COLLAGEN TRIPLE HELIX REPEAT CONTAINING 1 INCREASES MELANOMA CELL MIGRATION, ADHESION AND SURVIVAL THROUGH MODULATION OF THE ACTIN CYTOSKELETON

by

Wency Wan Sze Ip

Bachelor of Science, The University of British Columbia, 2005

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

June 2009

Abstract

Background: Collagen Triple Helix Repeat Containing 1 (CTHRC1) is a recently discovered extracellular protein that can bind and activate Wnt signaling pathway. In previous gene expression profiling experiments, it was found to be aberrantly upregulated in metastatic melanoma and its expression level was correlated with melanoma progression and metastasis.

Objective: The purpose of this study is to understand the functional impact of CTHRC1 on cancer using melanoma cell lines as a model.

Experimental Methods: We transfected two melanoma cell lines, MMAN and MMRU, with plasmid vectors to create stable clones with high and low CTHRC1 expression to study the functional effects of CTHRC1 *in vitro*. Using these two cell lines, we assayed for melanoma migration, adhesion and survival using scratch wound healing assay, attachment assay and cell cycle analysis, respectively. In addition, the cells were stained for F-actin with AlexaFluor 594 labeled phalloidin to observe for actin organization.

Results: Using these two pairs of cell lines, we have found that CTHRC1 expression increased melanoma cell migration, enhanced melanoma cell adhesion to both tissue culture plastic and matrigel, and protected melanoma cells from serum deprivation induced apoptosis. Further, it was demonstrated that CTHRC1's pro-survival effect was dependent on cell adhesion, as the protection effect was lost when melanoma cells were cultured in suspension. Immunofluorescent

staining of F-actin revealed that CTHRC1 expression increased the formation structures such as focal complexes, lamellipodia and filopodia.

Discussion: The increased formation of the adhesion structures may be the key to CTHRC1 associated cell migration, adhesion and survival. These structures are likely regulated by the Rho family of proteins that act downstream of the Wnt/PCP pathway, with which CTHRC1 has been previously demonstrated to be involved as a co-receptor.

Conclusion: Results from this study suggest that CTHRC1 expression promotes cellular behaviours associated with tumour metastases. Therefore, inhibition of this protein may be able to block melanoma metastasis and may have value as a potential therapeutic.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACT	-	alpha-1 antichymotrypsin
Ap-1	-	activator protein 1
APC	-	adenomatosis polyposis coli
Bcl-2	-	B-cell CLL/lymphoma 2
BRAF	-	v-raf murine sarcoma viral oncogene homolog B1
BSA	-	bovine serum albumin
CDKN2A	-	cyclin dependent kinase inhibitor 2A
CTHRC1	-	collagen triple helix repeat containing 1
DAPI	-	4',6-diamidino-2-phenylindole
DMEM	-	Dulbecco's modified eagle's medium
DNMT		DNA methyltransferase
DTIC	-	dacarbazine
Dvl	-	dishevelled
E-cadherin	-	epithelial cadherin
ECL	-	enhanced chemiluminescence
EDTA	-	ethylenediaminetetraacetic acid
EMT	-	epithelial – mesenchymal transition
ERK	-	extracellular regulated kinase
ET-3	-	endothelin 3
FAK	-	focal adhesion kinase
FBS	-	fetal bovine serum
Fra1	-	fos-related antigen 1
Fzd	-	frizzled
GSK3β	-	glycogen synthase kinase 3 beta
HRP	-	horseradish peroxidase
JNK	-	c-Jun N-terminal kinase
LRP	-	low-density lipoprotein receptors
MAPK	-	mitogen-activated protein kinase
MITF	-	microphthalmia-associated transcription factor
MTS	-	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
N-cadherin	-	neural cadherin
N-RAS	-	neuroblastoma ras viral (v-ras) oncogene homolog
OPN	-	osteopontin
PBS	-	phosphate bufered saline
PCP	-	planar cell polarity

PFA	-	paraformaldehyde
PI	-	propidium iodide
PI3K	-	phosphatidylinositol-3-kinase
PIP3	-	phosphatidylinositol phosphate
PKC	-	protein kinase C
PLC	-	phospholipase C
poly-HEMA	-	poly- hydroxyethyl methacrylic acid
PPARδ	-	peroxisome proliferator-activated receptor delta
PTEN	-	phosphatase and tensin homologue
PVDF	-	polyvinylidene difluoride
Rac	-	ras-related C3 botulinum toxin substrate
Rho	-	ras homolog gene family
ROCK	-	rho-kinase
Ror2	-	receptor tyrosine kinase-like orphan receptor 2
RTK	-	receptor tyrosine kinase
SAPK	-	stress activated protein kinase
SDS-PAGE	-	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFG-β	-	transforming gorwth factor-beta
TMZ	-	temozolomide
TRAIL	-	tumour necrosis factor-related apoptosis-inducing ligand
Wnt	-	wingless-type MMTV integration site family

Acknowledgements

I would like to express my sincere gratitude and appreciation to my supervisor, Dr. Youwen Zhou, for his advice and guidance throughout my project. I would also like to thank my supervisory committee members Dr. Yuzhuo Wang and Dr. Jan Dutz for their input and support. Your knowledge and way of thinking has been of great value to me. I am deeply grateful for the support and assistance in various aspects from Dr. Mingwan Su and members of the Predictive Medicine and Therapeutics lab. Lastly, I would like to extend my special thanks to the Prostate Center for letting me use their flow cytometer, and Dr. Christopher Shaw's lab for letting me use their fluorescent microscope. It is my pleasure to thank each of the above who made this project possible.

Dedication

This thesis is dedicated to my family, who have offered me unconditional love and support throughout the course of my studies.

1. Introduction

The Laboratory of Predictive Medicine and Therapeutics has been actively using genomic approaches to better understand skin diseases. This thesis is a study following an initial genomic approach to screen for novel melanoma associated genes. It is aimed at characterizing the biological function of Collagen Triple Helix Repeat Containing 1 (CTHRC1), which was identified to be aberrantly upregulated in invasive and metastatic melanoma. There is limited knowledge about CTHRC1 and its function in cancer cells. This is the first study looking at the biological function in cancer cells using melanoma cells as the experimental model. Unveiling some of the functions of this protein will yield important information regarding melanoma progression, and may aid in the development of targeted therapies for melanoma.

1.1. Melanoma

Melanoma is an aggressive cancer which arises from the abnormal growth of epidermal melanocytes, which are the pigment producing cells of the skin. It accounts for 4% of all skin cancers at present; however, it is responsible for 80% of deaths associated with dermatological cancers. Although early stages of melanoma are highly curable, 20% of patients with primary melanoma may develop the metastatic disease. At present, less than 15% of metastatic melanoma patients are able to survive past 5 years due to the inability of current interventions to effectively manage the disease (Cancer facts & figures, 2008, Miller and Mihm, 2006, Nguyen, 2004).

Despite the fact that much effort has been put into understanding melanoma, treatment of the advanced disease remains extremely challenging. Therefore, novel insights and further

understanding of the molecular mechanisms metastatic melanoma cells acquire to maintain malignant properties is crucial for developing effective and specific therapies in the future. In addition, such knowledge of melanoma progression and its potential therapeutic strategies might be broadly applicable to other human solid cancers.

1.2. Melanoma progression

During melanoma progression, melanocytes gradually develop malignancy through acquiring various phenotypes. The Clark model for melanoma progression describes the histological changes that are associated with the transformation from normal melanocytes to melanoma (Clark et al, 1984). According to this model, the first step in malignant transformation is the development of benign nevi, where the cells proliferate more quickly than normal. Studies have associated this phenotypic change to the aberrant activation of mitogen-activated protein kinase/ extracellular regulated kinase (MAPK/ERK) signaling pathway (Welsh et al, 2001, Brunet et al, 1999, Lin et al, 1998), usually as a result of mutations in N-RAS or BRAF, which act upstream of MAPK/ERK (Patton et al, 2005, Michaloglou et al, 2005, Eskandarpour et al, 2005, Hingorani et al, 2003, Hingorani et al, 2003). However, these mutations alone do not usually lead to cancer, as most nevi remain static. Additional mutations must be acquired by the cells in order to progress to a malignant state.

The second step in malignant transformation is the development of dysplastic nevi. These lesions with random cytologic atypia may arise as a new lesion or from pre-existing

benign nevi. This phenotypic change is associated with abnormalities that affect cell growth, DNA repair, and the susceptibility to cell death. Molecular changes that occur include mutations in CDKN2A and PTEN (Guldberg et al, 1997). The CDKN2A encodes 2 tumour suppressor proteins INK4A and ARF (Flores et al, 1996, Nobori et al, 1994), which arrests cell cycle or induces apoptosis in cells with damaged DNA or activated oncogenes (You et al, 2002, Kamb et al, 1994, Hussussian et al, 1994, Sharpless and Chin, 2003, Serrano et al, 1996, Chin et al, 1997). PTEN (phosphatase and tensin homologue) regulates the phosphatidylinositol-3-kinase (PI3K) pathway through controlling levels of phosphatidylinositol phoasphate (PIP3) (Cantley and Neel, 1999). Overactivation of PI3K signaling, resulting from a loss or mutation in PTEN, would inactivate proteins that induce apoptosis and suppress cell cycle, leading to increased cell survival (Wu, Goel and Haluska, 2003, Li et al, 1997, Steck et al, 1997).

The third step in malignant transformation is the development of radial growth phase, which is usually accompanied by the failure of melanocytes to differentiate. Melanocyte differentiation requires exiting from cell cycle and change of gene expression to ones that are necessary for pigment production, and thus many nevi regress through this process. Microphthalmia-associated transcription factor (MITF) is the gene responsible for regulating differentiation and cell cycle arrest in normal melanocytes (Hodgkinson et al, 1993) and maintenance of melanocyte progenitor cells in adults (Nishimura, Granter and Fisher, 2005, Widlund and Fisher, 2003). On the other hand, MTIF acts as an oncogene in melanoma cells, where it contributes to survival by expressing Bcl-2, a key anti-apoptotic gene (McGill et al, 2002). In addition, overexpression of MITF is associated with poor prognosis and chemotherapy resistance (Garraway et al, 2005).

The last two steps in malignant transformation are the development of vertical growth phase, and finally, metastasis. These two phases are the main contributors to melanoma morbidity and mortality. In the vertical growth phase, cells undergo epithelial- mesenchymal transition (EMT) and acquire the ability to locally invade through the basement membrane into the dermal layer. Whereas in metastatic melanoma, tumour cells detach from the primary site, invade through the surrounding stroma, intravasate into the blood vessels, then extravasate and form tumours at distant sites (Haass et al, 2005). Invasive phenotypes are acquired from changes in cell adhesion and migration. Key molecular changes in these phases include change from epithelial-cadherin (E-cadherin) to neural-cadherin (N-cadherin) expression, and changes in integrin expression. Expression of N-cadherin allows for melanoma cells to change its interaction with epithelial cells (i.e. keratinocytes) to interaction with other N-cadherin expressing cells, such as dermal fibroblasts and endothelial cells (Hsu et al, 2000, Hsu et al, 1996, Valyi-Nagy et al, 1993, Danen et al, 1996, Scott et al, 1997). Integrins are the molecules responsible for mediating cell contacts with extracellular matrix proteins such as fibronectin, collagens, and laminin (Kuphal, Bauer and Bosserhoff, 2005). Aberrant expression of integrin receptors, which are common in metastatic melanoma, induces cells to express proteases (i.e. matrix-metalloproteinases) that degrade the matrix in the basement membrane, stimulate cell motility, and increases cell survival (Petitclerc et al, 1999, Li et al, 2001, Brooks et al, 1996, Hofmann et al, 2000)

1.3. Current interventions for melanoma

Melanomas are treated by different therapeutic means depending on their staging. Early stages of melanoma may be effectively cured by means of surgical excision with a clear margin.

On the other hand, treatment of metastatic melanoma is much more challenging. Many therapeutic approaches have been tested in the past; however, none of the therapies tested have been able to achieve response rates greater than 25%, leaving metastatic melanoma largely untreatable. The low efficacy of the current therapeutics is because of melanoma's high resistance to apoptosis. Radiotherapy and immunotherapy with IL-2 and Interferon-alpha (IFN-α) have been tested, and were found to be modestly effective against advanced melanoma (Mendenhall et al, 2008, Fang, Lonsdorf and Hwang, 2008, Kim et al, 2002). Other agents such as nitrosoureas, platinum analogs, vinca alkaloids and taxanes were tested and found to be only minimally active against advanced melanoma (Hocker, Singh and Tsao, 2008). The chemotherapeutic agent, Dacarbazine (DTIC), although only having complete response rates of 5%, remained the main single agent chemotherapy for advanced melanoma for many years (Eigentler et al, 2003). Temozolomide (TMZ), a derivative of DTIC, functions by methylating O6 in guanine. It has similar response rates as DTIC, but has been shown to be more effective against brain metastases due to its higher bioavailability and ability to penetrate the central nervous system (Boogerd, de Gast and Dalesio, 2007). Single agent chemotherapeutic approaches were not found to be effective against metastatic melanoma, thus setting off the research involving combination chemotherapy and immunotherapy.

With the extensive research done in understanding melanoma genetics, targeted and combination therapeutic approaches have been used to attempt to shut down the genetic aberrations that contribute to malignant behaviour and resistance to chemotherapy. Combination therapy, for example, of a cell death inducing agent and a compound that targets a specific gene to reduce melanoma's resistance to apoptosis, may have synergistic effects and have higher efficacy than single agent treatments. Therapies targeting the RAS signaling pathway, BRAF,

MEK, PI3K and Bcl-2 have been developed. The receptor tyrosine kinase inhibitor, imatinib mesylate, inhibits the melanocyte lineage gene, c-kit. Although this compound was effective against other tumours, the response rate in melanoma was very low (Wyman et al, 2006). Tipifarnib and lonafarnib, farnesyltransferase inhibitors, were developed to block Ras activation, but were not found to be effective as a monotherapy for melanoma. However, lonafarnib combined with cisplatin has demonstrated some promising effects against melanoma *in vitro* (Smalley and Eisen, 2003). Sorafenib, the BRAF inhibitor, was not found to be effective against melanoma as a monotherapy (Eisen et al, 2006). However, this compound showed promising results in combination therapy with carboplatin, paclitaxel, DTIC and TMZ (Flaherty et al, 2008, McDermott et al, 2008, Takimoto and Awada, 2008). MEK inhibitors, which are able to suppress downstream anti-apoptotic proteins in the Bcl-2 family, showed promising results in combination therapy in clinical trials (Koo et al, 2002). mTOR, a protein downstream of PI3K and Akt that regulates protein synthesis, cell-cycle, and angiogenesis, has also been targeted. However, results were not promising, as this protein is widely expressed and is difficult to specifically target melanoma cells (Hocker, Singh and Tsao, 2008, Dancey, 2006, Madhunapantula, Sharma and Robertson, 2007).

Another class of therapeutic, DNA methyltransferase (DNMT) inhibitors, targets to reverse the epigenetic gene silencing of tumor suppressors due to DNA methylation at the promoter regions. *In vitro* data revealed that DNMT inhibitors are able to enhance the sensitivity of melanoma cells to IFN-induced apoptosis (Bae et al, 2008). However, like mTOR inhibition, the major drawback of this treatment is its lack of tissue specificity. Inhibitors of Bcl-2 family have also been tested in combination therapy with DTIC, and this combination demonstrated higher response rate and increased overall survival (Bedikian et al, 2006). Of notable mention is

bortezomib, a proteasomal inhibitor that has effects on Bcl-2 family and NF-kB. Bortezomib is able to increase melanoma sensitivity to TMZ induced cell death (Cusack et al, 2001, Sunwoo et al, 2001).

Targeted and combination therapy are not only tested with chemotherapeutic agents. One study reports that the inhibition of melanogenesis with D-penicillamine and N-phenylthiourea sensitized melanoma cells to radiation therapy in advanced melanomas (Brozyna, VanMiddlesworth and Slominski, 2008). Bcl-2 inhibition was also found to enhance the killing of melanoma cells by immune cells, perhaps through Fas ligand and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) (Lickliter et al, 2007). Many molecules were also found to enhance cisplatin's effectiveness in melanoma (Liu, Chan and Ho, 2008, Tamura et al, 2003).

Despite the many approaches that attempt to increase the efficacy of melanoma therapies, an effective treatment for metastatic melanoma has not yet been identified. In general, most of the combination therapeutic strategies mentioned have either showed disappointing results at the clinical testing stage, or are still currently in pre-clinical development. Nonetheless, due to the inability of the current interventions to control the tumor cells' growth, local invasion and metastatic spread, most patients with metastatic melanoma still have poor prognosis. Therefore, it is important to continue to study advanced melanoma, so that new intervention strategies can be developed to increase melanoma patient survival.

1.4. Identification of genes with aberrant expression in melanoma

In attempt to further understand melanoma, our laboratory performed a cDNA microarray analysis to identify the molecular changes in gene expression profiles across samples corresponding to melanoma progression. The study comparing the mRNA expression levels between benign nevi, melanoma in situ, non-invasive primary and metastatic melanoma has revealed that many of the most strikingly up-regulated genes identified are related to matrix remodelling and cell-matrix interaction. Among these are osteopontin (OPN) (Zhou et al, 2005), endothelin-3 (ET-3) (Tang et al, 2008), alpha-1 antichymotrypsin (ACT) (unpublished data), and collagen triple helix repeat containing 1 (CTHRC1) (Tang et al, 2006).

1.5. Collagen triple helix repeat containing 1 (CTHRC1) protein

This section summarizes the current knowledge of CTHRC1. Since the discovery CTHRC1 in 2005, several groups of researchers have studied this protein. However, despite the new area of research, knowledge of CTHRC1 is limited at present.

1.5.1. General knowledge about CTHRC1

CTHRC1 was identified as one of the genes with striking aberrant expression in metastatic melanoma in our laboratory's previous gene array study. This protein was initially shown by other researchers to be involved in arterial wound healing and modulation of collagen matrix deposition (Pyagay et al, 2005). CTHRC1 is a highly conserved 28 kDa protein that contains many short repeats of collagen Gly-X-Y motifs, and is secreted by the cells into the extracellular environment. In addition, it was reported that CTHRC1 forms homotrimers. It has the ability to enhance cultured fibroblast migration and inhibit its collagen synthesis (Pyagay et

al, 2005, Durmus et al, 2006). Characterization of CTHRC1 temporal expression during mammalian development showed that the expression patterns are similar to the transforming growth factor-beta (TGF-β) family members, and suggests a function for CTHRC1 at the epithelial-mesenchymal interfaces. Moreover, CTHRC1 appears to regulate TGF-β signalling in a feedback mechanism in smooth muscle cell types (Durmus et al, 2006, LeClair and Lindner, 2007, LeClair et al, 2007). In addition, it was shown to be a positive regulator of osteoblastic bone formation (Kimura et al, 2008). A recent study reports that CTHRC1 acts as a Wnt (Wingless-type MMTV integration site family) cofactor protein that activates Wnt/ PCP pathway and stabilizes the interaction between CTHRC1-Wnt-Fz or CTHRC1-Wnt-Ror2 complex (Yamamoto et al, 2008). The interactions of the different complexes activate different domains of Dishevelled (Dvl), which then activates different branches of Wnt signaling (will be discussed in section 1.6).

1.5.2. CTHRC1's involvement with human cancer

Using melanoma tissue microarrays linked with clinical annotation of the melanoma biopsies, it was found that up-regulation of CTHRC1 occurs during the steps of melanoma tissue invasion and metastasis. Further, in a survey of 310 human tumour derived tissues representing 19 types of human solid cancers, CTHRC1 was found to be upregulated in 16 of the 19 cancers, suggesting that CTHRC1 may play a role in human cancer pathogenesis in general (Tang et al, 2006). The relevance of CTHRC1 to cancer was reinforced by the identification that CTHRC1 upregulation was highly significant in a large proportion of breast cancer biopsies (Turashvili et al, 2007). To date, there has been limited investigation into the roles of CTHRC1 protein in the development of cancer phenotypes.

1.6. Overview of Wnt signaling pathway and melanoma

A recent study revealed, for the first time, a potential mechanism of action for CTHRC1. In that report, CTHRC1 was found to co-immunoprecipitate with Wnt signaling components such as Fzd 3, Fzd 5, Fzd6, Ror2, Wnt 3a, Wnt 5a and Wnt 11. In addition, CTHRC1 was found to increase Rac1 and RhoA activation, which are two downstream effectors in Wnt/PCP signaling pathway (Yamamoto et al, 2008), an important pathway in embryonic development and neural crest induction. Since melanocytes originated from the neural crest and as there may be similarities between neural crest migration and cancer metastasis, CTHRC1 and the Wnt pathway may be important contributors to regulating key events in melanoma progression (De Calisto et al, 2005). This section provides a brief overview of Wnt signaling and its potential relevance to melanoma progression.

Wnt signalling can regulate diverse cellular processes and involves the interaction of proteins belonging to several different groups: Wnt ligands, frizzled (Fzd) receptors, and Wnt co-receptors. The phenotypic outcome of Wnt signaling varies according to the combination of proteins involved in the activation of the pathway. To initiate Wnt signaling activation at the cellular membrane, Wnt ligands bind to frizzled receptors, which are serpentine G-protein coupled receptors (GPCR) (Slusarski, Corces and Moon, 1997). Frizzled co-receptors such as low-density lipoprotein receptors (LRP), derailed and Ror2 may also be involved. Upon binding of the Wnt ligands to their receptors and other associated proteins, G-proteins are activated, which in turn activate the cytoplasmic phosphoprotein dishevelled (Dvl). Different domains of Dvl are activated by different combinations of proteins. The Wnt signal then diverges to the canonical Wnt signaling pathway (β -catenin pathway), planar cell polarity pathway (Wnt/PCP

pathway), and the Wnt/Ca^{2+} signaling pathway downstream of Dvl (Figure 1-1) (Weeraratna, 2005, Morin and Weeraratna, 2003, Cadigan and Liu, 2006, Habas and Dawid, 2005).

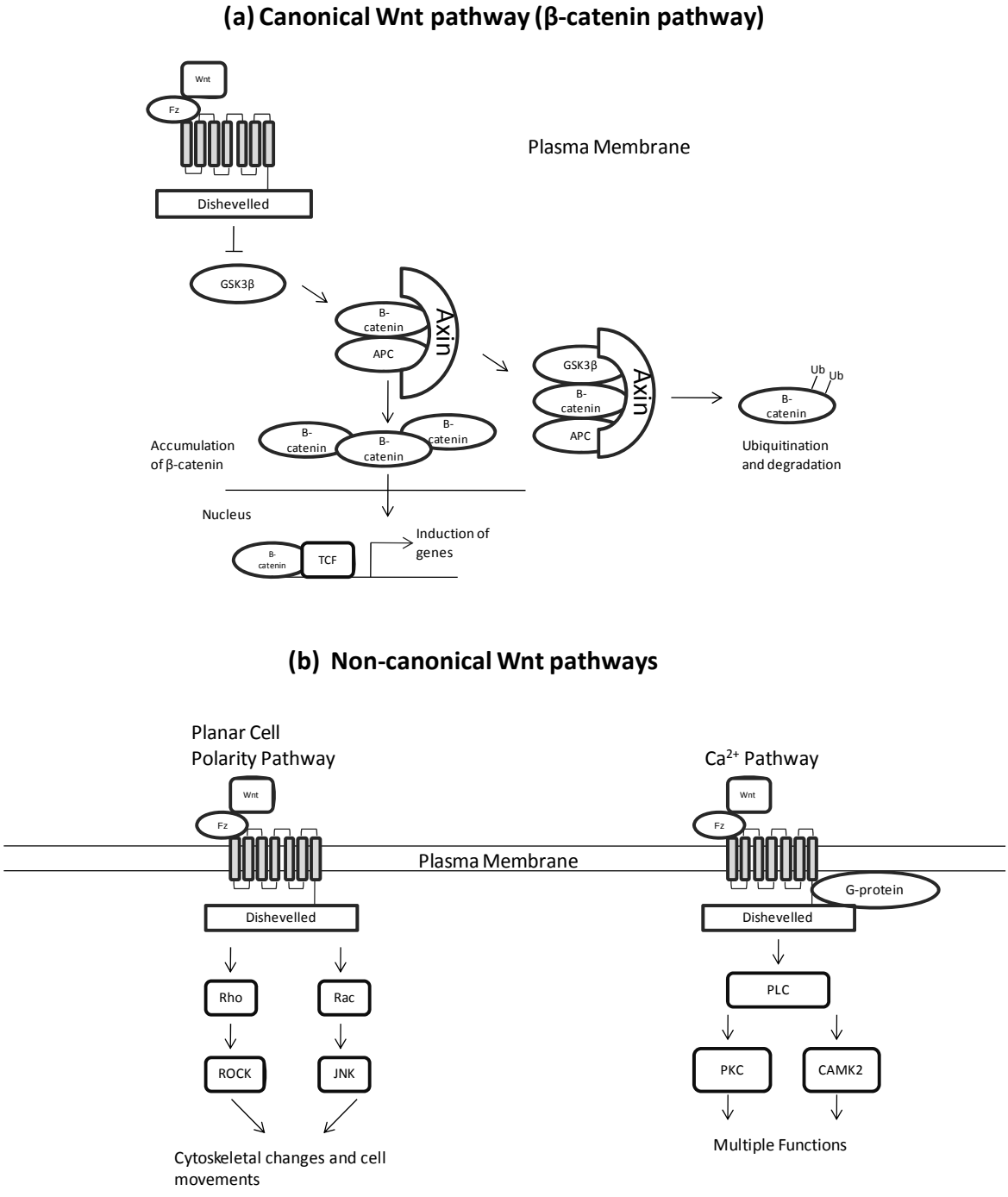

Figure 1-1. Wnt signaling pathways

The canonical Wnt signaling pathway is the best characterized among the three Wnt pathways. In this pathway, Dvl is activated at the PDZ and Dix domain. This activation releases β –catenin from the axin/APC complex and inhibits GSK3β from phosphorylating β–catenin. This leads to the stabilization and accumulation of β -catenin, allowing it to translocate into the nucleus (Seidensticker and Behrens, 2000, Behrens et al, 1998, Hart et al, 1999). β –catenin can then act as a transcription factor along with TCF to regulate genes responsible for diverse cellular processes, such as c-Myc, cyclin D1, PPARδ, matrix metalloproteinase, matrilysin, Ap-1, c-jun and fra1 (He et al, 1999, He et al, 1998, Tetsu and McCormick, 1999, Brabletz et al, 1999, Mann et al, 1999). It is speculated that β –catenin is important for early stages of melanoma, as Wnt/β-catenin signaling can increase melanoma proliferation and survival through BRAF activation. Interestingly, it was found that the overexpression or mutation of β-catenin did not correlate with melanoma progression (Demunter et al, 2002), but the overexpression of phosphorylated nuclear β-catenin did (Kielhorn et al, 2003). In addition, one study identified the loss of β-catenin to contribute to melanoma progression (Demunter et al, 2002) .

The non-canonical Wnt pathways are less well characterized compared to the β–catenin pathway, therefore, its roles in melanoma progression require further elucidation. Experimental results to-date suggest that these pathways may have significant overlaps and may also play crucial roles in the establishment of invasive and metastatic tumours. For example, Wnt5a, a Wnt ligand belonging to both the Wnt/PCP and Wnt/Ca^{2+} pathway, is strongly correlated with aggressive melanomas (Bittner et al, 2000).

The planar cell polarity (PCP) pathway is activated when Dvl is activated at the DEP domain (Axelrod et al, 1998). This can then activate GTPases Rho and Rac, which in turn regulates Rho-kinase (ROCK) and c-Jun N-terminal kinase (JNK) respectively (Strutt, Weber and Mlodzik, 1997, Strutt, 2001, Boutros et al, 1998). Activation of ROCK leads to modulation of cytoskeleton, resulting in the increased formation of actin stress fibers, and increased adhesion and cell spreading (Strutt, 2001, Narumiya, Tanji and Ishizaki, 2009, Gimona, 2008). JNK, also known as stress activated protein kinase (SAPK), acts upstream of the JNK/MAPK pathway, and regulates cell proliferation, differentiation and apoptosis (Dhanasekaran and Reddy, 2008).

The Wnt/Ca^{2+} pathway can activate phospholipase C (PLC), which triggers secondary messengers that induce Ca^{2+} release from the endoplasmic reticulum (Jafri and Keizer, 1995). The intracellular Ca^{2+}can then upregulate the expression and activity of calmodulin, calmodulin kinases, and protein kinase C (PKC), leading to changes in cell motility, morphogenesis, apoptosis, cytoskeleton and differentiation (Kuhl et al, 2000a, Kuhl et al, 2000b, Sheldahl et al, 1999). Nonetheless, researchers have found that the aberrant regulation of these pathways can play a role in the progression of melanoma.

1.7. Hypothesis

Since CTHRC1 is positively correlated with melanoma progression, we hypothesize that CTHRC1 must have an important role in melanoma metastasis and that CTHRC1 inhibition may reduce metastatic phenotypes. The purpose of this study is to identify the functional roles of CTHRC1 in metastatic melanoma. Also, since CTHRC1 was previously identified as a Wnt /PCP cofactor protein, we examine the potential of downstream activation of Wnt/PCP pathway

by CTHRC1 in melanoma. Finally, we evaluate the possible impact of inhibiting CTHRC1 function on melanoma cells' survival.

2. Materials and Methods

2.1. Cell lines and growth conditions

Melanoma cell lines MMAN (no endogenous CTHRC1 expression) and MMRU (high endogenous CTHRC1 expression) were used. Both MMAN and MMRU are metastatic melanoma cell lines originally derived from the inguinal and cervical lymph nodes of patients, respectively (Byers et al, 1991). Both cell lines are wild-type for p53, a tumour suppressor commonly mutated in cancers. Both cell lines were kind gifts from Dr. Gang Li (University of British Columbia), who received the cell lines from Dr. H.R. Byers (Boston University School of Medicine) as a gift (Li et al, 1995). All melanoma cell lines were cultured in Invitrogen's Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 units/ mL penicillin, 100 μg /mL streptomycin, and 10% fetal bovine serum (FBS) at 37^{o}C with 5% CO_2. All cultures were provided with fresh medium 3 times weekly, and replenished with fresh medium 24 hours prior to treatments.

2.2. Establishing stable transfection clones of melanoma cells with altered CTHRC1 protein expression

1.5 x 10^5 MMAN cells, which lack endogenous expression of CTHRC1, were transfected with pCI-neo mammalian expression vector-based plasmid construct (Promega) containing CTHRC1 sense and neomycin (G418) resistance genes. Conversely, MMRU cells, which abundantly express CTHRC1 protein, were transfected with pCI-neo-antisense CTHRC1 construct (Figure 2-1) to knock down the expression of CTHRC1. For both cell lines used, an

empty vector of the plasmid construct with only the neomycin resistance gene was transfected into the cells as a mock-transfection control.

The CTHRC1 sense and antisense plasmids were generated by Dr. Mingwan Su (PMT lab) by using the cDNA synthesized from human melanoma tissue. For the synthesis of the sense plasmid, the CTHRC1 sense sequence from 98-830 kb (CTHRC1 98-830), with Xho1 site at 98kb and Not1site at 830kb, was inserted into pCR2.1-TOPO according to Invitrogen's TOPO cloning kit. The CTHRC1 fragment is then excised from the TOPO vector with Xho1 and Not1 and inserted into pCI –Neo vector (Figure 2-1). As for the antisense construct, after TOPO cloning, the antisense fragment is excised with Not1 only and inserted into pCI-NEO. The orientation of the sense and antisense clones were then confirmed by cutting it with Xho1 restriction enzyme and checking it on agarose gel.

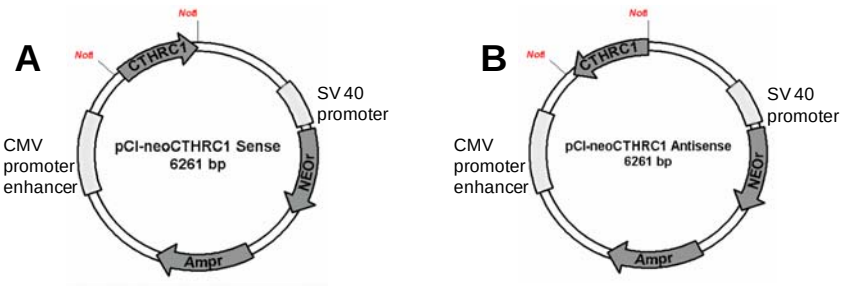

Figure 2-1. Plasmid vectors used for stable transfections

siPORT Amine (Ambion) was mixed with OPTIMEM (Invitrogen), and 5 μg of plasmid DNA was added to the mixture of transfection reagents. After 10 minutes, the transfection mixture was added dropwise to the culture medium of MMAN or MMRU cells. Cells were then seeded at 1/10 and 1/20 dilution 24 hours after transfection. Transfected cells were cultured in media containing 500 μg/mL G418 (Invitrogen) for 3-4 weeks to select for stably transfected

cells. During the selection period, fresh media containing 500 μg/mL G418 were supplied to the cells every 3 days. After the initial selection phase, individual colonies were isolated.

To isolate the antibiotic resistant clones, UV sterilized petroleum jelly was applied to the bottom of a small glass separator ring. The separator was then placed around the isolated colony, and 20 μL of 1x Trypsin (Invitrogen) was added. After the cells have detached from the plate, 80 μL of media containing serum was added to neutralize the trypsin, and the colony was transferred to a separate well in a 24-well plate. Once the cells have grown to 70% confluency, they were transferred to a 6-well plate. When the isolated clones have grown to confluency, they were assayed for CTHRC1 protein expression by western blotting. After selection, stable clones of MMAN and MMRU cells were maintained in regular culture medium plus 300 μg/mL G418.

2.3. Western blotting

Protein was extracted from cells for western blotting analysis by directly lysing cell pellets in RIPA buffer (Phosphate Buffered Saline (PBS), 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate). Protein concentration was measured by creating a standard curve from 0 to 1000 μg/mL using bovine serum albumin (BSA), adding the Bradford Reagent (Bio-Rad) at 1:50 ratio, and reading the absorbance at 595 nm. Protein concentrations of the samples were then extrapolated based on standard curve readings. Fifteen micrograms of total protein were then separated by 12% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After transferring, the proteins immobilized on the PVDF membrane were stained with 1x Ponceau S to ensure equal protein loading. The membrane was then blocked in 5% skim milk in PBS (pH 7.4) at 4^{o}C for overnight, and then probed with primary antibody in

3% BSA in PBS (see Table 1 for details). After 1 hour of incubation in primary antibody, the corresponding secondary antibody conjugated to horseradish peroxidase (HRP) (AbCam) was used to probe the membrane. After the secondary antibody incubation, the membranes were washed several times in PBS + 0.04% Tween 20 between incubations in each antibody. The signals were detected by enhanced chemiluminescence (ECL) detection (Amersham Biosciences).

Table 2-1. List of antibodies used for Western blot

Antibody	Type	Source	Concentration Used	Secondary Antibody
CTHRC1	Rabbit Polyclonal	Dr. Zhou's Laboratory (PMT Lab) with the assistance of Immunochem Biopharm Inc (Burnaby, BC) (Tang et al, 2006)	650 ng/mL	goat anti-rabbit IgG-HRP at 1:1000
Actin	Mouse Monoclonal	AbCam	500 ng/mL	goat anti-mouse IgG-HRP at 1:1000
Beta-catenin	Rabbit Polyclonal	AbCam	15 μg/mL	goat anti-rabbit IgG-HRP at 1:1000

2.4. Assay for cell number

To study the effect of CTHRC1 on the growth in reduced serum and survival in serum free conditions of the 2 melanoma lines, 2000 cells (for growth assay) and 5000 cells (survival assay) were seeded in 96-well plates. At each of the timepoints, cell numbers in triplicate wells were counted by trypan blue exclusion assay. Trypan blue exclusion assay is a simple assay used for the detection of viable cell numbers. Cells were detached from the tissue culture plate

with the addition of 0.05% trypsin (Gibco), and neutralized with 3x the volume. 10 μL of the cell suspension was then mixed with an equal volume of trypan blue (Invitrogen). The cells were then counted using a hemocytometer. The total cell number and the number of viable cells, which do not stain with trypan blue, were recorded.

For qualitative observation of cell morphology and cell density, a parallel set of cells were fixed and stained. 4% paraformaldehyde (Sigma) was added to the cells and incubated for 15 mins at room temperautre to fix the cells onto culture plates. After fixation, cells were stained with 0.04% toludine blue (Sigma) for 15 mins at room temperature. Cells were then washed extensively with water to remove the stain afterwards. The plates were then air dried and stored at room temperature.

2.5. Cell cycle analysis

Apoptosis and proliferation of MMAN and MMRU cells under various growth conditions was assayed for by staining the cells with propidium iodide (PI) and analyzing for cell cycle distribution by flow cytometry. For identification of changes in cell cycle due to serum deprivation, $1.5x10^5$ cells were grown in 6-well tissue culture plates. At 24 and 48 hours after serum withdrawal treatment, the cells were harvested and stained according to the following procedures.

Cells were harvested by adding 0.05% trypsin EDTA (Gibco) and allowing them to detach from the tissue culture plate. Once the cells were detached, 5x the volume of DMEM + 10% FBS were added to neutralize the trypsin, and then the cell suspension was centrifuged at 1200 RPM for 5 mins to collect the cells to the bottom of the tube. After the cells were

harvested from their treatment, they were washed with PBS and then quickly fixed by adding 70% ethanol dropwise to the cells while vortexing. The fixed cells were then placed at -20^{o}C and stored until analysis. The cells were analyzed once all samples from different time points were collected.

PI was chosen as the dye to use for cell cycle analysis because of its ability to bind and intercalate between bases in the DNA and emit a strong fluorescent signal. The fluorescence intensity reflects the amount of DNA present, and thus flow cytometry can be used to analyze the fluorescent signal of each cell and yield information about the proportion of cells in each phase of the cell cycle in a sample. PI staining is done by first washing off the fixative in the sample, and then incubating the cells in 50 μg/mL PI, 0.1 mg/mL RNaseA, 0.05% Triton X-100 in PBS. After staining, the cells were washed with 5x the volume of PBS, and resuspended in PBS and kept in the dark until analysis. Samples were then analyzed in the PI channel of the FACS Canto II (BD Biosciences). A plot of PI-W vs PI-A was generated, and single cell population was selected for analysis. The phases of the cell cycle are identified as follows. The first sharp peak is designated as the G0/ G1 peak, while the second peak at double the intensity of the first peak is designated as the G2/M peak. The phase between G0/G1 and G2/M peaks is the S phase, since cells undergoing synthesis can have any amount of DNA between 1 and 2 sets of genetic material. Cells that are undergoing apoptosis would undergo DNA degradation, and thus, are designated as the population of cells that have less fluorescence intensity than cells in the G0/G1 phase. Gates were set up to analyze the percentage of cells in each phase of the cell cycle. The average percentage of cells in each phase of the cell cycle for each treatment condition was calculated.

2.6. Anoikis assay

Anoikis, a Greek word meaning "a state of homelessness", refers to the apoptosis of anchorage dependent cells caused by detachment from physical growth support. In order to measure anoikis, one must first force the melanoma cells to remain in suspension in culture. This is achieved by first coating the tissue culture plates with poly- hydroxyethyl methacrylic acid (poly-HEMA), which is a material to which cells cannot attach. Poly-HEMA was first prepared at 10 mg/mL in 95% ethanol, and then added to coat the tissue culture plates. The plates were then air dried to remove the ethanol. The plates were then washed extensively with PBS prior to using it for the anoikis assay.

To assay for anoikis, $5x10^5$ cells were seeded in the poly-HEMA coated tissue culture plates. Cells were then collected for cell cycle analysis to assay for the proportion of cells undergoing apoptosis. Collection and preparation of samples for cell cycle analysis was performed as of the procedure described in section 2.6 cell cycle analysis. The only exception in the procedure is that trypsin treatment was not used to harvest the cells as cells were already in suspension and could be collected by directly pipetting out of the wells. Both MMAN and MMRU cells were used for this experiment.

2.7. Attachment assay

MMAN and MMRU stable clones were seeded onto 96-well tissue culture plates and matrigel coated tissue culture plates at 7000 cells per well. At 30, 60, and 180 minutes, the wells were washed three times with 1x PBS to remove unattached cells. The cells were then detached and counted by the addition of 0.05% trypsin, neutralizing with DMEM + 10% FBS, and

counting using hemocytometer. At each of the time points, a parallel set of cells were fixed in 4% paraformaldehyde (PFA), and stained with 0.04% toluidine blue.

2.8. Matrigel attachment assay

Matrigel attachment assay was performed in a very similar way as the attachment assay for regular tissue culture plates. MMAN and MMRU stable clones were seeded onto 96-well tissue culture plates and matrigel coated tissue culture plates at 7000 cells per well. At 15, 30, and 60 minutes (for MMAN), and 30, 60 and 120 minutes (for MMRU), the wells were washed three times with 1x PBS to remove unattached cells. Different time points were chosen because of the difference in the ability of the cells to attach to matrigel. The cells were then fixed in 4% PFA, and stained with 0.04% toluidine blue. Photos of 4 random low-power fields are then taken for each of the quadruplicate wells for each condition. The number of cells in each picture are counted using Image J software, and averaged for each time point.

2.9. Migration assay

Scratch assay was used to measure the effects of CTHRC1 on melanoma migration (Liang, Park and Guan, 2007). This was done by seeding $5x10^5$ cells into 6-well plates, and then allowing time for the cells to attach and form a confluent monolayer. A scratch was then made with a P200 pipette tip that is hard enough to remove the attached cells, but not hard enough not to damage the plastic layer so that the cells can migrate and recover the gap. Pictures of the gaps created were then taken at 0 and 5 hours at the same marked areas. For each time point, 12 pictures were taken for each cell line. 10 random distances between each gap were then

measured in each photo using Image J software to generate an average gap distance value. The migratory capacity was then measured by observing the closing of the gap, and thus the decrease in distance between the gap. The 5 hour timepoint was chosen in order to lower the influence of cell proliferation to the closing of the gaps.

2.10. MTS assay

To detect viable cells within a cell sample, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was used. MTS (CellTiter 96® AQ_{ueous} One Cell Proliferation Assay, Promega) was prepared and added to the culture medium according to the manufacturer's protocol, and incubated for 2 hours with the melanoma cells. Reduction of MTS to formazan occurs when mitochondrial enzymes are active, and thus is an indication of viable cells in culture. The coloured formazan can then be measured at 490 nm by a spectrophotometer, with the measurement at 650 nm as a background control.

2.11. Immunofluorescence

To prepare coverslips for immunofluorescence staining, 200 μL of 0.01% poly-L-lysine (Sigma) was added to each coverslip. The coverslips were then left to coat with poly-L-lysine for 5-10 minutes, followed by extensive washing with dH_20. The coverslips were then air-dried in a dust free environment.

When the coverslips are ready, 1.0×10^5 cells were added to each slide and allowed to attach. The adherent cells were then fixed with ice-cold 4% PFA for 15 minutes at room

temperature. After fixation, the cells were permeated with 0.05% Triton-X 100 in PBS for 5 min, followed by blocking in 5% BSA + 0.01% Tween-20 in PBS for 20 min. F-actin staining was done by adding 5 μL of AlexaFluor 594 labelled phalloidin (Invitrogen) to every 200 μL of 2% BSA + 0.01% Tween-20 in PBS used to stain the slides. The coverslips were incubated with phalloidin for 45 minutes, and then washed extensively with PBS + 0.01% Tween-20. 4',6-diamidino-2-phenylindole (DAPI) was then added to the coverslips to stain DNA and incubated for 1 minute. After DAPI staining, the coverslips were washed with PBS + 0.01% Tween-20, and then mounted onto slides. The slides were viewed by Zeiss Axiovert 200M fluorescent microscope (courtesy of Dr. Christopher Shaw's Laboratory, UBC).

2.12. Chemosensitivity assay

MMAN and MMRU cells with high and low levels of CTHRC1 expression were tested for their sensitivity to temozolomide (TMZ), a conventional chemotherapeutic agent used to treat melanoma. The pairs of MMAN and MMRU cells were seeded in 96-well plates at 5000 cells/ well. After the cells have attached onto the tissue culture plates, 100, 500 and 1000 μM of TMZ were added to the wells. After 48 hours of treatment in TMZ, cell survival was assessed using MTS assay (as described in section 2.11). The level of survival of the treatment groups are expressed as percentages of the untreated cells (control).

2.13. Inhibition of exogenous CTHRC1

MMAN cells with and without CTHRC1 expression were blocked with CTHRC1 antibody and assayed for cell survival in serum free conditions. MMAN cells were seeded in a

96- well plate at 5000 cells/well. After the cells have attached onto the tissue culture plates, the cells were washed with PBS and replenished with serum free medium. CTHRC1 antibody was then added at 1 μg/mL and 10 μg/mL, and normal rabbit IgG at the same concentrations was added to a parallel set of cells as a control. Cell survival was measured using MTS assay at the start of the treatment, and at 48 hours of treatment. The level of survival was expressed as a percentage of the cell number at time 0.

2.14. Conditioned medium preparation

CTHRC1 containing conditioned media was prepared by growing the pair of MMAN cells with and without CTHRC1 in T-75 culture flasks to approximately 80% confluency. When the cells have reached the appropriate density, they were washed twice with 1x PBS. Fresh serum free media was then added to each of the flasks and incubated for 1 day.

Conditioned medium was collected by pipetting the medium out of the flask, followed by centrifugation at 1200 rpm for 5 minutes to spin down any detached cells. The conditioned medium was then concentrated by transferring it into the upper chamber of a spin column which has 20 kDa molecular cut off. The spin column was then centrifuged at 3500 rpm until there is approximately 1 mL volume left of the concentrated conditioned medium in the upper chamber. The concentrated conditioned medium, containing proteins with greater than 20 kDa, were then quantified using Bradford Assay.

3. Results

3.1. Confirmation of CTHRC1 expression in cell line model

Two model systems were developed to study the role of CTHRC1 in melanoma progression and metastasis. CTHRC1 expression was introduced into MMAN cells, which have no endogenous CTHRC1 expression, by transfecting it with the sense plasmid. On the other hand, CTHRC1 expression was knocked down in MMRU cells (with high endogenous CTHRC1 expression) by transfecting it with the antisense construct. The cells were transfected with an empty vector as a control. Multiple clones were isolated from the generation of stable MMAN and MMRU clones. Upon analysis of the CTHRC1 expression of these clones using Western blotting, the MMAN clone with the strongest CTHRC1 expression and the MMRU clone with the highest CTHRC1 knockdown were used for the following experiments (Figure 3-1).

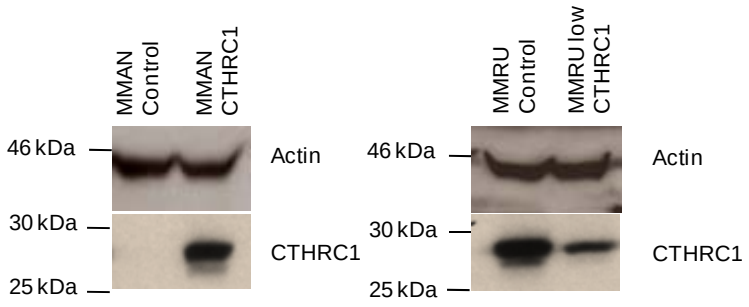

Figure 3-1. Regulating CTHRC1 expression in cultured melanoma cell lines.
Comparison of CTHRC1 expression levels of cell lines used in this study by Western blot.

3.2. CTHRC1 and melanoma cell migration

Previous studies by our group have shown that CTHRC1 expression is correlated with higher migration in KZ-28 cells using transient siRNA trasfection. This observation was also

confirmed by other groups. However, this phenotype has not been demonstrated in MMAN and MMRU cells with stable alteration of CTHRC1 expression. A scratch assay was then used to study the migration of MMAN and MMRU stable cells in vitro. The pro-migratory role was again demonstrated in MMAN and MMRU stable cells with differing CTHRC1 expression level. Cells which express higher levels of CTHRC1 among the pair migrate faster than its counterpart, confirming that CTHRC1 expression increases migration (Table 3- 1, Figure 3-2).

Table 3-1. Migration of cells expressing high and low levels of CTHRC1 as indicated by the percentage decrease in gap distance of scratch wound at 5 hours

Cell Line	Percentage Decrease in Gap Distance		% reduction with low CTHRC1 expression	p-value
	High CTHRC1	Low CTHRC1		
MMAN	5.41% ± 0.62%	1.86% ± 0.56%	66%	<0.05
MMRU	17.75% ± 1.64%	6.90% ± 1.22%	61%	<0.05

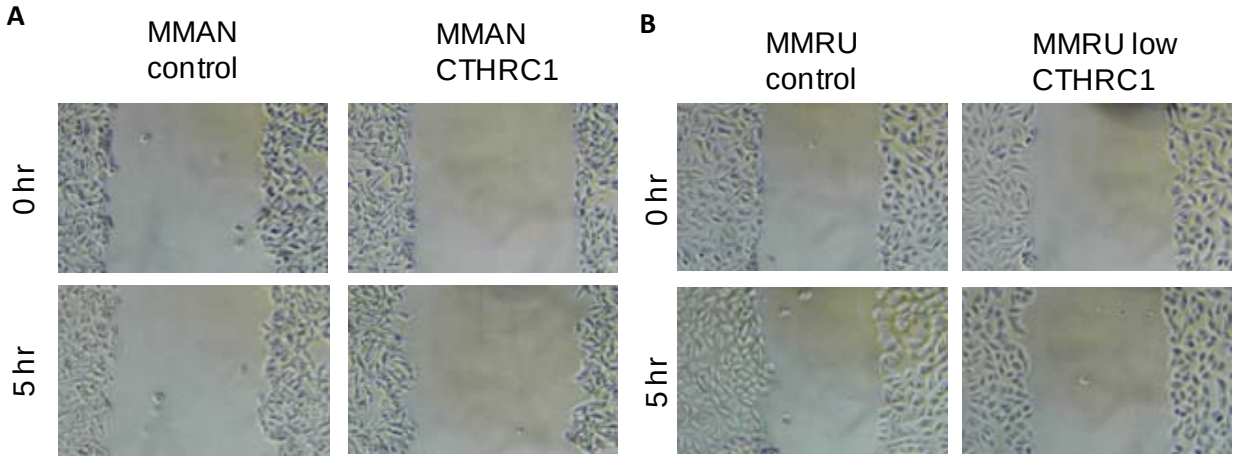

Figure 3-2. Effect of CTHRC1 on melanoma migration

Migration of MMAN (A) and MMRU (B) cells in the presence and absence of CTHRC1 as assessed by the scratch assay. (n=12 for each condition)

Migration of the cells was assessed at 5 hours in order to minimize the contribution of the cell proliferation to the closing of the gap. Although it is not clearly visible from the picture, quantitative measurements of the changes in gap distance indicated that MMAN cells with CTHRC1 expression migrated slightly faster than the control cells (without endogenous CTHRC1 expression). This effect was also demonstrated in MMRU cells. As seen from Figure 3-2, the MMRU cells at the edge migrated into the gap.

3.3. CTHRC1 and melanoma cell adhesion

3.3.1 Cell adhesion to regular tissue culture plates

An appropriate level of cell adhesion is required for cell migration. Since CTHRC1 appears to increase melanoma cell migration, it is probable that CTHRC1 may modulate melanoma cell adhesion. Pairs of stably transfected MMAN and MMRU cells were first seeded onto tissue culture plates. Then, the number of cells attached at regular time intervals was measured.

The MMAN cell line, which normally does not express CTHRC1, appears to have increased its cell adhesion with the introduction of CTHRC1. There were approximately 1.5 times more cells attached at 30 min, and at least 2 times more cells attached at 60 mins in the presence of CTHRC1. However, at 180 mins, almost all of the cells have attached, and there were no differences observed (Figure 3-3a,c). At 23 min, 50% of MMAN CTHRC1 cells have attached, while MMAN control cells required approximately 75 min to reach 50% saturation. This results in a difference of 52 min between the MMAN cells to reach 50% saturation.

The MMRU cell line displayed a very similar trend to the MMAN cells. At 30 and 60 minutes, there were approximately 2 times more control cells attached compared to the low

CTHRC1 expression clones. At 180 mins, there were no observable differences in the number of cells attached as all of the cells have attached by then (Figure 3-3b,d). At approximately 21 min, 50% of MMRU control cells have attached, while MMRU low CTHRC1 cells required approximately 75 min to reach 50% saturation. This results in a difference of 54 min between the MMRU cells to reach 50% saturation.

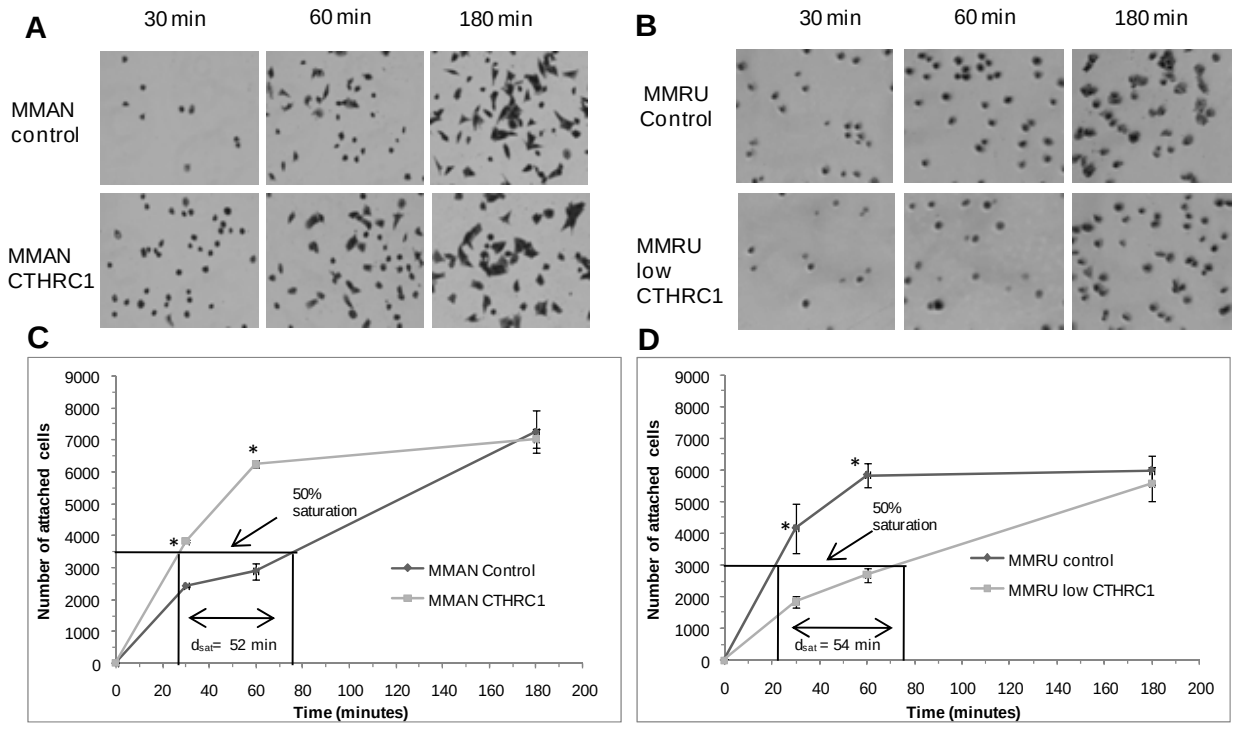

Figure 3-3. Effect of CTHRC1 on melanoma adhesion.

MMAN cells with and without CTHRC1 (A,C) and MMRU with high and low CTHRC1 (B,D) were seeded onto 96-well tissue culture plates. The cells were removed at 30, 60, and 180 minutes, and the wells were washed with 1x PBS. Cells attached to the wells were fixed with 4% paraformaldehyde and stained with 0.04% toluidine blue. The total numbers of cells in each well were counted. Values presented are the average number of cells attached ± SEM (n=3). * indcates $p<0.05$ as determined by the student's t-test.

Through this experiment, it was found that cells which have higher CTHRC1 expression among the pair appear to consistently have more cells attached at 30 and 60 min.

3.3.2 Cell adhesion to matrigel coated tissue culture plates

For melanoma cells to metastasize they must invade out of their microenvironment and interact with the extracellular matrix. To explore if CTHRC1 has a role in extracellular matrix interaction using *in vitro* methods, MMAN and MMRU stably transfected cells onto culture

plates coated with matrigel, which is a biologically active matrix that resembles the human cellular basement membrane. We then measured the number of attached cells at regular time-points.

MMAN cells appear to attach very quickly to matrigel, as almost all of the cells have attached by 60 minutes. However, at earlier time-points such as 15 and 30 min, we observed that there were about a third fewer MMAN control cells attached compared to the CTHRC1 expressing MMAN (Figure 3-4a,c). The difference in achieving 50% adhesion saturation is 4.5 min between the pair of MMAN cells with and without CTHRC1 expression. MMRU cells on the other hand, did not demonstrate a difference in matrigel attachment at the earlier time points. However, when almost all of the MMRU control cells have attached at 120 min, the amount of low-CTHRC1 expressing MMRU attached remained the same level as 60 min, and was approximately 3 times less than the control (Figure 3-4b,d). At around 62 min, 50% of the MMRU control cells attached to matrigel; however, the MMRU low CTHRC1 expressing cells did not reach 50% adhesion saturation at the time points tested. A parallel set of cells were seeded in regular tissue culture plates as an experimental control, and all of the MMRU control and MMRU low CTHRC1 expressing cells were attached by 120 min. In this experiment, CTHRC1 expression appears to increase cell adhesion to matrigel.

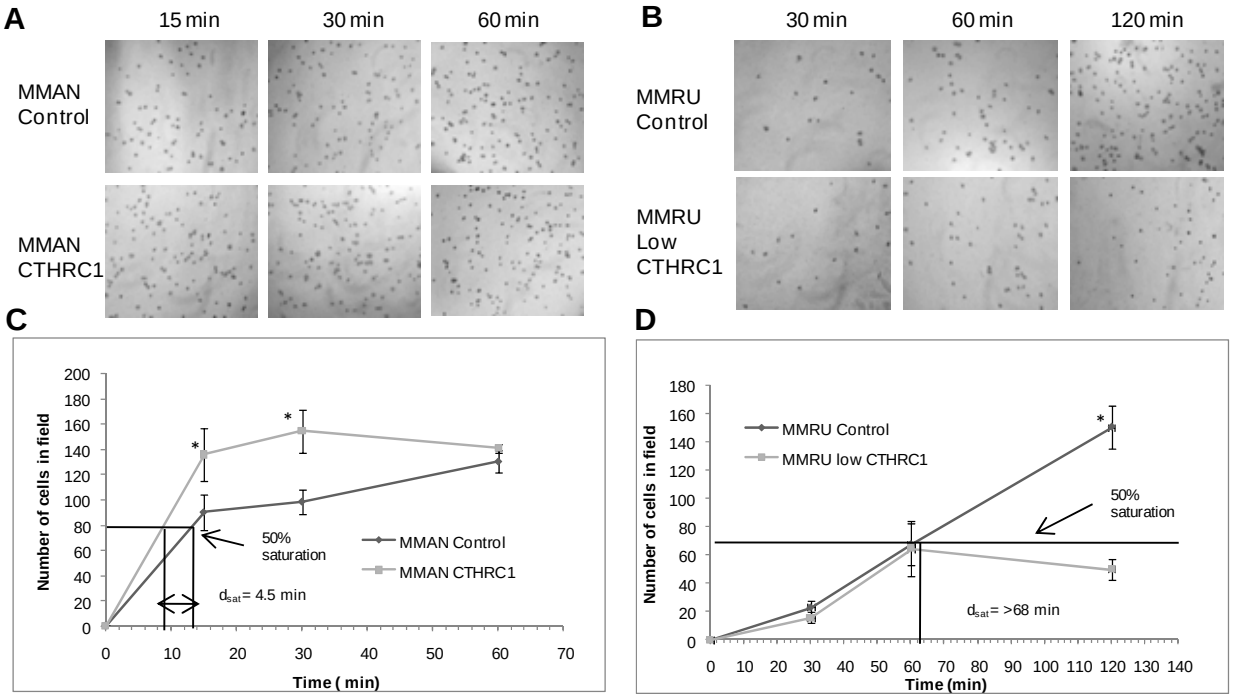

Figure 3-4. Effect of CTHRC1 on adhesion to matigel coated tissue culture plates.

MMAN cells with and without CTHRC1 (A,C) and MMRU with high and low CTHRC1 (B,D) were seeded onto 96-well matrigel coated tissue culture plates. The unattached cells were removed at 15, 30, and 60 minutes for MMAN cells, and at 30, 60, and 120 minutes for MMRU cells. The wells were washed with 1x PBS, and the attached cells were fixed with 4% paraformaldehyde and stained with 0.04% toluidine blue. The total numbers of cells in each well were counted based on photos taken from random fields of 4 replicates. Values presented here indicate the average number of cells per field ± SEM (n=4).

3.4. CTHRC1 and the survival of melanoma cells in monolayer culture

Preliminary studies have revealed that CTHRC1 has a pro-survival role in melanoma cells that are undergoing environmental stress. Since adhesion to physical growth support can deliver pro-survival signals to the cell, we investigate if a relationship exists between cell adhesion due to CTHRC1 and melanoma cells' survival. We compare the proportion of apoptotic cells in adhered and detached cells under serum deprived conditions.

In the first condition, when adhered melanoma cells were cultured as a monolayer in serum free media, cells which express CTHRC1 survived significantly better than cells with low CTHRC1. In the case of MMAN cells, the pro-survival effect of CTHRC1 can be observed starting from 1 day after the treatment. MMAN control cells (no CTHRC1 expression) started to decrease in cell number, while CTHRC1 expressing MMAN cells were able to proliferate to 1.2 times of the starting cell number. By the second day after the treatment, control cells were only at 40% of the cell number compared to day 1, while CTHRC1 expressing cells were at 80%. By day 4, almost all of the control MMAN cells were dead while more than 35% of the CTHRC1 expressing cells were still alive and attached to the tissue culture plate (Figure 3-5 a,c). However, the difference in apoptosis was more pronounced at 2 days post-treatment, with control cells at 60% apoptotic and CTHRC1 expressing cells at 40% apoptotic (Figure 3-6a).

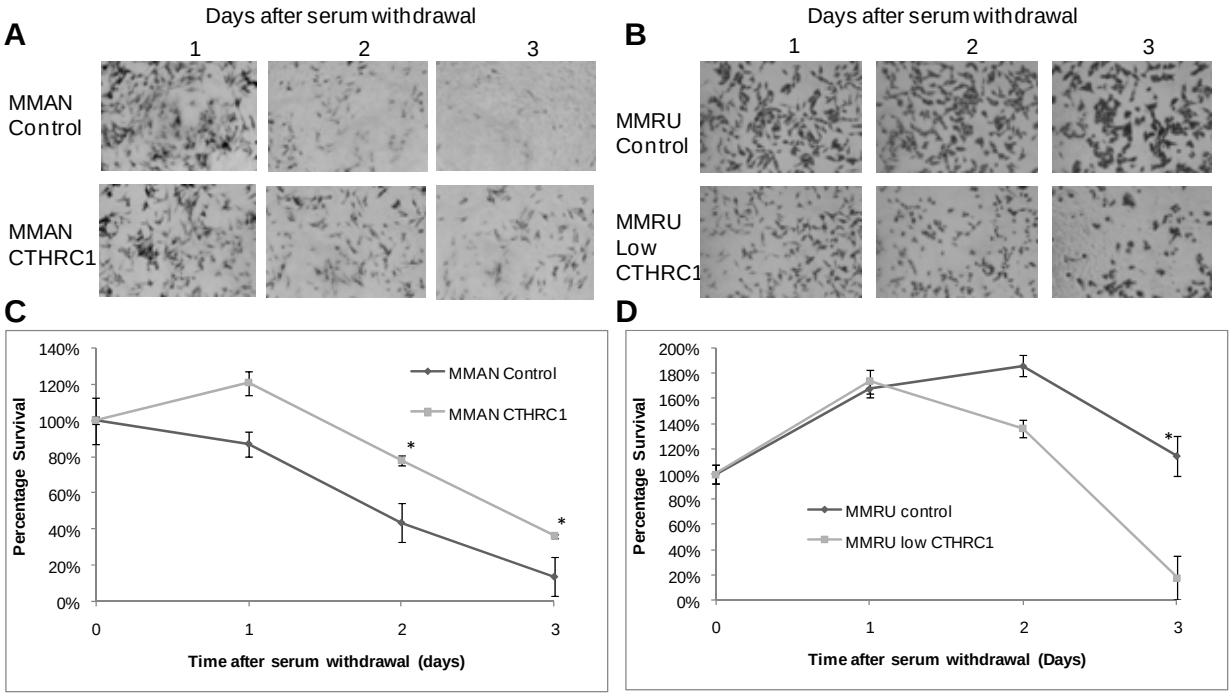

Figure 3-5. Effect of CTHRC1 on mealnoma survival in monolayer culture.

Survival of melanoma cells under serum deprivation. The pairs of MMAN and MMRU cells were cultured in serum free DMEM. Cells were fixed with 4% paraformaldehyde and stained with 0.04% toluidine blue (A,C). Cell numbers were obtained by trypan blue exclusion assay, and averaged over the number of replicates (n=3) (B, D). Values presented are the percentage of cell number compared the start of the experiment ± SEM. * indicates $p<0.05$ as determined by the student's t-test.

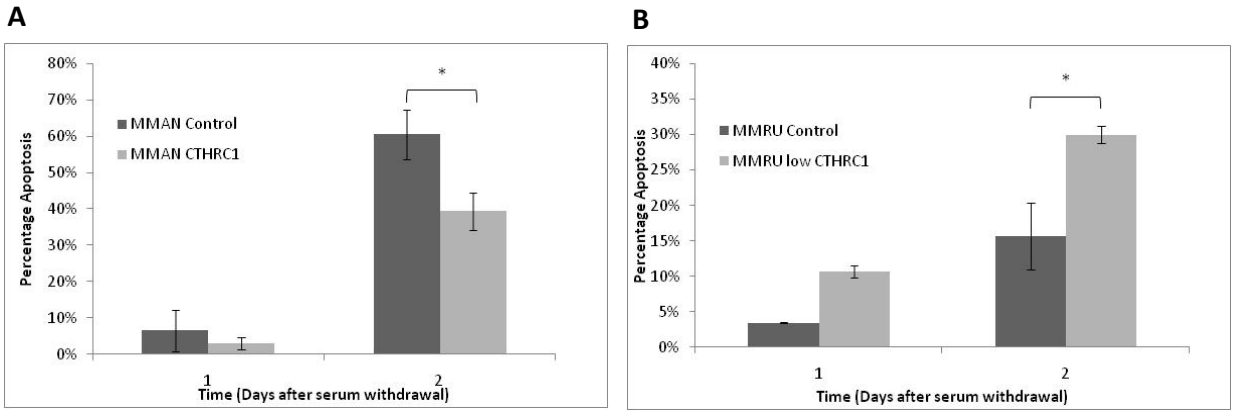

Figure 3-6. Effect of CTHRC1 on apoptosis of attached melanoma cells.

Cell cycle analysis of MMAN (A) and MMRU (B) cells under serum deprivation conditions as stained by propidium iodide and analyzed by flow cytometry. The percentage of cells in the sub-G1 population are calculated and averaged. Values presented are the average cell percentage ± SEM (n=3). * indicates $p<0.05$ as determined by the student's t-test.

The pro-survival effect of CTHRC1 was also evident in stably transfected MMRU cells. MMRU cells are able to survive well in serum free conditions for the first 2 days. At the second day after treatment, MMRU cells with low CTHRC1 expression decreased in cell number. The greatest difference was observed after 3 days, where MMRU control cells still had approximately 110% the starting number of cells but MMRU low CTHRC1 expressing cells had less than 25% surviving cells (Figure 3-5 b,d). Cell cycle analysis revealed that the cellular differences are correlated with changes in apoptotic rate when CTHRC1 level is altered. MMRU cells with low CTHRC1 expression had at least twice the amount of cells undergoing apoptosis when compared to their control counterpart, with the difference in apoptosis showing on the first day after serum withdrawal. MMRU control cells were only about 4% apoptotic, while MMRU low CTHRC1 expressing cells were approximately 10% apoptotic after 1 day of serum starvation. By 2 days

after treatment, MMRU control cells were 15% apoptotic, while MMRU CTHRC1 knockdowns were already at 30% (Figure 3-6b).

Cell fate is determined by the balance of survival and apoptosis signals within the cell. MMAN and MMRU cells were able to slightly proliferate for the first day after serum withdrawal is likely because there were more survival signaling proteins expressed than the apoptosis signaling proteins. This could be due to survival signaling remnant from the growth factors in the serum and also from CTHRC1. However, as cells were starved for a longer period of time, cell death can be observed because of the decrease in survival signal and the increase in apoptotic signal.

3.5. CTHRC1 and the survival of melanoma cells in suspension

Since adhesion to growth support surface can deliver pro-survival signals to the cells, we sought to determine if the CTHRC1 associated survival advantage was due to increased adhesion. Then, we assayed for the survival of unattached melanoma cells in the presence or absence of CTHRC1. MMAN and MMRU cells were seeded in tissue culture plates coated with poly-HEMA to prevent the cells from attaching. Cells were then collected at 1 and 2 days after the start of the treatment and analyzed for the percentage of apoptotic population in the sample by cell cycle analysis to assess cellular apoptosis. There appears to be no observable difference in anoikis according to the experimental results for both of the cell lines tested (Figure 3-7a,b).

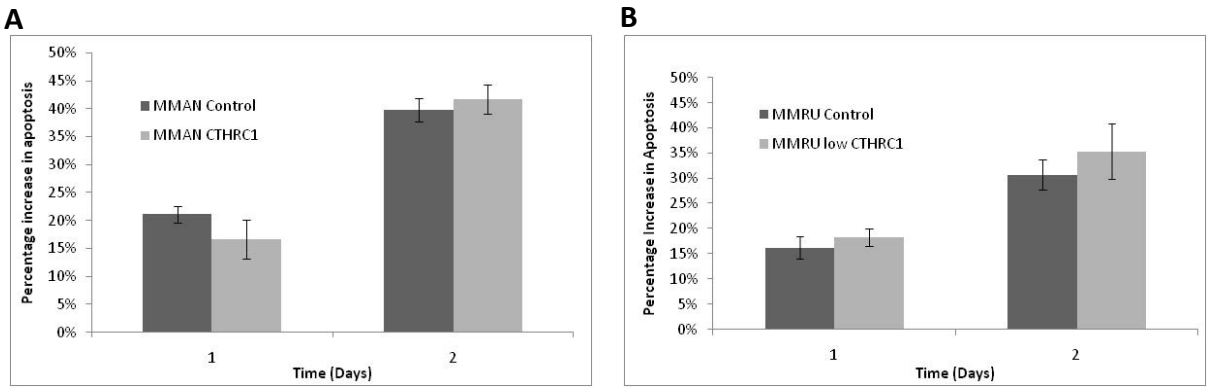

Figure 3-7. Effect of CTHRC1 on apoptosis of melanoma cells in suspension.

MMAN and MMRU stably transfected cells were grown in suspension by coating the culture plates with poly-HEMA. Cell cycle analysis was performed on these cells by propodium iodide staining followed by analysis by flow cytometry. CTHRC1 expression does not affect the apoptosis of the pairs of MMAN (A) and MMRU (B) stably transfected cells when grown in suspension. Values presented are the average cell percentage ± SEM (n=3).

3.6. Wnt signaling pathway in melanoma

A recent article reported that CTHRC1 selectively activates the planar cell polarity pathway of Wnt signaling by stabilizing the Wnt ligand-receptor complex HEK293T cells (Yamamoto et al, 2008). To examine if CTHRC1 functions in the same way in melanoma, we performed immunofluorescence staining on actin. The Wnt/PCP pathway activates RhoA and Rac and is able to exert various effects on cell morphology, as well be discussed later.

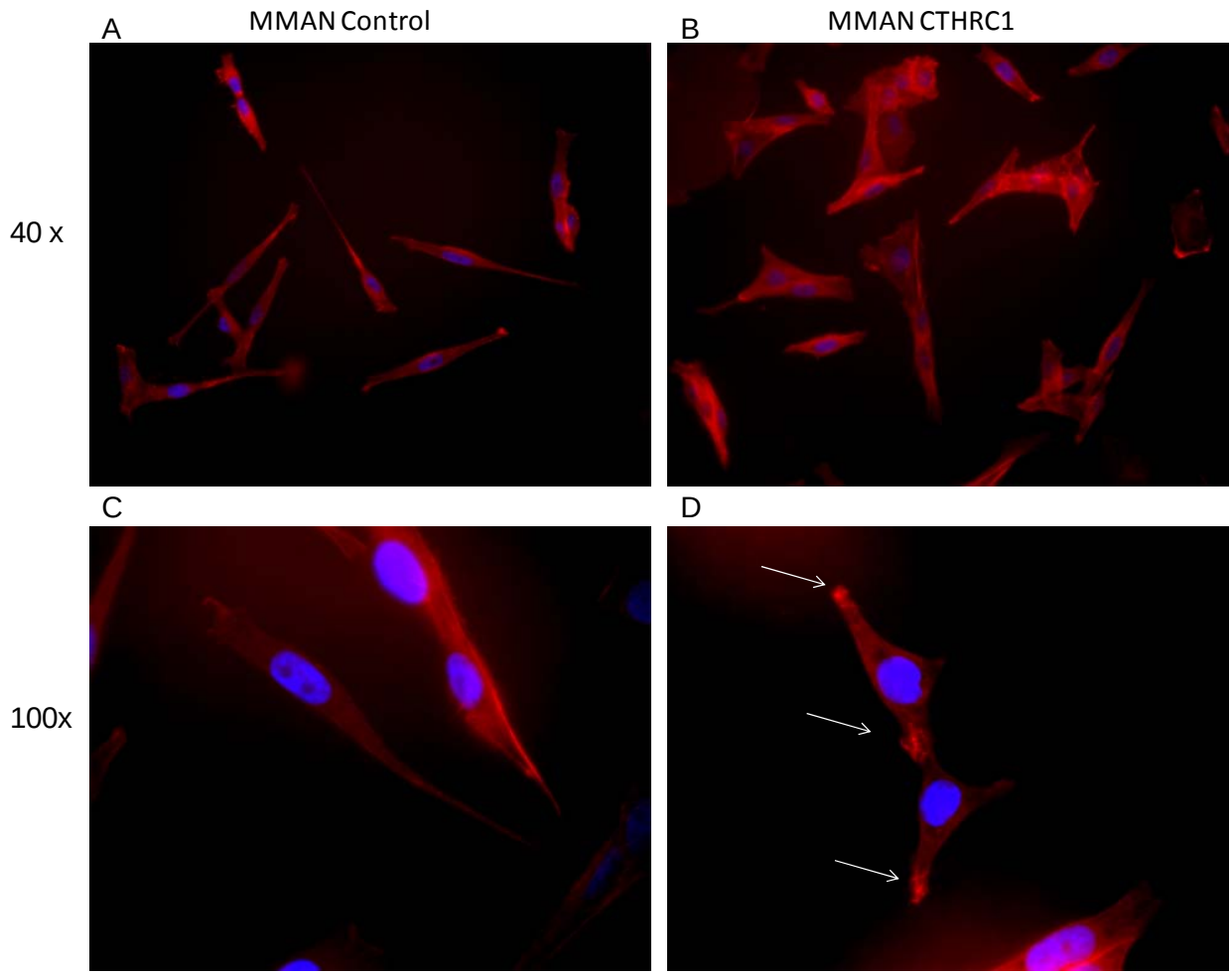

Figure 3-8. Effects of CTHRC1 on actin organization in MMAN cells.

MMAN cells without CTHRC1 expression at 40x (A) and 100x (C) magnification and MMAN cells with high CTHRC1 expression at 40x (B) and 100x (D) magnification were seeded on poly-L-lysine coated coverslips, allowed to attach, fixed with 4% PFA, and incubated with AlexaFluor 594 labelled phalloidin. Cell morphology was clearly different between the pair of MMAN stable clones. MMAN control cells were more elongated and contracted, while MMAN CTHRC1 cells were more spread out. Arrows show areas of dense actin structure resembling filopodia.

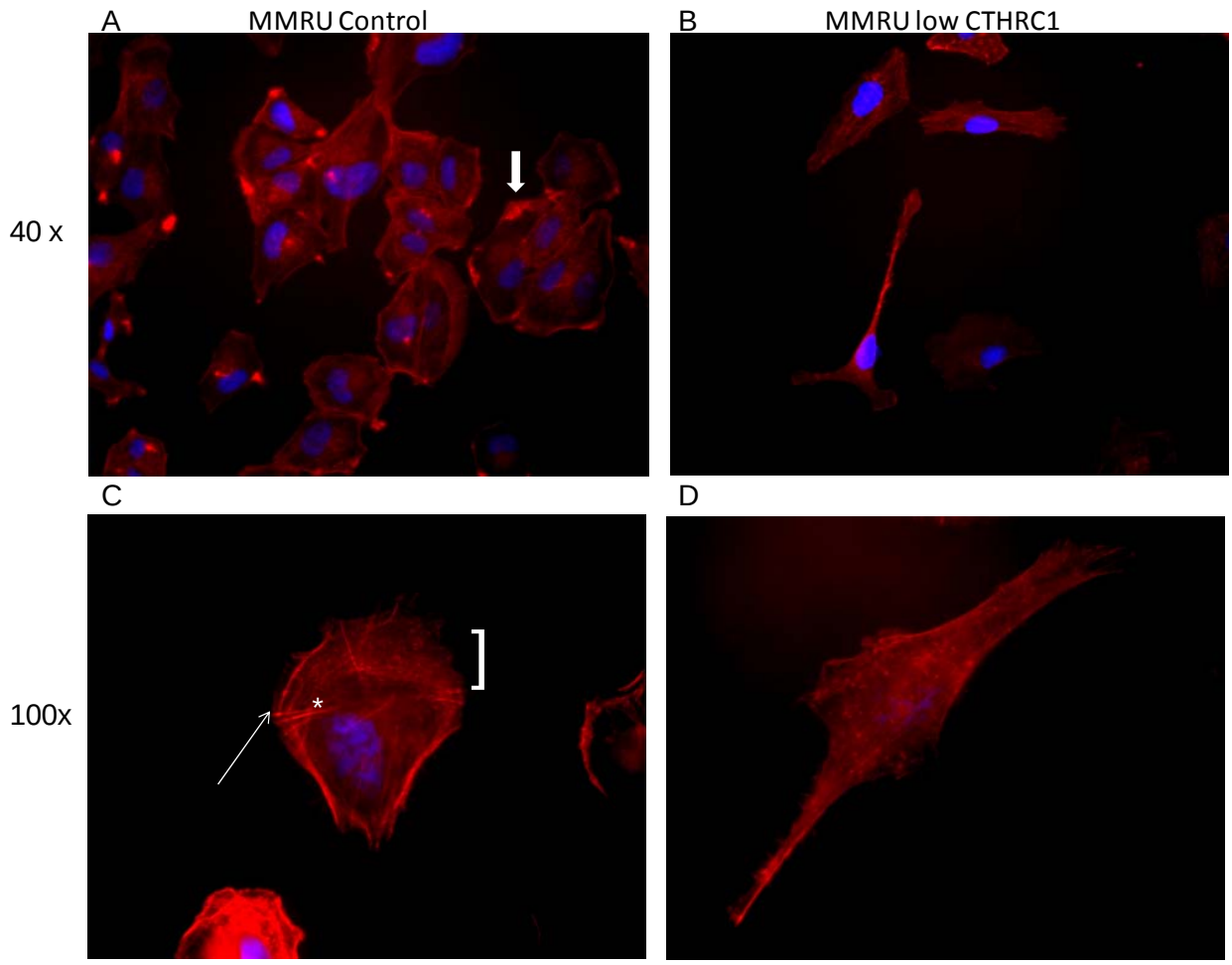

Figure 3-9. Effects of CTHRC1 on actin organization in MMRU cells.

MMRU cells with high CTHRC1 expression at 40x (A) and 100x (C) magnification and low CTHRC1 expression at 40x (B) and 100x (D) magnification were seeded on poly-L-lysine coated coverslips, allowed to attach, fixed with 4% PFA, and incubated with AlexaFluor 594 labelled phalloidin. CTHRC1 expression appears to have altered the cell morphology of MMRU cells. Structures shown: (*) network of short actin stress fibers (arrow) filopodia/ microspikes (square bracket) lamellipodium and supporting actin meswork (block arrow) membrane ruffling.

CTHRC1 appears to affect the morphology and actin organization in melanoma cells (Figure 3-8 and Figure 3-9). In both cell lines tested, the clones with low CTHRC1 expression were more contracted and elongated in shape. MMAN cells with CTHRC1 expression appear to be more spread out and more polygonal or stellate in shape. In addition, there appears to be

dense actin structures at the tips of the cells which resemble filopodia (Figure 3-8). These morphological differences were even more evident in MMRU cells. MMRU cells transfected with the empty vector are enlarged, more spread out, and have shorter actin stress fibers compared to the MMRU CTHRC1 knockdowns. These cells have areas of dense actin resembling that of lamellipodia (protrusions that are supported by a meshwork of actin filaments), membrane ruffling (folding of the lamellipodia), and filopodia (microspike protrusions that extend beyond the lamellipodium), features that are less evident in the low CTHRC1 counterpart. There are clear differences in the morphology and actin organization between the pairs of MMAN and MMRU cells tested, and they could be mediated by changes in Wnt/PCP signaling. The potential mechanisms by which they are modulated will be further discussed (see below).

The study by Yamamoto's group reported that CTHRC1 activation of Wnt/PCP pathway does not affect Wnt/β-catenin pathway signaling. Here, we examined the relationship between CTHRC1 expression and β-catenin stabilization, which is a key event downstream of Wnt/ β-catenin signaling (details described in introduction section). Briefly, upon canonical Wnt activation, β –catenin is released from the axin/APC complex, and is no longer targeted for degradation. Therefore, if CTHRC1 is involved with canonical Wnt signaling, we would observe an accumulation of β –catenin.

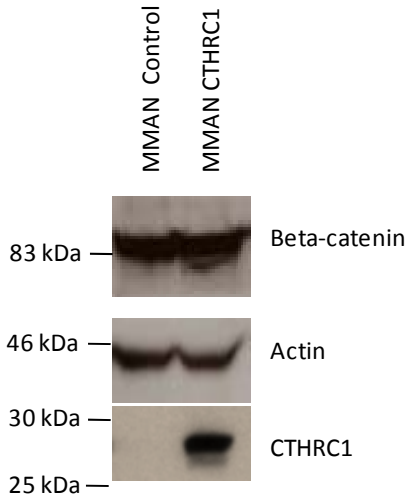

Figure 3-10. Comparison of beta-catenin expression in MMAN control and MMAN CTHRC1 expressing cells.

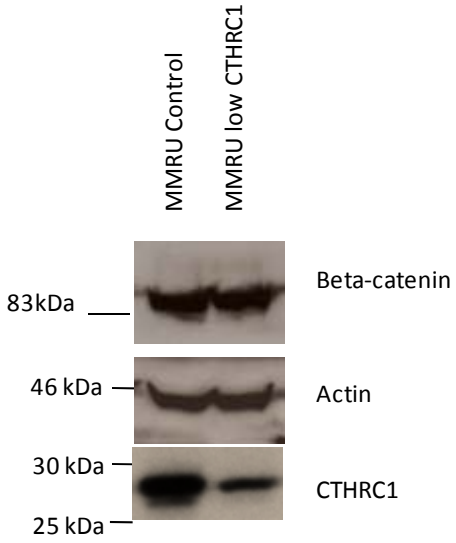

Figure 3-11. Comparison of beta-catenin expression in MMRU control and MMRU low CTHRC1 expressing cells.

According to the western blots in Figure 3-10 and Figure 3-11, CTHRC1 expression did not affect β-catenin levels in both MMAN and MMRU cells. This suggests that CTHRC1 did not have any effects on the canonical pathway.

4. Therapeutic implications of CTHRC1 modulation

The following few sections describe some of the preliminary investigations towards developing CTHRC1 inhibition as a therapy for melanoma.

4.1. CTHRC1 and melanoma sensitivity to temozolomide (TMZ)

The biochemical properties and biological functions CTHRC1 is associated with make it an interesting potential therapeutic target. It appears to play an important role in the melanoma metastasis process by influencing cell migration, survival and adhesion. Being an extracellular protein, it is easier to design therapeutics against because the drug candidate would not have to be delivered to inside the cell.

As mentioned in the introduction, melanoma cells are very resistant to chemotherapy and other conventional therapeutic treatments. Since silencing of CTHRC1 expression decreases melanoma survival, we hypothesize that inhibition of CTHRC1 will sensitize the melanoma cells' response to the chemotherapeutic agent temozolomide (TMZ). TMZ is the derivative of dacarbazine (DTIC), one of the few treatments to have some response in melanomas. Under physiological conditions, TMZ can spontaneously convert to the active metabolite 5-(3-methyl-1-triazeno)-imidazole-4-carboxamide (MTIC) (Agarwala and Kirkwood, 2000). MTIC then exerts cytotoxic effects to induce cell death by alkylating DNA, thereby inhibiting DNA synthesis and creating DNA strand breaks (Lunn and Harris, 1988, Maynard and Parsons, 1986). Based on CTHRC1's pro-survival effect, we hypothesize that CTHRC1 inhibition may act synergistically with TMZ.

The pairs of MMAN and MMRU cells with low and high CTHRC1 expression were treated with TMZ at concentrations of 100, 500, and 1000 μM. The survival of the cells was

assessed after 2 days with MTS assay. We expect cells with high CTHRC1 expression to survive better than their low-CTHRC1 expressing counterparts.

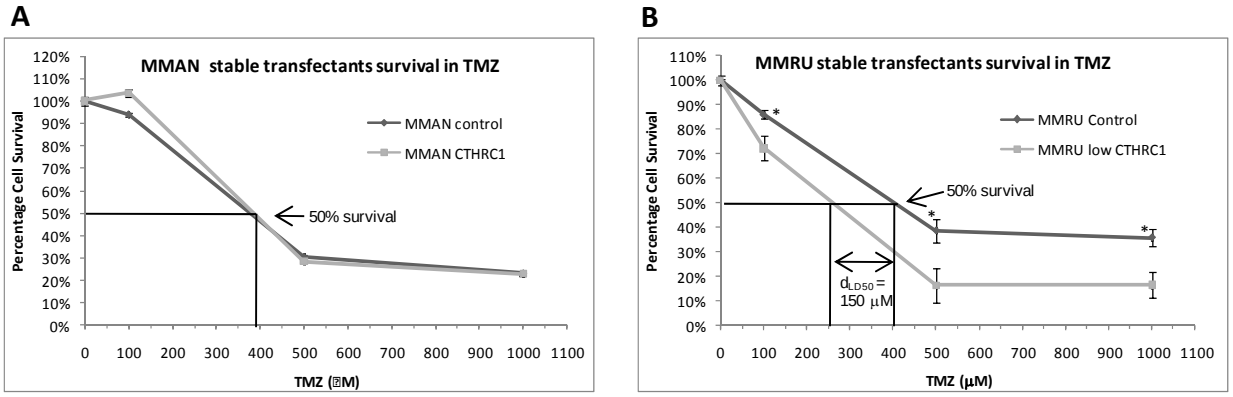

Figure 4-1. Effect of CTHRC1 on treatment of melanoma cells with temozolomide (TMZ)

Melanoma cells MMAN (A) and MMRU (B) were treated with 100, 500 and 1000 uM of temozolomide. Viability of the cells were measured by the MTS assay after the incubation period of 2 days. Results are expressed as the percentage of cells surviving relative to the untreated control cells. Values presented are the average ± SEM (n=3). * indicates $p<0.05$ as determined by the student's t-test.

There seems to be some effect of CTHRC1 expression on the susceptibility of MMRU melanoma cells to cell death induced by TMZ. The combination treatment of TMZ with CTHRC1 inhibition resulted in poorer cell survival in MMRU cells at all conditions tested (Figure 4-1). There was a significantly higher degree of cell death in MMRU cells with low CTHRC1 expression compared to the control (Figure 4-1b). At all of the concentrations of TMZ tested, there were around 20% less survival in cells with low CTHRC1 expression. The LD_{50} of the TMZ in MMRU control was around 400 μM, while the MMRU low CTHRC1 was decreased by about 150 μM to approximately 250 μM. This suggests that inhibition of CTHRC1 decreases the concentration of TMZ required to reach LD_{50}. The difference in MMAN cells; however, was not as apparent as in MMRU cells. The LD_{50} of TMZ in MMAN CTHRC1 was very similar to

the low CTHRC1 expressing control. At 100 μM of TMZ there were slightly more surviving cells in MMAN cells expressing CTHRC1 (Figure 4-1a). As the TMZ concentration in the treatment increased, both control and CTHRC1 expressing cells were dying, and did not demonstrate a difference in survival. Therefore, the difference in response observed here could be due to the fact that MMAN cells are more sensitive to high TMZ concentrations than MMRU cells.

4.2. Effects of CTHRC1 inhibitors and exogenous CTHRC1 on melanoma

4.2.1. Inhibition of extracellular CTHRC1 using CTHRC1 antibody

Since CTHRC1 is a protein that is both secreted and membrane anchored, its main biological function may be in the extracellular region. Inhibition of extracellular CTHRC1 may be able to inhibit its biological function. To test if extracellular CTHRC1 is involved with its mechanism of action, CTHRC1 antibody was added to the pair of MMAN cells with and without CTHRC1 expression to block extracellular CTHRC1. Antibody was added to the cells at concentrations of 1 μg/mL and 10 μg/mL. Normal rabbit IgG at the same concentrations were added as controls.

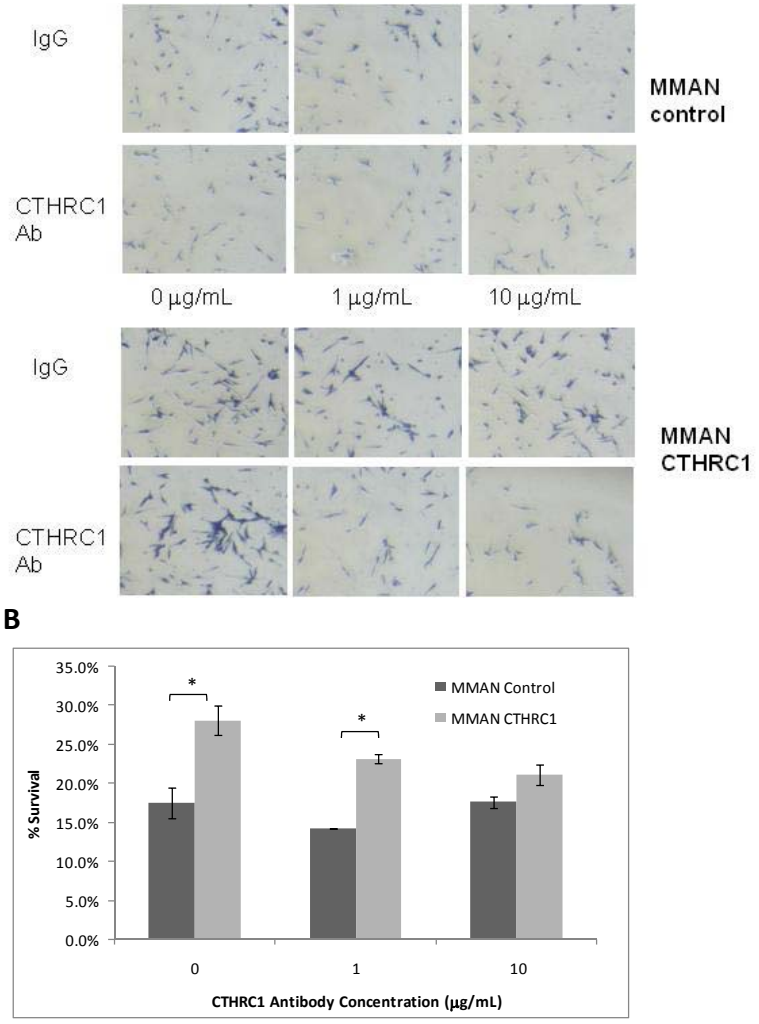

Figure 4-2. Effect of CTHRC1 antibody on the survival of MMAN cells with and without CTHRC1.

MMAN cells with and without CTHRC1 expression were treated with CTHRC1 antibody and normal rabbit IgG under serum free conditions. Cell survival was assessed after 2 days of treatment. (A) Toluidine blue staining of cells after 2 days of treatment. (B) Percentage survival of cells after 2 days of treatment in CTHRC1 antibody compared to day1. Values presented are the average percentage cell survival after 2 days of treatment ± SEM (n=3). * indicates $p<0.05$ as determined by the student's t-test.

The results from this experiment show that there is a dosage dependent decrease of cell survival in the MMAN cells tested. CTHRC1 antibody at concentration of 1 µg/mL was able to

significantly decrease the survival of MMAN cells; however, control normal rabbit IgG did not have any effects on the survival of melanoma cells at any of the concentrations tested (Figure 4-2). In addition, both normal rabbit IgG and CTHRC1 antibody did not have any effects on cell survival on MMAN control cells that do not express CTHRC1. Based on these results, it appears that CTHRC1 plays a functional role in the extracellular region, thus leading to the increased survival of melanoma cells.

Although this experiment suggests that CTHRC1 plays a role in the extracellular region, it does not generate solid proof for it. The CTHRC1 antibody used in the experiment is a rabbit polyclonal antibody, and may have non-specific effects. Therefore, other methods must be sought to inhibit extracellular CTHRC1 function.

4.2.2 Addition of CTHRC1 conditioned media to cells without endogenous CTHRC1

In this experiment, CTHRC1 was supplemented to cells without endogenous expression. Conditioned media from control and CTHRC1 expressing MMAN cells were collected and concentrated to approximately 1 mg/mL using Amicon's concentrator column, which separates proteins based on molecular weight. The concentrator column consists of an upper and lower chamber, separated by a filter of 20 kDa. When the sample is applied to the top chamber and subjected to centrifugation, proteins less than 20 kDa will pass through the filter, leaving the proteins greater than 20 kDa in the upper chamber. The sample remaining in the upper chamber was then collected after concentration, and quantified using Bradford assay. The presence of CTHRC1 in the conditioned medium was then verified by Western blotting (Figure 4-3).

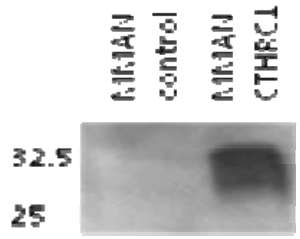

Figure 4-3. CTHRC1 conditioned medium.

The conditioned media was then sterilized it using a 0.2 uM filter syringe. Several concentrations ranging from 1 μg/mL to 100 μg/mL of the CTHRC1 conditioned medium was added to MMAN cells and normal melanocytes, both without endogenous CTHRC1 expression. The same concentrations of conditioned media from MMAN control cells (no CTHRC1 expression) were added as a negative control. The cells were incubated with the conditioned media for over a period of 2 days, and assayed for differences in cell survival. The viability of the cells was measured 2 days post-treatment by MTS assay. There appears to be no significant differences in viability between cells treated with CTHRC1 containing or control conditioned media (data not shown).

5. Discussion

In this study, CTHRC1 was found to increase cell migration, adhesion and survival under serum deprivation, which are important contributing factors in melanoma progression. CTHRC1 also appears to activate the Wnt/PCP pathway as demonstrated by the cytoskeletal changes that resemble changes in the Rho family of proteins, which act downstream of Wnt/PCP pathway signaling. This is consistent with the idea that in melanoma cells, CTHRC1 also functions as a cofactor protein for the Wnt/PCP pathway, as previously described by Yamamoto et al (Yamamoto et al, 2008). The following sections will discuss in detail how CTHRC1 may bring about the phenotypic changes observed in this study, and how these phenotypes are important to melanoma metastases.

5.1. CTHRC1's impact on cytoskeleton organization and cell adhesion

CTHRC1 expression affects various changes in the actin cytoskeleton, such as cell spreading, lamellipodia formation, filopodia formation, and actin reorganization. Such changes are the likely causes for the changes in cell adhesion, motility, and survival. The results in this study suggest that CTHRC1 expression likely leads to these phenotypic changes by activating the Rho family of GTPases, as these G-proteins are important contributors to cell motility and adhesion through the regulation of cytoskeletal rearrangements (Kolyada, Riley and Herman, 2003). Moreover, the Rho GTPases function downstream of Wnt/PCP signaling pathway, with which CTHRC1 has been shown to be associated as a cofactor protein.

Key Rho family members that could be responsible for modulating these phenotypes include RhoA, Cdc42 and Rac (Kolyada, Riley and Herman, 2003, Delaguillaumie et al, 2002).

RhoA and its downstream effector Rho kinase (ROCK) controls the formation of actin stress fibers and the attachment of actin bundles and myosin to the cell membrane at focal adhesion points, where integrin co-localizations are observed (Campos et al, 2009, Leblanc, Tocque and Delumeau, 1998, Nobes and Hall, 1995, Ridley and Hall, 1992). Cells with RhoA expression are contracted in shape, and have a dense array of actin stress fibers throughout the cytoplasm. Cdc42 is associated with the formation of filopodia and microspikes, and Cdc42 expressing cells are enlarged, have less actin stress fibers, and an increase in lamella and filopodia (Kozma et al, 1995, Olivo et al, 2000). Rac is associated with the formation of membrane ruffles and lamellipodia (Delaguillaumie et al, 2002, Ridley et al, 1992). Cells with Rac activation are polymorphic in shape with edges that are rich in membrane ruffles, and a decrease in stress fibers. Based on the observations, it appears that CTHRC1 may affect the expression of Cdc42, Rac1 and RhoA, as the phenotypes with CTHRC1 expression induced changes that are characteristic of the three proteins. The fact that these cells form lamellipodia and filodpida, and have shorter and less actin stress fibers throughout the cell body may imply that CTHRC1 expression may increase Rac and Cdc42, and decrease RhoA. Although there is strong evidence that the Rho family of proteins are involved in CTHRC1's function, further experiments must be done to precisely pinpoint how the proteins in the subfamily are involved.

Wnt/PCP may not be the only signaling pathway regulating Rho proteins, as Rho subfamily protein activation can also be regulated by other signaling pathways such as Wnt/ Ca^{2+} , Ras, receptor tyrosine kinase (RTK), integrin-linked kinae (ILK) and integrins (Delaguillaumie et al, 2002, Leblanc, Tocque and Delumeau, 1998, Wong et al, 2007, Yamada and Miyamoto, 1995). Of the above list of pathways that are able to modulate Rho proteins' expression, Wnt/ Ca^{2+} pathway is also likely to be involved since this pathway also involves Wnt5a and Wnt3a,

ligands that CTHRC1 binds to for Wnt/PCP pathway activation. This pathway is able to modulate Cdc42 activation through PKC phosphorylation. Since there are many cross-talks between signaling pathways, the involvement of the other pathways in mediating the observed results cannot be ruled out. Further work must be done to clarify exactly how CTHRC1 modulates the actin cytoskeleton.

5.2. CTHRC1's impact on cell migration

Migration, an important process involved in metastasis, depends on the physical interaction of the cell with its environment. It requires the dynamic regulation of actin to coordinate a series of events including the extending a protrusion, formation of new adhesions to the substratum, contraction, forward translocation of the cell body, de-adhesion and retraction at the rear of the cell (Gaggioli and Sahai, 2007, Ammer and Weed, 2008, Ridley, 2006). During migration, actin polymerization occurs at the migrating front of the cell, forming focal complexes containing lamellipodia and filopodia, thus allowing the cell to adhere and grip to the supporting substrate. Lamellipodia are protrusions at the motile edge of the cell. Within this structure, actin reorganizes to form microspikes and filopodia which extend beyond the cell edge. The combination of lamellipodia, filopodia, stress fiber and actomyosin regulation together facilitate the cell to move along the underlying support matrix (Gimona, 2008). CTHRC1 likely facilitates the migration process by enhancing the formation of protrusions and new adhesions to the substratum by affecting lamellipodia and filopodia formation.

Rho, Rac and Cdc42 contribute to the formation of focal complexes for migration by the formation of actomyosin contractile network/ actin stress fibers, lamellipodia and filopodia, respectively (Ammer and Weed, 2008). CTHRC1 perhaps facilitates migration by regulating the

expression of Rho, Rac and Cdc42 through the Wnt/PCP pathway, thus controlling the formation of structures important for cell migration. Cdc42 and Rac have been demonstrated to be important in formation of protrusions, while integrins are important in the formation of new adhesions, and RhoA/ROCK are important for cell contraction and translocation of the cell body through stress fiber and actomyosin formation (Gaggioli and Sahai, 2007). However, how CTHRC1 affects each of these protein expressions requires further elucidation.

As mentioned in the previous section, the Wnt/PCP pathway may not be the only pathway involved in CTHRC1's function. Wnt/Ca^{2+} non-canonical pathway may also have an important role in this process. Wnt/Ca^{2+} signaling activation by CTHRC1 may activate downstream proteins such as PKC, which has been previously demonstrated to have a role in cytoskeleton regulation. PKC can activate Cdc42, and is able to induce cells to increase dismantling of actin stress fibers, and increase in formation of filopodia and microspikes (Larsson, 2006, Vuori and Ruoslahti, 1993, Ridley and Hall, 1994, Brandt et al, 2002).

5.3. CTHRC1's effects on cell survival

CTHRC1 expression protected the melanoma cells from serum deprivation induced apoptosis in this study. Such pro-survival effect may be mediated by cell adhesion, as CTHRC1 fails to protect melanoma cells in suspension from anoikis, a special form of apoptosis in anchorage-dependent cells that is induced by detachment. During serum deprivation, actin depolymerises, leading to the loss of adhesion (Ruggieri, Chuang and Symons, 2001). CTHRC1 expressing cells, which have increased cell adhesion possibly due to activation of the non-canonical Wnt pathways, are able to better attach to the growth support surface and prevent apoptosis induced by the loss of attachment. In addition, the cell survival advantage may also

be mediated through other proteins and cell survival signaling such as JNK (Jun Kinase), which also acts downstream of the Wnt/PCP pathway (Dhanasekaran and Reddy, 2008, Ruggieri, Chuang and Symons, 2001, Minden and Karin, 1997); PI3K, which is involved with Rac1 activation (Ruggieri, Chuang and Symons, 2001); and integrin signaling. However, the possibility that CTHRC1's pro-survival effect is mediated through the latter mechanisms is less likely. This is because CTHRC1 failed to protect the cells from anoikis, suggesting its dependence on cell adhesion and not on cell survival signaling.

5.4. CTHRC1 and cell adhesion to extracellular matrix proteins

The formation of cell-ECM interactions was also shown to be an important process in invasion and metastasis (Yamada and Miyamoto, 1995, Schwartz and Horwitz, 2006, Dunehoo et al, 2006). During tumour progression and metastasis, the adhesion properties of the cancer cells undergo significant changes. The expression repertoire of cell adhesion molecules is strikingly different between the non-invasive radial growth phase and the potentially metastatic vertical growth phase (Haass et al, 2005, McGary, Lev and Bar-Eli, 2002). Such alterations in adhesion could enhance melanoma cells' interaction with other cell types such as endothelial and other non-epithelial cells during migration and invasion, as well as interaction with ECM components (Tsuji et al, 2002). The fact that CTHRC1 increased melanoma cell adhesion to the ECM proteins present in matrigel suggests that CTHRC1 contributes to melanoma cells' ability to adhere and survive in the extracellular matrix *in vivo*, a process that they must encounter and overcome during metastasis. CTHRC1 expressing melanoma cells may have enhanced attachment to ECM proteins by the mechanism of cytoskeletal rearrangement as

described in the previous section (section 5.1). However, it is probable that other cell adhesion molecules (CAM) are also involved.

While it is possible that CTHRC1 can either be directly or indirectly associated with the CAMs such as selectins, cadherins and the immunoglobulin superfamily, it is more likely that CTHRC1 enhanced cell-ECM interaction is associated with integrin function (Kuphal, Bauer and Bosserhoff, 2005, Dunehoo et al, 2006, Dalton, Marcantonio and Assoian, 1992, Hehlgans, Haase and Cordes, 2006). This is because cell-substratum attachment and cell-soluble protein interactions are often mediated by interactions between ECM molecules and specific cell surface integrins (Kurschat and Mauch, 2000). In addition, integrins are often recruited to sites of focal adhesion complexes. Integrins are able to transcribe the intracellular signals to alter the interaction with the environment, for example, by regulating adhesion and de-adhesion, and is often mutated in metastatic cancers (Haass et al, 2005, Tsuji et al, 2002). The enhanced adhesion to ECM may be modulated through the recruitment of integrins as a secondary event to Wnt/PCP signaling. If this were the case, the following metastasis and survival related integrins could be recruited: $\alpha v\beta 3$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 6\beta 1$, and those involving the $\beta 1$subunit, which dimerizes with various alpha chains to form complexes that recognize extracellular matrix proteins such as fibronectin, laminin, and collagens (Felding-Habermann et al, 2002, Felding-Habermann et al, 1992, Koistinen et al, 2004, Montgomery, Reisfeld and Cheresh, 1994, Natali et al, 1997, Natali et al, 1993, Wewer et al, 1987, Kramer and Marks, 1989). Depending on the pairs of alpha and beta integrins recruited, integrin signaling can activate kinases such as ILK and focal adhesion kinase (FAK), which in turn has downstream effects on cell survival, adhesion and motility. Cell survival can be regulated through PI3K signaling, while cell adhesion and migration can be regulated through altering the expression of Rho, Rac and Cdc42.

5.5. Potential roles of CTHRC1 other than being a Wnt co-receptor

Although CTHRC1 has been demonstrated by Yamamoto et al to form homotrimers and activate the Wnt/PCP pathway by stabilizing the ligand-receptor interaction between Wnt-Fzd and Wnt-Ror2 proteins (Yamamoto et al, 2008), this protein may have other functions beyond its role as a co-receptor on the cell membrane. CTHRC1 exists in different forms: intracellular, membrane bound and secreted forms. Secreted CTHRC1, like osteopontin (OPN), could perhaps act as a ligand for CAMs on the cell surface (Zhou et al, 2005, Geissinger et al, 2002). However, the possibility of this function is unlikely because supplementing secreted CTHRC1 to cells without endogenous expression did not have any effect on the survival of cells (section 4.2.2). Another possibility is that CTHRC1 binds to an extracellular matrix bridging molecule that mediates the binding with CAMs. An example of this mechanism is denatured collagen, which binds αv β1 integrin by binding to the bridging fibronectin, a molecule capable of binding a variety of molecules such as collagens, heparin, and cell surface receptors (Dalton, Marcantonio and Assoian, 1992, Tuckwell et al, 1994). Nonetheless, future work must be done to investigate if other potential roles of CTHRC1 exist.

5.6. Impact of described work

During the metastatic process, melanoma cells will encounter harsh conditions such as nutrient deprivation or hypoxia. Cells which are able to survive these conditions will be more successful in metastasizing to distant sites and resuming growth at the secondary site. A multi-step model of invasion suggests that cancer cells must first adhere to the ECM, then proteolytically degrade the matrix, migrate through the barriers and to distant sites (Albini, 1998,

Albini et al, 2004, Huber, Kraut and Beug, 2005, Quinones and Garcia-Castro, 2004). CTHRC1 has been demonstrated in this study to be a protein produced by melanoma cells to enhance their adhesion, migration and survival to facilitate metastasis. In addition, CTHRC1 expression is correlated with inhibition of collagen matrix deposition (Pyagay et al, 2005, Durmus et al, 2006), suggesting a role for CTHRC1 in ECM modulation. These results collectively suggest that inhibition of the function of this protein may be able to decrease melanoma metastasis.

5.7. CTHRC1 as a potential therapeutic target

As previously noted, melanomas are very resistant to conventional treatments largely because of its resistance to apoptosis. It is therefore important to actively seek new ways to improve melanoma's response to therapies. Since low CTHRC1 expression is correlated with a decrease in melanoma survival under harsh conditions, combining CTHRC1 inhibition with current treatments may be able to improve the efficacy of the therapy. Through treating melanoma cells with different levels of CTHRC1 expression with TMZ, it was found that MMRU cells were more susceptible to cell death when CTHRC1 is knocked down. MMAN cells with CTHRC1 expression, on the other hand, only demonstrated a small increase in survival over the control cells when treated with TMZ. The difference between the response of MMAN and MMRU cells could be due to the differences in cell lines. Different cell lines have different sets of gene expression, and may respond differently to therapies. These preliminary results on CTHRC1's enhancement of melanoma chemosensitivity are encouraging, although future work must be done to investigate the scope of the effectiveness of combining CTHRC1 inhibition with current therapies.

CTHRC1's involvement in metastatic phenotypes, the encouraging preliminary results on chemosensitivity, and its ease of accessibility on the cell membrane make it worthwhile to develop therapies against it. Blocking peptides and antibodies can be designed to inhibit CTHRC1's function. CTHRC1 inhibitors can then be combined with current therapeutics to increase its efficacy, as CTHRC1 inhibition increases cell death and renders the cells more fragile. Although future work must be done to clarify the precise role of CTHRC1 in melanoma metastasis, CTHRC1 has value as a target, for which we can design anti-cancer therapeutics against to decrease metastatic melanoma patient mortality.

5.8. Future directions

The identification of the association of CTHRC1 with metastatic phenotypes has opened up new areas of research. More work is required to further clarify precisely how CTHRC1 affects the Rho family of proteins. In addition, more work must be done to elucidate other potential roles of CTHRC1.

The pilot experiment with TMZ revealed that CTHRC1 inhibition sensitized MMRU cells to TMZ treatment. In order to identify if this effect is only evident in MMRU cells or applicable to all CTHRC1 expressing cells, future work must be done to test for TMZ sensitization effect in more CTHRC1 expressing melanoma cells. In addition, CTHRC1 inhibitors in the form of antibodies or small molecules may be developed for easier administration.

6. Conclusions

Metastatic melanoma has been difficult to treat because of its strong resistance to apoptosis. In this study, CTHRC1 expression has been demonstrated to be involved in metastatic phenotypes such as increased cell adhesion, cell migration and cell survival. It is likely that CTHRC1 functions through the Wnt/PCP pathway, activating downstream proteins belonging to Rho subfamily of GTPases and modulates the cytoskeleton. CTHRC1's association with metastatic phenotypes renders it a potential therapeutic target for melanoma.

References

Agarwala SS, Kirkwood JM (2000). Temozolomide, a novel alkylating agent with activity in the central nervous system, may improve the treatment of advanced metastatic melanoma. *Oncologist* 5: 144-151.

Albini A (1998). Tumor and endothelial cell invasion of basement membranes. the matrigel chemoinvasion assay as a tool for dissecting molecular mechanisms. *Pathol Oncol Res* 4: 230-241.

Albini A, Benelli R, Noonan DM, Brigati C (2004). The "chemoinvasion assay": A tool to study tumor and endothelial cell invasion of basement membranes. *Int J Dev Biol* 48: 563-571.

Ammer AG, Weed SA (2008). Cortactin branches out: Roles in regulating protrusive actin dynamics. *Cell Motil Cytoskeleton* 65: 687-707.

Axelrod JD, Miller JR, Shulman JM, Moon RT, Perrimon N (1998). Differential recruitment of dishevelled provides signaling specificity in the planar cell polarity and wingless signaling pathways. *Genes Dev* 12: 2610-2622.

Bae SI, Cheriyath V, Jacobs BS, Reu FJ, Borden EC (2008). Reversal of methylation silencing of Apo2L/TRAIL receptor 1 (DR4) expression overcomes resistance of SK-MEL-3 and SK-MEL-28 melanoma cells to interferons (IFNs) or Apo2L/TRAIL. *Oncogene* 27: 490-498.

Bedikian AY, Millward M, Pehamberger H, Conry R, Gore M, Trefzer U, et al (2006). Bcl-2 antisense (oblimersen sodium) plus dacarbazine in patients with advanced melanoma: The oblimersen melanoma study group. *J Clin Oncol* 24: 4738-4745.

Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, Wirtz R, et al (1998). Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. *Science* 280: 596-599.

Bittner M, Meltzer P, Chen Y, Jiang Y, Seftor E, Hendrix M, et al (2000). Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature* 406: 536-540.

Boogerd W, de Gast GC, Dalesio O (2007). Temozolomide in advanced malignant melanoma with small brain metastases: Can we withhold cranial irradiation? *Cancer* 109: 306-312.

Boutros M, Paricio N, Strutt DI, Mlodzik M (1998). Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. *Cell* 94: 109-118.

Brabletz T, Jung A, Dag S, Hlubek F, Kirchner T (1999). Beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. *Am J Pathol* 155: 1033-1038.

Brandt D, Gimona M, Hillmann M, Haller H, Mischak H (2002). Protein kinase C induces actin reorganization via a src- and rho-dependent pathway. *J Biol Chem* 277: 20903-20910.

Brooks PC, Stromblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, et al (1996). Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell* 85: 683-693.

Brozyna AA, VanMiddlesworth L, Slominski AT (2008). Inhibition of melanogenesis as a radiation sensitizer for melanoma therapy. *Int J Cancer* 123: 1448-1456.

Brunet A, Roux D, Lenormand P, Dowd S, Keyse S, Pouyssegur J (1999). Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. *EMBO J* 18: 664-674.

Byers HR, Etoh T, Doherty JR, Sober AJ, Mihm MC Jr (1991). Cell migration and actin organization in cultured human primary, recurrent cutaneous and metastatic melanoma. Time-lapse and image analysis. *Am J Pathol* 139:423-435

Cadigan KM, Liu YI (2006). Wnt signaling: Complexity at the surface. *J Cell Sci* 119: 395-402.

Campos S, Ashworth SL, Wean S, Hosford M, Sandoval R, Hallett MA, et al (2009). Cytokine induced F-actin reorganization in endothelial cells involves RhoA activation. *Am J Physiol Renal Physiol*.

Cancer facts & figures (2008). American Cancer Society. Atlanta, Georgia.

Cantley LC, Neel BG (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci U S A* 96: 4240-4245.

Chin L, Pomerantz J, Polsky D, Jacobson M, Cohen C, Cordon-Cardo C, et al (1997). Cooperative effects of INK4a and ras in melanoma susceptibility in vivo. *Genes Dev* 11: 2822-2834.

Clark WH,Jr, Elder DE, Guerry D,4th, Epstein MN, Greene MH, Van Horn M (1984). A study of tumor progression: The precursor lesions of superficial spreading and nodular melanoma. *Hum Pathol* 15: 1147-1165.

Cusack JC,Jr, Liu R, Houston M, Abendroth K, Elliott PJ, Adams J, et al (2001). Enhanced chemosensitivity to CPT-11 with proteasome inhibitor PS-341: Implications for systemic nuclear factor-kappaB inhibition. *Cancer Res* 61: 3535-3540.

Dalton SL, Marcantonio EE, Assoian RK (1992). Cell attachment controls fibronectin and alpha 5 beta 1 integrin levels in fibroblasts. implications for anchorage-dependent and -independent growth. *J Biol Chem* 267: 8186-8191.

Dancey JE (2006). Therapeutic targets: MTOR and related pathways. *Cancer Biol Ther* 5: 1065-1073.

Danen EH, de Vries TJ, Morandini R, Ghanem GG, Ruiter DJ, van Muijen GN (1996). E-cadherin expression in human melanoma. *Melanoma Res* 6: 127-131.

De Calisto J, Araya C, Marchant L, Riaz CF, Mayor R (2005). Essential role of non-canonical wnt signalling in neural crest migration. *Development* 132: 2587-2597.

Delaguillaumie A, Lagaudriere-Gesbert C, Popoff MR, Conjeaud H (2002). Rho GTPases link cytoskeletal rearrangements and activation processes induced via the tetraspanin CD82 in T lymphocytes. *J Cell Sci* 115: 433-443.

Demunter A, Libbrecht L, Degreef H, De Wolf-Peeters C, van den Oord JJ (2002). Loss of membranous expression of beta-catenin is associated with tumor progression in cutaneous melanoma and rarely caused by exon 3 mutations. *Mod Pathol* 15: 454-461.

Dhanasekaran DN, Reddy EP (2008). JNK signaling in apoptosis. *Oncogene* 27: 6245-6251.

Dunehoo AL, Anderson M, Majumdar S, Kobayashi N, Berkland C, Siahaan TJ (2006). Cell adhesion molecules for targeted drug delivery. *J Pharm Sci*.

Durmus T, Leclair RJ, Park KS, Terzic A, Yoon JK, Lindner V (2006). Expression analysis of the novel gene collagen triple helix repeat containing-1 (Cthrc1). *Gene Expr Patterns* 6: 935-940.

Eigentler TK, Caroli UM, Radny P, Garbe C (2003). Palliative therapy of disseminated malignant melanoma: A systematic review of 41 randomised clinical trials. *Lancet Oncol* 4: 748-759.

Eisen T, Ahmad T, Flaherty KT, Gore M, Kaye S, Marais R, et al (2006). Sorafenib in advanced melanoma: A phase II randomised discontinuation trial analysis. *Br J Cancer* 95: 581-586.

Eskandarpour M, Kiaii S, Zhu C, Castro J, Sakko AJ, Hansson J (2005). Suppression of oncogenic NRAS by RNA interference induces apoptosis of human melanoma cells. *Int J Cancer* 115: 65-73.

Fang L, Lonsdorf AS, Hwang ST (2008). Immunotherapy for advanced melanoma. *J Invest Dermatol* 128: 2596-2605.

Felding-Habermann B, Fransvea E, O'Toole TE, Manzuk L, Faha B, Hensler M (2002). Involvement of tumor cell integrin alpha v beta 3 in hematogenous metastasis of human melanoma cells. *Clin Exp Metastasis* 19: 427-436.

Felding-Habermann B, Mueller BM, Romerdahl CA, Cheresh DA (1992). Involvement of integrin alpha V gene expression in human melanoma tumorigenicity. *J Clin Invest* 89: 2018-2022.

Flaherty KT, Schiller J, Schuchter LM, Liu G, Tuveson DA, Redlinger M, et al (2008). A phase I trial of the oral, multikinase inhibitor sorafenib in combination with carboplatin and paclitaxel. *Clin Cancer Res* 14: 4836-4842.

Flores JF, Walker GJ, Glendening JM, Haluska FG, Castresana JS, Rubio MP, et al (1996). Loss of the p16INK4a and p15INK4b genes, as well as neighboring 9p21 markers, in sporadic melanoma. *Cancer Res* 56: 5023-5032.

Gaggioli C, Sahai E (2007). Melanoma invasion - current knowledge and future directions. *Pigment Cell Res* 20: 161-172.

Garraway LA, Widlund HR, Rubin MA, Getz G, Berger AJ, Ramaswamy S, et al (2005). Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. *Nature* 436: 117-122.

Geissinger E, Weisser C, Fischer P, Schartl M, Wellbrock C (2002). Autocrine stimulation by osteopontin contributes to antiapoptotic signalling of melanocytes in dermal collagen. *Cancer Res* 62: 4820-4828.

Gimona M (2008). The microfilament system in the formation of invasive adhesions. *Semin Cancer Biol* 18: 23-34.

Guldberg P, thor Straten P, Birck A, Ahrenkiel V, Kirkin AF, Zeuthen J (1997). Disruption of the MMAC1/PTEN gene by deletion or mutation is a frequent event in malignant melanoma. *Cancer Res* 57: 3660-3663.

Haass NK, Smalley KS, Li L, Herlyn M (2005). Adhesion, migration and communication in melanocytes and melanoma. *Pigment Cell Res* 18: 150-159.

Habas R, Dawid IB (2005). Dishevelled and wnt signaling: Is the nucleus the final frontier? *J Biol* 4: 2.

Hart M, Concordet JP, Lassot I, Albert I, del los Santos R, Durand H, et al (1999). The F-box protein beta-TrCP associates with phosphorylated beta-catenin and regulates its activity in the cell. *Curr Biol* 9: 207-210.

He TC, Chan TA, Vogelstein B, Kinzler KW (1999). PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell* 99: 335-345.

He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, et al (1998). Identification of c-MYC as a target of the APC pathway. *Science* 281: 1509-1512.

Hehlgans S, Haase M, Cordes N (2006). Signalling via integrins: Implications for cell survival and anticancer strategies. *Biochim Biophys Acta*.

Hingorani SR, Jacobetz MA, Robertson GP, Herlyn M, Tuveson DA (2003). Suppression of BRAF(V599E) in human melanoma abrogates transformation. *Cancer Res* 63: 5198-5202.

Hocker TL, Singh MK, Tsao H (2008). Melanoma genetics and therapeutic approaches in the 21st century: Moving from the benchside to the bedside. *J Invest Dermatol* 128: 2575-2595.

Hodgkinson CA, Moore KJ, Nakayama A, Steingrimsson E, Copeland NG, Jenkins NA, et al (1993). Mutations at the mouse microphthalmia locus are associated with defects in a gene encoding a novel basic-helix-loop-helix-zipper protein. *Cell* 74: 395-404.

Hofmann UB, Westphal JR, Van Kraats AA, Ruiter DJ, Van Muijen GN (2000). Expression of integrin alpha(v)beta(3) correlates with activation of membrane-type matrix metalloproteinase-1 (MT1-MMP) and matrix metalloproteinase-2 (MMP-2) in human melanoma cells in vitro and in vivo. *Int J Cancer* 87: 12-19.

Hsu M, Andl T, Li G, Meinkoth JL, Herlyn M (2000). Cadherin repertoire determines partner-specific gap junctional communication during melanoma progression. *J Cell Sci* 113 (Pt 9): 1535-1542.

Hsu MY, Wheelock MJ, Johnson KR, Herlyn M (1996). Shifts in cadherin profiles between human normal melanocytes and melanomas. *J Investig Dermatol Symp Proc* 1: 188-194.

Huber MA, Kraut N, Beug H (2005). Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 17: 548-558.

Hussussian CJ, Struewing JP, Goldstein AM, Higgins PA, Ally DS, Sheahan MD, et al (1994). Germline p16 mutations in familial melanoma. *Nat Genet* 8: 15-21.

Jafri MS, Keizer J (1995). On the roles of Ca2+ diffusion, Ca2+ buffers, and the endoplasmic reticulum in IP3-induced Ca2+ waves. *Biophys J* 69: 2139-2153.

Kamb A, Shattuck-Eidens D, Eeles R, Liu Q, Gruis NA, Ding W, et al (1994). Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. *Nat Genet* 8: 23-26.

Kielhorn E, Provost E, Olsen D, D'Aquila TG, Smith BL, Camp RL, et al (2003). Tissue microarray-based analysis shows phospho-beta-catenin expression in malignant melanoma is associated with poor outcome. *Int J Cancer* 103: 652-656.

Kim CJ, Dessureault S, Gabrilovich D, Reintgen DS, Slingluff CL,Jr (2002). Immunotherapy for melanoma. *Cancer Control* 9: 22-30.

Kimura H, Kwan KM, Zhang Z, Deng JM, Darnay BG, Behringer RR, et al (2008). Cthrc1 is a positive regulator of osteoblastic bone formation. *PLoS ONE* 3: e3174.

Koistinen P, Ahonen M, Kahari VM, Heino J (2004). alphaV integrin promotes in vitro and in vivo survival of cells in metastatic melanoma. *Int J Cancer* 112: 61-70.

Kolyada AY, Riley KN, Herman IM (2003). Rho GTPase signaling modulates cell shape and contractile phenotype in an isoactin-specific manner. *Am J Physiol Cell Physiol* 285: C1116-21.

Koo HM, VanBrocklin M, McWilliams MJ, Leppla SH, Duesbery NS, Woude GF (2002). Apoptosis and melanogenesis in human melanoma cells induced by anthrax lethal factor inactivation of mitogen-activated protein kinase kinase. *Proc Natl Acad Sci U S A* 99: 3052-3057.

Kozma R, Ahmed S, Best A, Lim L (1995). The ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in swiss 3T3 fibroblasts. *Mol Cell Biol* 15: 1942-1952.

Kramer RH, Marks N (1989). Identification of integrin collagen receptors on human melanoma cells. *J Biol Chem* 264: 4684-4688.

Kuhl M, Sheldahl LC, Malbon CC, Moon RT (2000a). Ca(2+)/calmodulin-dependent protein kinase II is stimulated by wnt and frizzled homologs and promotes ventral cell fates in xenopus. *J Biol Chem* 275: 12701-12711.

Kuhl M, Sheldahl LC, Park M, Miller JR, Moon RT (2000b). The Wnt/Ca2+ pathway: A new vertebrate wnt signaling pathway takes shape. *Trends Genet* 16: 279-283.

Kuphal S, Bauer R, Bosserhoff AK (2005). Integrin signaling in malignant melanoma. *Cancer Metastasis Rev* 24: 195-222.

Kurschat P, Mauch C (2000). Mechanisms of metastasis. *Clin Exp Dermatol* 25: 482-489.

Larsson C (2006). Protein kinase C and the regulation of the actin cytoskeleton. *Cell Signal* 18: 276-284.

Leblanc V, Tocque B, Delumeau I (1998). Ras-GAP controls rho-mediated cytoskeletal reorganization through its SH3 domain. *Mol Cell Biol* 18: 5567-5578.

LeClair R, Lindner V (2007). The role of collagen triple helix repeat containing 1 in injured arteries, collagen expression, and transforming growth factor beta signaling. *Trends Cardiovasc Med* 17: 202-205.

LeClair RJ, Durmus T, Wang Q, Pyagay P, Terzic A, Lindner V (2007). Cthrc1 is a novel inhibitor of transforming growth factor-beta signaling and neointimal lesion formation. *Circ Res* 100: 826-833.

Li G, Ho VC, Trotter MJ, Horsman DE, Tron VA (1995). P53 mutation in metastatic melanomas and primary melanomas from sun-exposed and sun-protected sites. *J. Eur. Acad. Dermatol. Venereol* 4: 48-53

Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, et al (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 275: 1943-1947.

Li X, Regezi J, Ross FP, Blystone S, Ilic D, Leong SP, et al (2001). Integrin alphavbeta3 mediates K1735 murine melanoma cell motility in vivo and in vitro. *J Cell Sci* 114: 2665-2672.

Liang CC, Park AY, Guan JL (2007). In vitro scratch assay: A convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc* 2: 329-333.

Lickliter JD, Cox J, McCarron J, Martinez NR, Schmidt CW, Lin H, et al (2007). Small-molecule bcl-2 inhibitors sensitise tumour cells to immune-mediated destruction. *Br J Cancer* 96: 600-608.

Lin AW, Barradas M, Stone JC, van Aelst L, Serrano M, Lowe SW (1998). Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes Dev* 12: 3008-3019.

Liu X, Chan SY, Ho PC (2008). Comparison of the in vitro and in vivo effects of retinoids either alone or in combination with cisplatin and 5-fluorouracil on tumor development and metastasis of melanoma. *Cancer Chemother Pharmacol* 63: 167-174.

Lunn JM, Harris AL (1988). Cytotoxicity of 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC) on mer+, Mer+Rem- and mer- cell lines: Differential potentiation by 3-acetamidobenzamide. *Br J Cancer* 57: 54-58.

Madhunapantula SV, Sharma A, Robertson GP (2007). PRAS40 deregulates apoptosis in malignant melanoma. *Cancer Res* 67: 3626-3636.

Mann B, Gelos M, Siedow A, Hanski ML, Gratchev A, Ilyas M, et al (1999). Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proc Natl Acad Sci U S A* 96: 1603-1608.

Maynard K, Parsons PG (1986). Cross-sensitivity of methylating agents, hydroxyurea, and methotrexate in human tumor cells of the mer- phenotype. *Cancer Res* 46: 5009-5013.

McDermott DF, Sosman JA, Gonzalez R, Hodi FS, Linette GP, Richards J, et al (2008). Double-blind randomized phase II study of the combination of sorafenib and dacarbazine in patients with advanced melanoma: A report from the 11715 study group. *J Clin Oncol* 26: 2178-2185.

McGary EC, Lev DC, Bar-Eli M (2002). Cellular adhesion pathways and metastatic potential of human melanoma. *Cancer Biol Ther* 1: 459-465.

McGill GG, Horstmann M, Widlund HR, Du J, Motyckova G, Nishimura EK, et al (2002). Bcl2 regulation by the melanocyte master regulator mitf modulates lineage survival and melanoma cell viability. *Cell* 109: 707-718.

Mendenhall WM, Amdur RJ, Grobmyer SR, George TJ,Jr, Werning JW, Hochwald SN, et al (2008). Adjuvant radiotherapy for cutaneous melanoma. *Cancer* 112: 1189-1196.

Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, et al (2005). BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* 436: 720-724.

Miller AJ, Mihm MC,Jr. (2006). Melanoma. *N Engl J Med* 355: 51-65.

Minden A, Karin M (1997). Regulation and function of the JNK subgroup of MAP kinases. *Biochim Biophys Acta* 1333: F85-104.

Montgomery AM, Reisfeld RA, Cheresh DA (1994). Integrin alpha v beta 3 rescues melanoma cells from apoptosis in three-dimensional dermal collagen. *Proc Natl Acad Sci U S A* 91: 8856-8860.

Morin PJ, Weeraratna AT (2003). Wnt signaling in human cancer. *Cancer Treat Res* 115: 169-187.

Narumiya S, Tanji M, Ishizaki T (2009). Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion. *Cancer Metastasis Rev*.

Natali PG, Hamby CV, Felding-Habermann B, Liang B, Nicotra MR, Di Filippo F, et al (1997). Clinical significance of alpha(v)beta3 integrin and intercellular adhesion molecule-1 expression in cutaneous malignant melanoma lesions. *Cancer Res* 57: 1554-1560.

Natali PG, Nicotra MR, Bartolazzi A, Cavaliere R, Bigotti A (1993). Integrin expression in cutaneous malignant melanoma: Association of the alpha 3/beta 1 heterodimer with tumor progression. *Int J Cancer* 54: 68-72.

Nguyen TH (2004). Mechanisms of metastasis. *Clin Dermatol* 22: 209-216.

Nishimura EK, Granter SR, Fisher DE (2005). Mechanisms of hair graying: Incomplete melanocyte stem cell maintenance in the niche. *Science* 307: 720-724.

Nobes CD, Hall A (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81: 53-62.

Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA (1994). Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* 368: 753-756.

Olivo C, Vanni C, Mancini P, Silengo L, Torrisi MR, Tarone G, et al (2000). Distinct involvement of cdc42 and RhoA GTPases in actin organization and cell shape in untransformed and dbl oncogene transformed NIH3T3 cells. *Oncogene* 19: 1428-1436.

Patton EE, Widlund HR, Kutok JL, Kopani KR, Amatruda JF, Murphey RD, et al (2005). BRAF mutations are sufficient to promote nevi formation and cooperate with p53 in the genesis of melanoma. *Curr Biol* 15: 249-254.

Petitclerc E, Stromblad S, von Schalscha TL, Mitjans F, Piulats J, Montgomery AM, et al (1999). Integrin alpha(v)beta3 promotes M21 melanoma growth in human skin by regulating tumor cell survival. *Cancer Res* 59: 2724-2730.

Pyagay P, Heroult M, Wang Q, Lehnert W, Belden J, Liaw L, et al (2005). Collagen triple helix repeat containing 1, a novel secreted protein in injured and diseased arteries, inhibits collagen expression and promotes cell migration. *Circ Res* 96: 261-268.

Quinones LG, Garcia-Castro I (2004). Characterization of human melanoma cell lines according to their migratory properties in vitro. *In Vitro Cell Dev Biol Anim* 40: 35-42.

Ridley AJ (2006). Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends Cell Biol* 16: 522-529.

Ridley AJ, Hall A (1994). Signal transduction pathways regulating rho-mediated stress fibre formation: Requirement for a tyrosine kinase. *EMBO J* 13: 2600-2610.

Ridley AJ, Hall A (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 70: 389-399.

Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* 70: 401-410.

Ruggieri R, Chuang YY, Symons M (2001). The small GTPase rac suppresses apoptosis caused by serum deprivation in fibroblasts. *Mol Med* 7: 293-300.

Schwartz MA, Horwitz AR (2006). Integrating adhesion, protrusion, and contraction during cell migration. *Cell* 125: 1223-1225.

Scott RA, Lauweryns B, Snead DM, Haynes RJ, Mahida Y, Dua HS (1997). E-cadherin distribution and epithelial basement membrane characteristics of the normal human conjunctiva and cornea. *Eye* 11 (Pt 5): 607-612.

Seidensticker MJ, Behrens J (2000). Biochemical interactions in the wnt pathway. *Biochim Biophys Acta* 1495: 168-182.

Serrano M, Lee H, Chin L, Cordon-Cardo C, Beach D, DePinho RA (1996). Role of the INK4a locus in tumor suppression and cell mortality. *Cell* 85: 27-37.

Sharpless E, Chin L (2003). The INK4a/ARF locus and melanoma. *Oncogene* 22: 3092-3098.

Sheldahl LC, Park M, Malbon CC, Moon RT (1999). Protein kinase C is differentially stimulated by wnt and frizzled homologs in a G-protein-dependent manner. *Curr Biol* 9: 695-698.

Slusarski DC, Corces VG, Moon RT (1997). Interaction of wnt and a frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. *Nature* 390: 410-413.

Smalley KS, Eisen TG (2003). Farnesyl transferase inhibitor SCH66336 is cytostatic, pro-apoptotic and enhances chemosensitivity to cisplatin in melanoma cells. *Int J Cancer* 105: 165-175.

Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, et al (1997). Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* 15: 356-362.

Strutt D (2001). Planar polarity: Getting ready to ROCK. *Curr Biol* 11: R506-9.

Strutt DI, Weber U, Mlodzik M (1997). The role of RhoA in tissue polarity and frizzled signalling. *Nature* 387: 292-295.

Sunwoo JB, Chen Z, Dong G, Yeh N, Crowl Bancroft C, Sausville E, et al (2001). Novel proteasome inhibitor PS-341 inhibits activation of nuclear factor-kappa B, cell survival, tumor growth, and angiogenesis in squamous cell carcinoma. *Clin Cancer Res* 7: 1419-1428.

Takimoto CH, Awada A (2008). Safety and anti-tumor activity of sorafenib (nexavar) in combination with other anti-cancer agents: A review of clinical trials. *Cancer Chemother Pharmacol* 61: 535-548.

Tamura S, Bito T, Ichihashi M, Ueda M (2003). Genistein enhances the cisplatin-induced inhibition of cell growth and apoptosis in human malignant melanoma cells. *Pigment Cell Res* 16: 470-476.

Tang L, Dai DL, Su M, Martinka M, Li G, Zhou Y (2006). Aberrant expression of collagen triple helix repeat containing 1 in human solid cancers. *Clin Cancer Res* 12: 3716-3722.

Tang L, Su M, Zhang Y, Ip W, Martinka M, Huang C, et al (2008). Endothelin-3 is produced by metastatic melanoma cells and promotes melanoma cell survival. *J Cutan Med Surg* 12: 64-70.

Tetsu O, McCormick F (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* 398: 422-426.

Tsuji T, Kawada Y, Kai-Murozono M, Komatsu S, Han SA, Takeuchi K, et al (2002). Regulation of melanoma cell migration and invasion by laminin-5 and alpha3beta1 integrin (VLA-3). *Clin Exp Metastasis* 19: 127-134.

Tuckwell DS, Ayad S, Grant ME, Takigawa M, Humphries MJ (1994). Conformation dependence of integrin-type II collagen binding. inability of collagen peptides to support alpha 2 beta 1 binding, and mediation of adhesion to denatured collagen by a novel alpha 5 beta 1-fibronectin bridge. *J Cell Sci* 107 (Pt 4): 993-1005.

Turashvili G, Bouchal J, Baumforth K, Wei W, Dziechciarkova M, Ehrmann J, et al (2007). Novel markers for differentiation of lobular and ductal invasive breast carcinomas by laser microdissection and microarray analysis. *BMC Cancer* 7: 55.

Valyi-Nagy IT, Hirka G, Jensen PJ, Shih IM, Juhasz I, Herlyn M (1993). Undifferentiated keratinocytes control growth, morphology, and antigen expression of normal melanocytes through cell-cell contact. *Lab Invest* 69: 152-159.

Vuori K, Ruoslahti E (1993). Activation of protein kinase C precedes alpha 5 beta 1 integrin-mediated cell spreading on fibronectin. *J Biol Chem* 268: 21459-21462.

Weeraratna AT (2005). A wnt-er wonderland--the complexity of wnt signaling in melanoma. *Cancer Metastasis Rev* 24: 237-250.

Welsh CF, Roovers K, Villanueva J, Liu Y, Schwartz MA, Assoian RK (2001). Timing of cyclin D1 expression within G1 phase is controlled by rho. *Nat Cell Biol* 3: 950-957.

Wewer UM, Taraboletti G, Sobel ME, Albrechtsen R, Liotta LA (1987). Role of laminin receptor in tumor cell migration. *Cancer Res* 47: 5691-5698.

Widlund HR, Fisher DE (2003). Microphthalamia-associated transcription factor: A critical regulator of pigment cell development and survival. *Oncogene* 22: 3035-3041.

Wong RP, Ng P, Dedhar S, Li G (2007). The role of integrin-linked kinase in melanoma cell migration, invasion, and tumor growth. *Mol Cancer Ther* 6: 1692-1700.

Wu H, Goel V, Haluska FG (2003). PTEN signaling pathways in melanoma. *Oncogene* 22: 3113-3122.

Wyman K, Atkins MB, Prieto V, Eton O, McDermott DF, Hubbard F, et al (2006). Multicenter phase II trial of high-dose imatinib mesylate in metastatic melanoma: Significant toxicity with no clinical efficacy. *Cancer* 106: 2005-2011.

Yamada KM, Miyamoto S (1995). Integrin transmembrane signaling and cytoskeletal control. *Curr Opin Cell Biol* 7: 681-689.

Yamamoto S, Nishimura O, Misaki K, Nishita M, Minami Y, Yonemura S, et al (2008). Cthrc1 selectively activates the planar cell polarity pathway of wnt signaling by stabilizing the wnt-receptor complex. *Dev Cell* 15: 23-36.

You MJ, Castrillon DH, Bastian BC, O'Hagan RC, Bosenberg MW, Parsons R, et al (2002). Genetic analysis of pten and Ink4a/Arf interactions in the suppression of tumorigenesis in mice. *Proc Natl Acad Sci U S A* 99: 1455-1460.

Zhou Y, Dai DL, Martinka M, Su M, Zhang Y, Campos EI, et al (2005). Osteopontin expression correlates with melanoma invasion. *J Invest Dermatol* 124: 1044-1052.