapeutic target (Loeffler et al., 2006). As mentioned, molecules that are associated with CAFs or involved in the tumour-stroma interactions may play a role in tumour metastases at a particular stage, however they may inhibit tumour progression in another tumour stage or microenvironment setting. For this reason, thorough assessment of interaction between melanoma and its microenvironment is essential for successful targeting of particular molecules in CAFs. This includes comprehending the dynamics of melanoma cancer cell survival, growth, and subsequent invasion, all of which are associated with CAFs. The use of novel genetic, molecular and cellular methods will certainly aid and accelerate this process.

Importantly, agents targeting certain growth factor signalling in CAFs, such as HGF, PDGF and TGFβ are currently under preclinical or clinical trials for a number of cancers, including breast cancer, melanoma and chronic myelogenous leukaemia (Gonda et al., 2010; Xing et al., 2010). The modes of action of these drugs are to either directly block fibroblast growth or target signalling pathways associated with CAF-tumour cell interactions (Gonda et al., 2010; Togo et al., 2013). It was reported that lack of Notch1 in mouse embryonic fibroblasts (MEFs) leads to an increase in their proliferation and survival. Recently, it was shown using cell culture and a xenograft mouse model that Notch1 pathway activation in CAFs inhibits melanoma cell growth (Shao et al., 2011). Furthermore, CAFs treated with imatinib, a tyrosine

kinase inhibitor that blocks PDGFR signalling, reduced cervical cancer growth and invasion in mice (Pietras et al., 2008).

The current treatment options for metastatic melanoma patients still lack the ability to improve the overall survival rate and significant challenges still lie ahead in order to implement stromal targeting in a clinical setting. Understanding tumour stroma-dependent resistance to therapy may have a great effect on therapeutic strategies that target the microenvironment. Furthermore, targeting various parts of the tumour may represent a solution to evade resistance and achieve positive responses.

2 Aims and Objectives

1. Analysis of the role of NG2 in AMT. We aimed to study mechanisms in which NG2 activates Rho/ROCK signalling. We hypothesised that NG2 interacts with MUPP1, as well as the Rho GEF Syx, and this leads to the activation of Rho in cancer cells. Subsequently, this may promote an amoeboid morphology.
2. Analysis of the role of cancer associated fibroblasts (CAFs) in melanoma cell invasiveness. We aimed to study effect of CAFs and factors secreted by CAFs on human melanoma cell invasiveness. Recently, IL-6 and IL-8 were identified as two genes that are associated with melanoma progression. It was shown that CAFs interaction with melanoma cells elevated IL-6 and IL-8 levels in transcriptomic analysis. These two cytokines have been linked with increased tumour growth and metastasis, and could thus contribute to the pathogenesis of melanoma. One of the main setbacks in melanoma is distant metastasis. We investigated whether direct interaction of CAFs and melanoma cells leads to elevated levels of IL-6 and IL-8, and whether this interaction promotes invasion of melanoma cells, in a 3D environment.

3 Materials and Methods

All chemicals are from Sigma, unless otherwise stated.

3.1 Cell lines and culture conditions

BLM, A2058, WM3629 and WM3670, A375m2 melanoma cells, human fibroblasts (HFP3) and cancer-associated fibroblasts (Mel Fib) were cultured in DMEM (Sigma), supplemented with 10% foetal bovine serum (FBS) (Sigma), 1% antibiotic-antimycotic solution (Sigma) and 50µg/ml Gentamicin (Sigma), at 37 ^{o}C with 5% CO2. K2 and A3 cells were cultivated in MEM with Hanks' salts (HMEM) supplemented with 10% bovine serum (ZVOS), 0.09% sodium bicarbonate, 0.12 g/l sodium pyruvate, and 1 mmol/l glutamine at 37 °C with 5% CO2.

3.2 Conditioned Media Preparation

1 x 10^5 fibroblasts (HFP3 or Mel Fib) were seeded on 10cm dishes, and for the co-cultured conditions, fibroblasts (HFP3 or Mel Fib) were plated with melanoma cells (BLM or A2058) on 10cm dishes at a ratio of 1:10. Cells were cultured in DMEM with 10% FBS for 48 hours, the medium was then replaced and the cells cultured for a further 24 hours. The conditioned media (CM) was then collected and filtered using a 0.22 µm syringe filter (TRP), and stored at -80 ^{o}C until required.

3.3 Spheroid Invasion Assay

Melanoma cells were grown as spheroids using Microtissues® 3D Petri Dish® (12-81 large spheroids) according to manufacturer's instructions. Spheroids were then embedded in 2 mg/ml Collagen R (Serva) solution, which contains DMEM (Sigma), 5% NaHCO3 (Sigma) and 10% FBS (Sigma). Using a 48 well plate, one spheroid was embedded per well, and once collagen polymerized, DMEM or CM was added to the

wells. Images were taken immediately, and after 48 hours using a Nikon-Eclipse TE2000-S (4×/0.13 PHL objective, 10×/0.13 MHC objective) and NIS-Elements software. They were analysed using ImageJ.

3.4 Cell invasion and morphology assays in 3D collagen

The 3D collagen invasion assay was performed using a 200 µl solution containing 2 mg/ml Collagen R (Serva) and 4 mg/ml collagen G (Biochrom). The mixture was prepared on ice, and it contained 100 µl of collagen R (Serva), 100 µl of PBS, 200 µl of collagen G (Biochrom), 46 µl of $NaHCO_3$, 46 µl HMEM and 8 µl of NaOH. 10 µl of the collagen solution was added into each well of µ-Slide Angiogenesis plate (Ibidi) and polymerized at 37 °C. 50 µl of cell suspension (2×10^5 cells/ml) was added on top of a collagen gel. After 2 days, invasion was scored as an average invasion depth of the cells in selected field of view using Nikon-Eclipse TE2000-S (20×/0.40 HMC objective) and NIS-Elements software. For each experiment, 10 µm optical z-sections were analysed in 6 fields of view and the average invasion depth was assessed. In order to compare between individual experiments, average invasion depth was normalized to that of untreated cells.

To analyse cell morphology in 3D collagen, cells were trypsinised, washed in complete medium, counted and 10^5 cells were mixed with 500 µl of 3 mg/ml Collagen R in complete medium. The suspension of cells in collagen (500 µl) was loaded to a well in a 12-well plate, the gel was allowed to polymerize at 37 °C for 30 minutes and overlaid with complete medium. After 24 h the morphology of cells in 3D collagen was analysed using Nikon Eclipse TE2000-S microscope (20×/0.40 HMC objective). Cell morphology was classified on the basis of the elongation index. The elongation index was calculated as the length divided by the width. Cells whose elongation index was more than 3 were considered mesenchymal. Intermediate cells had an elongation

index of 2–3; for amoeboid cells, the index was 1–2. Dividing cells were excluded from the analysis. For each condition a minimum of 300 cells per experiment were analysed for morphology in 3D collagen.

3.5 Gelatin degradation assay

Dry coverslips were coated with a thin layer of FITC-conjugated gelatin and immediately overturned on a drop of 0.5% ice-cold glutaraldehyde in PBS for 15 minutes in the dark. Coverslips were then transferred to a 12-well plate, with the coated side up and gently washed three times with PBS, incubated with sodium borohydride (5 mg/ml) in PBS for 3 minutes, and then finally washed with PBS. The cover slips were sterilized in 70% ethanol for 1 min, dried for 10 minutes in a sterile hood and quenched in complete medium for 1 hour at 37 ^{o}C. 3×10^4 cells were plated on fluorescent gelatin-coated coverslips in media containing 10 μM Batimastat (Tocris Biosciences, UK) and allowed to adhere at 37 °C overnight. Cells were washed three times with medium to remove the inhibitor and incubated another 3 h in complete medium. After 3 hours, the coverslips were washed, fixed in 4% paraformaldehyde and permeabilised with 0.1% Triton X-100 in PBS for 15 minutes. After blocking for 30 minutes in 5% BSA, F-actin was stained with Alexa594-conjugated phalloidin. Coverslips were mounted using Mowiol reagent and representative fields were documented by photomicroscopy using a Nikon Eclipse TE2000-S microscope (20×/0.75 Plan Apo objective) mounted with VDS Vosskühler CCD-1300 camera. To quantify areas of degradation for each independent experiment, we analysed 10 random fields. The value of each degradation area was measured using the public domain software ImageJ; the total area degraded in the captured field was then normalized for the number of cells. The effect of each

treatment was expressed as relative degradation: degradation normalized to control untreated cells.

3.6 Flow cytometry

Confluent cells were harvested, washed twice in ice cold FACS buffer (HBSS buffer supplemented with 20 mM glucose, 2% fetal bovine serum (Sigma), 2% antibiotic–antimycotic (GIBCO)), resuspended at 10^7/ml in FACS buffer, and 10^6 cells were incubated with anti-NG2 antibody. After 30 min at 4 °C, cells were washed and stained in dark for 20 min with Alexa Fluor-488 conjugated goat anti-mouse antibody. Flow cytometry was performed using a BD FACSVantage SE system (BD Biosciences).

3.7 Wound Healing Assay

BLM or A2058 cells were grown to confluency on 6-well plates. Consistently shaped wounds were made using a P200 pipette tip across each well, creating a cell-free area. The cultures were then washed with DMEM in order to remove loose cells. The cells were then cultured in DMEM with 10% FBS or CM, and images were taken at 0, 6 and 12 hours. At least 3 images of the scratched area were captured using a Nikon-Eclipse TE2000-S (10×/0.13 MHC objective) and NIS-Elements software. The same scratched area was selected for measurements at each time of study.

3.8 Proliferation Assay

6 x 10^3 BLM or A2058 melanoma cells were plated on a 96-well plate overnight, media was then removed, cells were washed with PBS and DMEM with 10% FBS or CM was added to the cells. Proliferation was analysed using alamarBlue® (Life

Technologies) according to manufacturer's instructions. Absorbance was measured using TECAN infinite® m200 PRO and i-control™ software.

3.9 Enzyme-Linked Immunosorbent Assay (ELISA)

IL-6 and IL-8/CXCL8 levels in conditioned media, and during invasion of BLM and A2058 cells from spheroids in collagen, were measured using sandwich ELISA according the manufacturer's protocol (R&D Systems). For statistical analysis, conditioned media and media from invading cells in collagen were collected three times independently.

3.10 Immunohistochemistry

Melanoma samples were obtained and routinely processed at Department of Dermatology, First Medical Faculty, Charles University in Prague under written informed consent of patients with respect to Declaration of Helsinki. Formaldehyde fixed paraffin embedded sections were routinely rehydrated and heat induced epitope retrieval in citrate buffer (pH 6, DAKO, Glostrup, Denmark) was consequently performed. Endogenous peroxidase activity was blocked by 1% hydrogen peroxide (20 minutes) and non-specific interaction of immunoglobulins was blocked by incubation in diluted 10% non-immune goat serum (20 minutes). Primary antibodies diluted according to manufacturer´s instructions and incubated overnight at 4°C (IL-6, mouse monoclonal, Ab 9324; IL-8, mouse monoclonal, Ab18672; Abcam, Cambridge, UK). Visualisation of immunohistochemical reaction was performed using HRP polymer kit with DAB (Histofine Simple Stain MAX PO Multi, Nichirei,Bioscience, Tokyo Japan) and counterstained with haematoxyline.Negative controls were performed using specie-specific isotype controls.

3.11 Immunoblotting and Rho pull-down assays

Confluent cell cultures were washed with phosphate buffered saline (PBS) and lysed in modified RIPA buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP40, 1% sodium deoxycholate, 50 mM NaF, 1% aprotinin and 0.1 mM Na3VO4). Protein concentrations in lysates were determined using the BCA assay (Pierce). For immunoblotting, samples were separated on 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Non-specific activity was blocked by incubating 45 min at room temperature in Tris-buffered saline containing 0.05% Tween-20 (TBST) and 4% bovine serum albumin (BSA). Membranes were then incubated overnight in primary antibody, washed extensively with TBST, and then incubated for 1 hour at room temperature with HRP-conjugated secondary antibody. After extensive washing in TBST, the blots were developed by enhanced chemiluminescence and exposed by using LAS 4000 Luminiscent image reader (Fujifilm Life Sciences). RhoA pull-down assays were performed using by GST-rhotekin and Rho pull-down detection kit according to the manufacturer's instructions (Thermo Scientific). The levels of total and active RhoA were revealed using a Rho-specific antibody.

Antibodies against NG2 and HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology Inc. Anti-pY421 Cortactin, Alexa Fluor 594-phalloidin, anti-rabbit (Alexa Fluor-594) and anti-mouse (Alexa Fluor-488) were from Life Technologies. Antibody against Rho was from Thermo Scientific (Pierce Biotechnology, USA).

3.12 Statistical Analysis

The significance of differences was analysed with ANOVA followed by Tukey's honest significant difference test, as well as an unpaired, two tailed, Student's *t* test. Values of $p < 0.05$ were considered to be statistically significant.

4 Results

4.1 Analysis of the Role of NG2 in Amoeboid-Mesenchymal Transition

The project was based on previous reports which showed, using RNA expression microarray analysis, that NG2 was greatly upregulated in the amoeboid chicken fibrosarcoma A3 cell line, as compared to K2 cells, its parental, non-invasive, mesenchymal counterpart (Cavanna et al., 2007). The NG2 proteoglycan mediates adhesion to collagen and other ECM components in a manner independent of integrins (Tillet et al., 2002), and could therefore represent the non-integrin ECM receptor of amoeboid cells.

In this project, my role included performing Rho pull-down assays, gelatin degradation and flow cytometry. A supplement of the publication corresponding to these results is attached at the end of the thesis.

We used the highly invasive and metastatic A3 cell line, and its parental, non-invasive K2 cell line, as well as metastatic melanoma cell line, A375m2. A3 cells predominantly have an amoeboid morphology and express high levels of NG2, whereas K2 cells are mesenchymal in morphology with undetected levels of NG2. We manipulated NG2 cells in these cells using siRNA-mediated knockdown and NG2 overexpression and re-expression. We then analysed the effect of these alterations on cell morphology and invasion in 3D, as well as Rho-GTP levels and ECM degradation.

Knockdown of NG2 in A3 cells, using siRNA, led to AMT. There were significantly lower levels of amoeboid A3 cells, and an increase in mesenchymal cells. Subsequently, siRNA-mediated knockdown of NG2 in A3 cells led to decreased levels of invasion and reduced levels of Rho-GTP levels. On the other hand, overexpression of NG2 in K2 cells resulted in increased invasion, higher levels of

Rho-GTP and MAT, which was inhibited by the ROCK inhibitor Y27632 (Fig.4). Thus further highlighting the role of Rho/ROCK signalling in NG2-dependent MAT.

We then confirmed the results observed in the fibrosarcoma cells in human melanoma A375m2 cells. These cells demonstrate a primarily amoeboid morphology in 3D, however they maintain their ability to form invadopodia and degrade ECM. We analysed the effect of siRNA-mediated knockdown of NG2 in A375m2 cells. This led to decreased levels of amoeboid cells and an increase in mesenchymal cells in 3D, as well as reduced levels of invasion. Both effects were rescued by NG2 re-expression. Knockdown of NG2 in A375m2 cells also led to decreased levels of Rho-GTP, which was also rescued by NG2 re-expression.

We then analysed the effect of NG2 on invadopodia formation in the amoeboid A375m2 cells and mesenchymal K2 cells using the gelatin degradation assay (Figure 5). siRNA-mediated knockdown of NG2 led to increased levels of gelatin degradation, which was rescued by NG2 re-expression. In K2 cells, which have undetected levels of NG2, overexpression of NG2 led to decreased levels of gelatin degradation.

These results suggest that NG2 proteoglycan plays an important role in amoeboid migration and invasion of cancer cells, which is associated with enhanced Rho-GTP levels. Furthermore, NG2 expression greatly inhibits matrix degradation. The results were published in European Journal of Cell Biology, in 2012.

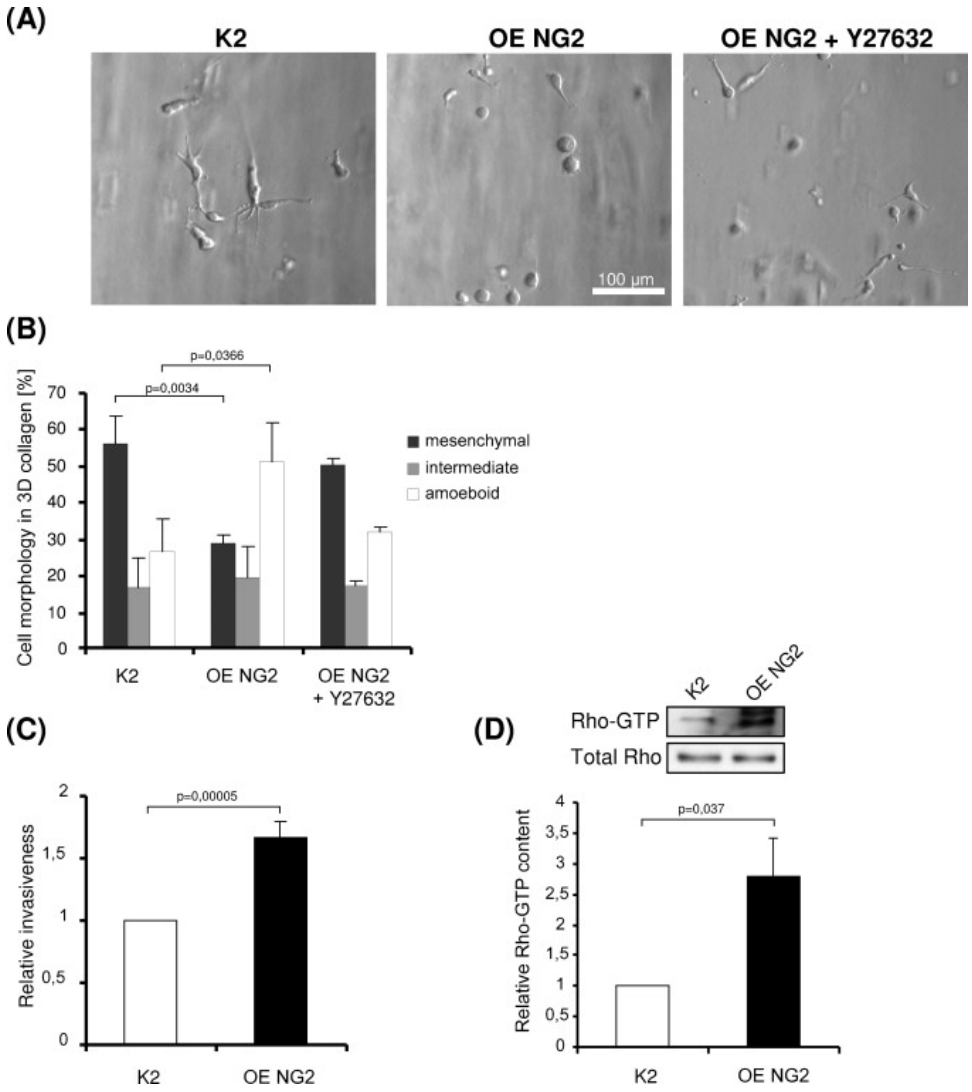


Figure 4. Effect of NG2 expression in K2 sarcoma cells. (A) Cells were grown in 3D collagen and after 24 h cell morphology was analyzed and documented using photomicroscopy. Left panel: K2 cells; medium panel: K2 cells overexpressing NG2; right panel: K2 cells overexpressing NG2 treated with 10 µM Y27632. (B) Quantitative analysis of cell morphology. Cells were grown in 3D collagen and after 24 h cell morphology was analyzed using photomicroscopy and classified on the basis of the elongation index as described in section "Materials and methods". (C) 3D collagen invasion assay. Cells were seeded on top of a collagen gel and their invasion was scored as described in section "Materials and methods". Bars represent the mean invasion depth, normalized to that of untreated K2 cells. (D) Rho-GTP analysis. Cell lysates of each cell variant were prepared, and total GTP-bound Rho was enriched by absorbing cell lysates to GST-RBD beads and measured by Western blotting using pan-Rho antibody. Densitometric ratios of total Rho and Rho-GTP signal were determined and normalized to that of untreated K2 cells. The error bars represents standard deviations and p values indicate statistical significances. Representative immunoblots of GTP-loaded (upper panel) and total Rho (lower panel) are shown above the graph. (Taken from Pankova et al., 2012)

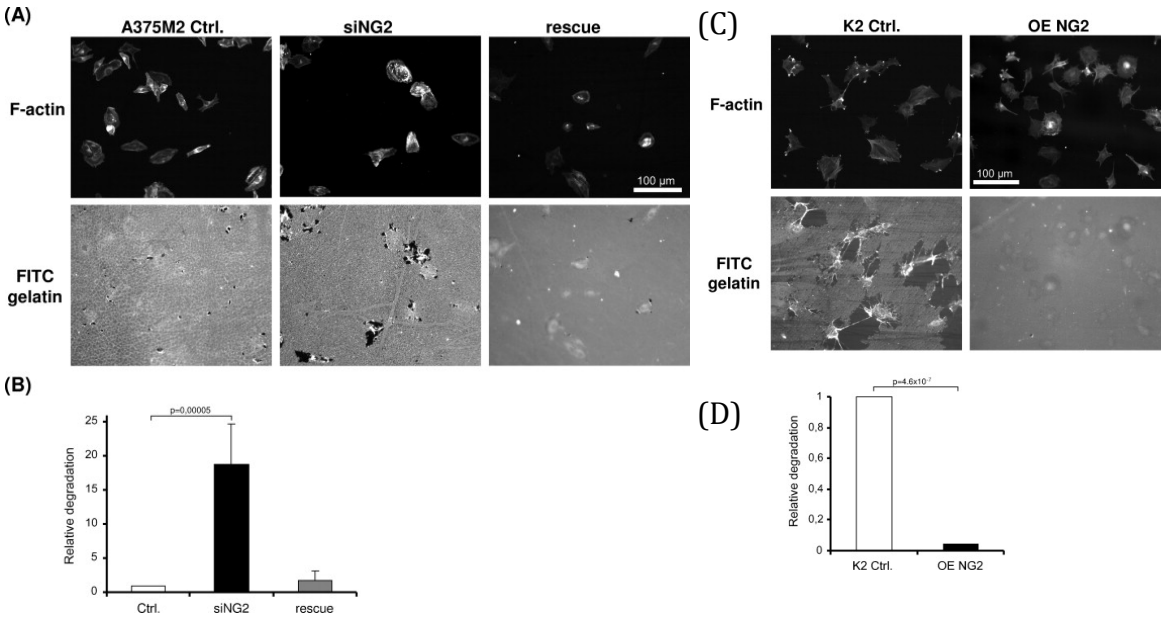


Figure 5. Effect of NG2 on matrix-degrading activity in A375M2 melanoma cells (A) and (B), and in K2 sarcoma cells (C) and (D). (A) and (C) Cells were plated on fluorescent gelatin-coated coverslips and subjected to gelatin degradation assay as described in section "Materials and methods". Representative fields were documented by photomicroscopy. (B) and (D) The quantification of matrix-degrading activity was performed as described in section "Materials and methods". Bars represent mean relative degradation obtained from 3 independent experiments and normalized to that of untreated A375m2 and K2 cells, the error bars represents standard deviations and p values indicate statistical significances. (Taken from Pankova et al., 2012)

Taking all this together, we set out to further address the initial aim of this thesis, which was to analyse the mechanism in which NG2 activate Rho/ROCK signalling. We hypothesised that NG2 interacts with MUPP1, a multivalent scaffold, via its PDZ binding domain. MUPP1 also interacts with the Rho GEF Syx, which may lead to subsequent activation of Rho in cancer cells, and thereby promoting an amoeboid morphology. However, in 2013, Biname and colleagues demonstrated in the published article results confirming our hypothesis that NG2 interacts with MUPP1 and Syx. In addition, they also confirmed that via its interaction with the MUPP1/Syx complex, NG2 stimulates RhoA/ROCK signalling (Biname et al., 2013). Thus, we changed the aim of my PhD project to analyse the role of cancer-associated fibroblasts (CAFs) in melanoma cell invasiveness.

4.2 Analysis of the Role of Cancer-Associated Fibroblast on Melanoma Cell Invasion

For the second aim, we focused on the tumour microenvironment, specifically, the role of a major component of the tumour stroma, which are cancer-associated fibroblasts (CAFs) (Kalluri & Zeisberg, 2006), and their effect on melanoma cell invasion. The invasion and metastasis of tumour cells plays a vital role in tumour progression. There is increasing evidence that the tumour microenvironment communicates constantly with tumour cells, and this leads to its active role in cancer development and progression (Okochi et al., 2013). Cancer cells and fibroblasts in the microenvironment secrete numerous factors, which can affect cell behaviour. CAFs have been shown to promote proliferation of cancer cells, as well as produce specific stimuli, which lead to modifications in the environment that enhance the growth of cancer cell subsets at distant sites (Gaggioli et al., 2007; Östman and Augsten, 2009; Martin et al., 2000; Comito et al., 2012). The mechanisms used by CAFs in order to promote invasion and metastasis, particularly at the distant site, are not fully understood.

Recently, several studies have demonstrated the upregulation of chemokines and cytokines in CAFs, in melanoma (Li et al., 2009; Kolar et al., 2012). Importantly, melanoma patients commonly demonstrate elevated serum levels of interleukin-8 (IL-8) and interleukin-6 (IL-6), which is involved in tumour progression (Kolar et al., 2012; Kucera et al., 2015). IL-6 and IL-8 are important in melanoma, as their levels of expression are correlated with overall patient survival, which indicates that they may be fundamental mediators of events involved in tumour cell invasion (Linnskog et al. 2014; Yao et al. 2014; Osuala et al. 2015).

We hypothesised that CAF and melanoma cell direct interaction may lead to increased levels of IL-6 and IL-8, which enhances melanoma cell invasion. We used

conditioned media (CM) from normal fibroblasts, CAFs, and normal and cancer-associated fibroblasts co-cultured with melanoma cells. We then used these conditioned media to analyse the effects of melanoma cell and CAF interaction on proliferation, migration and 3D spheroid invasion. In addition, we analysed the levels of IL-8 and IL-6 in conditioned media, as well as in human melanoma samples. We then analysed the effect of IL-8 and IL-6 neutralising antibodies on melanoma 3D spheroid invasion, individually and in combination.

These results were published in 'Histochemistry and Cell Biology' in April 2016. The publication is attached at the end of the thesis.

4.2.1 Conditioned media from CAFs co-cultured with melanoma cells stimulates melanoma cell invasion and migration

Cancer progression includes the modification of normal mechanisms, which constrain the migration and invasion of cells. In order to study the influence of factors secreted by CAFs on melanoma invasion, we analysed the effect of conditioned media on the invasive potential of two human melanoma cell lines; the highly invasive BLM cells, and the less invasive A2058 cells (Li et al., 2009). To better resemble the host-tumour microenvironment interaction *in vivo*, we used the spheroid invasion assay. In this assay, multicellular tumour spheroids are embedded in collagen, in order to simulate the three-dimensional (3D) structure of an *in vivo* tumour mass.

We analysed the invasion of melanoma cells from the spheroid into the surrounding collagen matrix, with the addition of different CM. As shown in Fig.6 a-b, treatment with CM from CAFs (Mel Fib) increases invasion significantly, and CM from CAFs co-cultured with BLM cells (Mel Fib + BLM) also significantly increases invasion of BLM cells, but at greater magnitude. CM from normal fibroblasts, and normal fibroblasts co-cultured with BLM cells does not increase invasion of BLM cells,

compared with that of the control. On the other hand, A2058 melanoma cells demonstrated a significant in invasion when treated with condition media from all conditions, in comparison to the control (Fig.6 c-d). Conditioned media from CAFs, and CAFs co-cultured with A2058 cells led to a greater increase in invasion, a 2- and 2.8-fold increase, respectively.

In order to invade, cancer cells must have the ability to migrate and penetrate the surrounding tissue. We then analysed the effect of CM on basal melanoma cell migration. Analysis of cellular migration, using the wound healing assay, showed that in BLM cells, CM from CAFs and CAFs co-cultured with BLM cells significantly increased migration, but CM from normal fibroblasts and normal fibroblasts co-cultured with BLM cells did not have an effect on migration, compared to the control (Fig.7 a). CM of all conditions, compared to the control, increased A2058 migration significantly (Fig.7 b).

To eliminate the possibility that CM-dependent increase in migration and invasiveness is due to CM-dependent stimulation of growth rates, we analysed the effect of CM on proliferation. The proliferative effect of CM on BLM and A2058 cells was analysed using the AlamarBlue® Assay. There was no significant impact on proliferation of both BLM and A2058 cells (Fig.6 e-f). These results indicate that CM from CAFs, and CAFs co-cultured with melanoma cells especially, enhance invasion and migration, but the effects of CM are independent of proliferation. Our results also demonstrate that A2058 melanoma cells are more responsive to CM-dependent increase in invasiveness, in comparison to BLM cells.

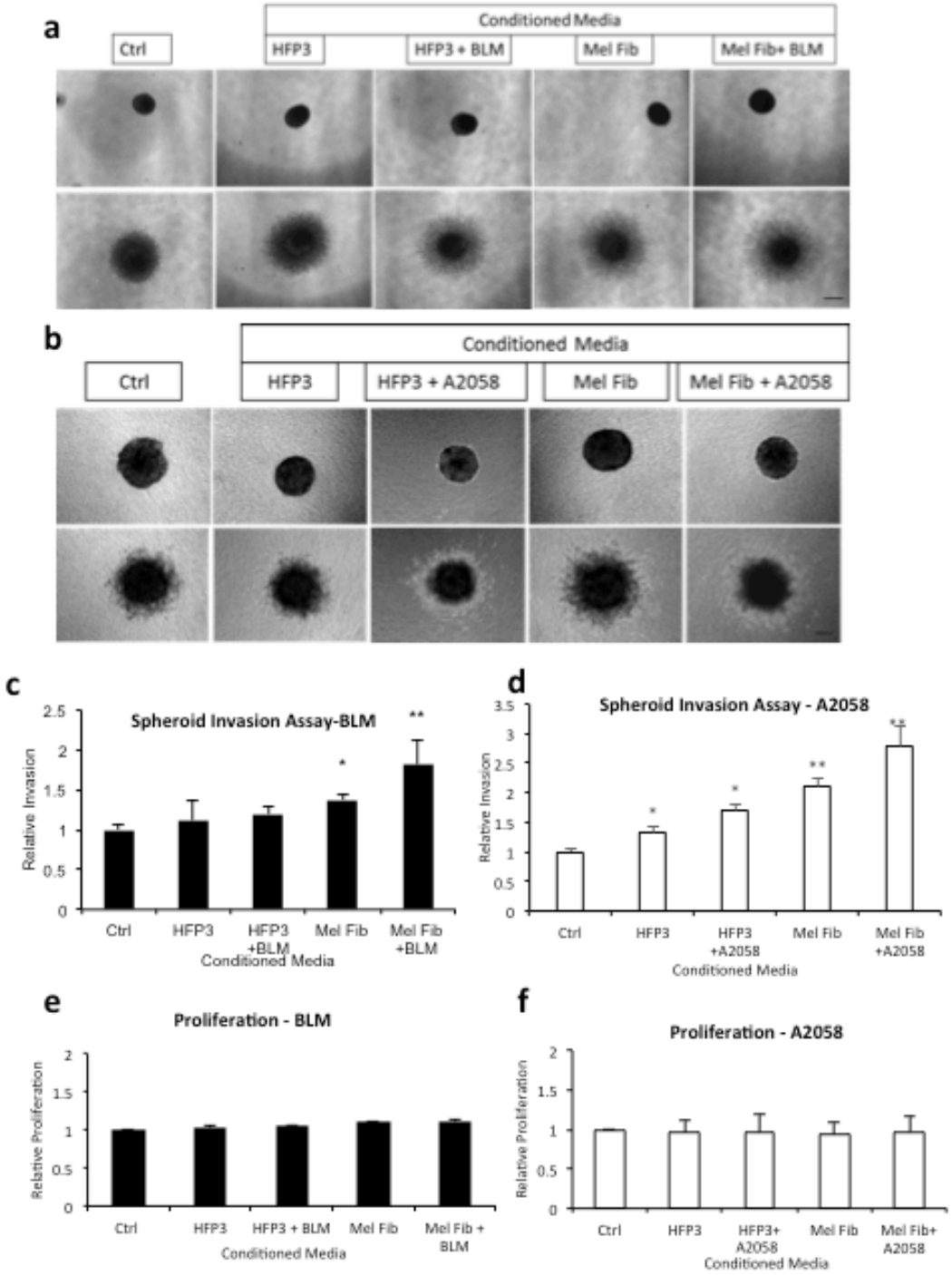


Figure 6. Conditioned media from CAFs increases invasion but not proliferation. (a, b) BLM and A2058 spheroids were embedded in collagen and conditioned media was added. The top panel are the spheroids at 0 hours, and the bottom panel are the spheroids at 48 hours after incubation with conditioned media. . Ctrl = DMEM; HFP3 = CM from Normal dermal fibroblasts; HFP3 + BLM = CM from Normal fibroblasts co-cultured with BLM melanoma cells; Mel Fib = CM from human melanoma derived CAFs; Mel Fib + BLM = CM from CAFs co-cultured with BLM melanoma cells. (c, d) Graphs show relative invasion of cells; each graph is representative of one experiment. 10 spheroids were observed per condition, and the experiment was repeated at least three times. *$p<0.05$; **$p<0.01$ compared to the control (e, f) AlamarBlue®Proliferation assay to measure the effect of CM on growth of BLM and A2058 cells. Scale Bar (a) 200um, (b) 100um. (Taken from Jobe et al., 2016)

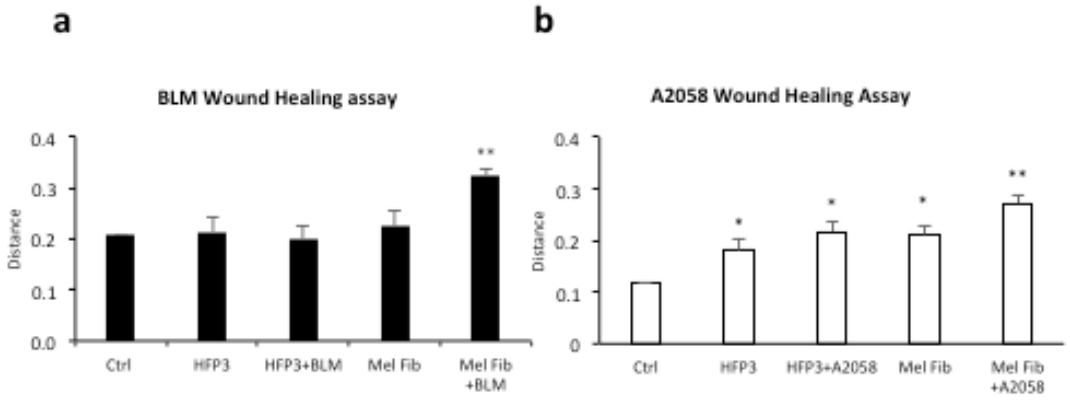


Figure 7. Conditioned Media from CAFs co-cultured with melanoma cells increases 2D melanoma migration. The effect of conditioned media on BLM (a) and A2058 (b) cell migration was measured using a scratch wound assay. *p<0.05, **p<0.01 compared to the control. These findings are representative of three independent experiments. (Taken from Jobe et al., 2016)

4.2.2 Analysis of IL-6 and IL-8 levels of secretion in mono- and co-cultures

Melanoma patients frequently have elevated levels of IL-6 (Na et al., 2013), which is correlated with poor prognosis. Recently, it was demonstrated that IL-6 induces *in vitro* invasion of melanoma cells, as well as lung metastasis in mice (Na et al., 2013). In addition, CAFs have also been implicated in the stimulation of cancer cell metastasis at distant sites, in an IL-6-dependent manner (Sasser et al., 2007). Furthermore, IL-8 was also shown to play a significant role in melanoma cell migration, as well as distant metastasis (Wu et al., 2012). Taken together, we hypothesised that the CM-dependent elevated invasion may be due to greater levels of IL-8 and IL-6. We thus analysed the levels of these two cytokines in conditioned media, in 2D (Fig.8 a-d). Basal amounts of IL-6 by BLM, and especially A2058 cells, in monocultures was significantly lower than in fibroblasts, which suggests that CAFs and normal fibroblasts are major producers of IL-6 in 2D (Fig.8 c,d). The basal IL-6 levels were similar in both fibroblast lines. Conversely, CAFs co-cultured with A2058 or BLM cells resulted in a significant elevation in IL-6 levels. In contrast, normal

fibroblasts responded by enhanced secretion of IL-6 only when co-cultured with BLM cells (Fig.8 c). Co-cultivation of normal fibroblasts with A2058 led to a decline in IL-6 production (Fig.8 d). The analysis of amounts of IL-8 levels media showed that melanoma cells are predominant producers of IL-8 in 2D (Fig.8 a,b). The fibroblasts produced insignificant amounts of IL-8. However, when fibroblasts were co-cultured with melanoma cells, the levels of IL-8 in the media were raised to levels comparable to melanoma cells cultured alone. This suggests that fibroblasts co-cultured with melanoma cells significantly enhances secretion of IL-8 in the co-culture, as melanoma cells only represent approximately 10% of the starting population (Fig. 8 a,b).

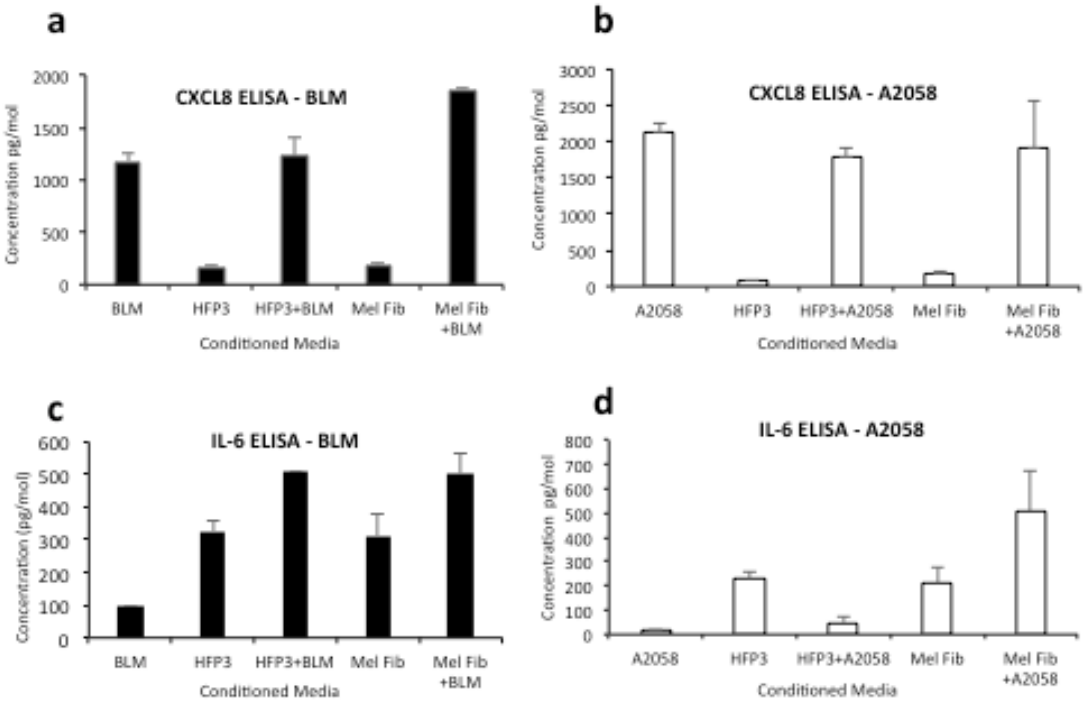


Figure 8. Co-culture melanoma cells and CAFs promotes IL-8 and IL-6 production. (a-b) IL-8 levels in CM were measured using ELISA, (c, d) IL-6 levels in CM were measured using ELISA. (Taken from Jobe et al., 2016)

4.2.3 Blocking IL-8 and IL-6 inhibits CAF-mediated increase in invasion

Our results suggest that co-cultivating fibroblasts and especially CAFs with melanoma cells potentiates IL-6 and IL-8 secretion, which suggests that increased IL-6 and IL-8 levels in CM may be important in fibroblast-mediated increase in melanoma cell invasiveness. To examine the effects of IL-8 and IL-6 in CM on invasion, we blocked IL-8 and IL-6 using neutralising antibodies, individually and in combination (Figures 9-11). We used anti-GFP antibody as a non-specific control antibody in all experiments, which had no effect on melanoma invasion. In both A2058 and BLM cells incubated with CM supplemented with IL-6 neutralising antibody, invasion was not significantly different from the invasion of cells in control media, except in CM from normal fibroblasts and CAFs co-cultured with melanoma cells, in BLM cells, and CM from normal fibroblasts and normal fibroblasts co-cultured with melanoma cells, in A2058 cells (Fig. 9). These results indicate that the IL-6 neutralising antibody inhibited the proinvasive effect of CM from CAFs in both melanoma cells, and CM from co-cultured CAFs with melanoma cells in A2058 cells only (Fig. 6). Likewise, we did not detect any significant difference in invasion between BLM and A2058 cells incubated with CM supplemented IL-8 antibody and cells incubated with control media (Fig. 10). This suggests that the IL-8 neutralising antibody also inhibited the proinvasive effect of CM. Notably, IL-8 neutralising antibody also resulted in partial inhibition of BLM cell invasion, and a significant decrease in invasion of A2058 cells, when added to normal media, compared to control (Fig. 10). These results highlight the increased basal level of IL-8 production in melanoma cells (Fig. 8 a, b) and suggest that some threshold level of IL-8 is necessary for basal invasion.

We then analysed the combinatory effect of both IL-8 and IL-6 antibodies, which resulted in a decrease in invasiveness to the level of control cells without CM, in both A2058 and BLM cells (Fig.11). These results suggest that IL-8 and IL-6 play a significant role in CAF-induced invasion of both A2058 and BLM.

To evaluate whether the observed effect of blocking IL-8 and IL-6 on BLM and A2058 invasion is a general representation of melanoma cells, we analysed two additional melanoma cells lines, WM3629 and WM3670. We found that CAF-dependent invasion of both cell lines is inhibited when IL-8 and IL-6 neutralising antibodies are added to CM (Fig.12).

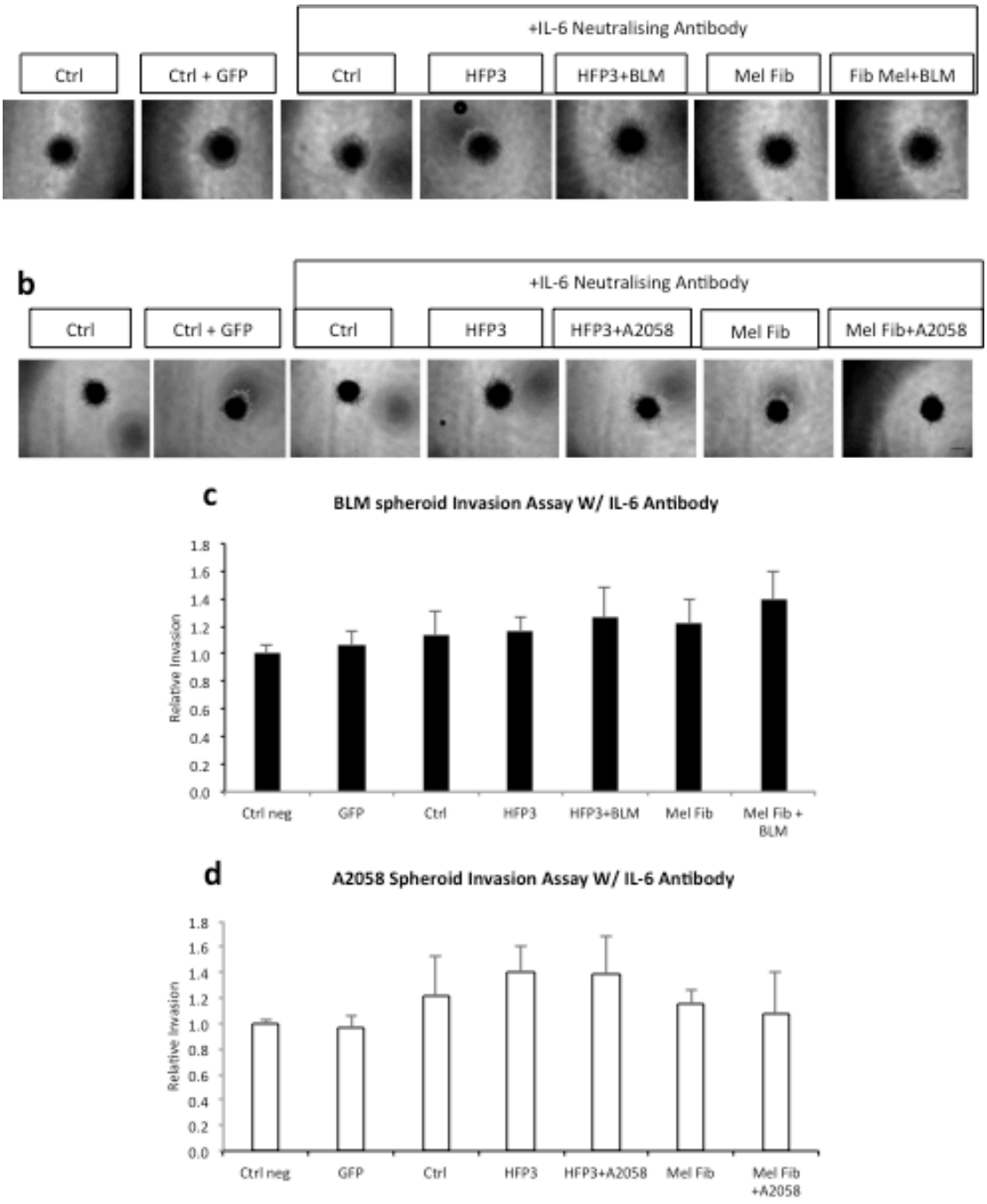


Figure 9. Blocking IL-6 decreases CAF-induced invasion of melanoma cells. BLM (a) and A2058 (b) melanoma spheroids were embedded in collagen, and incubated with conditioned media supplemented with IL-6 blocking antibody at a concentration of 200 ng/ml. The graphs (c, d) show relative invasion after 48 hours. These results are representative of three independent experiments. *$p<0.05$. Scale bar 200 um. (Taken from Jobe et al., 2016)

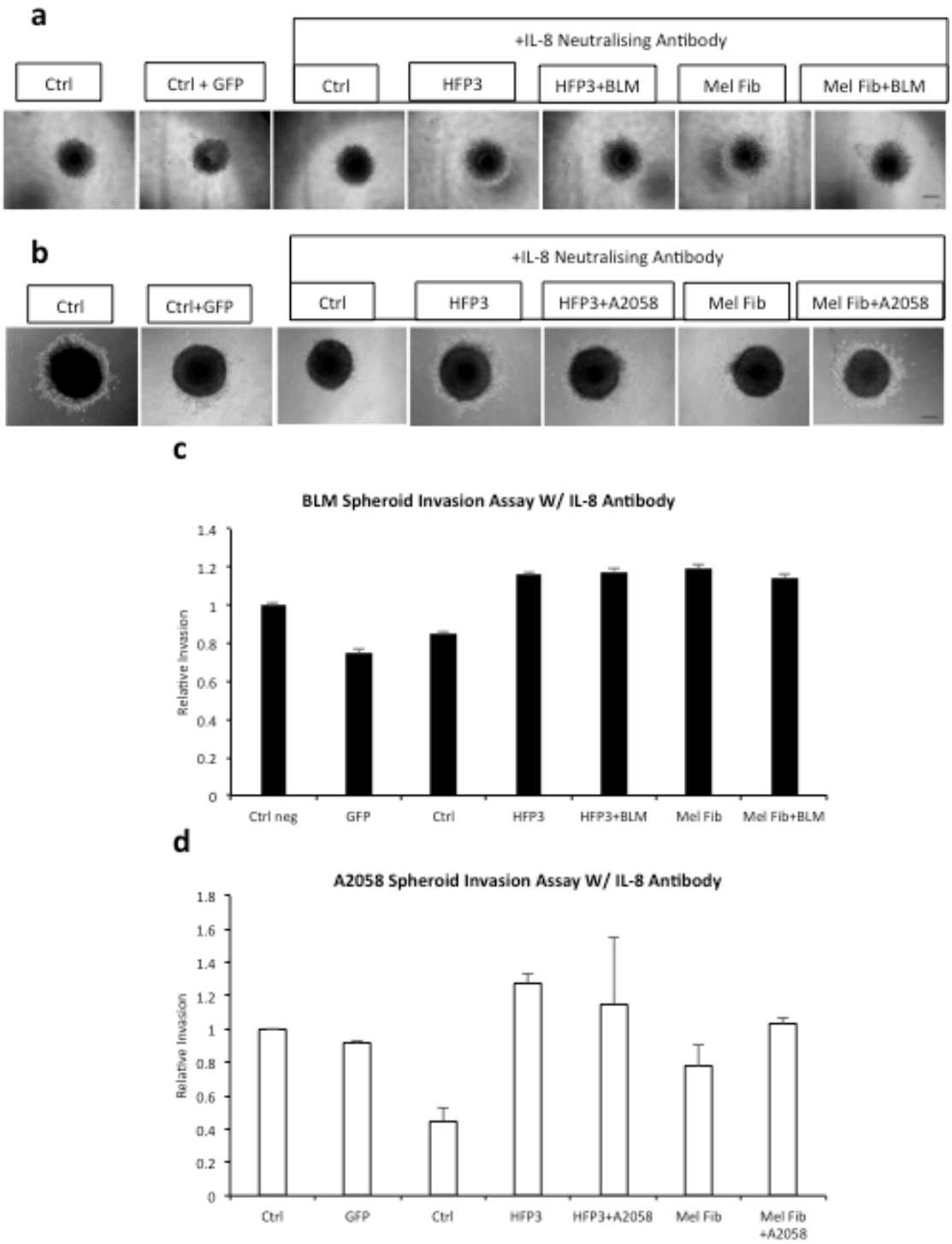


Figure 10. Blocking IL-8 in conditioned media significantly reduces basal invasion of melanoma cells. BLM (a) and A2058 (b) melanoma spheroids were embedded in collagen, and incubated with conditioned media supplemented with IL-8 blocking antibody at a concentration of 200 ng/ml. The graphs (c, d) show relative invasion after 48 hours. This is representative of three independent experiments. Scale Bar (a) 200uM, (b) 100um (Taken from Jobe et al., 2016)

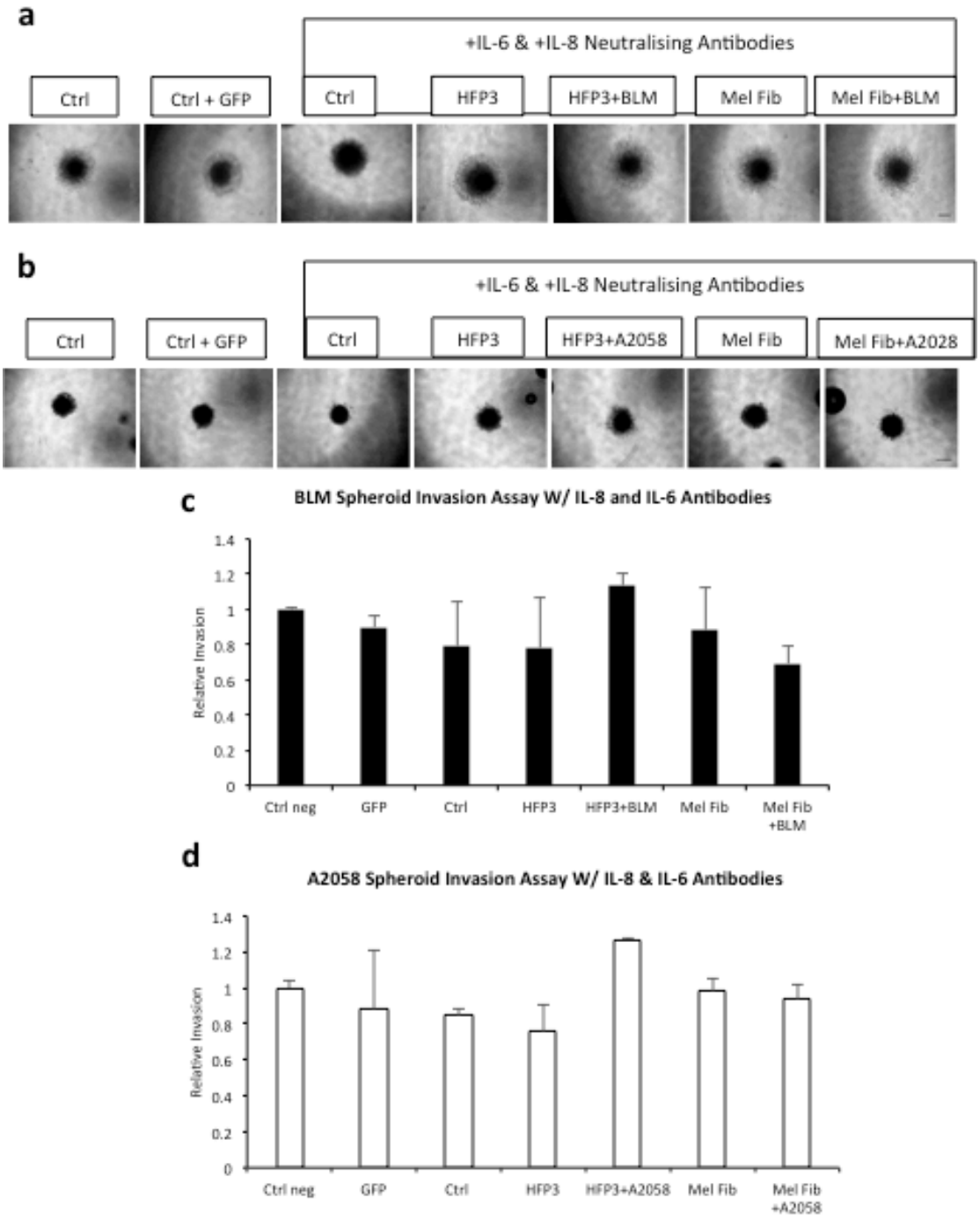


Figure 11. Blocking IL-6 and IL-8 in conditioned media fully inhibits CAF-induced invasion. BLM (a) and A2058 (b) melanoma spheroids were embedded in collagen, and incubated with conditioned media supplemented with IL-6 and IL-8 blocking antibody at a concentration of 200 ng/ml, each. The graphs (c, d) show relative invasion after 48 hours. These results are representative of three independent experiments. Scale bar 200 um. (Taken from Jobe et al., 2016)

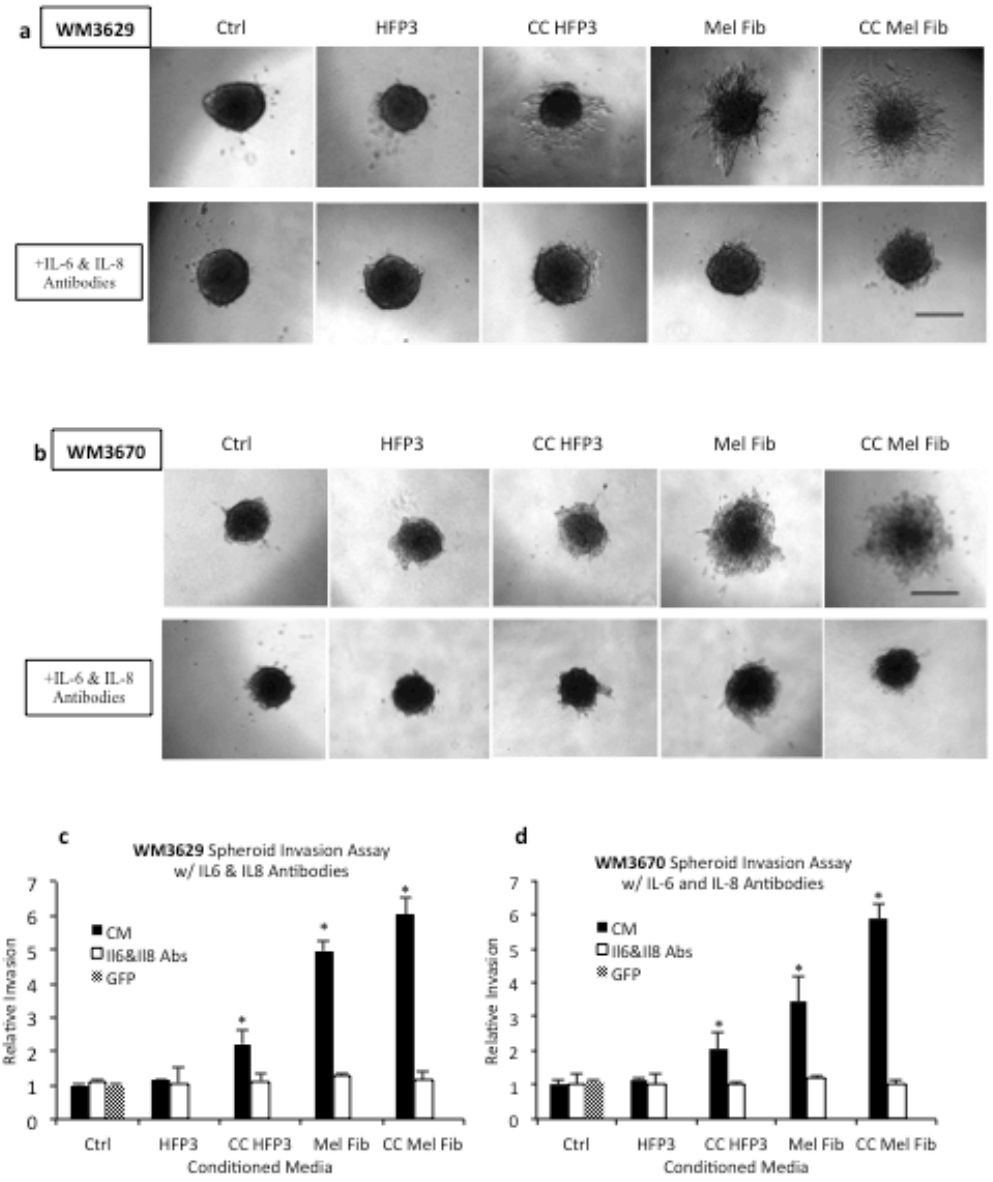


Figure 12. Blocking IL-6 and IL-8 in conditioned media fully inhibits CAF-induced invasion. WM3629 (a) and WM3670 (b) melanoma spheroids were embedded in collagen, and incubated with conditioned media (top panel) and conditioned media supplemented with IL-6 and IL-8 blocking antibody (bottom panel) at a concentration of 200 ng/ml, each. The graphs (c, d) show relative invasion after 48 hours. CC HFP3 and CC Mel Fib is conditioned media from co-culture of melanoma cells with normal fibroblasts and CAFs, respectively. These results are representative of three independent experiments. Scale bar 400 um. . *p<0.05 (Taken from Jobe et al., 2016)

4.2.4 Conditioned media significantly alters the sustained levels of IL-8 and IL-6, in a 3D environment

Our results have demonstrated that CM from CAFs induces invasion and migration. In addition, we observed that co-cultured CAFs and melanoma cells increases IL-8 and IL-6 levels in CM and that neutralising IL-6 and IL-8 antibodies inhibit the stimulating effect of CM on invasion. However, the effect of blocking antibodies did not clearly correlate with levels of IL-6 and IL-8 in CM (for example, despite differences in invasiveness we did not observe any difference in IL-8 levels in CM from CAFs co-cultured with melanoma cells and normal fibroblast co-cultured with melanoma cells). The amounts of IL-6 and IL-8 in CM only reflect the cytokines that were initially added to the system and does not indicate the levels of the cytokines present throughout the 3D invasion experiment. Hence, we analysed the levels of IL-6 and IL-8 in the complete volume of collagen and media at the final time point of the invasion assay. We observed that BLM cells, in 3D, showed high levels of both IL-8 and IL-6, and addition of CM did not have any significant effect on final IL-8 and IL-6 levels (Fig.13). On the contrary, A2058 cells, in 3D, did not secrete detectable levels of IL-6 and secretion of IL-8 was low, when compared to the 2D environment (Fig.13 b,d). Addition of conditioned media significantly increased final IL-6 and IL-8 levels in the system, with a greater effect observed for IL-6 levels when supplemented with CM from CAFs co-cultured with A2058 cells.

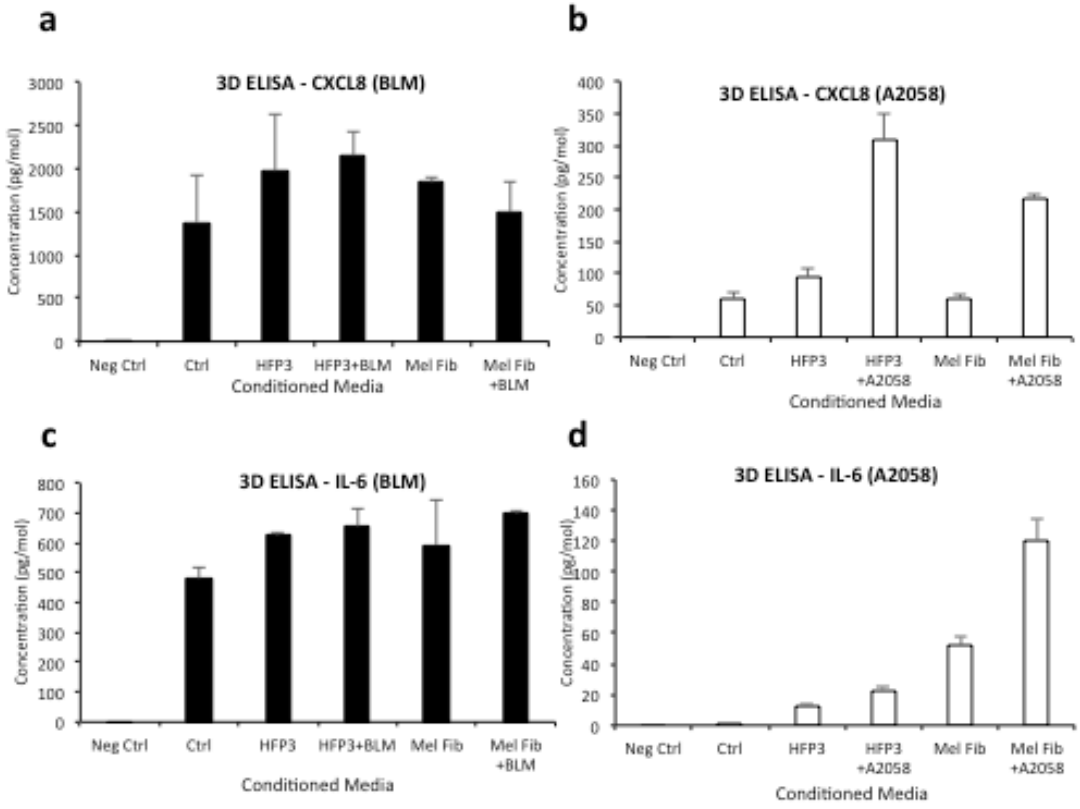
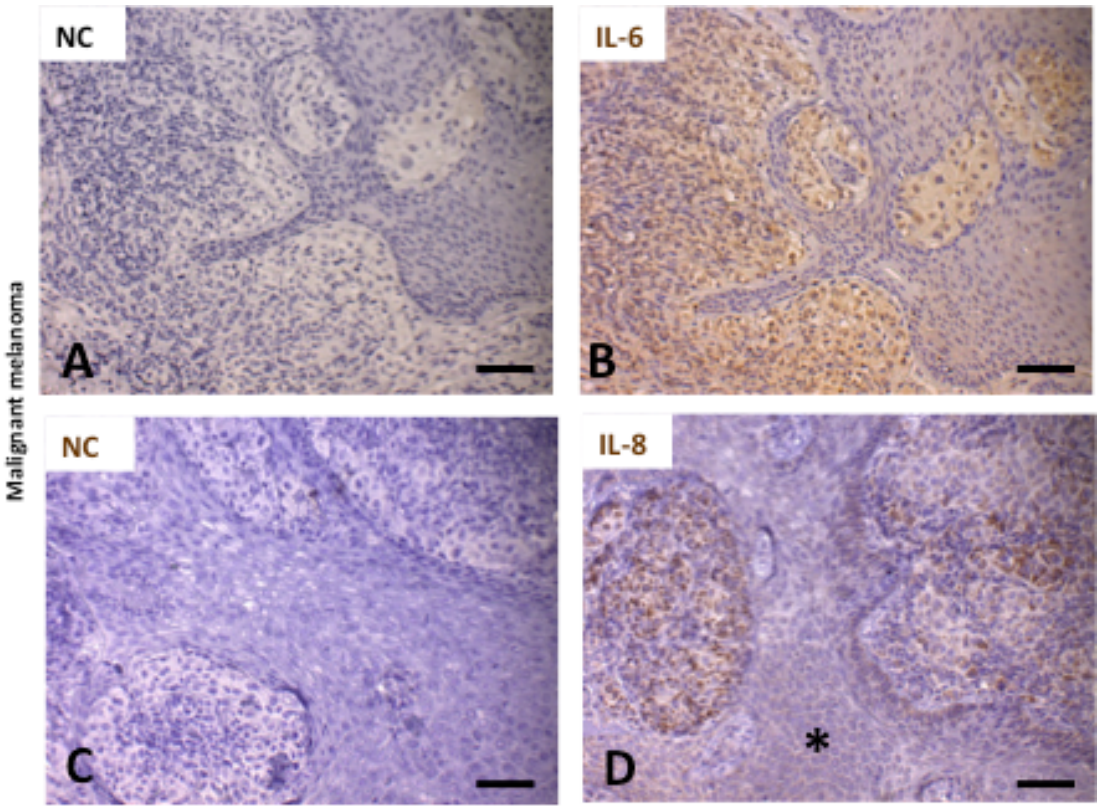


Figure 13. Co-cultivation of melanoma cells with CAFs induces IL-6 secretion in 3D. Total media from the spheroid culture was collected after 48 hours, and IL-8 (a, b) and IL-6 (c, d) levels were measured using ELISA. (Taken from Jobe et al., 2016)

4.2.5 IL-8 and IL-6 are produced by cancer cells, in human melanoma

Professor Smetana and colleagues performed these results in order to complement our *in vitro* results. Our results analyzing IL-8 and IL-6 production have demonstrated that in 2D monocultures, the melanoma cells secrete high levels of IL-8, but small amounts of IL-6, and CAFs are the main producers of IL-6 in 2D monocultures. We have further shown that, remarkably, in a 3D environment, the BLM spheroids secrete high levels of both IL-8 and IL-6. To reveal which of these conditions resembles the in vivo situation, Professor Smetana's group analysed IL-8 and IL-6 expression in human melanoma specimens. Consistent with the results obtained in BLM cells in the 3D model, the analysis of IL-8 and IL-6 expressions in human melanoma specimens showed both expression of IL-8 and IL-6 to be associated with tumour cells (Fig.14). Taken together, these results indicate that melanoma cells, *in vivo*, show high levels of IL- 6, as well as IL-8 expression, which is consistent with our observation of melanoma cell lines cultured in a 3D environment.



	Negative -	Positive +	Positive ++	Positive +++
IL-6	2	5	3	0
IL-8	4	4	2	0

Figure 14. No signal was observed in negative control of section of human melanoma (A,C). 10 samples of human melanoma were tested. 8/10 exhibited signal for IL-6 in melanoma cells v (exhibiting diffuse cytoplasmic or fine granular staining pattern B). 6/10 samples were focally positive for IL-8 (D). Adjacent epidermis surrounding melanoma buds presented also weak IL-8 positivity (D - asterisk). Haematoxyllin counterstained. Magnification 200x. Inserted length bar corresponds to 100um. (Taken from Jobe et al., 2016)

4.3 Review on 3-Dimensional Models Used in Invadopodia Drug Targeting

In addition to primary research, I was primary author in, and predominantly wrote a review on the use of complex 3D model used to study invadopodia drug targets. A supplement of the publication corresponding to this review is attached at the end of the thesis. In this review we discussed invadopodia, actin-based membrane protrusions that are involved in ECM proteolysis, and their role in metastasis. In addition, we highlighted the most popular 3D biological model systems that are used in invadopodia-targeted therapy. In addition to our research, using 3D model systems to study cancer cell invasiveness and some of the factors involved, this review also highlights the relevance of 3D model systems in drug targeting. We also indicate that "life-like" systems are of increasing biological interest, as the systems resembling the in vivo situation are more reliable, especially when it comes to drug discovery.

4.4 Unpublished Results

4.4.1 Cancer-Associated Fibroblast and Keratinocyte interaction promotes melanoma progression

As a follow up project, we are still collaborating with Professor Smetana and his group to analyse the effect of keratinocytes, and their association with CAFs, on melanoma progression. Recently, Kodet *et al.* showed that melanoma cells have the ability to promote keratinocyte differentiation, *in vivo* and *in vitro* (Kodet et al., 2015). The melanoma microenvironment, in addition to CAFs, also contains keratinocytes, which have been implicated in disease progression and drug resistance (Kodet et al., 2015). Previously, the same group showed that keratinocyte association with CAFs leads to increased levels of cytokines, and other immuno-modulatory factors (Kolar et al., 2012). Their previous data, as well as our results shown here,

have led to the analysis of keratinocyte and CAF association, two major cell types found in the melanoma tumour microenvironment. Professor Smetana's group prepared all the CM, and I performed the spheroid invasion assays. Preliminary results have shown that CM from keratinocytes co-cultured with normal fibroblasts and CAFs leads to increased BLM melanoma cell invasion. On the other hand, only CM from CAFs co-cultured with keratinocytes increases A2058 melanoma cell invasion (Fig.15). We are currently analysing the factors involved in keratinocyte-fibroblast-dependent enhanced invasion, using transcriptomics. These results also reflect the differences between the two melanoma cell lines, as discussed below (Chapter 5), due to the different response observed with conditioned media from normal dermal fibroblasts and keratinocytes (HFP3-HK). Furthermore they highlight the role of the tumour microenvironment in disease progression. In addition, we are also analysing the effect of conventional treatment on keratinocytes, and how this affects melanoma cell invasion.

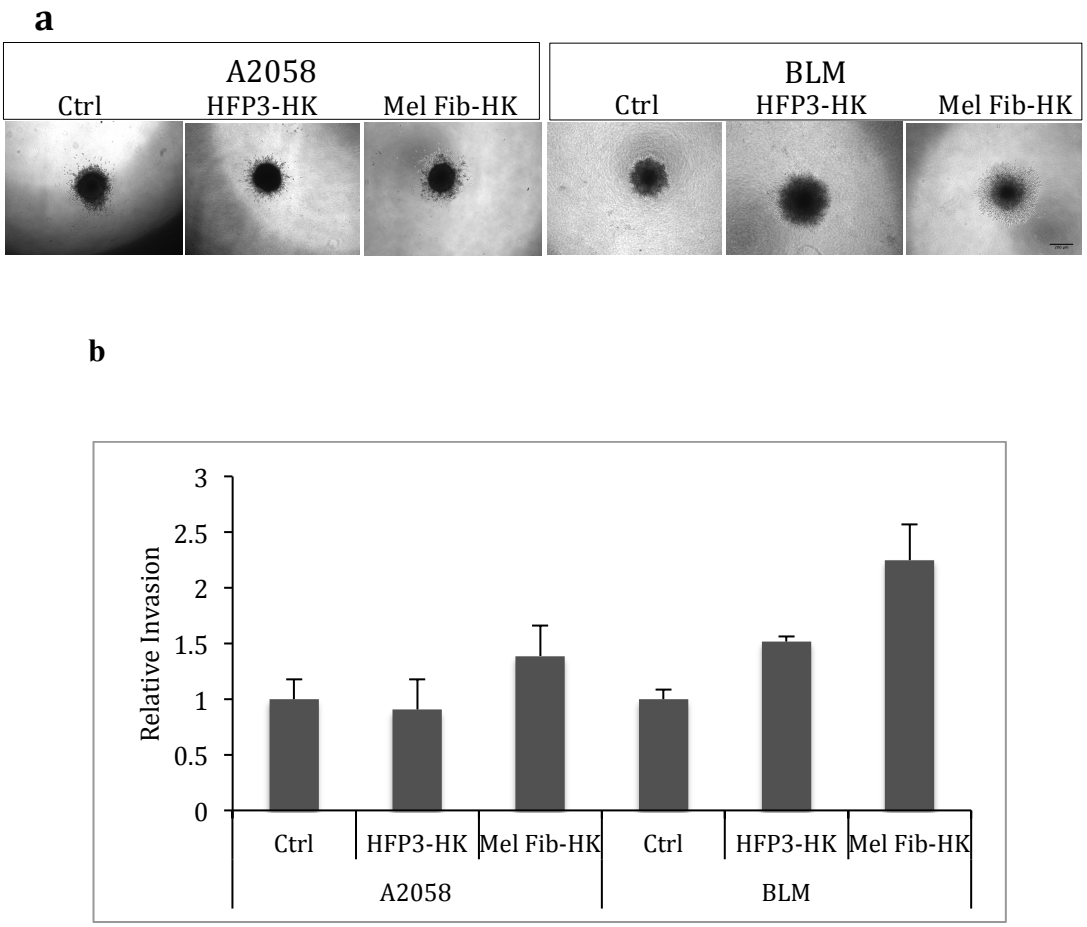


Figure 15. Co-culture CAFs with keratinocytes significantly increases melanoma cell invasion. A2058 and BLM melanoma cells were grown as spheroids and embedded in 2 mg/ml collagen I, and incubated with CM from human melanoma keratinocytes (HK) co-cultured with normal dermal fibroblast (HFP3) and CAFs (Mel Fib). Scale bar is 200 um.

4.4.2 Curcumin as a candidate for melanoma therapy

In addition to our work analysing the factors involved in cancer cell invasion, we are also exploring treatment options. I have highlighted the problems in melanoma therapeutics in this thesis, and in order to overcome these setbacks, we believe that more research on natural compounds is required. We collaborated with Dr Sunil Kumar, of the Godavari Pharma Group, Hyderabad, India, who has provided us with extracted and purified curcuminoids from turmeric, as we want to analyse the roles of curcuminoid, the active ingredient in turmeric, in its most natural state. Our results show that 25μM of purified curcumin inhibits A2058 and G361 melanoma cell invasion (Fig. 16). However curcumin only inhibits the proliferation of G361 cells, and not A2058 (Fig.16 e, f). These results show that curcumin may inhibit melanoma cell invasion by targeting different pathways. Our future plans include exploring other purified derivatives of curcumin, as recommended by our collaborators, and their effects on invasion. Further analysis *in vivo*, using mouse models, is required, in order to fully consider natural curcumin as a desirable candidate for melanoma therapy.

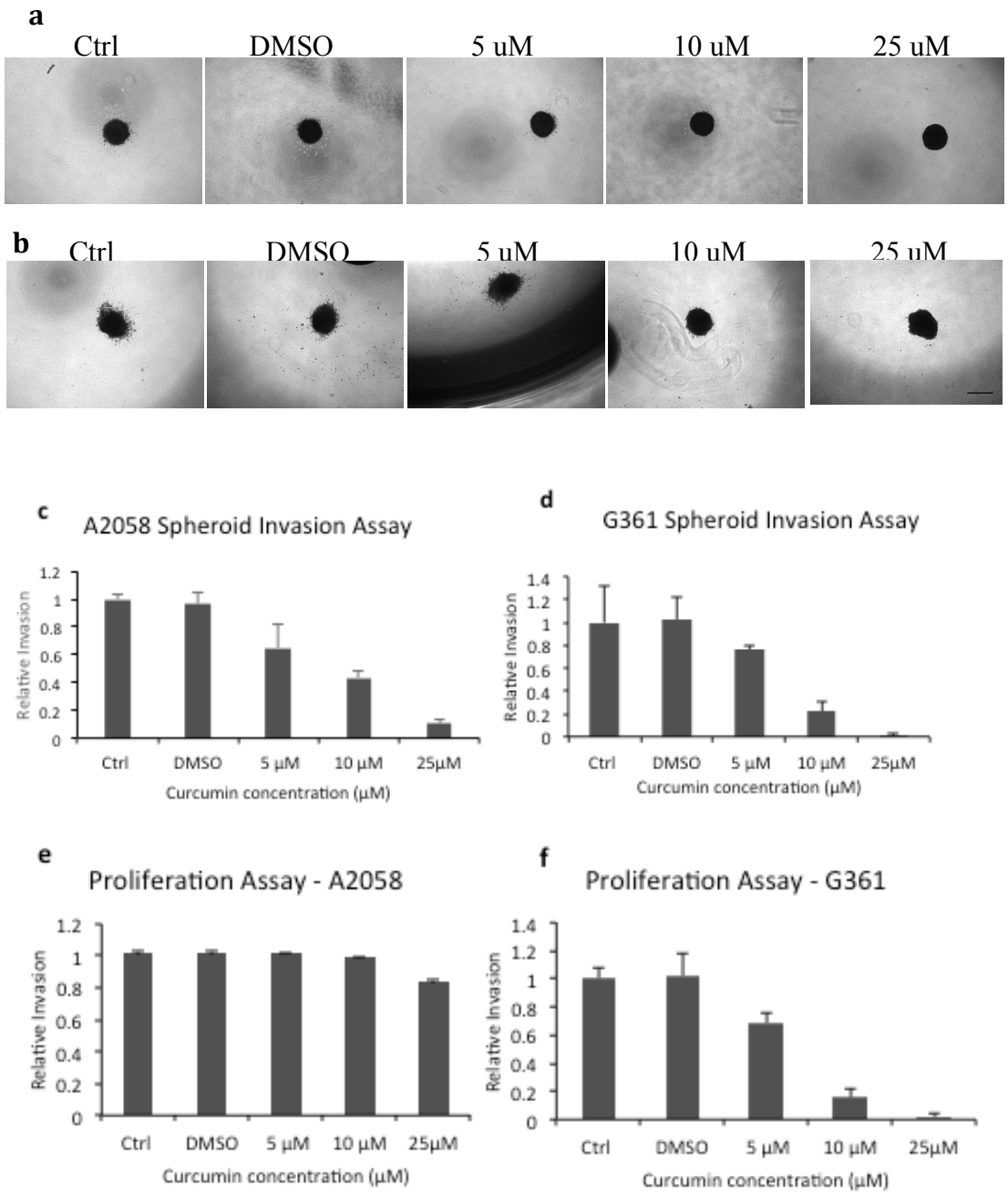


Figure 16. Curcumin inhibits melanoma cell invasion. (a, c) A2058 and (b, d) G361 melanoma cells were grown as spheroids and embedded in collagen containing different concentrations of curcumin. (e, f) Alamar Blue® Proliferation assay to measure the effect of curcumin on A2058 and G361 melanoma cell growth. Scale bar is 200 um.

5 Discussion

5.1 Analysis of the role of NG2 proteoglycan on cancer cell morphology and invasiveness

For the first aim of my thesis, we analysed the role of NG2 in cancer cell morphology and invasiveness. NG2 is known as a marker of undifferentiated cells, and its expression declines with reduced grades of malignancy (Chekenya et al., 1999). NG2 expression is downregulated upon terminal differentiation of immature precursor cells, which suggests that NG2 may be involved in cell proliferation, differentiation, or both (Burg et al., 1998). Results supporting this idea have shown that NG2 promotes ECM-cell interaction. For example, several reports have suggested that NG2 has the ability to control responses to platelet-derived growth factor (PDGF) (Grako & Stallcup, 1995;Nishiyama et al., 1996), which also plays a role in precursor cell transformation (Chen et al., 2015). NG2 was shown to bind to several ECM ligands, such as laminin, tenascin, and collagen types II, V and VI (Burg et al., 1996). Collagen VI is the most characterised of these interactions with NG2, and results have suggested that NG2 acts as a cellular receptor for collagen VI (Tillet et al., 1997; Stallcup et al., 1990). In addition, NG2 may control the ability of cells to bind and migrate on collagen VI (Tillet et al., 1997; Stallcup et al., 1990).

Previous reports have suggested that NG2 expression in mouse melanoma cells leads to a reduction in the strength of cell-cell, as well as cell-ECM binding (Burg et al., 1998). This is in agreement with our results, which show that NG2 promotes an amoeboid morphology in rat sarcoma K2 and A3 cells, as well as in human melanoma A374m2 cells. In the highly metastatic, amoeboid A3 and A375m2 cells, siRNA-mediated knockdown of NG2 promoted AMT, which was associated with reduced invasion into 3D collagen and Rho-GTPase inactivation. On the contrary,

overexpression of NG2 in the mesenchymal sarcoma K2 cells, as well as in A375m2 cells, led to an enhanced amoeboid morphology, which was associated with increased invasion into 3D collagen and elevated Rho-GTP levels. In addition, unpublished data from our laboratory has frequently shown, using next generation sequencing of cancer cells, that NG2 is a major protein upregulated in amoeboid cells (Cermak et al., unpublished results). Furthermore, these results show that NG2 also plays an important role in MAT.

Our results further highlight the role of NG2 in Rho GTPase signalling, as increased NG2 levels led to enhanced Rho activation, and downregulation of NG2 decreased Rho-GTP levels, significantly. NG2-mediated intracellular signalling is primarily dependent on the C-terminus of the cytoplasmic domain. The MUPP1 protein, which is a multivalent scaffold that interacts with the Rho-GEF syx (Ernkvist et al., 2009), is an adaptor protein that interacts with the PDZ domain of NG2 (Barritt et al., 2000). This interaction led to localised activation of Rho at the leading edge in endothelial cells (Ernkvist et al., 2009). NG2 was also shown to influence the activity of Rho GTPase Cdc42 in melanoma, and Rac in astrocytoma (Eisenmann et al., 1999; Majumdar et al., 2003). We therefore hypothesised that NG2-mediated Rho activation was via interaction with the polarity complex proteins MUPP1/Syx/PATJ. Recently, Biname and colleagues proved this hypothesis, as they showed that NG2 activates RhoA at the membrane via MUPP1/Syx interaction, which mediates directional migration (Biname et al., 2013). They also showed that NG2, alternatively, stimulates Rac activation via the polarity complex protein, PATJ and the Rho GEF, Tiam1 (Biname et al., 2013).

The expression of NG2 in cancer has shown that NG2 levels do not differ between primary and metastatic lesion during early stage cancers, however NG2 levels are

significantly greater in metastatic lesions during later stages (Burg et al., 1998). This suggests that NG2 plays a role in the migratory potential of cancer cells and poor prognosis. In addition, studies have suggested that NG2 may play an anti-adhesive role on the cell surface, where adhesion of melanoma cells to specific ligands for CD44 and α4β1 integrin is significantly reduced with NG2 overexpression, as NG2 interferes with integrin mediated adhesion (Rutka et al., 1988). NG2 was also shown to inhibit the adhesion and neurite outgrowth from cerebellar neurones on laminin substrates (Burg et al., 1998). This is in agreement with our results, which show that NG2 overexpression reduced matrix degradation, and siRNA-mediated downregulation of NG2 leads enhanced matrix degradation. Therefore, NG2 may inhibit integrin-dependent adhesion to ECM, which is in accordance with its amoeboid-promoting characteristics.

As NG2 is a cell surface proteoglycan that is not only expressed in cancer cell, but also cancer stem cells (Bao et al., 2006), it may be a desirable therapeutic target for metastatic cancers, which demonstrate increased expression of NG2. Monoclonal antibodies (mAbs) may be ideal in NG2-specific immunotherapy, and several studies have shown that cancer cell progression can be inhibited by an NG2-specific mAb (Rivera et al., 2012; Campoli et al., 2010). Although a better understanding of the role NG2 in malignancies will further aid the development of targeted therapeutic agents specifically and directly against NG2, in order to avoid limitations.

Our results, in addition to the work of others, suggest that NG2 plays a major role in Rho GTPase activation and localisation. NG2 may also play a role in integrin-independent adhesion. This explains the role of NG2 in migration and invasion of cancer cells, and in MAT. NG2 may also be a suitable immunotherapeutic target as it

is a transmembrane protein, and is upregulated in metastatic lesions. Further work in understanding the exact role of NG2 in promoting MAT and cancer cell invasion is required.

5.2 Analysis of the Role of Cancer-Associated Fibroblasts on Melanoma Cell Invasion

Secondly, we analysed the role of cancer-associated fibroblasts (CAFs) on melanoma invasion. We collaborated with Professor Smetana, from the Department of Anatomy, at the First Faculty of Medicine, and his group, and the project was based on their published findings that normal and malignant epithelial cells co-cultured with normal fibroblasts led to increased levels of IL-6 and IL-8 in media. This was based on whole-genome transcriptome profiling (Kolar et al., 2012). They provided us with cells (A2058, BLM, Mel Fib and HFP3), as well as enough IL-6 and IL-8 neutralising antibody for two experiments. It should be noted that I solely performed all the *in vitro* experiments; these were then complemented by the analysis of melanoma patient samples using immunohistochemistry, which was performed by our collaborators from Professor Smetana's laboratory.

5.2.1 The 3D Spheroid Invasion Model is closer to the In Vivo Condition than 2D models

Invasion and migration of cancer cells are hallmarks of tumour metastasis. The significant of studying cancer cell invasiveness in an environment that is resembles the *in vivo* situation and cellular context is becoming increasingly acknowledged (Yin et al., 2012). Tumour masses grow in a three dimensional (3D) spatial manner, which leads to varying exposure to oxygen and nutrients, as well as other physical and chemical strains. Cancer cell growth and hypoxia are mutually exclusive *in vivo*, excluding areas that experience temporary changes in perfusion where hypoxic tumour cells that are nonproliferating have been recognised (Pampaloni et al., 2007). The limitations in the distribution of oxygen, nutrients and signalling proteins are not mirrored in 2D single layer cultures (Pampaloni et al., 2007). In addition to probable stimulation of chemical gradients in 3D cultures, it is now accepted that 3D cell-cell

interactions can modify cell morphology, adhesion, mechanotransduction, as well as signal transduction, in a manner different from 2D culture systems (Baker & Chen, 2012). The 3D models enable the mimicking of various types of cancer cell heterogeneity seen *in vivo*. Hence, 3D cultures formed solely by cancer cells may show various phenotypes such as those of quiescent versus proliferating cells depending on the chemically induced gradients (Pampaloni et al., 2012). *In vitro* 3D cell culture models systems also have the structural design required to analyse cellular interactions (Okochi et al., 2013). Our spheroid invasion model mimics the *in vivo* situation better than two-dimensional (2D) model systems, as the cells are embedded in type I collagen, thereby interacting in a more biological way with the environment (Wiercinska et al., 2011; Frantz et al., 2010). In metastatic melanoma, as well as other cancers, increased production of type I collagen and other ECM components stimulates ECM remodelling and this facilitates tumour cell growth and migration, and is correlated with greater incidence of metastasis and poor prognosis (Shimoda et al., 2010; van Kempen et al., 2008). A number of 3D models that have been developed have concentrated on a variety of characteristics of metastasis, such as morphology (Petersen et al., 1992; Weiswald et al., 2015). Our spheroid invasion assay focuses predominantly on invasive potential and complements these 3D models (Fig. 15). It is simple, as well as quantitative, and can be used in screening assays (Vultur et al., 2014; Vinci et al., 2012).

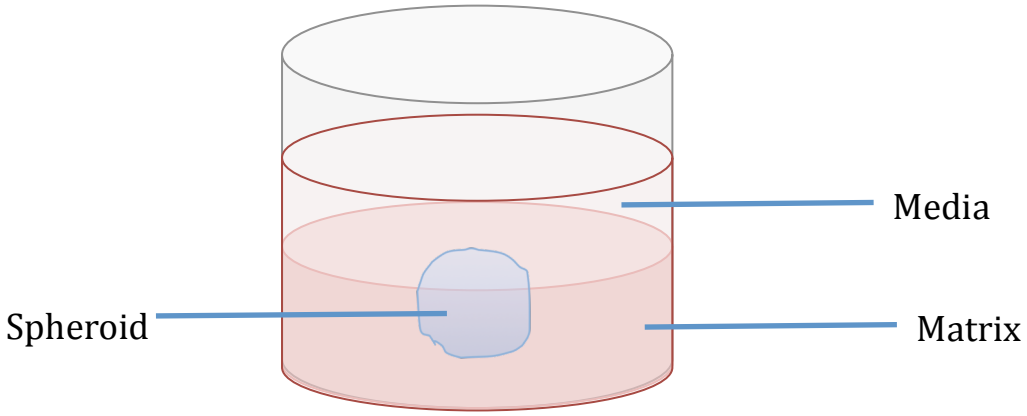


Figure 15. Schematic representing our 3D spheroid invasion assay. Cancer cells are grown as spheroids and embedded in collagen matrix.

5.2.2 Enhanced secretion of IL-6 and IL-8 in Conditioned Media is CAF-dependent and increases invasion and migration

Research on the tumour microenvironment has shown extensive deregulation of gene expression when compared to the normal stroma. The importance of the role of CAFs, in the tumour niche, in tumour progression has been indicated in the literature (Zhou et al., 2015; Harper & Sainson, 2014; Cirri & Chiarugi, 2012; Kalluri & Zeisberg, 2006). Studies analysing the interaction between melanoma cells and CAFs have demonstrated that fibroblasts only modify melanoma cell gene expression slightly, while melanoma cells greatly affect the gene expression array of CAFs (Gallagher et al., 2005). Recently, with great emphasis, it was shown in malignant melanoma that increased levels of interleukin-8 (IL-8) and interleukin-6 (IL-6) in the tumour microenvironment and patient serum play and important role in malignancy (Whipple & Brinckerhoff, 2014; Kucera et al., 2015). Furthermore, *in vitro* analysis also showed that CAFs produce IL-6 and IL-8, amongst other cancer-stimulating factors, especially when cultured in media conditioned by cancer cells (Kolar et al., 2012; Li et al., 2009). We co-cultured both melanoma cells (BLM and A2058) and CAFs, in order to assess the secretory effects of direct interaction, as observed in the tumour

stroma. Conditioned media (CM) from co-cultured CAFs with melanoma cells led to elevated melanoma cell invasion and migration, in addition to IL-6 and IL-8 levels in CM, in comparison to CAFs cultured alone. Normal dermal fibroblasts (HFP3) cultured with A2058 cells led to enhanced IL-8 levels in CM, but not IL-6. Further analysis of CM indicated that fibroblasts, CAFs and NFs, are the predominant producers of IL-6, and the melanoma cells predominantly produce IL-8, however, in co-culture, the precise source remains elusive. These results further highlight that CAFs are an ideal therapeutic target in melanoma, as combination therapy targeting various components of the tumour stroma would be a suitable scenario.

5.2.3 Melanoma Cells May Use Different IL-6 and IL-8 Upregulating Pathways

Previously, it was shown that BLM and A2058 cell lines have different gene expression profiles (Li et al., 2009), and for this reason, these cells were used to investigate the effect of CAFs on melanoma cells with varying phenotype and genotype. This difference was emphasised when the general effect of CM on melanoma cell production of IL-6 and IL-8 was analysed in melanoma spheroids embedded in 3D collagen. Our results demonstrated, in A2058 cells, that CM from co-cultured CAFs and A2058 significantly increased final IL-6 and IL-8 levels in the system, with a greater increase seen in IL-6 levels when conditioned media from CAFs co-cultured with A2058 cells was used. Notably, the final amounts of IL-6 in the A2058 3D invasion assay displayed a comparable trend as the effect of CM on A2058 spheroid invasion. This implies that IL-6 levels in CM from co-cultured CAFs and A2058 is responsible for increased levels of A2058 invasiveness.

Elevated levels of both IL-6 and IL-8 were observed in BLM spheroids embedded in 3D collagen, and CM did not have any significant effect on final IL-8 and IL-6 levels,

which suggests that the effect of CM on BLM invasion is not directly dependent on IL-6 and IL-8 levels in CM. Nevertheless, due to their high invasive potential, BLM cells may require a minimum concentration of IL-6 and IL-8 in order to promote invasiveness. This also signifies that other factors may be involved in CAF-stimulated BLM cell invasion, as well as migration. One possible factor of great importance is IL-1B. IL-1B is a secreted form of IL-1, and its expression has been implicated in the tumour stroma and metastasis in human melanoma. IL-1B mainly plays a role in promoting angiogenesis and cancer cell invasion, by stimulating the expression of pro-inflammatory molecules, which includes IL-8 (Li et al., 2009; Kim et al., 2010). BLM cells, and not A2058, were previously shown to greatly increase IL-1B production when co-cultured with CAFs (Li et al., 2009). In a positive feedback manner, the increased in IL-1B secretion could then be responsible for enhanced production of IL-8 in BLM cells. In a recent study, it was also demonstrated that greater levels of IL-1 and IL-6 are associated with poor prognosis (Fernandez-Garcia et al., 2016). In addition, it was also shown that paracrine IL-6 signalling between precancerous cells and CAFs plays an important role in initiating cancer progression (Osuala et al., 2015). Further work using array and proteomic technologies will aid the identification of other factors involved in the communication of melanoma cells and CAFs in the tumour microenvironment.

5.2.4 IL-6 and IL-8 in Combination is More Effective in Inhibiting CAF-dependent Increase in Invasion

In A2058 cells, basal invasion levels were suppressed by the IL-8, but not IL-6 neutralising antibody. Both antibodies decreased CAF-stimulated invasion of A2058 cells to basal level, when used alone or combined. This implies that A2058 cells produce high levels of IL-8 and IL-6, which is enhanced by CM from only CAFs and

CAFs co-cultured with A2058 cells, thereby stimulating invasion. A2058 cells in culture with CAFs enhanced IL-6 and IL-8 levels, as observed in a previous report (Kolar et al., 2012). These results suggests that A2058 cells, which possess a $BRAF^{V600E}$ mutation, well as other melanoma cells with BRAF mutations, such as WM3629 and WM3670, can interact with nearby stromal cells in the tumour milieu and stimulate melanoma progression, by increasing the production of lL-6 and IL-8, as well as other tumour-inducing cytokines and chemokines (Whipple & Brinckerhoff, 2014).

Our results emphasise that CM from CAFs, and CAFs co-cultured with melanoma cells enhances melanoma cell invasiveness, and this effect is inhibited with the use of neutralising antibodies against IL-6 and IL-8. Previously, it was shown that IL-6 has the ability to stimulate IL-8 expression, and this plays an important role in enhancing invasiveness (Okamura et al., 2013). We also demonstrate that the indirect interaction between CAFs and melanoma cells significantly elevated melanoma cell invasiveness, further highlighting the significant of the extended tumour microenvironment in cancer progression.

5.2.5 IL-6 and IL-8 may be Potential Therapeutic Targets to use in combination with traditional treatment

Cancer immunotherapy, an increasingly important and preferred method of therapy, refers to a number of methods that target immune system stimulation, in order to promote an objective response and disease maintenance. Melanoma is an ideal target for such an approach, although immunotherapy against melanomas has proven to be problematic. Partially, this is due to the complex nature of the tumour microenvironment, which includes CAFs, which are known to secrete numerous soluble factors that contribute to tumour progression. CAFs are also known to secrete

transforming growth factor-β (TGF- β), as well as other immunosuppressive cytokines, thereby creating an environment in which antitumour responses are compromised.

The BRAF mutation is present in approximately 60% of melanomas (Fofaria et al., 2015). Vemurafenib, and other BRAF-inhibiting therapies have proven unsatisfactory, as patients develop resistance to the inhibitors (Fofaria et al., 2015). Furthermore, some patients also develop squamous cell carcinoma, and others show tumour relapse (Su et al., 2012; Perez-Lorenzo & Zheng, 2012). In a recent study, it was shown that fibroblasts can protect melanoma cells from vemurafenib-dependent cytotoxicity, however combination therapy with vemurafenib and phosphoinositol-3 kinase (PI3K) inhibition led to tumour regression, *in vivo* (Federenko et al., 2015). Importantly, PI3K signalling is also a key signalling pathway downstream of IL-8.

Patients that responded to chemotherapy in metastatic melanoma showed decreased serum levels of IL-8, compared to patients with progressive and unresponsive disease (Brennecke et al., 2005). Another study also showed decreased and increased IL-8 levels in the serum of patients with positive responses or progressive disease, respectively, following treatment with BRAF inhibitors or ipilimumab (Sanmamed et al., 2014). Ipilimumab is a cytotoxic T-lymphocyte antigen-4 (CTLA4) blocking antibody, which is frequently used in melanoma patients with wild-type BRAF (Vilgelm et al., 2016). In addition to two-drug combination therapies, which may increase survival and reduce toxicity, triple combination therapy may also be the way forward with melanoma. The use of BRAF and MEK inhibitors, as well as IL-8 and IL-6 inhibitors, which are important in melanoma, may be worth exploring. This exact combination of agents needs to be elucidated further, as this may increase the chance

of treating advanced melanoma in an individualised manner, in order to avoid crucial problems of chemoresistance and metastasis.

5.2.6 Curcumin may be a potential candidate for metastatic melanoma therapy

Recently, due to problems with cytoxicity and resistance, scientists are turning to natural compounds in order to treat diseases. As demonstrated by the Nobel Peace Prize winner in 2015 that discovered a natural compound to treat popular parasitic diseases (website: nobelprize.org). The active compounds in plant extracts have long been considered as desirable candidates to treat a number of diseases, although natural products or their synthetic analogues may results in severe side effects (Thangapazham et al., 2013). As natural compounds usually show decreased levels of toxicity compared to synthetic products, they have been an increasingly popular research interest, especially in cancer therapeutics and its complications (Panahi et al., 2014). Curcumin is a natural polyphenol that is extracted form rhizomes of the *Curcuma longa* L. (turmeric) plant (Mirzaei et al., 2016). Increasing evidence has shown that curcumin plays an important role in a number of biological processes, and possesses various pharmacological properties, which are advantageous in the treatment of human diseases. These pharmacological effects include, but are not limited to, antioxidant, anti-inflammatory, anti-depressant, cardio-protective, anti-diabetic and cognition enhancing (Mirzaei et al., 2016). These indicate that curcumin can affect a number of cytokines, protein kinases, transcription factors, as well as inflammatory modulators. The treatment of metastatic melanoma has been problematic, and conventional treatments are often unsuccessful due to multi-drug resistance and tumour reoccurrence. One possible therapeutic agent in metastatic melanoma is curcumin. Curcumin is known to exert anti-inflammatory action in a

number of cell lines via a number of pathways, which include NF-kB and MAPK signalling pathways (Shishodia et al., 2005). Importantly, inflammatory responses to TNFa, which also plays a role in melanoma cell invasion and migration (Katerinaki et al., 2003), are mediated via direct stimulation by IL-1 expression, or distal and indirect stimulation by proinflammatory cytokines, such as IL-6 and IL-8 (Schottelius et al., 2004). NF-kB or MAPK pathways primarily mediate the expression of these inflammatory cytokines (Aggarwal et al., 2006). Thus, general anti-inflammatory effect of curcumin may be achieved via the inhibition of TNFa, NF-kB, or MAPK pathways. Interestingly, in human melanoma cell lines, we found that curcumin, naturally extracted from turmeric and purified, inhibits melanoma 3D spheroid invasion (Jobe et al., unpublished results).

6 Conclusion

Tumour cell invasion and metastasis is an important hallmark of cancer. We highlight here the role of NG2 proteoglycan in AMT and invasion, in a Rho-dependent manner, and NG2 may be involved in Rho activation and localisation in cancer. NG2 may be potential therapeutic target in cancers that possess elevated levels of NG2, as it is associated increased cancer cell invasion and poor prognosis. In addition, we also show the importance of CAF interaction with melanoma cells, which leads to the secretion and production of factors that stimulate melanoma cell invasion. These factors include IL-6 and IL-8, which play an important role in melanoma progression, and are considered desirable therapeutic target. Blocking IL-6 and IL-8 in CM led to a decrease in CAF-dependent invasion. Furthermore, CAF interaction with keratinocyte also led to melanoma invasion. The interaction between cancer cells and the tumour microenvironment needs to be fully elucidated, in order to limit disease progression. Metastatic melanoma therapeutics have not been successful, however, IL-6 and IL-8 signalling may be potential targets to use in combination with conventional treatment. Another prospective therapeutic is the natural compound curcumin, which was shown to inhibit melanoma cell invasion, in 3D. Taken together, our results indicate the importance of the tumour microenvironment in melanoma progression, and potential therapeutic targets, which may complementary to current treatment strategies, in order to successfully inhibit tumour invasion and metastasis.

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8 Supplementary