School of Pharmacy

The role of the sFRP4 protein in modulation of cancer growth and anti-neoplastic drug sensitivity

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Declaration

"To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university."

Signature Vanathi Perumal

Date: 11/11/2014

Conference/Symposium Presentations

Curtin University- Australia

1. Actively participated 15th Australian and New Zealand Microcirculation Society (ANZMS) symposium Margaret River 2011 Perth.
2. Candidacy presentation: Modulation of Wnt Signalling Pathways in Cancer Cells to Enhance Anti-Neoplastic Drug Sensitivity on October 26, 2011
3. 3 Minute Thesis Competition presentation: Heat One 30th July 2012 Curtin University on The role of the sFRP4 protein in modulation of cancer growth and anti-neoplastic drug sensitivity.
4. Journal club presentation on Secreted Frizzled-Related Protein 4 Reduces Insulin Secretion and Is Overexpressed in Type 2 Diabetes: research article Cell Metabolism 16, 625–633, November 7, 2012
5. Participated as registered participant CHIRI's annual conference on Thursday 8 and Friday 9 November 2012
6. Actively participated Cancer Council 4th Biennial Research Symposium 29th in November 2012
7. Oral presentation on Therapeutic Approach to target Mesothelioma Cancer cells Using the Wnt Antagonist sFRP4: Metabolic State of cancer cells at the Metabolic Health 'Program of Research' Research Symposium on 10th December 2012 CHIRI-Pharmacy/Biomedical Science Research Precinct, Curtin University
8. Poster presentation on Therapeutic Approach to target Mesothelioma Cancer Cells Using the Wnt Antagonist sFRP4: Metabolic State of Cancer Cells at Lorne cancer conference on 15th Feb 2013
9. Oral presentation at the 8th State cancer Conference 24th October 2013-Cancer Council Conference on localisation and aggregation of sFRP4 and its associated domains in malignant mesothelioma cells
10. Poster presentation on the role of the Secreted frizzled related protein (sFRP4) protein and its associated domains CRD and NLD in modulation of malignant mesothelioma and anti-neoplastic drug sensitivity. Presented at EMBO Workshop on Wnt signalling: Stem cells | Development | Disease on October 6-9, 2014, Broome, Western Australia.
11. Poster presentation on Netrin like domain (NLD) of Secreted frizzled related protein (sFRP-4) has vesicle association signal but not cysteine rich domain (CRD) Presented at the EMBO Workshop on Wnt signalling: Stem cells | Development |Disease on October 6-9, 2014, Broome, Western Australia.
12. Poster presentation on the Delivery of Secreted frizzled related protein-4(sFRP4) expression constructs by chitosan nanoparticles enhances expression and at the EMBO Workshop on Wnt signalling: Stem cells | Development | Disease on October 6-9, 2014, Broome, Western Australia.
13. Co-authored in poster presentation of Epigenetic Regulation of the secreted frizzle protein family in human glioblastoma multiforme at the EMBO Workshop

on Wnt signalling: Stem cells | Development | Disease on October 6-9, 2014, Broome, Western Australia.

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15. Oral presentation at the Cancer Council 5th Biennial Research Symposium, 20th November 2014 at the Perth Convention and Exhibition Centre on the role of the Secreted frizzled related protein (sFRP4) protein and its associated domains CRD and NLD in modulation of malignant mesothelioma and anti-neoplastic drug sensitivity

Publications:

1) Fox SA, Richard AK, Kusumah I, Perumal V, Bolitho EM, Mutsaers SE, Dharmarajan AM (2013). Expression profile and function of Wnt signalling mechanisms in malignant mesothelioma cells. Biochemical Biophysical Research Communications, 440(1) 82-87.

2) Schiefer, L, Visweswaran, M, Perumal, V, Arfuso, F, Groth, D, Newsholme, P, Warrier, S, and Dharmarajan A (2014). Epigenetic regulation of the secreted frizzled-related protein family in human glioblastoma multiforme. Cancer Gene Therapy, 21 (7), 297-303.

3) Pohl SO, Scott R, Perumal, V, Arfuso, F and Dharmarajan, A (2014). Secreted Frizzled Related Protein 4 and its implications in cancer and apoptosis: A review, Tumor Biology (Epub:December 13, 2014).

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2) Vanathi Perumal, Yan Chan, Simon Fox and Arun Dharmarajan
   Delivery of Secreted frizzled related protein-4(sFRP4) expression constructs by chitosan nanoparticles enhances expression
3) Vanathi Perumal, Arun Dharmarajan and Simon Fox
   The role of secreted frizzled related protein 4(sFRP4) in malignant Mesothelioma

Dedications

I dedicate this thesis to my family and well-wishers. A special feeling of gratitude to my mother whose words of encouragement ring in my ears and inspire me to achieve more , dad who always pushed me to be the best and giving strong basics and my husband Raj for emotionally supporting me through the hard times and for taking care of my children.

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TABLE OF CONTENTS

Abbreviations

APC- adenomatous polyposis coli

(ECL) Enhanced Chemiluminescence

β-Catenin-Beta Catenin

BSA-Bovine Serum Albumin

CO2-Carbon Dioxide

CRD-Cysteine Rich Domain

CS-DS Chitosan Sulfate and Dextran Sulfate

DMSO- Dimethyl Sulfoxide

DNA- Deoxyribonucleic Acid

DNAse-Deoxyribonuclease

DTT- Dithiothreitol

Dvl-3-Dishevelled 3

FBS-Fetal bovine serum

GFP-Green Fluorescent Protein

JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethyl-benzimidazolcarbocyanine iodide)

JU77, LO68 and ONE58

MMP-Mitochondrial Membrane Potential

MTT- (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

NLD-Netrin Like Domain

NB-Number and brightness

PBS-Phosphate Buffered Saline

pEGFP-plasmid Enhanced Green Fluorescent Protein

RNA- Ribonucleic Acid

RPMI- Roswell Park Memorial Institute

SDS- Sodium Dodecyl Sulfate

SDS-PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SFRP4-Secreted Frizzled Related Protein- 4

SOP- Standard Operating Procedure

TBS-Tris Buffered Saline

TGX- Tris Glycine

Abstract

Malignant mesothelioma (MM) is a highly aggressive cancer associated with past asbestos exposure that is characterized by rapid progression, late metastases and poor prognosis. There is a need for much improved and novel therapies for this cancer through improved biological understanding of the disease. There is convincing evidence in a number of different cancers that chronic activation of Wnt signalling is important in tumorigenesis and this has fuelled interest in targeting this pathway. The Wnts are secreted glycoproteins (at least 19 in humans) that binding to specific frizzled (Fzd) receptor complexes which activate canonical or non-canonical pathways. Aberrations of Wnt signalling have been described in mesothelioma and there is a need to further investigate the pathway in this disease. Previous studies have shown downregulation of the secreted frizzled-related protein (sFRP) family of Wnt regulators in mesothelioma and in particular one member of this family, sFRP4, may suppress growth and induce apoptosis in mesothelioma cells. sFRPs were first identified as antagonists of Wnt signalling by binding directly to Wnts. They comprise two domains, a Fzd-like cysteine rich domain (CRD) and a netrin-like domain (NLD). The aim of this project was to investigate the effect of differential expression of sFRP4 upon proliferation, migration and apoptosis in mesothelioma and explore the mechanisms responsible. In addition experiments were also performed to investigate some fundamental aspects of sFRP4 signalling regulation using MM models. In particular, the role of the domains of sFRP4 in the biological effects of this protein were studied.

In chapter 3 the effect of recombinant sFRP4 and its interaction with Wnt3a was investigated in mesothelioma cell models. Recombinant sFRP4 inhibited both proliferation and migration of MM cells as well as abrogating the stimulatory effect of recombinant Wnt3a. Morphologically sFRP4 induced a cytopathic effect which was characterised by loss of attachment and rounding up of cells. Further investigation showed that the effect of sFRP4 upon cell viability was independent of both caspase-3 activation and mitochondrial depolarisation. sFRP4 induced disruption of nuclear morphology which was quite distinct from apoptosis and was consistent with mitotic catastrophe. The combination of sFRP4 with cisplatin

enhanced cytotoxicity. These results indicated that downregulation of sFRP4 in mesothelioma plays a role in the biology of this tumour.

To further investigate the above mechanisms in Chapter 4 the effect of endogenous overexpression of sFRP4 in the JU77 and ONE58 mesothelioma cell models was determined. In addition the role of the netrin like (NLD) and cysteine rich (CRD) domains of sFRP4 in these effects was also investigated. Overexpression of sFRP4 in these cell lines displayed similar effects as endogenous protein upon cell viability, migration and nuclear morphology as seen in Chapter 3. The cytotoxic effects of sFRP4 were characterised by formation of cell membrane vesicular structures. Overexpression of the individual domains of sFRP4 displayed quite distinct effects. The NLD domain exhibited similar effect to the full length protein upon proliferation, migration and cell morphology whereas the CRD only had limited effect.

The aim of the Chapter 5 study was to evaluate the effects of chitosan nanoparticles as a delivery system for sFRP4-GFP fusion protein gene, the CRD and NLD; and the anti-cancer effectiveness of these systems in malignant mesothelioma cell models. Chitosan-dextran sulphate (CS-DS) nanoparticle formulations of sFRP4, CRD, and NLD were prepared by a complex coacervation technique. Entrapment efficiency was performed by centrifugal ultrafiltration. Mobility shift was performed using agarose gel electrophoresis. The mesothelioma cell lines JU77 and ONE 58 were used to evaluate the anti-cancer effects of these nanoparticles. Cytotoxicity was determined using an MTT assay, while GFP fluorescence was evaluated by both fluorescence microscopy and confocal live cell imaging. The sFRP4, CRD, and NLD CS-DS nanoparticle formulations maintained high integrity and entrapment efficiency. Gene delivery of sFRP4 and its domains by CS-DS nanoparticle produced enhanced biological effects in both JU77 and ONE58 cell lines, which is stronger than that of non-liposomal transfection FUGENE® HD reagent alone. Control nanoparticle delivery of GFP expression alone showed low cytotoxicity. The results of cytotoxicity assays revealed that NLD nanoparticle delivery significantly reduced proliferation relative to the control. The biological effects of the sFRP4/domain gene delivery were seen at an earlier time point and with lower DNA concentrations using CS-DS nanoparticles than with a conventional transfection reagent. Furthermore, live cell imaging and confocal laser microscopy demonstrated morphological changes,

which were characterised by the formation of membrane associated vesicles and the respective domain GFP fusion fluorescence.

Many aspects of the biology of the sFRP family of proteins remain to be elucidated and the experiments described in Chapter 6 addressed some of these questions. The number and brightness (N&B) method, a technique based on fluorescence fluctuation analysis was used in JU77 cells to characterise the intracellular aggregation and trafficking of sFRP4 domains. sFRP4 and its' domains were expressed as EGFP fusions and then the effect of exogenous Wnt3a characterised by fluorescence confocal imaging. Vesicular trafficking of sFRP4 and was observed and it was shown that the NLD domain has a vesicular association signal. It was found that sFRP4 and the CRD formed oligomeric aggregates in the perinuclear region while the NLD was distributed evenly throughout the cell with a larger proportion of aggregates. Most significantly intracellular redistribution of sFRP4 was observed in response to Wnt3a suggesting that Wnt3a can modulate intracellular localisation and secretion of sFRP4.

In summary, these results indicate that downregulation of sFRP4 in mesothelioma plays a role in the biology of this tumour and the cytopathic effects of sFRP4 was mediated by alternative cell death pathways. In particular it was found that the NLD domain mediated these effects which have implications for our understanding of sFRP4 since conventionally the CRD domain is considered to interact with Wnt ligands. The effectiveness of CS-DS nanoparticles for delivery of sFRP4, CRD and NLD based genes was also shown. This study also demonstrates the potential of using nanoparticles for targeting this pathway in mesothelioma. The results of microscopy analysis studies reveal a number of novel findings regarding sFRP4 which are likely to have relevance to this wider family. Overall this study suggests that the Wnt pathway may prove a promising target for therapy in mesothelioma.

CHAPTER 1

General Introduction

1.1 General Introduction

Cancer

The defined life span of the organism that is a property that extends to the individual somatic cells whose growth and division undergo a high regulation is the primary feature of all higher eukaryotes. Cancer cells, which arise as variants that have lost their usual growth control provide a noteworthy exception. These cancer cells constitute together to form a mass of tissue called a tumor. Malignant (cancerous) tumor cells are abnormal and divide without control or order hence can invade and damage nearby tissues. Also, these cancer cells can break away from a malignant tumor and spread to the other parts of the body by a process of metastasis. Proto-oncogenes are normal genes that are involved in cell growth and division. Changes in the regulation of these genes can lead to the development of oncogenes, which promotes excessive cell growth and division.

Tumor suppressor genes are involved in controlling cell division hence when tumor suppressor genes dysfunction, cells grow and divide abnormally which can lead to tumor growth. The production of abnormal proteins can result from the deregulation of genes or genetic changes that are not corrected by the cells. Damaged proteins may not respond to standard signals, may over-respond to normal signals, or otherwise fail to carry out their normal functions. The malfunctions of these proteins can lead to disruption of normal functions. These malfunctions of proteins further lead to disruption of normal crosstalk between the signalling components of cell division machinery. Normal cell growth and division fall under the control of a network of chemical and molecular signals. Disruption of the signalling process that results in abnormal growth and uncontrolled division of cells is called “cancer”, which is one of the major causes of death worldwide; Including Australia. According to the Australian Institute of Health and Welfare (AIHW) at the end of 2007, 3.6 % of the Australian population lived with a diagnosis of cancer where some prevalence for some cancers like breast cancer, melanoma of the skin, prostate and bowel cancer was particularly high. Survival rates for these cancers seemed to increase progressively except for pancreatic cancer and mesothelioma that already

had a low survival rate, with the lowest 5-year relative survival for pancreatic cancer being 5.2% and 6.2% for mesothelioma as indicated in Fig1.

(Cancer survival and prevalence in Australia: Period estimates from 1982 to 2010 Australian Institute of Health and Welfare Australian Institute of Health and Welfare, (Canberra, Australian Capital Territory, Australia).

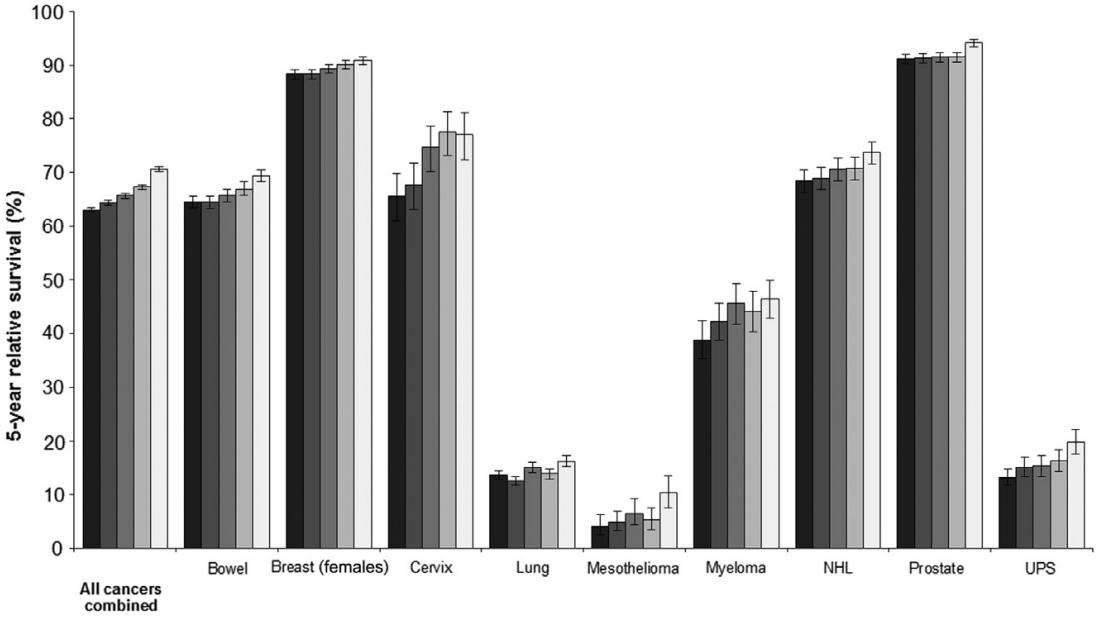


Fig1.1 Five-year relative survival by socioeconomic status, selected cancers, Australia, 2006–2010, showing quintile 1 (lowest), quintile 2, quintile 3, quintile 4, quintile 5 (highest). NHL, non-Hodgkin's lymphoma; UPS, unknown primary site. Error bars represent 95% confidence intervals.

Based, on projection, cancer deaths will continue to rise with an estimation of 11.4 million dying by 2030. In Australia prostate cancer is the most common type of cancer occurring in males whereas uterine and breast cancer are common in females.

1.2 Cancer Types

Histologically there are hundreds of different cancers, which are grouped into five categories: carcinoma, sarcoma, myeloma, leukaemia and lymphoma, in addition, there are also some cancers of mixed types. Carcinoma refers cancer of epithelial origin or of the internal or external lining of the body. Carcinomas account for 80 to 90% of all cancer cases. Sarcoma originates in supportive and connective tissues such as bone, tendon, cartilage, muscle and fat. Sarcoma tumors usually resemble the tissue in which they grow. Myeloma is cancer that originates in the plasma cells of bone marrow, Leukemias ("liquid cancer" or "blood cancers") are cancers of the bone marrow (the site of blood cells production) Lymphomas develop in the glands or nodes of the lymphatic system, a network of vessels, nodes and organs (specifically the spleen, tonsils and thymus) that purity bodily fluids and produce infection-fighting white blood cells, or lymphocytes. Unlike the leukemias which are sometimes called liquid cancers, "lymphomas are solid cancer". Lymphomas may also occur in specific organs such as the stomach, breast and or brain.

1.3 Cancer treatment

The rational treatment of cancer quotes "All substances are poisonous; there is none that is not a poison: the right dose differentiates a poison from a remedy". Paracelsus (Auroleus Phillipus Theostratus Bombastus von Hohenheim), alchemist and physician, 1538. "Doctors are men who prescribe medicines of which know little, to cure diseases of which they know less, in human beings of who they know nothing". Voltaire (Francois-Marie Arouvet), author and philosopher 1760.

Treatment of cancer may involve chemotherapy, radiation therapy, surgery, hormonal therapy, biological therapy or some combination of these. Chemotherapy is the use of anti-cancer drugs that destroy cancer cells by stopping growth or multiplication at some point in their life cycles. Chemotherapy is often given in cycles of alternating treatment and rest periods. Radiation therapy is with ionizing radiation, which destroys cells or the genetic material of cells in the area being

treated, thereby making it impossible for these cells to grow. Surgery involves removal of the tumour. Sometimes, surrounding tissue and lymph nodes are also removed. Hormone therapy is the use of hormones, to change the way hormones help cancers to grow in the body. Biological therapy (Immunotherapy) makes use of the body's immune system, either directly or indirectly, to fight cancer. The most advanced forms of treatment may produce a 5 – year survival rate of 75% or more for certain types of cancer, e.g. cancer of the uterine corpus, breast, testis and melanoma. By contrast, the survival rates in cancer of the pancreas, liver stomach and lung are generally less than 15%.

1.4 Chemotherapy

Chemotherapy is the most effective and widely used form of cancer treatment to date. The most well -studied and effective chemotherapy agents are Cisplatin, Doxorubicin, Etoposide, Hydroxyurea, Imitinab, Methotrexate, Paclitaxel and Vinblastine. Most anticancer drugs act by inhibiting DNA synthesis or some other process in the cell cycle. While chemotherapy can be quite effective in treating cancer, these agents do not effectively differentiate normal healthy cells from cancer cells and as a result leading to various side effects. The way in which the other cells are affected determines the side effects of the individual drugs. Other cells affected include blood cells, hair follicles and cells that line the digestive tract. As a result, side effects may include loss of hair, poor appetite nausea vomiting, diarrhoea or mouth lip sores. Moreover, continuous exposure to these chemotherapeutic agents leads to the development of drug resistance, because of which the patients may fail to respond to chemotherapy or suffer reoccurrence of tumours.

1.5 Chemotherapy and drug resistance

Drug resistance in tumour cells may be due to three major mechanisms: first, decreased uptake of drugs; second various changes in the cells that affect the capacity of cytotoxic drugs to kill cells, including alterations in cell cycle, increased repair of DNA damage, reduced apoptosis and altered metabolism of drugs; and third, increased energy-dependent efflux of drugs. Of these mechanisms, the one that

most commonly encountered in drug resistant conditions is the increased efflux of a broad class of cytotoxic drugs that is mediated by a family of energy dependent transporter, known as ATP- binding cassette (ABC) transporters.

Hannahan and Weinberg considered the 'cancer cell' or cancer as a dynamic heterogeneous system which undergoes many biological and biochemical changes which are common to most cancers, and some are specific to distinct tumors. The eight hallmarks of cancer were well defined as, self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion, metastasis, metabolic reprogramming, and evasion of the immune system (Hanahan, D & Weinberg, RA 2000) fig 2. Surprisingly, the fundamental characteristic of cancer cells, the loss of differentiation which distinguishes the benign and malignant tumors was not considered as distinct hallmark (Floor, Dumont, Maenhaut, & Raspe, 2012). Furthermore, the concept of cancer stem cells able to self-renew and maintain the tumorigenic nature but then again able to differentiate suggests that their behaviour dependent on external signals such as Wnts.

1.6 Therapeutic targeting

Targeted therapeutics and efficacy of the drug can be classified according to their effects on one or more hallmark capabilities of tumour inhibition and progression as illustrated in Fig 2 examples. The quality of the drug has been directed toward specific target inhibitory activity. Fewer off-target effects and less nonspecific toxicity leads to momentary clinical responses followed by resistance and recurrence of the disease. In response to therapy cancer cells reduce their dependence on a particular hallmark capability, becoming more dependent on another, this represents a quite different form of acquired drug resistance. The concept is explained clearly by recent discoveries answering the unexpected responses to antiangiogenic therapies. The effective inhibition of angiogenesis would be expected to lead to tumour ceasing and suppression (Folkman & Kalluri, 2004). Alternatively, the

clinical responses to antiangiogenic therapies have been found to be temporary (Azam, Mehta, & Harris, 2010).

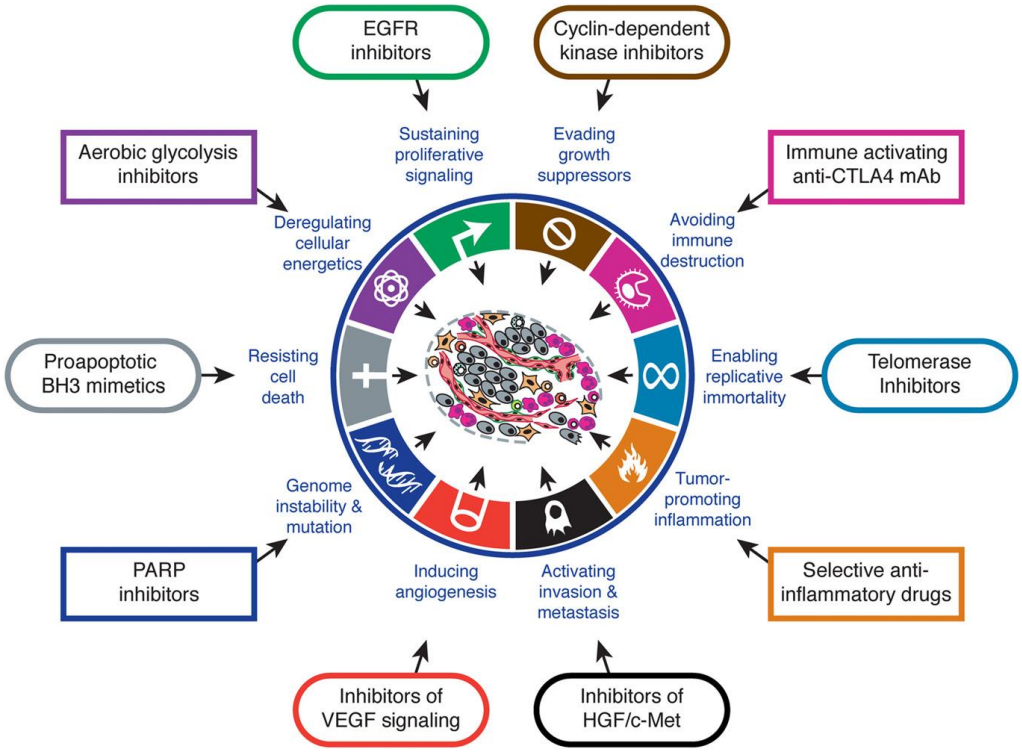


Fig2 The Eight Hall Marks of Cancers

Adapted from (Hanahan & Weinberg, 2000) The hall marks of cancer.

1.7 Malignant Mesothelioma

A mesodermally derived single-cell layer named mesothelium serosally lines the pleural, peritoneal and pericardial cavities as shown in (Fig-3). In males, mesothelium also lines the sac surrounding the testes.

The name mesothelium was first coined by Minot in 1890 (Mutsaers, Prele, Brody, & Idell, 2004). The primary malignancy arising from the mesothelium was proposed as mesothelioma by (Eastwood & Martin, 1921) Malignant mesothelioma was not considered a clinical entity until Wagner described asbestos as a causal pathogenic factor in the 1960's (Wagner, Hackanson, Lübbert, & Jung, 2010).

1.8 Prevalence and epidemiology

Malignant Mesothelioma is considered a rare disease. In industrialised countries ,the usage of asbestos increased from the end of World War II until the end of 1970s. The current incidence of mesothelioma is 2 per million in females and 10-30 per million in males per year. In Western Australia, an endemic region, the incidence rate is higher as 66 in a million men aged 35 or above are affected In the United States and the Western Europe respectively 3000 to 5000 people are affected annually. The latency period between asbestos exposure and mesothelioma development ranges from 30 to 40 years. Hence, epidemiological studies have estimated that the number of male deaths due to mesothelioma will double in Western Europe by 2015 over the next 10-20years (Peto, Decarli, La Vecchia, Levi, & Negri, 1999). The male-female ratio of mesothelioma occurrence is about 4:1 and 80% of the mesotheliomas arise from the pleural layers. Demographically, the mean age of mesothelioma patients is approximately 60 years, but the occurrence of the disease is independent of age, hence there is a possibility of its occurrence even in childhood (Grundy & Miller, 1972).

1.9 Etiology and pathogenesis

Malignant mesothelioma occurrence shares a strong relationship to the exposure to asbestos fibres. Various epidemiological surveying has revealed that 80 % occurrences of mesothelioma are attributed to their previous asbestos exposure (Chahinian et al., 1982). The oncogenicity of asbestos was established in animal studies. The various types of asbestos are divided into two major subgroups: serpentine and amphiboles. The serpentine has curly and flexible properties and is primarily comprised of crysotile. The amphiboles are stiff and straight in nature and are composed of crocidolite, and amosite, anthophylite, tremolite and actinolite.

Crysolite found in white asbestos; amosite in brown asbestos and crocidolite found in blue asbestos are the serpentine and amphiboles (Tweedale, 2002).

The exact processes of how asbestos acts a causal factor for asbestos-induced malignant mesothelioma have not been completely made clear. Suggestions for the mechanisms include asbestos being responsible for DNA damage through direct physical interaction or an indirect action of reactive oxygen species(ROS) produced in response to asbestos by inflammatory cells (Hesterberg, Ririe, Barrett, & Nettesheim, 1987). Mutations deriving from DNA strand breaks and deletions are inducted by this ROS. The autophosphorylation of the EGF receptor (EGFR) in mesothelial cells is stimulated by asbestos, this stimulation is responsible for an increased activity of the activator protein-1 (AP -1) and cell apoptosis (Robledo & Mossman, 1999). The increased susceptibility of the cells to DNA damaging agents and genetic instability and the extension of transformed cell populations is a function of growth factors such as EGF, TGF- ALPHA, IGF-I and II, HGF, PDGF and keratinocyte growth factor (KGF) acting as an autocrine or paracrine growth stimuli of mesothelial cell proliferation.

1.10 Genetic alteration

Irrespective of what causes mesothelioma, be it asbestos, SV40, genetics or radiation, mesothelial cells become increasingly malignant due to the accumulation of a number of genetic alterations. Deletions involving discrete chromosome regions lead to the most cytogenetic aberrations in mesothelioma while the pathogenesis of mesothelioma can be attributed to the accumulated loss and or inactivation of several tumor suppressor genes TSGs in chromosome arms 1p, 3p, 6q, 9p, 13q, 15q and 22q. In mesotheliomas, three TSGs are frequently deleted, which are p16INK4a, p14 ARF located at 9p21 and NF2 located at 22q12 (Murthy & Testa, 1999).

Considering the above information, it can be taken that malignant mesotheliomas are sarcomas that interact with various environmental carcinogens like asbestos and erionite, SV40 virus, radiation, and genetics to cause malignancy. Further isolating

the specific mesothelioma susceptibility gene provides the opportunity to understand the precise pathogenesis mechanisms of mesothelioma.

1.11 Pathologic morphology

Conventional histological evaluations have categorised malignant mesothelioma into three subtypes (fig-4) such as epithelioid, sarcomatoid and biphasic (Attanoos & Gibbs, 1997).

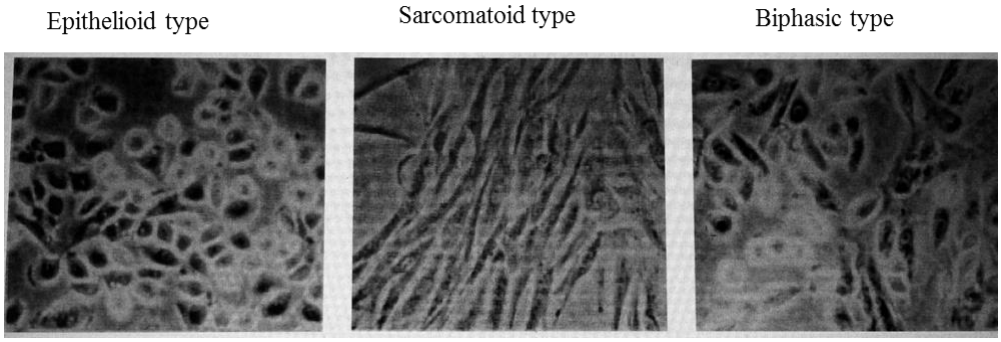


Fig-1.4 Types of Mesothelioma (Attanoos & Gibbs, 1997)

Malignant mesotheliomas are classified into epithelioid, sarcomatoid and biphasic subtypes based on conventional histological examination (Attanoos & Gibbs, 1997). Approximately 50% pleural and 75% peritoneal mesotheliomas are of epithelioid subtype; 30% are biphasic subtype and the remainder is pure sarcomatoid subtype(15% to 20%) (Attanoos & Gibbs, 1997).

The mesothelial cell bundle arrangement serves as the distinguishable factor among these subtypes. The epithelioid subtype contains mesotheliomal cells arranged in a manner that they form a tubulopapillary or trabecular shapes and may be morphologically identical to a number of adenocarcinomas. The sarcomoid subtype triggers a range of spindle-cell sarcoma morphotypes and has a presence of malignant osteoid and cartilage. The biphasic subtype is depicted by poorly differentiated mesothelioma imbibing the features of both sarcomatous and epithelial morphologies (Wick & Mills, 2000).

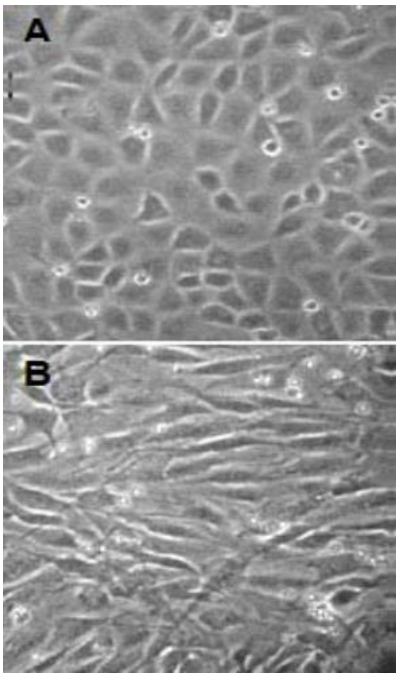


Fig-1.5 Malignant mesothelioma cells grown in a culture exhibit a property of growing as a substrate adherent monolayer that displays the morphology of epithelial, fibroblast or biphasic cells (Mutsaers et al., 2004). The invitro morphology of the malignant mesothelioma cells can be altered by different serum supplement additives (Klominek, Robért, Hjerpe, Wickström, & Gahrton, 1989).

1.12 Biological and invasive behaviour

Malignant mesothelioma unlike other solid tumours ,starts as a localised tumor only to spread rapidly along the mesothelially lined surfaces such as pericardium, contralateral hemithorax, and peritoneal cavity by invading through the diaphragm. The resultant tumor forms a diffuse thickening of the surfaces involved, unlike other neoplasms that develop a solitary rounded lesion (Semb, 1962).

The traditional features of malignant mesothelioma include an incision in the thoracic wall and a needle invasion through biopsy tracts (Chahinian et al., 1982; Hillerdal, 1983).In up to 75% of patients, the underlying basement membranes are also invaded by malignant mesotheliomas and metastases are produced (Ruffie et al., 1989). The mesothelioma cells must interact with ECM proteins and growth factors

embedded in it during the invasion process. Further interactions with the stromal cells that participate in synthesis and modifications of this micro-environments is also observed.

1.13 Clinical features and treatment

The genesis of mesothelioma proceeds in a steady, gradual manner with severe depletive effects where the only prominent presenting symptom is persistent localised pain. Symptoms such as chest pain, cough dyspnea and weight loss also frequently appear in pleural mesothelioma. In up to 95 % of the patients a high level of hyaluronan in the pleural effusion is also present. (Hillerdal, 1983), (Burgers & Damhuis, 2004)Main clinical findings associate local pain and abdominal distension with ascites in peritoneal mesothelioma (Chahinian et al., 1982).Depending on the histological subtype, the median survival time ranges from 4 to 12 months where a five year survival has a less than 5 % chance.

Malignant mesothelioma exhibits resistance to radiotherapy, but to prevent mesothelioma cells from spreading through needle biopsy tracks local radiotherapy is highly suggested (Rusch et al., 2001). Complete surgical resection is near impossible because of the biological and invasive features of the malignancy. Chemotherapy, which is an option, still continues to be challenging to administer and measure success. Inspite of the evaluation of various cytotoxic agents in malignant mesothelioma, the yielded response rates are no better than 20% Palliative treatment approaches remain the sole choice in a majority of late stage cases. Nevertheless, improved response rates and prolonged survival time in patients with malignant pleural mesothelioma has been recently published in a phase III study of pemetrexed (a new antifolate) plus cisplatin. This study showed that response rates of 41.3% and prolonged survival time in has been noted patients treated with pemetrexed/cisplatin as compared with cisplatin treated alone could be achieved (Vogelzang et al., 2003).

Even so overall survival time remains weak (9-12 of months) and the improvements with this regimen are modest. Current studies are focusing on several new anticancer

agents in a variety of malignant tumours of which some agents are responsible for target signal transduction from EGFR. Transmembrane glycoprotein EGFR is overexpressed in malignant mesothelioma (Dazzi et al., 1990). The intracellular tyrosine kinase (TK) domain is activated by ligand binding which triggers EGFR tyrosine autophosphorylation that is essential in the regulation of cell proliferation, survival, angiogenesis, cell movement and metastasis. Novel low molecular weight EGFR-TK inhibitors such as ZD1839, OSI-774 and CI-1033 are known to have shown inhibitory effectiveness in malignant mesothelioma experimentally (LIU & KLOMINEK, 2004)Hence, a potential therapeutic target for treatment of malignant mesothelioma can be inspired by understanding the blockade of EGFR signalling pathway.

1.14 Newer drugs under investigation

The oral administration of Everolimus (RAD001) in combination with cisplatin showed synergistic effect and being evaluated in 2 phase II trials inhibiting PI3K/AKT/mTOR pathway. Currently, in combination with pemetrexed and cisplatin MORAB-009 - monoclonal antibody targets mesothelin, this has been observed in the clinical trials conducted on MPM patients.

For second-line therapy of advanced malignant pleural mesothelioma, a Phase II study of belinostat (PXD101), a histone deacetylase inhibitor was carried out. J Thorac Oncol 4(1); 97-101) observed increased efficacy of the treatment on MPM patients in the Belinostat in phase II trial when vorinostat was combined with carboplatin and paclitaxel, and another HDACi was evaluated. CBP501, a G2 checkpoint abrogator, as monotherapy and in combination with cisplatin were treated in patients with advanced solid tumors. Shapiro GI, Tibes R, Gordon MS, Wong BY, Eder JP, Borad MJ et al (2011). An antibody IMC-A12 targeting the insulin-like growth factor (IGF-1) decreased proliferation. The self-renewal of MPM CSC and an increased combination therapy with cisplatin, pemetrexed and the FAK inhibitor VA-4718.

1. 15 Wnt signal pathway

Wnts are lipidated to a family of secreted glycoproteins that undergoes conservation by several residues of cysteine are named Wnts. Wnts play a crucial role in the determination of the fate of the cell, its proliferation and cell patterning during embryogenesis. Despite the signalling's importance in normal developmental processes, Wnt pathway's aberrant activation shares a close association with tumorigenesis. Out of 19 human Wnt genes, several of them are encoded as additional, alternatively spliced isoforms. Based on the activity of Wnt proteins in cell lines or invivo assays they are categorised into two classes – canonical and noncanonical. Stablisation of β-catenin by canonical Wnts (eg.Wnt1, Wnt3A, and Wnt8) activates the transcription of TCF/LEF target genes. The result is a secondary axis formation in xenopus embryos and some mammalian cell lines undergoing morphological transformations.

Other signaling pathways also undergo activation by noncanonical Wnts such as Wnt4, Wnt5A and Wnt11. The planar cell polarity (PCP)-like pathway responsible for guiding cell movements during gastrulation (Heisenberg et al 2000) and the Wnt/Ca2+ pathway are activated by the noncanonical Wnts. Noncanonical Wnts can act as antagonists of the canonical pathway. However, both canonical and noncanonical properties are exhibited in several Wnt proteins. For eg, Wnt5A, a noncanonical Wnt when co-expressed with its receptor induces secondary axis formation (He et al., 1997). Hence, the functional classification of Wnts is dependent on the repertoire of Wnt receptors in particular cell type.

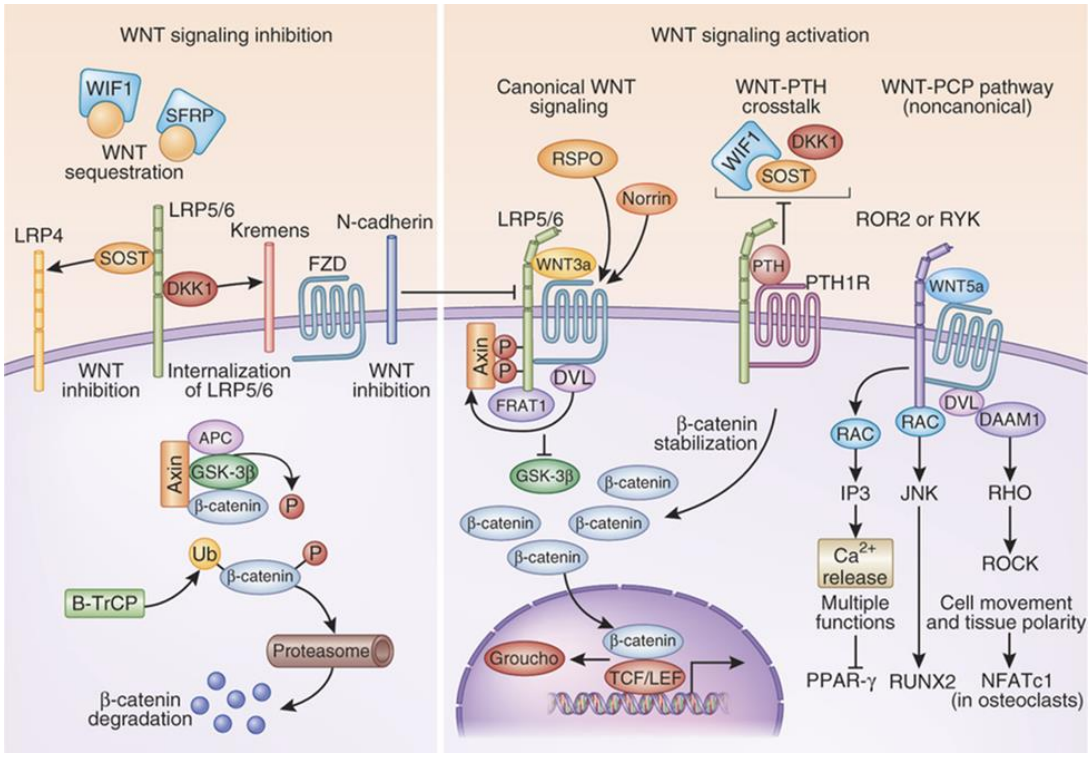


Fig 1.9 Wnt signalling canonical pathway (Baron & Kneissel, 2013)

The canonical pathway is activated by the Wnt receptor complex that contains a member of frizzled (Fz) family (there are 10 of these seven-transmembrane-span proteins in humans) and either one of two single-span transmembrane proteins, low-density-lipoprotein-receptor-related proteins LRP-5 and LRP-6 (Pinson, Brennan, Monkley, Avery, & Skarnes, 2000; Wehrli et al 2000; Tamai et al 2004). The Fz family mediates the activation of the noncanonical Wnt pathways, but the requirement of LRP5/LRP6 is unclear. Wnt antagonists are divided into two functional classes, the SFRP class whose members include the sFRP, family WIF-1 and Cerberus that bind directly to Wnts to alter their ability to bind to Wnt receptor complex and the Dickkopf class, whose members contain Dickkopf family proteins that inhibit Wnt signalling on binding to the LRP5/LRP6 component of the Wnt receptor complex. Hence, theoretically sFRP class antagonists will inhibit both canonical and non-canonical pathways, while Dickoff class antagonists show specific inhibition to the canonical pathway.

1.16 Wnt Signalling at the Plasma Membrane Wnt and Frizzleds: Monomers, Heterodimers, and Homodimers

The role of the Wnt-binding domain of the frizzled receptor homologous to sFRPs N -erminal cysteine -rich domain (CRD) in binding of Wnt ligand by sFRPs, is still unclear (Kawano & Kypta, 2003). On an interesting note, it was observed that the cysteine -rich domains of sFRPs have the capacity to dimerize with other sFRPs and they bind to the Frizzled itself hinting that the Wnt signalling by sFRPs could occur through the soluble Wnt ligand binding or by forming non-functional receptor complexes with cell-surface frizzled receptors acts. (Bafico et al., 1999) Evidence for Frizzled dimers and multimers also exists. The concept of how the analysis of a particular Wnt ligand or Frizzled receptor acts in a certain biological context requires the investigation of the transduction mechanisms of the Wnt signal to be established by further investigation. This leads us to question more about how is Wnt signaling initiated at the plasma membrane? And how is specificity achieved?

1.17 Discovery of the sFRP family

Secreted frizzled–related proteins (sFRPs) were first identified around 1996 and postulated to be potential modulators of Wnt signalling (Hoang et al., 1996; Rattner et al., 1997) Currently, there are five members of the sFRPs family in humans, although sFRP3 is many times designated as FrzB (Frizzled) motif associated with bone development), reflecting its original name before the reclassification of these proteins supported the notion that they could bind Wnts and thus act as modulators of Wnt/ βcatenin signalling (Lin et al., 1997) (Wang, Krinks, Lin, Luyten, & Moos Jr, 1997); (Bafico et al., 1999; Hoang, Moos, Vukicevic, & Luyten, 1996) sFRPs are antagonists that directly bind to Wnts and have been given different names in the literature, reflecting their simultaneous discovery by different approaches(Jones & Jomary, 2002) (Bovolenta, Esteve, Ruiz, Cisneros, & Lopez-Rios, 2008) report that sFRPs not only antagonises Wnt, but can inhibit each other activity by binding to

frizzled receptors and enhance axon guidance, regulating BMP signalling by binding to the receptors and matrix molecules as proteinase inhibitor.

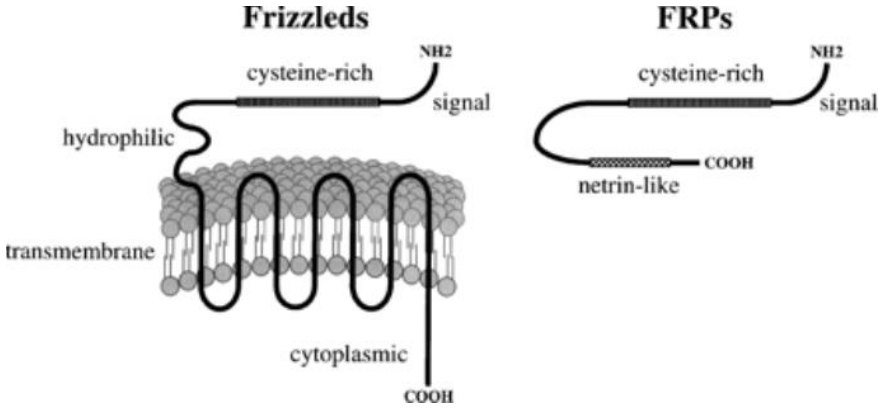


Fig1.7 Structure of frizzled receptors and sFRPs Adapted from (Jones & Jomary, 2002)

Frizzled (Fzd) receptors are characterized by an N-terminal signal peptide, an extracellular cysteine-rich domain (CRD) followed by a hydrophilic linker, seven transmembrane domains, and a cytoplasmic tail. Frizzled-related proteins (FRPs) are secreted proteins with a CRD similar to Fzd receptors, and a netrin-like domain (NLD). FRPs lack the transmembrane domains seen in the Fzd receptor. Adapted from (Wodarz &Nusse, 1998).

sFRP1, sFRP2 and sFRP5 are placed in a subgroup based on the sequence homology. sFRP3 and sFRP4 are also placed in the subgroup though they have slight distant relation to the other sFRPs. The subgroup contains the sizzled, sizzled2 and crescent. The reports of the ability of Sizzled to inhibit Wnt signaling are conflicting. (Bradley et al., 2000; Collavin & Kirschner, 2003) It is noteworthy that with a single exception (Illies et al., 2002), in invertebrates sFRPs (and the other Wnt antagonists) have not been found. Inspite of this, demonstrations of them inhibiting the activity of the Drosophila Wnt homologue Wingless (Wg) have been made. Inactivation of SFRP1 and SFRP2 prevents Wnt/β-catenin signalling activation of the mouse optic cup periphery (Esteve et al., 2011).

Considering the importance of Wnt signalling in cell-fate determination, it is plausible to conclude that this pathway undergoes regulation by a varied array of strategies. This behaviour of sFRPs is consistent with their frequent downregulation pattern in carcinomas and also their role as tumor supressors. Interaction with of the sFRP molecules with both Wnts and FZDs have been documented as evidence. However recent evidence makes claims the role of sFRPs is much more complex and is dependent on context and cell type and also has the capacity to potentiate Wnt signalling under certain circumstances. sFRP4 belongs to a family that have a conflicting role in cancer having contradictory reports for both down and upregulation in tumour cells and tissues.

1.18 Wnt/Frizzled signalling in Mesothelioma

Wnt signalling is so significant in determining of cell fate it is not surprising that this pathway is tightly regulated. One mechanism is secretory factors including the secreted frizzled-related proteins (sFRPs). They are frequently downregulated in cancers and are suggested to be tumour suppressors. There is evidence that sFRP molecules may interact with both Wnts and Fzds. Recent studies have showed that the role of sFRPs might be more complex and depend upon expression of other molecules since they may also potentiate Wnt signalling under some circumstances (Jones & Jomary, 2002). Epigenetic silencing of sFRPs has been reported as an important mechanism for aberrant activation of Wnt signalling in a number of cancers (Suzuki et al., 2004). sFRP4 is a member of this family whose role in tumour progression is unclear with reports of either down or upregulation in different tumour cell types. sFRP4 has been implicated in apoptosis (Ilyas, 2005) regulation of proliferation and tumour progression (Horvath et al., 2004; Ko et al., 2002).

1.19 Wnts Frizzled receptor, secreted frizzled-related proteins and its domains Cystein Rich Domain (CRD) and Netrin Like Domain (NLD)

Wnts activate Fz in the presence (canonical signaling, e.g., Wnt1, Wnt3a, Wnt8) or

absence (noncanonical signaling, e.g., Wnt4, Wnt5a, Wnt11) of LRP5/6 (Rehn M, 1998 et al) cross-linking of Fz with LRP5/6 may not be essential for all types of Fz signalling. The structure of XWnt8/Fz8-CRD complex is crucial for signalling and Dishevelled signalosome assembly.

Wnt protein binds to the extracellular domains of both LRP and Frizzled receptors, forming membrane- associated hetero-oligomers that interact with both Dishevelled (via the intracellular portions of Frizzled) and Axin (via the intracellular domain of LRP) (Cong, Schweizer & Varmus 2004)

The frizzled like extracellular cysteine-rich domain (Fz-CRD) has been identified in FZDs and other proteins. The Glycosylation site and several conserved motifs in FZDs may be related to Wnt interaction. (Yan et al., 2014)

The CRD domain of Frzb seemed to be necessary and sufficient for both activities: Wnt binding and inhibition. The CRDs of sFRPs show 30%– 50% sequence similarity with those of Fz receptors and contain 10 conserved cysteine residues (Rehn, Pihlajaniemi, Hofmann, & Bucher, 1998), which form a pattern of disulphide bridges (Chong, Üren, Rubin, & Speicher, 2002). In contrast more recent reports of sFRP1structure and function using Wnt reporter assays indicate that both protein domains, CRD and NTR, are necessary for optimal Wnt inhibition (Bhat, Stauffer, Komm, & Bodine, 2007). The crystal structures of the CRDs from mouse Fz8 and mouse sFRP3 have been determined and this analysis has revealed the potential for the CRDs to dimerize (Dann et al., 2001).

The sFRP3 and sFRP4 contain NTR domains with a different cysteine spacing and disulphide linkage pattern (Lin et al., 1997) (Lopez-Rios, Esteve, Ruiz, & Bovolenta, 2008) (Chong et al., 2002). If NLD modules have functional significance for sFRPs, the differences observed in the cysteine spacing and inferred disulphide bonding patterns could result in contrasting activities among the various family members (Chong et al., 2002). The Netrin like domain (NLD) is homologous to TIMP(Leclère & Rentzsch, 2012) and possess protease inhibitory activity, including the axon guidance. Very little is known about the overall function of the NLD, yet there is some evidence that the NLD may also play a role in Wnt binding suggesting that multiple Wnt binding sites may exist on SFRP molecules. Alternatively those SFRPs

are exhibiting differential affinities for Wnt ligands according to the different SFRP conformational and post-translational modifications (Bhat et al., 2007; Yan et al., 2014) (Chong et al., 2002).

Furthermore, a recent study on sFRP1 combining biochemical and functional assays in cell culture and medakafish embryos shows that its NTR domain mimics the function of the entire molecule in binding Wnt8 and inhibiting Wnt signalling (Lopez-Rios et al.,2008).

1.20 sFRP4 structure and new dimension of its biological role

A differential display cloning technique was used to isolate the Secreted Frizzled-Related Protein 4 (sFRP4) also known as Frappe (Abu-Jawdeh et al., 1999). The differential display method from the corpus luteum of the rat was originally used to isolate the Y81 or DDC4, a FRPAP, frizzled-related apoptosis associated protein. (Rattner et al., 1997) identified expression of this protein in regressing tissues like corpus luteum, mammary gland and prostate. After hormonal withdrawal, particularly in mammary gland and ventral prostate, expression of sFRP4 has been found in these regressing tissues. Investigations of SFRP4, Gas-1 and integrin-associated protein (IAP) expression and involvement in tissue remodelling and apoptosis associated functions have been undertaken in the corpus luteum of the cycling, pregnant, lactating and forced weaning rat.

The short arm of chromosome 7 (7p14.1) that comprises of coding exons, spanning 10.99kb is the location of the SFRP4 gene (Carmon & Loose, 2008). The least structural homology as compared to other members of the SFRP family is exhibited by SFRP4. (Carmon & Loose, 2010) This is because of the existence of a region of 6-conserved cysteine residues that is linked by a disulphide bridge (Carmon & Loose, 2008). SFRP4 is the largest member of the SFRP family a molecular weight of 39.9kDa and 346 amino acids in length (Carmon & Loose, 2008).

SFRPs contain a terminal domain and an N-terminus domain that act independently of each other. The N-terminus is made of a 120 amino acids long cysteine-rich domain (CRD) that is comprised of conserved region of 10-cysteine residues [8]. A 20-30 amino acid signal peptide is also present. A the netrin-like domain (NLD), is also contained in the C-terminal domain, that is preceded by a conserved region of 6 cysteine residues.

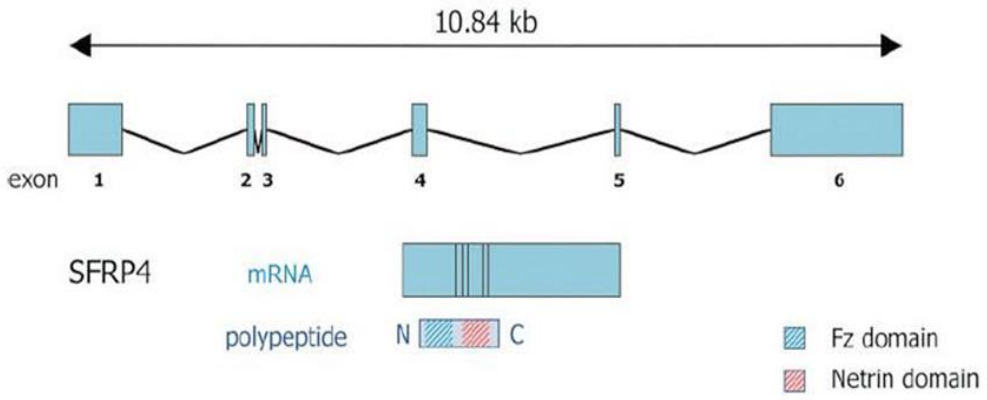


Fig 1.8 Representative diagram of the human SFRP4 gene and polypeptide (Adapted from (Jones and Jomary, 2002)

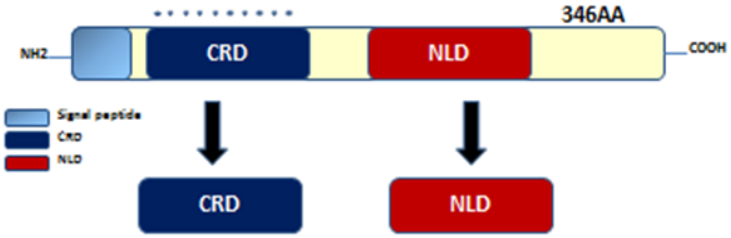


Fig 1.9 sFRP4 and Domains (Cysteine Rich Domain (CRD) and Netrin Like Domain (NLD)

Intracellular calcium levels that lead to the activation of the Wnt/Ca2+ signalling pathway are increased by the CRD and the NLD of sFRP4 (Longman, Arfuso, Viola, Hool, & Dharmarajan, 2012). The coimmunoprecipitation of Fzd5, Fzd10, and SFRP4 in Ishikawa cells was found by using Wnt 7a interactors (Carmon & Loose, 2008). Inhibition of endometrial cancer cell growth in vitro has been observed both by the stable overexpression of SFRP4 and recombinant SFRP4 protein treatment (Hrzenjak et al., 2004). A mechanism by which the nature of the Wnt7a signal in the endometrium is dependent on the Fzd repertoire of the cell and can be influenced by regulation through SFRP4 was supported by these findings (Carmon & Loose, 2008).

Constitutive Wnt signalling that arise as a result of mutational events has been vividly described in various cancers that focussed on β-catenin encoding genes itself or direct regulators such as adenomatous polyposis coli (APC) and axin (Ilyas, 2005) beta-catenin gene's active mutations haven't been widely described in mesothelioma in a study by (Abutaily, Collins& Roche, 2003). 63 primary MM tissues were screened, and no mutations were found in the CTNNBI gene. An additional smaller study in mesothelioma cell lines' and two tumors did not discover any activating mutations in CTNNB1, although it was concluded that the majority of the gene was deleted in one MM cell line (NCI-H28). This finding has little significance to mesothelioma as the tumors are invariably positive for β-catenin (Uematsu et al., 2003), (Orecchia, Schillaci, Salvio, Libener, & Betta, 2004).

Immunohistochemistry of 10 pleural malignant mesotheliomas patient tissues showed intense nuclear β-catenin expression with faint cytoplasmic staining, similarly the stromal cell staining was cytoplasmic membranous and submembranous (Anani et al., 2011).

Despite the implications of the loss of function mutations in axin and APC in tumorigenesis for many cancer types, thus far no investigation of these genes has taken place in mesothelioma (Abutaily et al., 2003). Abutaily study has provided descriptions of a possible of APC gene abnormality in 23% of a cohort of 63

mesothelioma tumors that underwent immunohistochemistry investigations. However, no reports of confirmatory genetic studies have been made.

Along with mutational events, many molecular components of Wnt signalling pathways perturbed expression has been expressed in a variety of cancers. The literature regarding the expression of individual molecules in MM are sporadic although a systematic MM cell line survey has been recently published by our laboratory. (Fox et al., 2013)

In at least one study of Wnt1, overexpression was demonstrated in a range of tumor cell types including five mesothelioma cell lines. This depicted that Wnt1 increased apoptosis, even though the blocking experiments were not conducted in mesothelioma cells (He et al., 2005). Wnt specific gene array found that Wnt2 was also been upregulated by a study of matched normal pleura and tumours in 8 patients. These investigations were extended to mesothelioma cell lines. Reduced cytosolic βeta-catenin and dishevelled-3 (Dvl-3) (Dai, Bedrossian, & Michael, 2005) along with reducing proliferation and increasing apoptosis was observed following treatment with Wnt2 antibody or Wnt2 RNA-i. There was a lack of clarity of the mechanism involved in Wnt2 upregulation, and the similar evidence concerning Wnt2's role in other cancers provides a prospect for further examination of the molecule contained in mesothelioma. In MM tissue, a report of increased Dvl-3 levels relative to normal pleura has also been made (Uematsu et al., 2003). However, since there is evidence that Wnt2 regulated Dvl-3. Expression of Wnt 1, 2, 3a, 7a, 8a, 10a, and 16 transcripts were not detected in either tumor or primary cell in these cell lines (Fox et al., 2013) it is not clear as to the whether increased (Abutaily et al., 2003) Dvl-3 represented a genetically determined overexpression of this gene in isolation or if it is a mere indication of the general upregulation of Wnt signaling.

1.21 sFRP4 in mesothelioma

Implications for Sfrp4 have been made in apoptosis regulation of proliferation (Lee, He, You, Dadfarmay, et al., 2004) and tumor progression. (He et al., 2005) reported the usage of RT-PCR to identify sFRPs 1, 3, 4 and 5 expressions and found that these

sFRPs were detectable in normal pleura but underwent an apparent downregulation mesothelioma tissue samples and two cell lines. Evidence from overexpression (Lee, He, You, Dadfarmay, et al., 2004) and RNAi experiments (He et al., 2005) have indicated that sFRP4 has a role in growth suppression and apoptosis induction in MM. Our own laboratory researchers have shown that sFRP4 was expressed in 4/4 primary human mesothelial cell cultures but only 1/4 human MM cell lines (Fox et al., 2013). Similar mouse model examinations of mesothelioma have discovered downregulated expression of sFRPs compared to normal Mesothelial cells (Fox & Dharmarajan, 2006).

1.22 Non-canonical signaling in malignant mesothelioma

Up until now, most of the studies concerning Wnt signaling in cancer have discussed the canonical pathway that has the best characterization to date. However, independent of the beta-catenin and the varied downstream consequences involved in morphogenesis, there are a number of alternate pathways through which Wnts may signal. The planar cell polarity (PCP) and the Wnt/Calcium pathways are the two non-canonical pathways typically described, but these pathways aren't well defined and suggestions for them to be divided into least three distinct pathways have been made in recent researches. The transduction of Wnt/FZD signals include heteromeric GTP-binding proteins, calcium-dependent kinases (CamKII and PKC) and JNK are brought upon by the diverse mechanisms in these non-canonical pathways. It has been discussed that these alternate pathways have a role in canonical signalling regulation and in the mechanisms of antagonism of β-catenin signalling by calcium dependent crosstalk. The identification of a β-catenin null cell line (Omori, Matsumoto, & Ninomiya-Tsuji, 2011) in MM has played a role in provoking the investigation of non-canonical pathways in these cells. An extracellular regulator of Wnt/FZD signalling - Dickkopf-1 (Dkk-1) is believed inhibit canonical Wnt signalling by interacting with LRP5/6 and FZDs (Lee, He, You, Xu, et al., 2004).

MM cell studies show that Dkk-1 has a role in the proliferation inhibition and apoptosis indication by a β-catenin independent mechanism that is mediated by JNK similar sFRP4 studies have indicated that this molecule has the potential to inhibit

proliferation and induce apoptosis in both β-catenin expressing and non-expressing cell lines (Omori et al., 2011). It was indicated that that downstream signalling induced by this Wnt1 had a non-canonical component because of the blockade of Wnt1 signaling in β-catenin null cells, the non-canonical compound was which was mediated by JNK that contributed to proliferation and apoptotic resistance (Orecchia et al., 2004). Cells deficient in β-catenin have been useful in the demonstration that gamma-catenin, another member of the catenin family also has the potential to activate Wnt signalling in the absence of β-catenin. Despite the lack of clarity of the physiological relevance of these findings, to β-catenin positive MM cells, useful insights into non-canonical pathways have been provided by these studies.

1.23 Scope of my Thesis

Treatment for MM is currently ineffective and new therapeutic approaches are required. Wnt signalling is increasingly considered an important therapeutic target in some cancers. This project proposed to employed more relevant in vitro models and experimental techniques not previously employed in MM to investigate the role of this Wnt signalling pathway in mesothelioma growth and resistance to chemotherapeutic drugs.

The precise molecular targets of sFRP4 have not been determined to date; and it was hoped that this project could provide new insights into the functioning of sFRP4 which would likely be of relevance not only in cancer but also in stem cell biology where Wnt signalling also plays an important role.

1.24 Hypothesis

In cancerous mesothelioma cells sFRP4 is turned off or downregulated. SFRP4 prevents or slows the cell growth. Reintroducing the sFRP4 protein will restore this function, slow cell growth and make them more sensitive to treatment.

The purpose of this project was to investigate the effect of differential expression of sFRP4 upon proliferation and apoptosis in malignant mesothelioma. The availability of MM cell lines which are null or express low levels of sFRP4 made the fundamental experiments feasible and means that the results will be relevant to other

cancers where sFRP4 is downregulated but not absent. In addition the intention was also to investigate some fundamental aspects of sFRP4 signalling regulation using MM models.

1.25 Aims

1) To investigate the effect of both exogenous sFRP4 protein upon proliferation, migration and apoptotic signalling in malignant mesothelioma cells
2) To investigate the effect of over expression of sFRP4 and its domains upon proliferation, migration and apoptotic signalling in malignant mesothelioma cells
3) To investigate the effects of sFRP4 upon Wnt signalling induced by exogenous Wnt ligands
4) To investigate the effect of sFRP4 upon chemo sensitivity of mesothelioma cells
5) To identify and characterise the functional role of structural domains of sFRP4 protein in mesothelioma cells.

1.26 Structure of this thesis

This thesis is composed of 7 chapters. Apart from this chapter which gives context to the study there is one chapter containing all of the materials and methods used (Chapter 2) followed by 4 results chapters (3-6) and finally a chapter of discussion (Chapter 7). Since the overall experimental program was focussed and had a common theme discussion of the results is restricted to this final chapter.

CHAPTER 2

Materials and Methods

2.1 Cell culture and reagents

The malignant mesothelioma cell lines JU77, LO68 and ONE58 were used in this study. These cell lines were originally derived from pleural effusions of different patients presenting with malignant pleural mesothelioma (Manning et al., 1991). All cells were cultured and maintained in medium R5, which is RPMI 1640 plus 5% heat-inactivated foetal bovine serum (FBS), 300 mM L-glutamine, 120 µg/ml penicillin and 100 µg/ml gentamicin (all from Thermofisher Hyclone, Vic., Aust). All cell cultures were grown at 37°C in a 5% CO2 humidified atmosphere. Manning et al indicated that the characteristics of these cell lines remained stable despite repeated passaging of cells in culture. In this project, the cells from a limited range of passages were used to minimise changes in cell line properties that may occur with prolonged culture. These were JU77 (p89-100), LO68 (p116-128) and ONE58 (p50-60).

2.2 General Chemicals and Reagents

Cisplatin (Oncotain, Mayne Pharma, VIC, Australia) was used at a concentration of 0 to100µM/ml. The drug was diluted by 10-fold serial dilutions of the stock solution in R-5.

Recombinant proteins

Recombinant human Wnt 3a was obtained from R&D Systems (MN, USA).

Recombinant human sFRP4 was obtained from R&D Systems (MN, USA).

Transfection Reagent

FUGENE® HD reagent (Promega, NSW, Australia)

Antibodies

Primary

Monoclonal rabbit anti-human β-Catenin (Cell Signalling Technology, MA, USA) Polyclonal rabbit DVl-3(Cell signalling Technology, MA, USA) Monoclonal mouse anti- Actin (BD Transduction Laboratories, North Ryde, Australia)

Secondary

Goat anti-mouse IgG HRP conjugate (Jackson Immunosearch, PA, USA)

Goat anti-rabbit IgG HRP conjugate(Jackson Immunosearch, PA, USA)

2.3 MTT assay

Cell viability, following exposure to cisplatin or other treatments, was quantitated by the MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) assay. Cells were seeded into 96-well plates at a density of 10,000-20,000 cells/well, depending on experiment, in 100µl of media. Following 24h incubation, cisplatin or other treatments were added at concentrations depending on experiments and cells incubated for a further 24-72h (depending on experiments). The assay was terminated by aspirating the media, 100µl of 1mg/ml MTT in RPMI was added and the plates were incubated for 120 minutes at 37°C. The supernatant was removed, 100µl of DMSO (Chemo-Supply, Gillman, SA, Australia) was added to solubilize the dye, and the absorbance measured at 595 nm with a microplate reader (Enspire, Perkin Elmer).

2.4 Data analysis

For each treatment, the mean absorbance for the replicates was calculated. The control sample (untreated cells) was set to 100% viable cells and all other data were normalised to this value based on absorbance value. The data was expressed as mean ± standard deviation. Unpaired t-test was performed using Microsoft Excel:Mac 2004 (Microsoft Corporation, Redmond, WA, USA).

2.5 Proliferation

Cells were seeded at a density of 5 x 10^4 cells/well in 24 well plates and treatments added at 24 hours. The cells were harvested 48hrs later by trypsinization and evaluated by a trypan blue assay using a Countess Automated Cell Counter (Life Technologies).

2.6 Cell Migration Assay

Scratch wound assays are an established method for assessing cell migration especially when the duration of the experiment is relatively short (Liang et al. 2007). Scratch wound assays were undertaken to assess the effect of exogenous SFRP4 and endogenous SFRP4, the CRD or the NLD domain on mesothelioma cell migration. Six well plates were seeded with JU77 and ONE58 cells at 300,000 cells per well. The plates were incubated overnight. The next day the monolayer was scratched as a cross line by running a P10 pipette tip down the whole monolayer. The media were aspirated and the monolayers were washed once with 1 x PBS to remove any non-adherent cells and cell debris. All treatments and transfections were made up with growth media. The monolayers were imaged using a Nikon inverted microscope after 0 and 6 hours. The field of view was kept constant by drawing a line on the well bottom perpendicular to the centre of T scratch site and using this as a reference point. The area of scratch recovered by JU77 and ONE58 cell migration after 6 hours was quantified using Tscratch assay and the TScratch software (Gebäck et al, Biotechniques. 2009 46:265) to automatically analyse wound healing images.

2.7 Real-time RT-PCR

Total RNA was prepared from cell cultures using (MO BIO Laboratories –Spin Column USA) and contaminating DNA was removed using RQ1 DNAse (Promega, NSW, Aust). cDNA synthesis was carried out using a Superscript III first strand synthesis kit (Life Technologies). Gene specific PCR primers were designed using the Primer 3 software Table 1). Conventional RT-PCR was performed essentially as previously described. Real-time PCR was performed using a standard protocol from the Sensimix SYBR Kit (Bioline, NSW, Aust) and run on a ViiA™ 7 Real-Time PCR System | Life Technologies). Gene expression data normalisation and analysis

were performed using ViiA™ 7 Software (Life Technologies) and NormFinder programme. (Anderson et al, 2004 cancer research 64:5245).

2.8 Detection of the mitochondrial potential sensor JC-1

JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethyl-benzimidazolcarbocyanine iodide) is a catonic dye that exhibits potential dependent accumulation in mitochondria by fluorescence emission shift from green (~525 nm) to red (~590nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. Cells were seeded into black 96 well plates (Greiner, Germany) at a density of 10 000 cells/well in 100 µl R5. Following 24h incubation, cisplatin was added at concentrations from 0-100 µM and the cells were incubated for a further 24h. The media was then aspirated and 50 µl of staining solution added (33 µM JC-1 Invitrogen in serum free R-5), and the plates incubated for 1h at 37°C, 5% CO2. The staining solution was then removed and 200 µl of PBS with 5% BSA were added. After further 5-minute incubation, PBS/BSA was removed and 100µl PBS was added. The plates were read on a Fluostar Optima plate reader (excitation filter 485nm and emission filter 520nm) (BMG laboratories, VIC, Australia). Data is presented as the ratio of red to green.

2.9 Caspase-3 activity assay

Activation of effector caspases during apoptosis was determined using the Caspase-3 Assay Kit#2 (Molecular Probes, USA). With a protocol modified for a direct homogenous assay (3.1.4), cells were seeded into black 96-well plates (Greiner, Germany) at a density of 20,000 cells/well in 100µl medium. Following 24h incubation, cisplatin was added at concentrations from 0-100µM and the cells incubated for a further 24h. The assay was terminated by the addition of 100µl of reagent buffer (5µl 20x cell lysis buffer, 20µl 5x reaction buffer, 0.5µl substrate-Z-DEVD-R110, 0.5µl of 1M DTT, 74µl H_2O) and the fluorescent signal measured after 1h using a Fluostar Optima plate reader (BMG Laboratories, NSW, Australia). Appropriate blank and control samples were included in each run as recommended by the assay manufacturer.

2.10 Protein Extraction

For experiments of protein analysis 300,000 cells were plated into 6 well plates in growth media. The dishes were incubated for approximately 24 hours at 37^{o}C / 5% CO2. The media was removed and relevant transfection or treatment performed. The plates were incubated for the relevant time. Once treatment of cells was complete, cells were washed with PBS and lysed with 100ul of 1x SDS loading buffer (BioRad) containing 2x SDS loading buffer stock diluted in sterile water and 50ul/mL beta-mercaptoethanol (Sigma-Aldrich). Cell monolayer was disrupted using a pipette tip and each well of cells was transferred to a 1.5mL microfuge tube (on ice). Samples were sonicated (Misonix sonicators, QSonica, LLC) 3x at 40amp for 15 seconds, with intervals of 30 seconds. Samples were then centrifuged at 10,000 x g for 5min. After centrifugation, samples (20µl) were transferred to 0.6mL microfuge tubes and denatured at 95°C for 5min.

2.11 Western Blotting

Sample proteins were separated by size using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then immunoblotting was used to locate specific proteins and quantify their expression relative to the housekeeping protein β-actin. SDS electrophoresis was performed on 4% to 10% BioRad Mini Protean TGX gels using 1X Tris- Glycine Running Buffer. For β-catenin, Dvl-3 western blot analysis. Gel electrophoresis was performed according to the manufacturer's instructions (Biorad, CA, USA).

Gels were transferred to nitrocellulose membrane (Biorad) according to the manufacturer's recommendations. The transfer apparatus was then run for 60 min and 30V constant to transfer protein to the membrane. After protein transfer, the membranes were removed from the apparatus and washed twice with TBS-T. The membranes were stained with Ponceau S dye (Sigma Aldrich) for 5 minutes to verify consistent protein loading and then membranes were rinsed with distilled water and blocked.

2.12 Immunoblotting

The detection procedure for β-catenin Dvl-3 and β-actin (loading control).

Table 2.1 Immuno-detection protocol Step	**procedure**	**Duration and conditions**
Blocking	5% BSA diluted in 1X TBST	1 hour at room temperature
Primary antibody	1:1000 in 5% BSA diluted in 1X TBST	Overnight at 4^{o}C with gentle rocking
Washing	1% TBST	3 x 5 minutes
Secondary antibody	1:2000 in 1X TBST	10ml TBS/T for 1hr at RT
Washing	1X TBST	3 x 5 minutes
(ECL) Enhanced chemiluminescence	1:1 ratio of enzyme substrate	5 minutes at RT

2.13 Live cell imaging with Hoechst 33342

Live mesothelioma cells plated on 96-well plates treated with relevant treatment and transfection and at the end of the experiment the media was aspirated and, incubated with Thermo Scientific Hoechst 33342 Solution (Thermo Scientific) was added at 1µg/mL for 5 minutes before imaging the cells were washed with 1X PBS and fresh media added before imaging and imaged with the Olympus inverted microscope (Obj 10X).

Primer sequence for Wnt target genes

Table 2.2. Primers were purchased from Gene-Works (SA, Australia)

Gene		Primer Sequence 5'-3'	GenBank
Cyclin D1	F	AACTACCTGGACCGCTTCCT	NM_053056
	R	CCACTTGAGCTTGTTCACCA	
cMyc	F	CCTACCCTCTCAACGACAGC	NM_002467
	R	[illegible]	
LEF1	F	GACGAGATGATCCCCTTCAA	NM_016269
	R	AGGGCTCCTGAGAGGTTTGT	
APC	F	TGCGAGAAGTTGGAAGTGTG	NM_000038
	R	GATTTGCCTGTGGTCCTCAT	
Axin2	F	CTCCCCACCTTGAATGAAGA	NM_004655
	R	TGGCTGGTGCAAAGACATAG	
G3PDH	F	ACCACAGTCCATGCCATCAC	NM_002046
	R	TCCACCACCCTGTTGCTGTA	
hHPRT1	F	TGACACTGGCAAAACAATGCA	KJ891389.1
	R	GGTCCTTTTCACCAGCAAGCT	
hUBC	F	ATTTGGGTCGCGGTTCTT	AB009010.1
	R	TGCCTTGACATTCTCGAT	

2.14 Nanoparticle Encapsulation

Determination of particle size and zeta potential

Particles size of empty and loaded CS-DS nanoparticles was determined using photon correlation spectroscopy using a Zetasizer 3000HS (Malvern Instruments, Malvern, Worcestershire, UK). The measurements were performed at 25ºC and the raw data were correlated to Z average mean size using a cumulative analysis by the Zetasizer 3000HS software package. Zeta potential analysis was performed using small volumes of samples analysed by a Nano ZS (nanoseries, Malvern Instruments,

UK). The sample was fed into a clear disposable zeta cell with folded capillary (DTS 1060) using a syringe. Measurements were taken at 25°C and using the pre-set standard operating procedure (SOP), with a mean of 10 measurements for each analysis. All determinations were performed in triplicate.

2.15 Preparation of SFRP4 and domains constructs loaded CS-DS nanoparticles

CS-DS nanoparticles were prepared by the complex coacervation technique described previously (Y Chen, Basavaraj, Chan, & Benson, 2008; Sharma, Benson, Mukkur, Rigby, & Chen, 2013), employing biodegradable polymers of CS and DS which carry opposed charges in a weight ratio of 2:1. Briefly, CS-DS nanoparticles containing SFRP4 , SFRP4 CRD, SFRP4 NLD and pEGFP were prepared by drop wise addition of 100 µl of DS solution (0.1% w/v in Milli-Q water) into a 200 µl CS solution containing 100 µl solution of DNA mixed with 100 µl CS solution (0.2% w/v in 0.4% v/v acetic acid solution) in a glass vial with controlled stirring on a magnetic stirrer (IEC, Australia) with the speed maintained at level 6 at room temperature (~ 25 $^{\circ}$C). The mixture was stirred for 20 minutes before being used for any further studies. Empty nanoparticles were prepared in the same fashion without inclusion of SFRP4 and its constructs.

2.16 Entrapment efficiency of SFRP4 plasmid DNA in nanoparticles

The quantity of SFRP4 and its related constructs in the nanoparticles was determined by calculation of the difference between the total quantity of DNA added during the nanoparticle formation and the quantity of non-entrapped DNA remaining in the aqueous filtrate. The non-entrapped DNA was separated from nanoparticles following the centrifugation of nanoparticles at 3000 rpm (approximate 630 x g) for 10 min using filtration units (Vivaspin® 500, Cole-Parmer, USA) fitted with a membrane of 1 million molecule weight cut-off (MWCO). The filtrate which contains non-entrapped DNA was then analysed by Nanodrop 1000 (Thermo Scientific, Wilmington, USA). The filtrate of empty nanoparticles was used as the control/blank. The particles were washed twice and filtration was repeated in between each washing. The entrapment efficiencies of SFRP4 and its related constructs were calculated from the following equation:

$$\% \text{ Entrapment efficiency} = \frac{\text{Quantity added} - \text{Nonentrapped quantity}}{\text{Quantity added}} \times 100$$

2.17 Plasmid Constructs

Expression vectors for the human sFRP4 gene and the CRD and NLD domains were a kind gift from Prof. Roberts Friis, University of Bern, Switzerland. The sFRP4 constructs were prepared by PCR and cloning into the pEGFP-N1 vector (Clontech, CA, USA) so that the full length sFRP4, the CRD domain or the netrin C terminal domain were expressed in frame as amino terminal fusions to the GFP. The sFRP4, CRD and Netrin constructs retained the Kozak and signal sequences of sFRP4. The parental pEGFP-N1 vector expressing GFP was used as a control. Plasmid DNA for transfection was prepared using a HiSpeed Plasmid Midi Kit (Qiagen, Vic., Australia).

Chamber cover glass slide

8 well Lab Tek chambered #1.0 Borosilicate cover glass system w/cvr (ThermoScientific).

2.18 Transfection

Transient transfections were performed on JU77 and One58 cells using FUGENE® HD reagent and the pEGFP-N1 plasmid vector constructs. Cells were seeded at a density of 80% confluency in 250ul RPMI growth medium in 8 well Lab Tek chambered #1.0 Borosilicate cover glass system w/cvr (ThermoScientific) on the day of transfection. Following transfection with Reagent:DNA in a ratio of 3:1 for 24 hrs, a further 48 hrs of incubation was allowed for the protein to be expressed. The transfection reagent was removed from the cells and replaced with standard complete RPMI medium after 48 hrs. For the Wnt3a treatment group, 48 hrs post transfected cells in chambers were replenished with recombinant human Wnt3a (R&D systems) 250pg/mL in RPMI complete medium for 6 hrs and imaged under confocal microscope for imaging studies (Chapter 6) The GFP-only vector was also expressed to act as a control. Transfected JU77 and One58 cells expressing GFP-fusion protein fluorescence were measured using a Nikon-A1+ confocal microscope (Nikon, Tokyo, Japan with 488nm laser point scanning). For studies described in Chapter 4

cells were transfected as above except that the appropriate culture vessel was used (ie 96 well microplate or 6 well plate) and then treated as outlined in the relevant figure legend.

2.19 Confocal live cell imaging and Number and Brightness analysis

Number and brightness (NB) analysis was performed on the transfected samples and measured by the time series of 100 frames of 256 × 256 pixels, obtained with a HV 96, offset-8 and laser 1.0 setting was used to obtain barely bright enough, best focus, and visible images to see the same GFP fluorescence for all the groups using a scan speed of 1/2. The pixel size was 50 nm with a pixel dwell time of 23.5 ìs, and the pinhole was set at 33.0 ìm. These image stacks were analyzed using SimFCS software (Laboratory for Fluorescence Dynamics, University of California, and Irvine CA). The detector calibration was obtained from 100 frames of background image taken before and after the experimental image stacks using the exact same settings but with the laser turned off as described previously (Dalal et al. 2008). Brightness (B) of fluorescence particle, B= 1 values represent the immobile fraction of the image, and B> 1 values represent the mobile fraction (Dalal et al. 2008). To obtain the molecular brightness in photons/molecule/s, the B= 1 value was divided by the pixel dwell time. The B vs intensity plot was analysed using two cursors. A red cursor selects all the pixels of the image that have B values between 1 and 1.5 and paints the pixels of the image in red. A second green cursor was used to select these pixels that have B values between 1.5 and 4 which were painted in green. There are essentially no pixels with B values above 4. Therefore the B images show in green the regions of the cell where larger aggregates form (larger B values). For each image the fraction of green and red pixels was also calculated. Changes upon stimulation of Wnt3a were also calculated by normalizing the difference of green pixels in the image before and after Wnt3a activation by the total number of pixels above a minimum of intensity threshold.

CHAPTER 3

Effect of exogenous sFRP4 and Wnt3a on mesothelioma cells

3.1 Introduction

Previous studies have shown that SFRPs in general and SFRP4 in particular are down regulated in mesothelioma tissue and cell lines (Kohno et al., 2010; Lee, He, You, Dadfarmay, et al., 2004). These studies also demonstrated promoter methylation of SFRP4. Mechanistic studies of SFRP4 have been carried out in mesothelioma cell models showing induction of apoptosis and growth inhibition (He et al, 2005). However this study uses a mesothelioma cell line which was β- catenin deficient and canonical signalling not present (He et al., 2005). Most mesothelioma tumours and cell lines have been found to express β-Catenin (Abutaily et al., 2003 ; Kohno et al., 2010; Orecchia et al., 2004). In our laboratory we have previously investigated SFRP expression in our mesothelioma cell models and found SFRP4 in particular down regulated (Fox et al., 2013). These cell lines express β-Catenin and therefore more accurately reflect most mesotheliomas. We have also shown that conditioned media from SFRP4 over expressing cells suppressed growth in 2/3 mesothelioma cell lines. At the time of those studies recombinant SFRP4 was not available to us. The first aim was to characterise the effect of purified recombinant SFRP4 and Wnt3a in mesothelioma cell models as this has not been reported. We examined the effect of this molecule alone and in combination upon cell proliferation, morphology and migration. To further understand the effects of these proteins on biochemical and morphological changes associated with cell death were characterised. The effects upon signalling were also assayed by western blotting for Wnt signalling proteins and real-time PCR of target genes.

3.2 Effect of exogenous SFRP4 and Wnt3a on Mesothelioma cell proliferation.

Based upon previous studies in our laboratory which showed that SFRP-4 conditioned media downregulated mesothelioma cell proliferation, initial experiments were conducted to examine the effects of purified recombinant SFRP-4 upon these cells. Recombinant Wnts has not been previously examined on these cell lines and so this was also tested. In our collaborators laboratory it had been found that optimal inhibition of proliferation and apoptosis induction occurred at picogram concentrations of SFRP-4. It was found that recombinant SFRP4 did in fact cause inhibition of cell viability/proliferation in JU77, ONE58 and LO68 cells at concentration of 250pg/mL (Figure 3.1). The effect of Wnt3a was also determined in a similar experiment and interestingly it was found that while 250pg/mL at recombinant Wnt3a did appear to up regulate proliferation at concentrations above 1ng/ml there was an inhibition (Figure 3.2) As a result for further experiments the concentrations of 250pg/mL were used for both SFRP-4 and Wnt3a. To further investigate, a trypan blue assay was used to confirm and extend these findings. These experiments showed that Wnt3a significantly up regulated proliferation in both JU77 and ONE58 (Figure 3.3). Also SFRP4 inhibited proliferation in all these cell lines and was able to block the effects of Wnt3a (Figure 3.3).

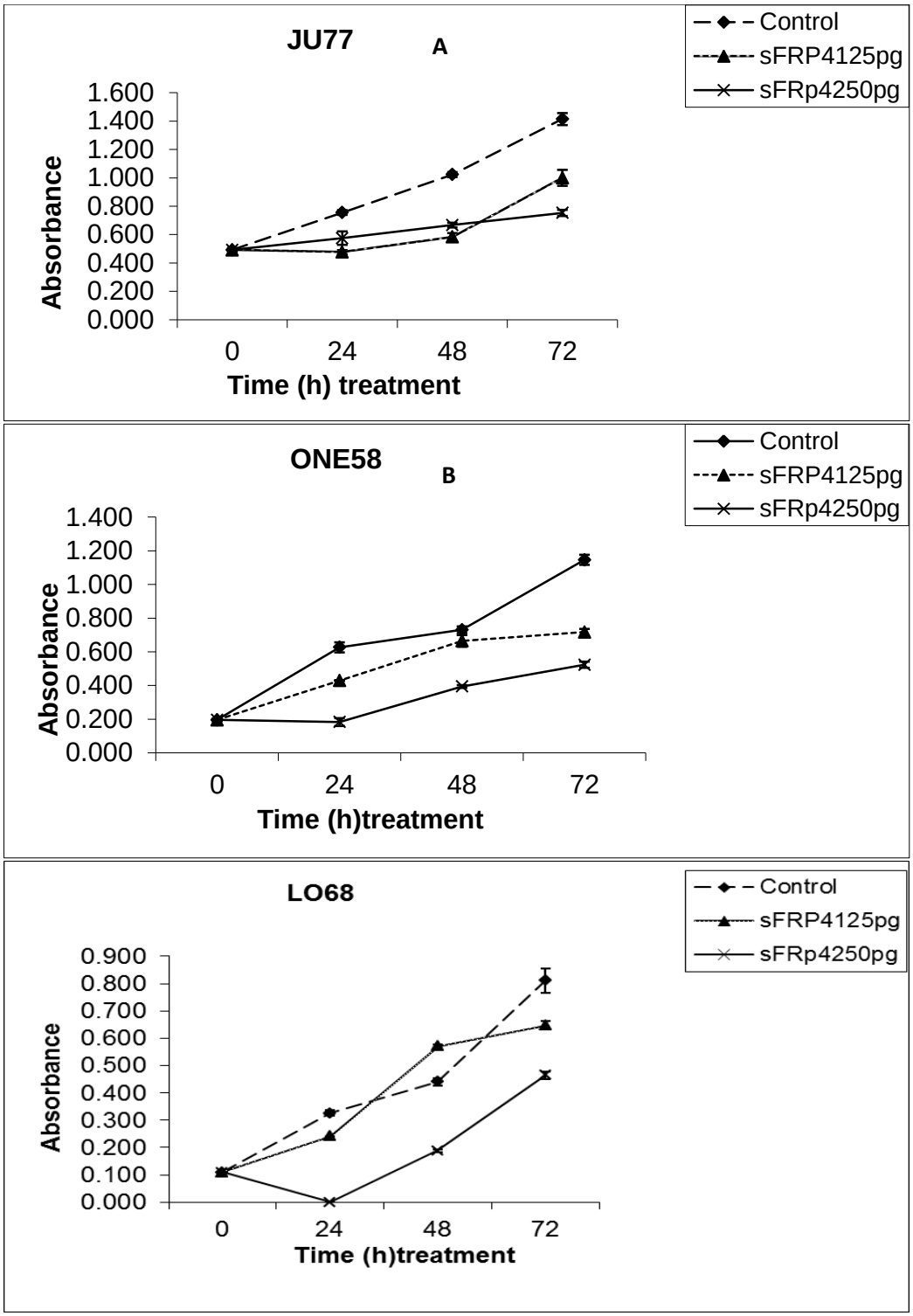


Figure 3.1 Effect of recombinant sFRP4 on mesothelioma cells. JU77, ONE58, LO68 cells were treated with sFRP4 125pg/mL or sFRP4 250pg/mL, and assayed for proliferation by MTT assay at 0, 24, 48 and 72 hours. Data absorbance was measured at 595nm

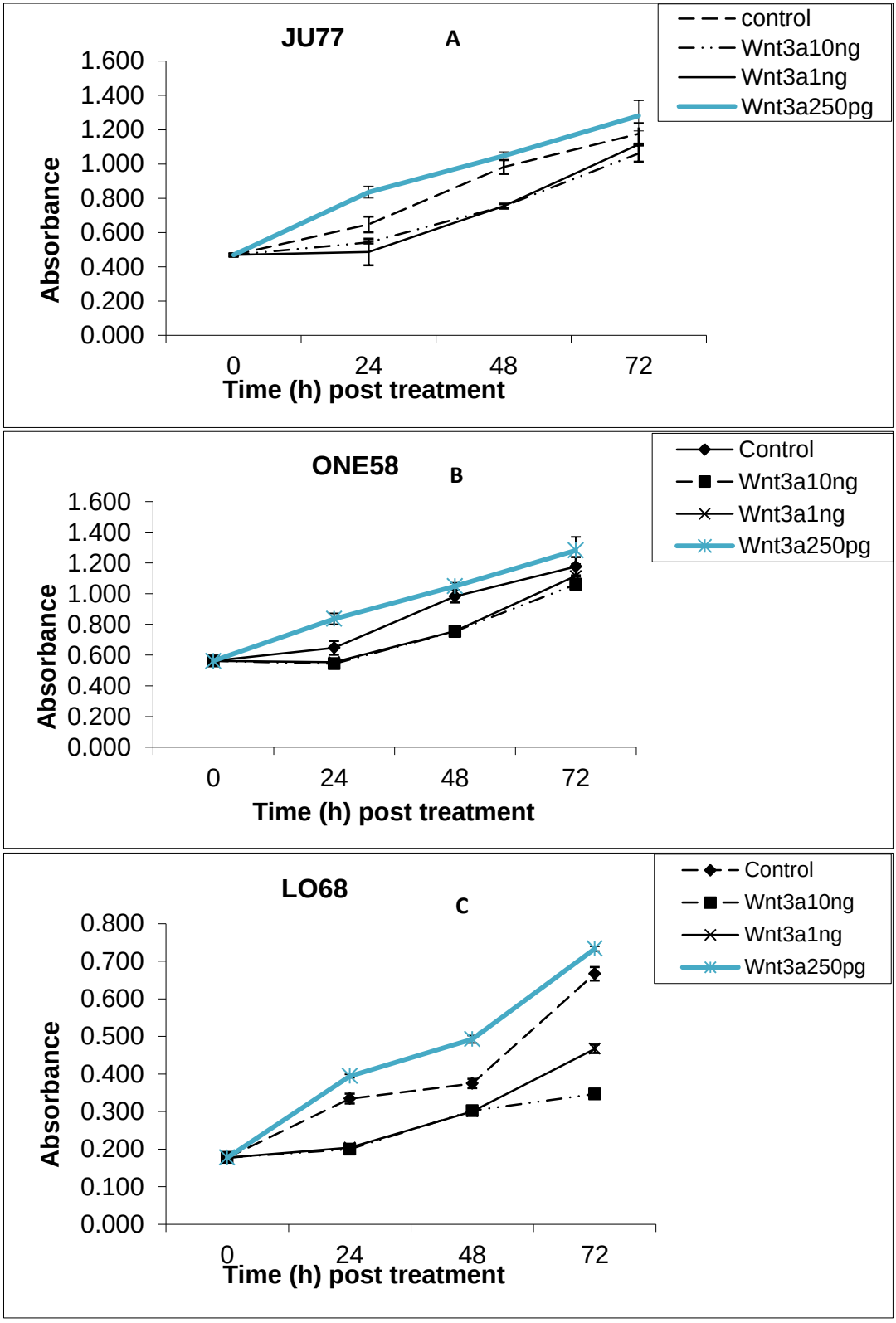


Figure 3.2 Effect of recombinant Wnt3a on mesothelioma cells. JU77, ONE58, LO68 cells were treated with Wnt3a 250pg, 1ng, 10ng/mL and assayed for proliferation by MTT assay at 0, 24, 48 and 72 hours. Data absorbance was measured at 595nm.

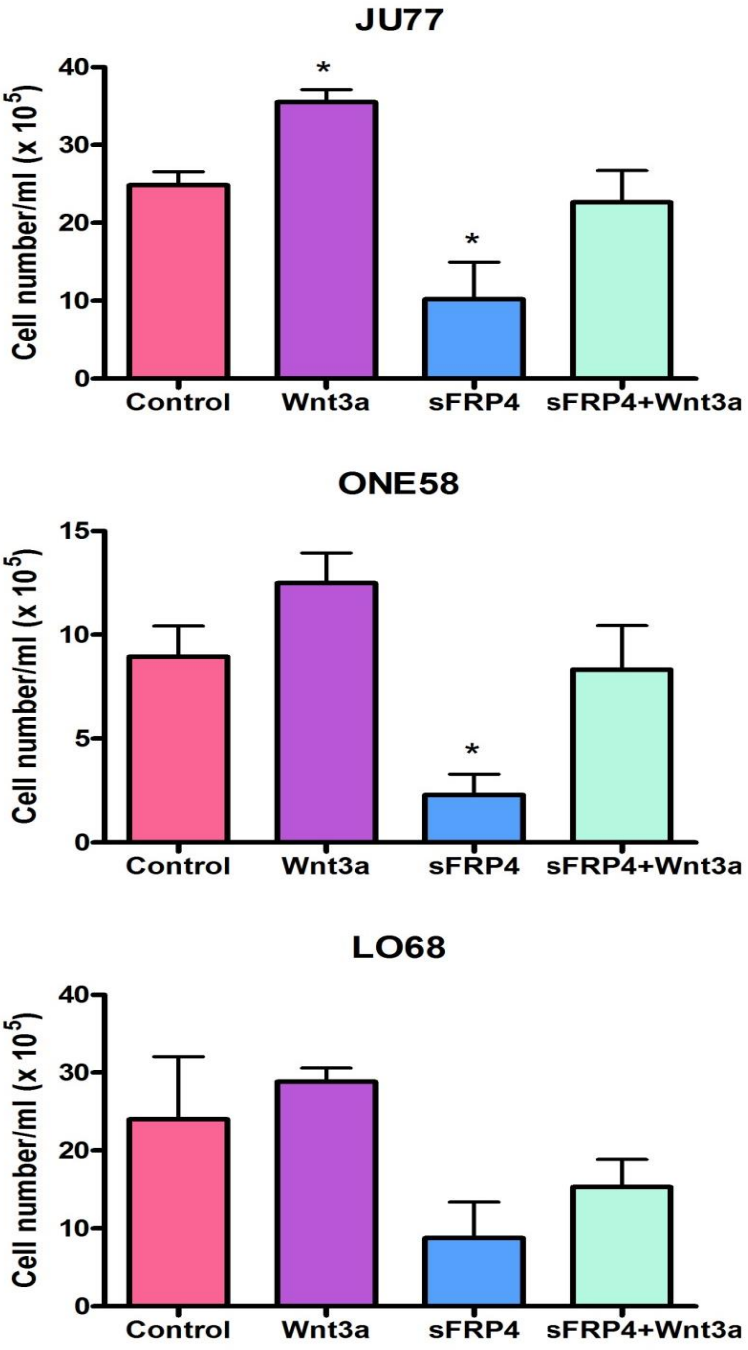
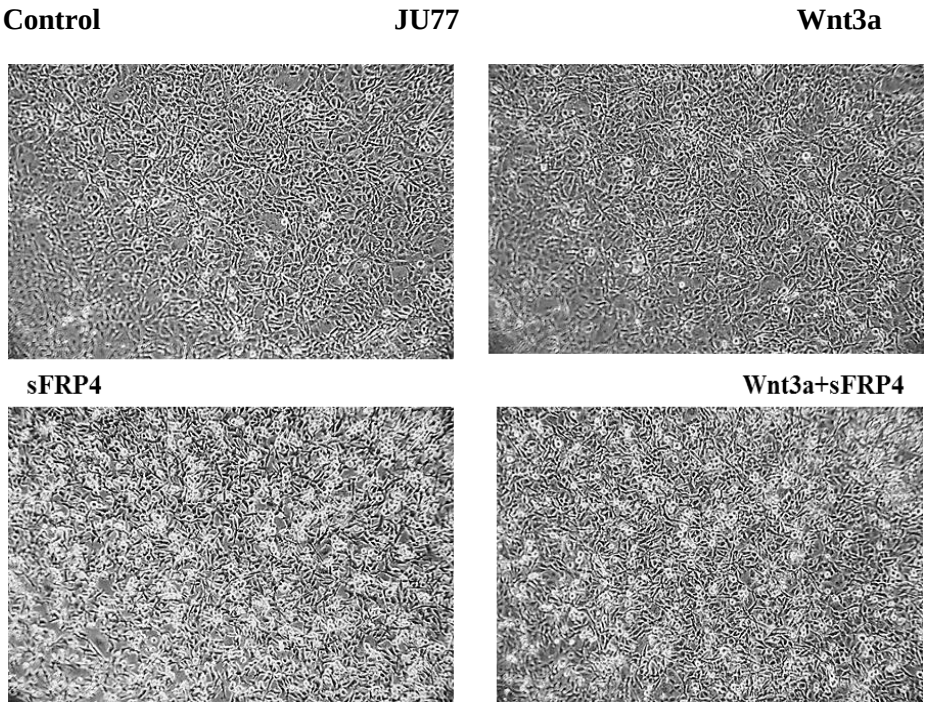
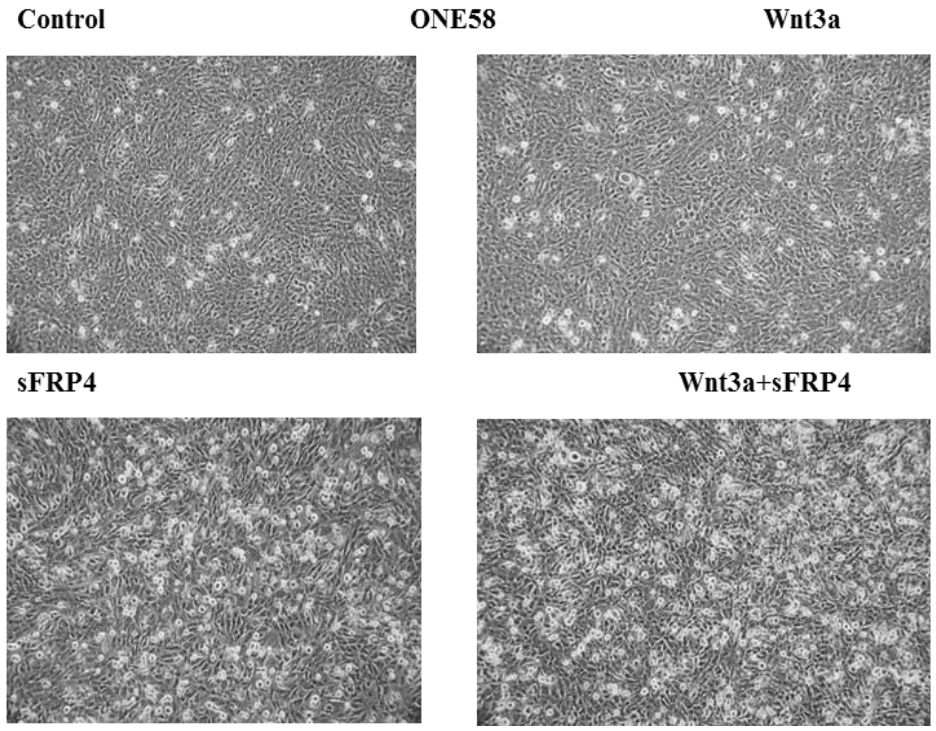


Figure 3.3 Effect of recombinantWnt3a 250pg and sFRP4 on mesothelioma cell viability. Cultures were treated with 250pg/mL Wnt3a or sFRP4 or combination for 48 hrs and viability determined by trypan blue assay.(A) JU77 (B) ONE58 and (C) LO68. Data is mean (SD),*$p<0.05$).

3.3 Effect of SFRP-4 on cell morphology

To further investigate the effects of SFRP4 upon cell proliferation/viability cell cultures been treated with SFRP4 and Wnt3a were observed for microscopic appearance. In all three cell lines treatment with SFRP4 had significant morphological effects upon the cultures (Figures 3.4). Many cells showed a characteristic loss of attachment and rounding up appearance after SFRP4 treatment compared to control untreated cells. Combined SFRP4 and Wnt3a treated cells also showed moderate characteristic loss of attachment and rounding up appearance compared to SFRP4 alone treated cells. As expected Wnt3a induced cell proliferation and maintained intact cell morphology similar in appearance to the control cultures. These findings showing loss of attachment and rounding up of cell morphology suggested that as has been found in other cell types SFRP4 was inducing cell death rather than just inhibiting cell proliferation.





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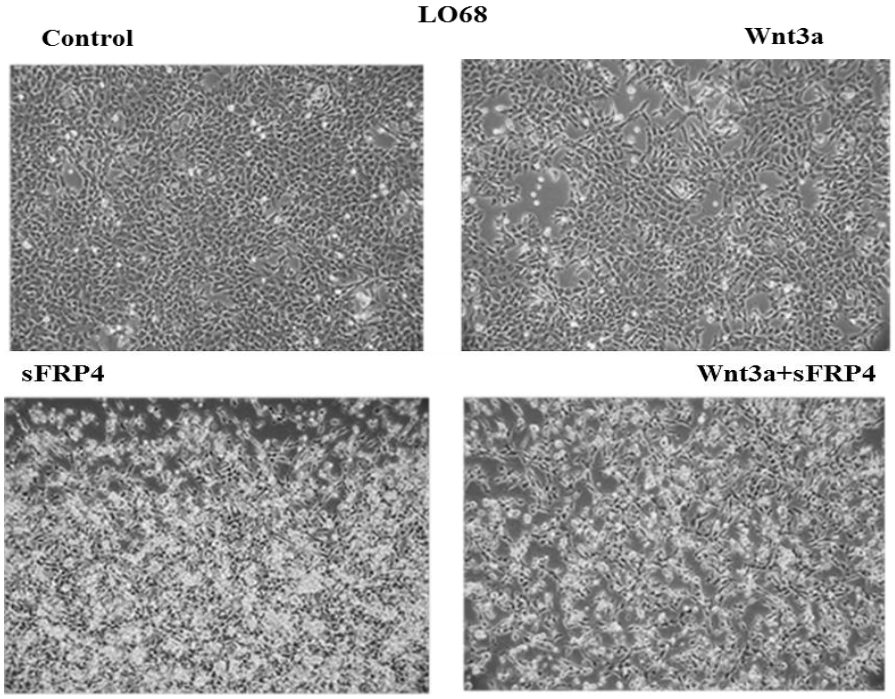
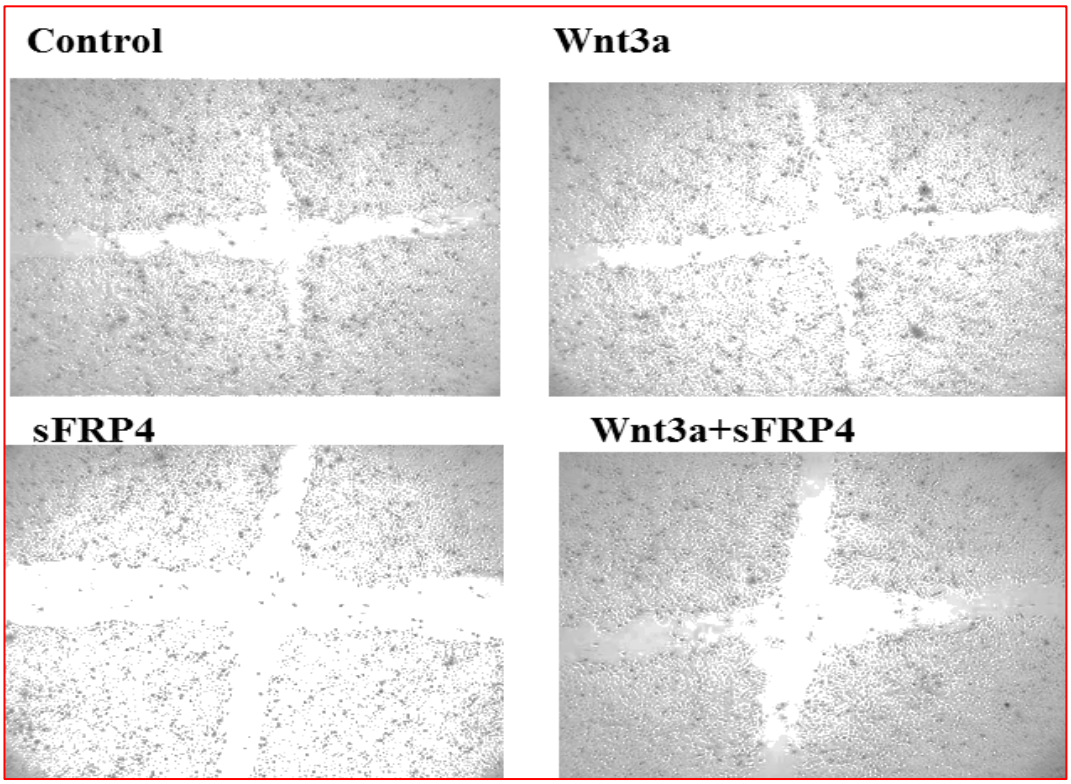


Figure 3.4 Effect of recombinant Wnt3a and sFRP4 upon mesothelioma cell morphology. JU77, ONE58 or LO68 cells were treated with 250pg/mL of Wnt3a, sFRP4 or combination and images at 48 hrs using Nikon inverted microscope.

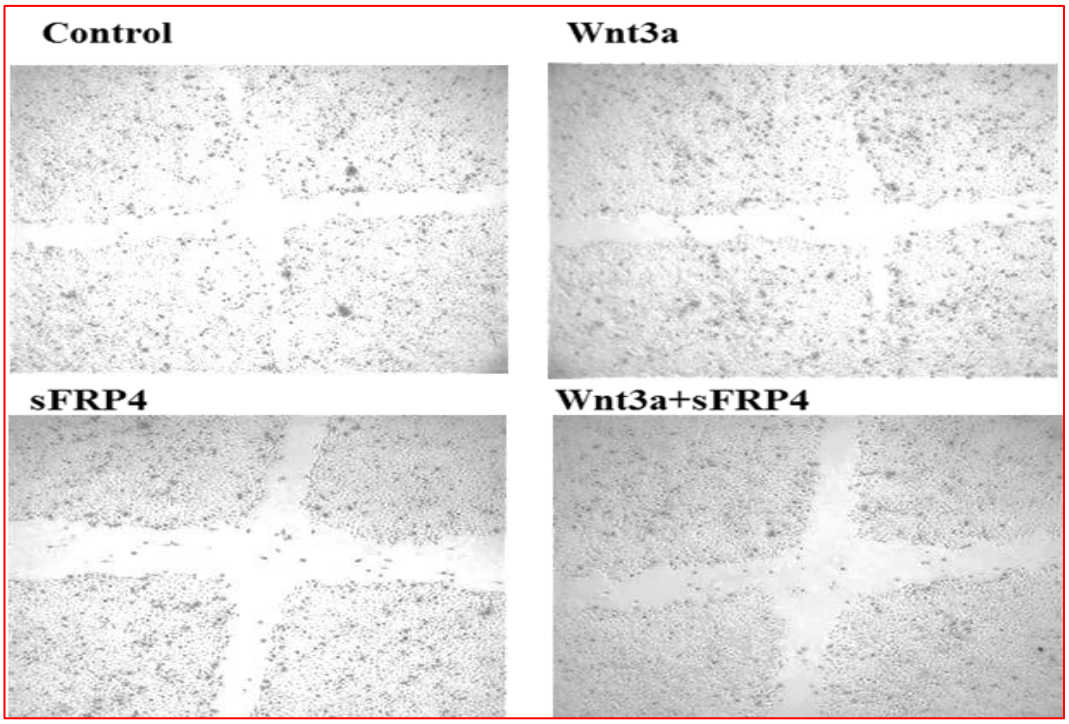
3.4 Effect of SFRP4 on migration of mesothelioma cells

Previous studies have suggested that SFRPs can affect migration in cancer cells (Roth et al 2000). Therefore the effect of SFRP4 and WNT3a on the migration of malignant mesothelioma cells was investigated using a scratch or wound healing assay. Since the migration of these cell lines has not been studied before in our laboratory preliminary experiments were conducted to optimize cell seeding, culture conditions and timing for each cell line (results not shown). Initial experiments using single wounds proved quite problematic due to variability in measurements. However, eventually the automated image analysis method was adopted (Gebäck, Schulz, Koumoutsakos, & Detmar, 2009). Overall both JU77 and ONE58 were quite rapidly able to close wounds in the monolayer. While LO68 required much longer incubations (Figure 3.5). In all three cell lines sFRP4 appeared to inhibit wound closure (Figure 3.5) and this result was significant as confirmed by open wound area image analysis (Fig.3.6). Interestingly, Wnt3a had little effect upon wound closure in JU77 and ONE58 (Figure 3.6 a, b) cells although a significant effect was seen in LO68 (Figure 3.6c). Also observed was that in JU77 and ONE58 where Wnt3a had little effect on its own in , it did appear to reduce the effect of sFRP4 (Figure3.6) Reference:(Gebäck et al., 2009).

JU77



ONE58



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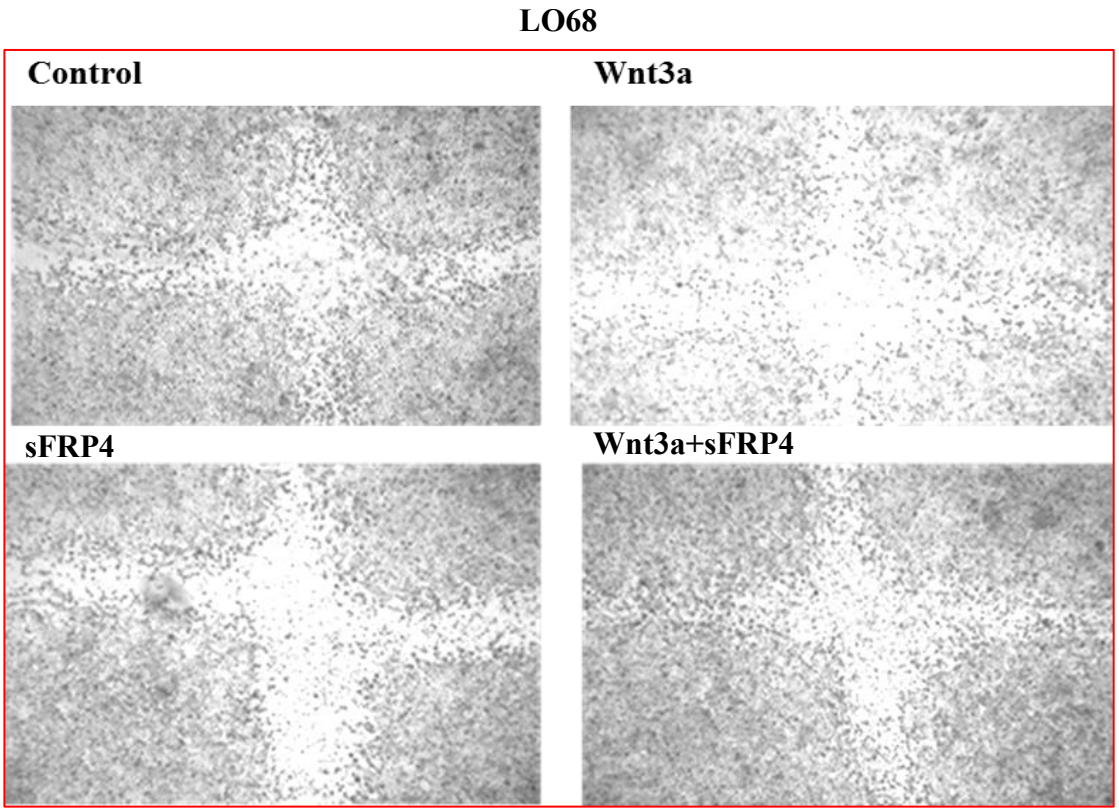


Figure 3.5 Effect of recombinant Wnt3a and sFRP4 upon mesothelioma cell migration JU77, ONE58 or LO68 cells were treated with 250pg/mL of Wnt3a, sFRP4 or combination and images at 48 hrs using Nikon inverted microscope.

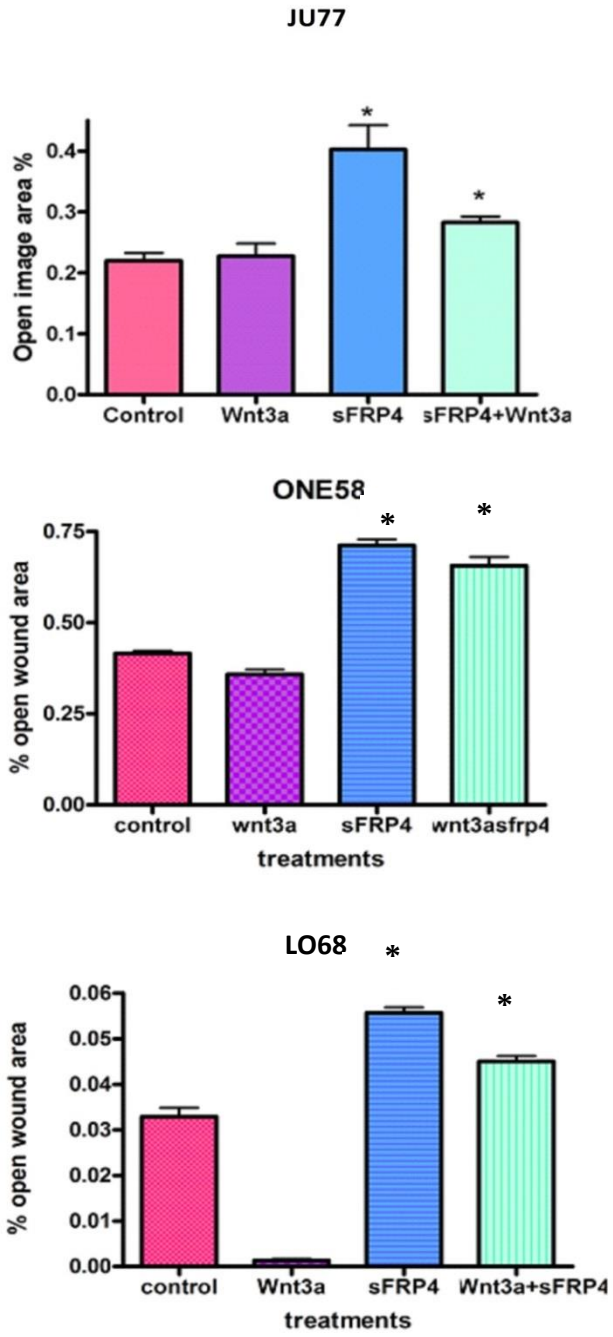


Figure 3.6 Effect of recombinant Wnt3a and sFRP4 on mesothelioma cell migration. (A) JU77, (B) ONE58 and (C) LO68 cells were analysed by scratch assay following treatment with 250pg/mL and Wnt3a, sFRP4 or combination for 4 hrs (JU77, ONE58) and 72 hrs (LO68). Analysis by Tscratch software. Mean (SD)*$p<0.05$)

3.5 Effect of exogenous sFRP4 treatment upon cisplatin sensitivity of Mesothelioma cells

Cells were treated with 0.001μM to 100 μM cisplatin for 48 hours with and without 250pg/mL recombinant sFRP4 and assayed for cell proliferation using MTT. As shown in Figure 3.7 sFRP4 did sensitise both JU77 and ONE58 cells to cisplatin. In JU77 cells the effect was greater compared to ONE58 even at lower concentrations of 0.001 to 0.1μM cisplatin. This was especially noticeable at cisplatin concentrations of 1 and 10uM. At 1uM cisplatin and for the combination of cisplatin and sFRP4 there was approximately 30% less viable cells (as measured by MTT) compared to cisplatin alone in both JU77 and ONE58 cells. This data is supportive that the Wnt pathway inhibition by sFRP4 may sensitise mesothelioma cells to chemotherapeutic agents.

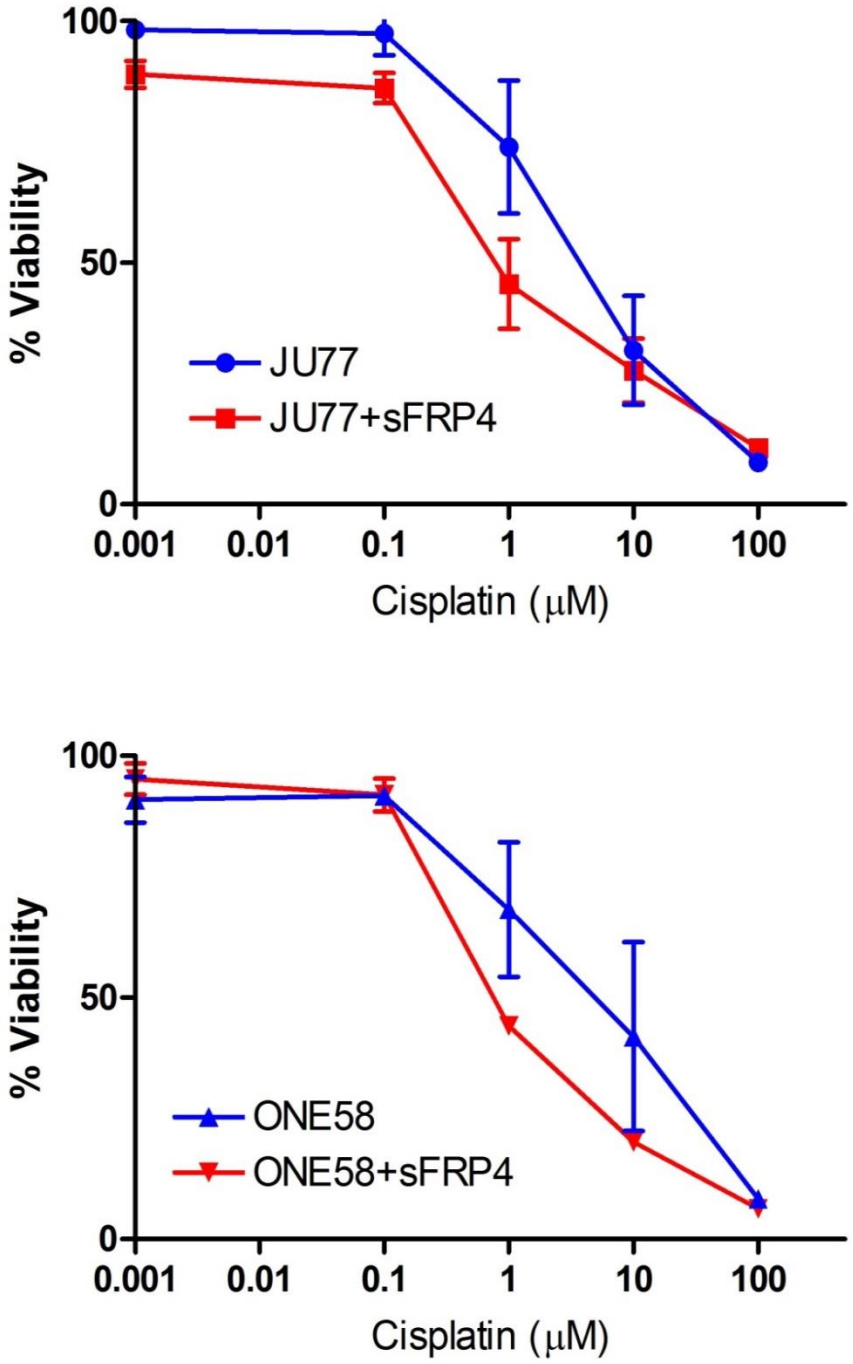


Figure 3.7 Effect of sFRP4 250pg/mL on cisplatin sensitivity in Mesothelioma cells. Cells were treated with 0.001μM to100 μM cisplatin for 48 hours with and without 250pg/mL recombinant sFRP4 and assayed for cell proliferation using MTT.

3.6 Mechanisms of sFRP4 induced cell death in mesothelioma cells.

To examine in more detail the cytopathic effect of sFRP4 observed in cell viability and morphology assays experiments were carried out to characterize cell death mechanisms in response to sFRP4. These and all subsequent experiments were carried out in JU77 and ONE58 cells for practical reasons since although sFRP4 and Wnt3a did affect LO68 this cell line was generally slow growing and more difficult to work with. Using a caspase 3 assay we measure a caspase 3 activation in response to sFRP4 or Wnt3a and we did not see any significant effect (Figure 3.8). An important event in apoptotic signalling is disruption of the mitochondrial membrane and loss of the membrane potential. Cells were treated with various treatments and assayed after 24 hrs using the fluorescent mitochondrial depolarization and there was a modest statistically significant decline in all treated cultures. However, this did not approach the level of the positive control decouplings agent FCCP (Figure 3.9)

Cells undergoing apoptosis show characteristic changes in nuclear morphology with chromatin condensation and nuclear fragmentation. The nuclear morphology of JU77 and ONE58 cells was observed by Hoechst 33342 Staining after 48 hrs of treatment (Figure 3.10). Interestingly, in cells treated with sFRP4 characteristic apoptotic changes were not seen although there were distinctive changes in nuclear morphology. These changes of multinucleated cells with chromosome vesicles were characteristic of mitotic catastrophe (Vakifahmetoglu, Olsson, & Zhivotovsky, 2008). These changes were consistently observed in both JU77 and ONE58 cells in response to sFRP4. Apoptotic nuclei were not observed.

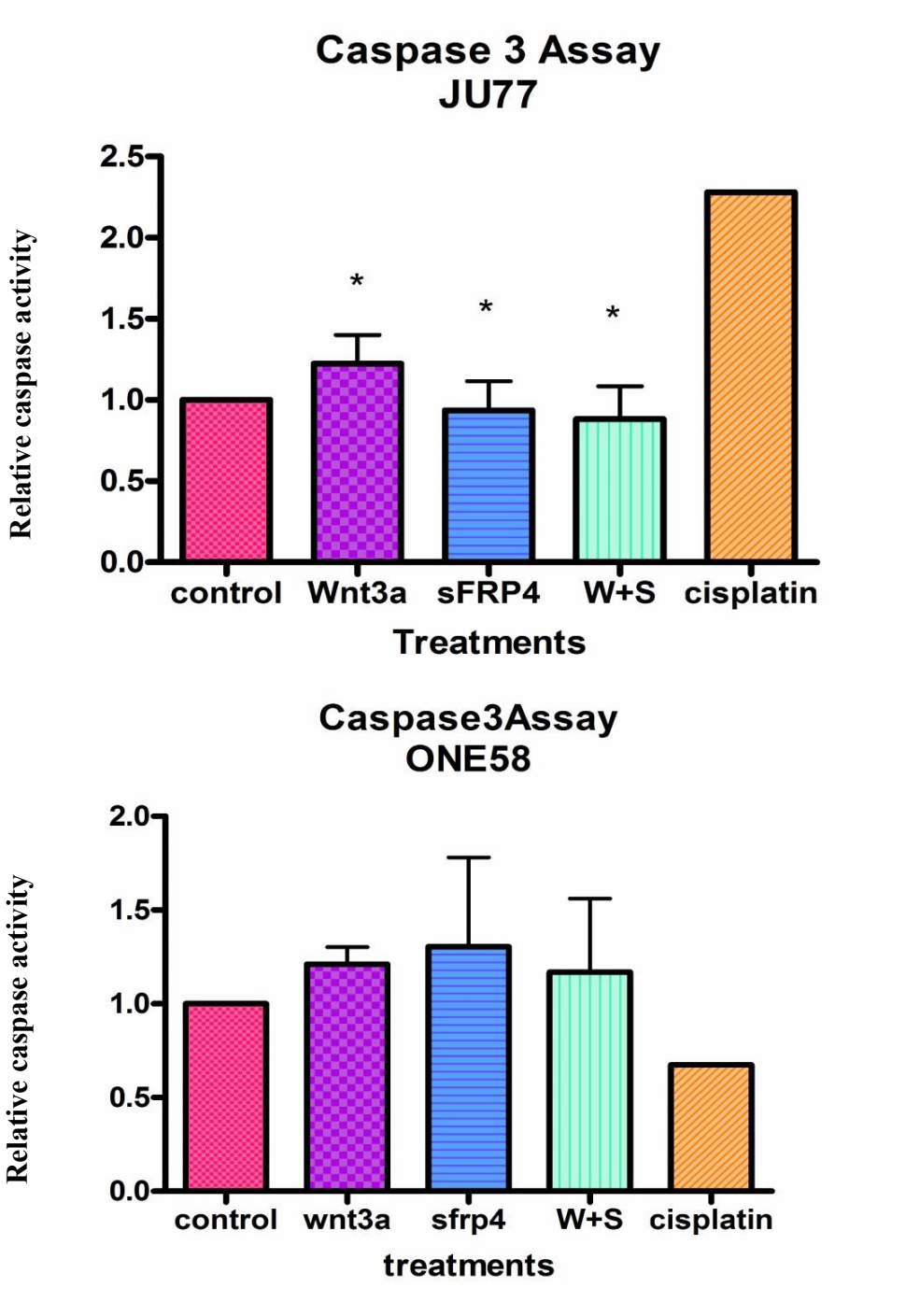


Figure 3.8 Assay of caspase activation in response to recombinant Wnt3a and sFRP4. Caspase activity was measured in (A) JU77 and (B) ONE58 following 24 hour treatment with 250pg/mL Wnt3a, sFRP4 and combination, Cisplatin (10µM). Data is fold increase in fluorescence relative to untreated cultures. Mean (SD) of two independent experiments

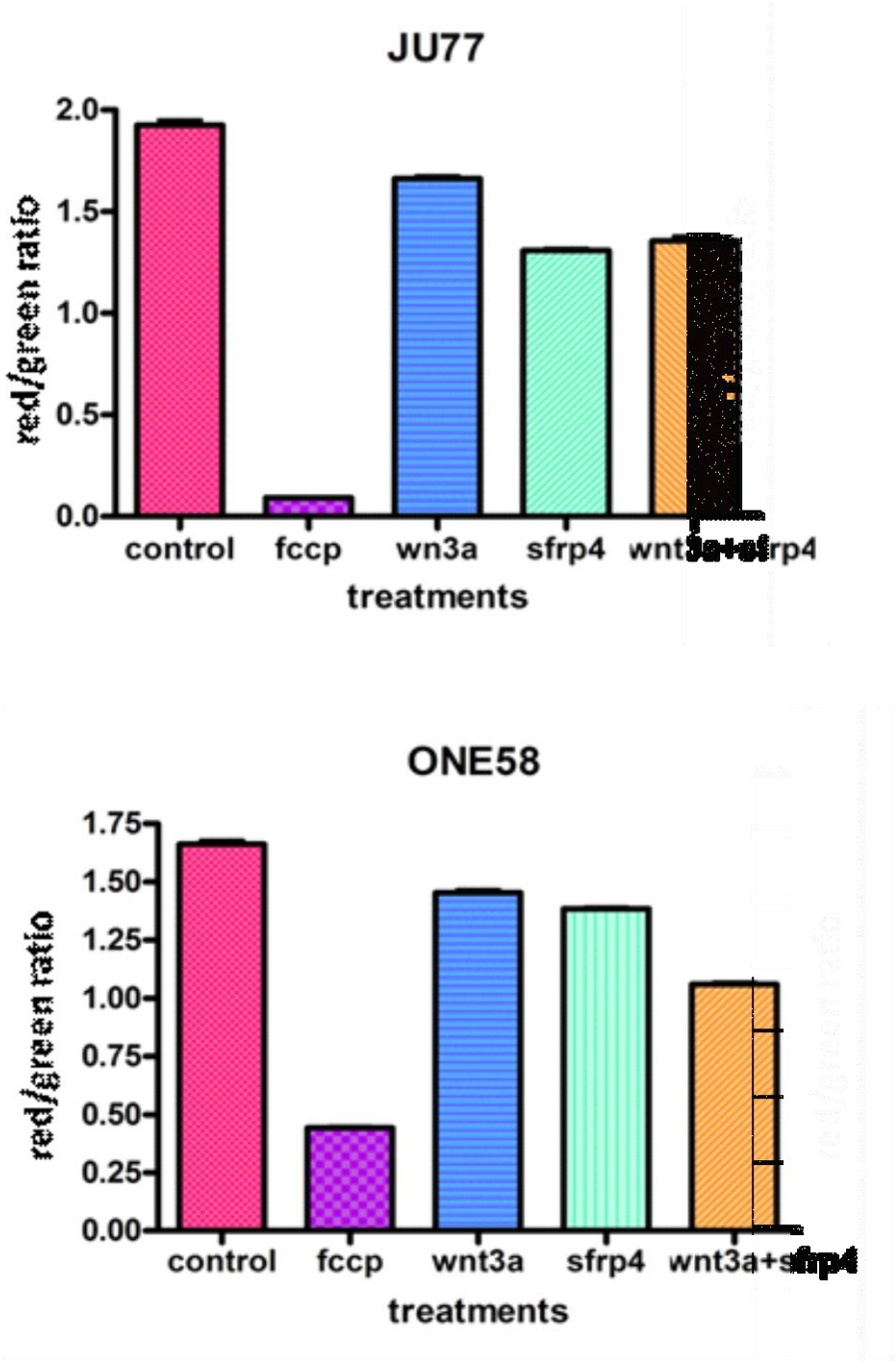


Figure 3.9 Effect of sFRP4 upon mitochondrial membrane integrity in mesothelioma cells. (A) JU77 and ONE58 cells were treated with 250pg/mL Wnt3a, sFRP4 and combination for 24 hrs and mitcochondrial depolarisation measure by JC1 accumulation. Results are mean (SD) of at least 3 independent experiments. FCCP-positive control.

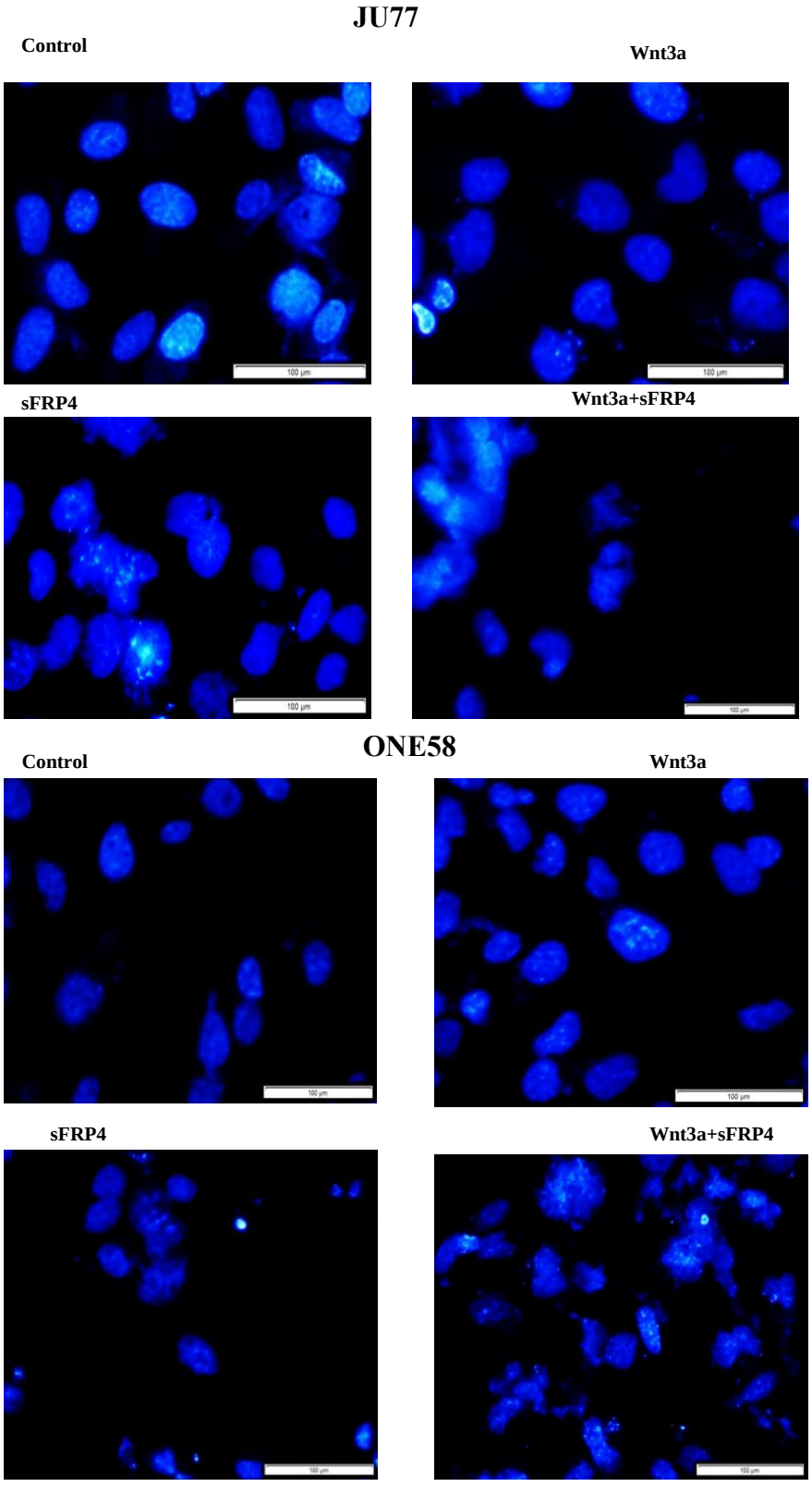


Figure 3.10 Effect of Wnt3a 250pg/mL and sFRP4 250pg/mL on nuclear morphology by Hoechst staining on JU77 and ONE58 cells showing apparent clusters misaggregated chromosomes. Nuclear morphology assessed by Hoechst 33258 staining after 48 hrs.

3.7 Wnt signaling in response to sFRP4.

To further understand the effects of sFRP4 upon mesothelioma cells β-catenin and Dvl-3 were assayed by western blotting. As shown in Figure 3.11 in both JU77 and ONE58 Wnt3a treatment upregulated β-catenin levels (Figure 3.11). While in JU77 sFRP4 downregulated β-catenin this is was not observed in ONE58 although basal β-catenin levels were quite low in this cell line. Similarly, in JU77, Wnt3a induced apparent Dvl-3 phosphorylation while sFRP4 down regulated Dvl-3. Surprisingly, these effects were not seen in ONE58 despite other assays showing similar responses to sFRP4 in both cell lines.

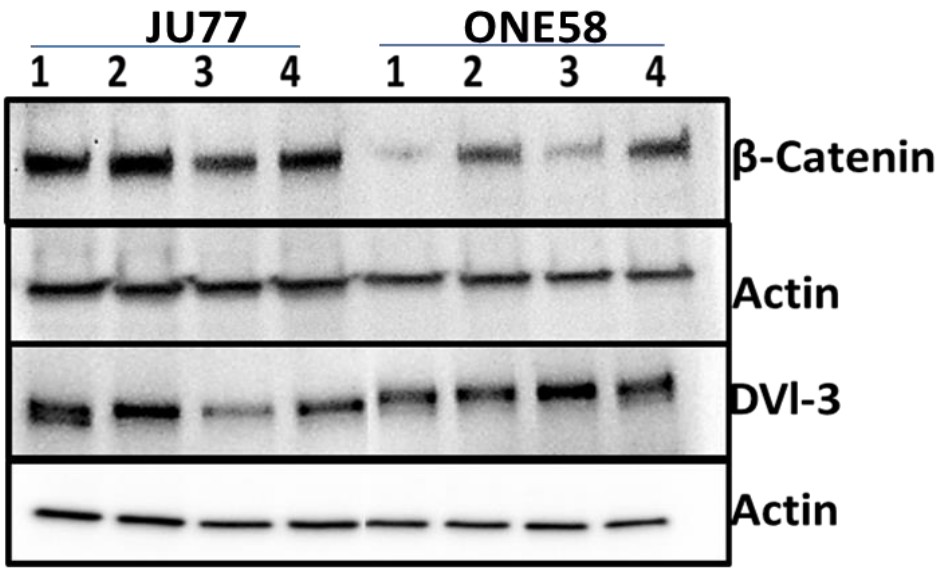


Fig 3.11 Wnt signalling proteins in response to sFRP4.
Representative immunoblots showing β-catenin and Dvl-3 in cell lysates treated with 1.Control. 2. Wnt3a (250pg/mL) 3.sFRP4 (250pg/mL) 4.Wnt3a+sFRP4 (250pg/mL) for 6 hrs.

3.8 Changes in gene expression in response to sFRP4 treatment.

To further investigate as studied by Carmon and Loose (2010) the effects of sFRP4 on mesothelioma cells gene expression of previously described, canonical Wnt target genes were assayed. The rationale for looking Axin2, APC, LEF1, cMYC and Cyclin D1 is because they are the target genes for canonical Wnt signalling.

Cultures were treated for 24 hrs with 250pg/mL sFRP4 and then relative gene expression changes were measured by real time PCR. Overall changes in gene expression observed were unexpectedly small. Neither Axin2, APC nor LEF1 mRNA levels were changed by sFRP4. (Figure 3.12) The expression of cMYC were upregulated in JU77 but down regulated in ONE58. Surprisingly, Cyclin D1 was upregulated in both cell lines (Figure 3.12)

Based on our findings and others studies we know the canonical Wnt signalling plays an important role in mesothelioma cells. The effect may vary due to basal expression of endogenous sFPR4 in the cell and may be cell specific.

Gene expression data did not reveal many changes consistent with activation of canonical signalling. There are a number of possible reasons behind this including that these may not be the appropriate target genes in these cells and also that the timing of expression changes can vary.

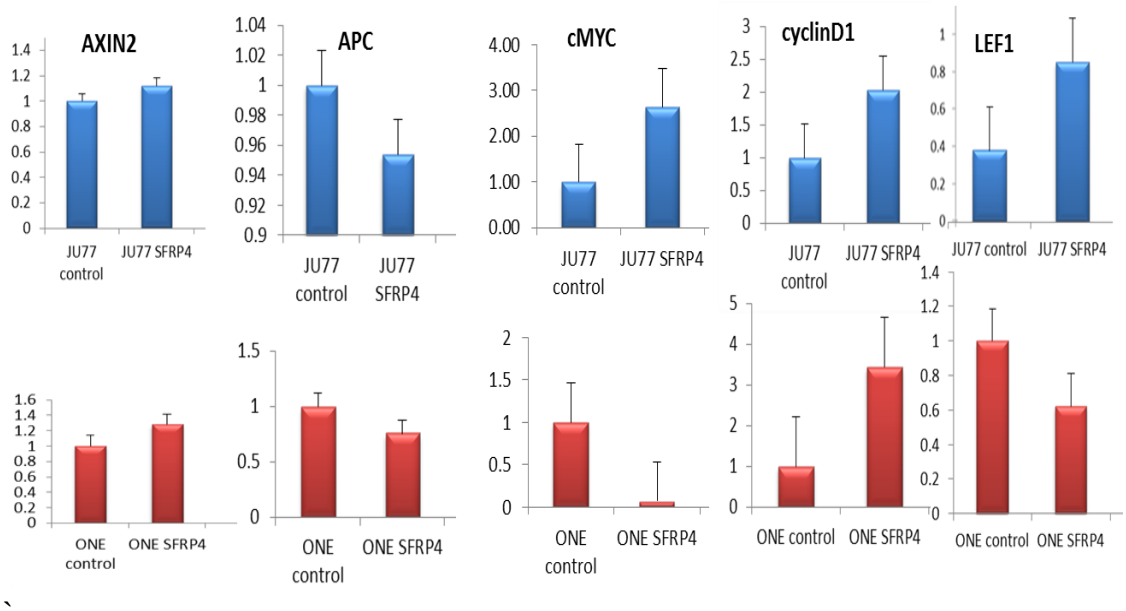


Fig 3.12 Effect of recombinant sFRP4 on Wnt target gene expression.

Basal gene expression of Axin2, APC, cMYC, cyclinD1, LEF1 in JU77 and ONE58 cells. Total RNA isolated from cells were analysed by 2-step real time RT-PCR using gene specific primers. Basal gene expression is expressed as mRNA levels relative to the respective control cultures following normalisation by reference gene expression. Results are mean ± SD of three biological experiments.

3.9 Conclusion

The results of this chapter experiments were sFRP4 both down regulated mesothelioma proliferation and antogonised the effects of Wnt3a alone. Interestingly, the dose response study of Wnt3a revealed a biphasic response in proliferation. The microscopic observation of sFRP4 treated mesothelioma cell lines showed cell rounding up morphology. However, further investigation of caspase activation and mitochondrial membrane potential (MMP) failed to show clear evidence of apoptosis although there was moderate reduction in MMP. The findings of nuclear morphology were also not consistent with apoptosis. Actually they were most similar to the changes in the nuclear morphology seen in mitotic catastrophe. Migration of mesothelioma cells was greatly inhibited by sFRP4 as demonstrated by the wound healing assay data. Interestingly, Wnt3a had little effect upon migration as a single agent. It did, however reduce the effect of sFRP4 on migration especially in JU77 cells. This suggests that what sFRP4 is interacting with to have its effect on migration may be competed by Wnt3a. When the effect of sFRP4 on cisplatin sensitivity was determined, it was also found that sFRP4 increased sensitivity to the drug.The results for western blotting were consistent with sFRP4 down regulating canonical signalling especially in JU77 which was generally the most responsive cell line in the experiments. Surprisingly, gene expression data did not reveal many changes consistent with activation of canonical signalling. There are a number of possible reasons behind this including that these may not be the appropriate target genes in these cells and also that the timing of expression changes can vary.

CHAPTER 4

Effect of endogenous sFRP4 and overexpression of sFRP4 and its domains on mesothelioma cells

4.1 Introduction

The experiments described in the previous chapter showed SFRP4 could inhibit mesothelioma cell proliferation and migration. These results influenced us to further investigate the mechanisms by which SFRP4 had these effects. As with other members of the family SFPR4 is comprised of two domains: a cysteine rich domain (CRD) and a netrin like domain (NLD). The role of individual domains in the function of SFRPs is unclear as evidence in the literature is conflicting. Some studies have suggested that the CRD interacts with Wnts and based on its structural similarities with the CRD of Fzd receptors acts to inhibit Wnt binding to the Fzds (Lin etal, 1997). Other studies have shown that NLD is able to bind to Wnts and inhibit Wnt signalling (Bhat et al., 2007 ; Lopez-Rios et al., 2008). With regard to SFRP4 the evidence is limited about the CRD and NLD.

In the only relevant study of endothelial cells, it was found that SFRP4 NLD may induce apoptosis in endothelial while the SFRP4 CRD inhibited tube formation in an angiogenesis assay (Longman et al., 2012). This study did not directly over express the domains in endothelial cells but used conditioned media. The experiments described in this chapter aimed to further investigate the role of SFRP4 in mesothelioma biology and to characterize the effects of over expression of NLD and CRD in mesothelioma cell models. Determining the contribution of these domains to the effects of SFRP4 could give clues about the functions of this protein.

4.2 Over expression of SFRP4 and its domains in mesothelioma cells.

Initial experiments aimed to show over expression of SFRP4 and domains in the JU77 and ONE58 cell lines. The expression constructs used were the same as those reported in an earlier study (Longman et al., 2012). Transfection efficiency was optimized by varying conditions of DNA and transfection reagent and monitored by EGFP fluorescence. JU77 and ONE5858 cells were cultured and transfected with the optimal protocol as described in section 2.18. Transfections were assessed by fluorescence microscopy for GFP. Transfection efficiencies were similar for all four constructs. Efficiency was assessed visibly by fluorescence microscopy/bright field counting (Figure 4.2) and approached 80% in some experiments. Efficiency was assessed in this way routinely in all following transfection experiments.

Overall all constructs were successfully expressed in the two cell lines (Figure 4.1) at 48 hour post transfection.

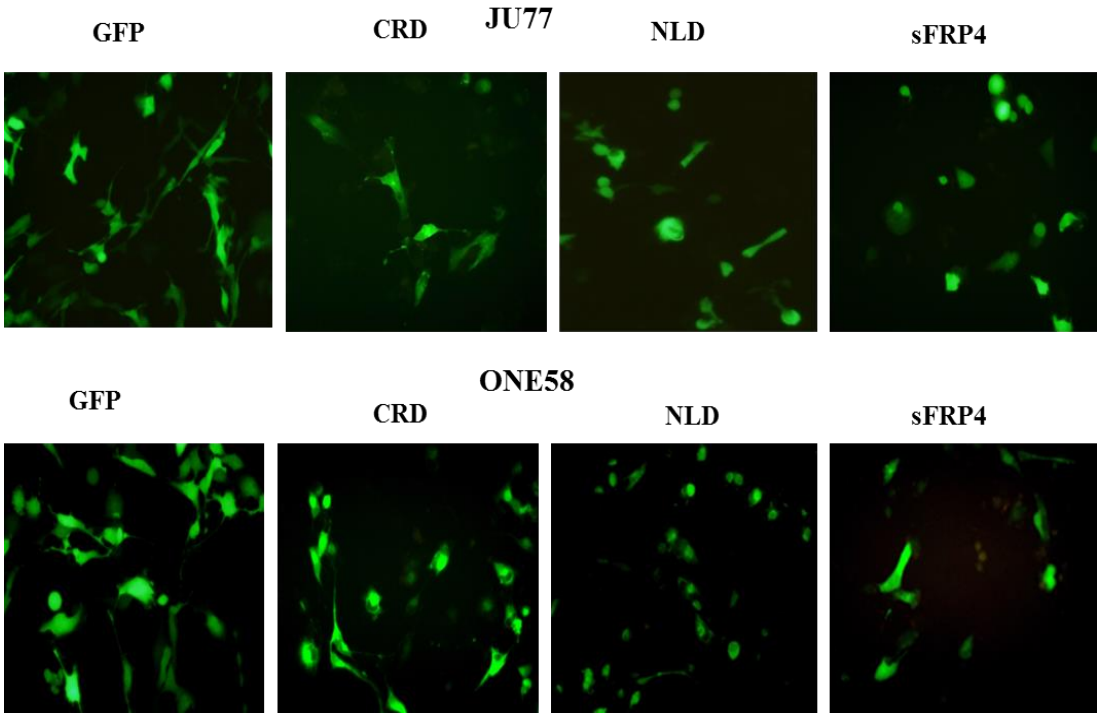
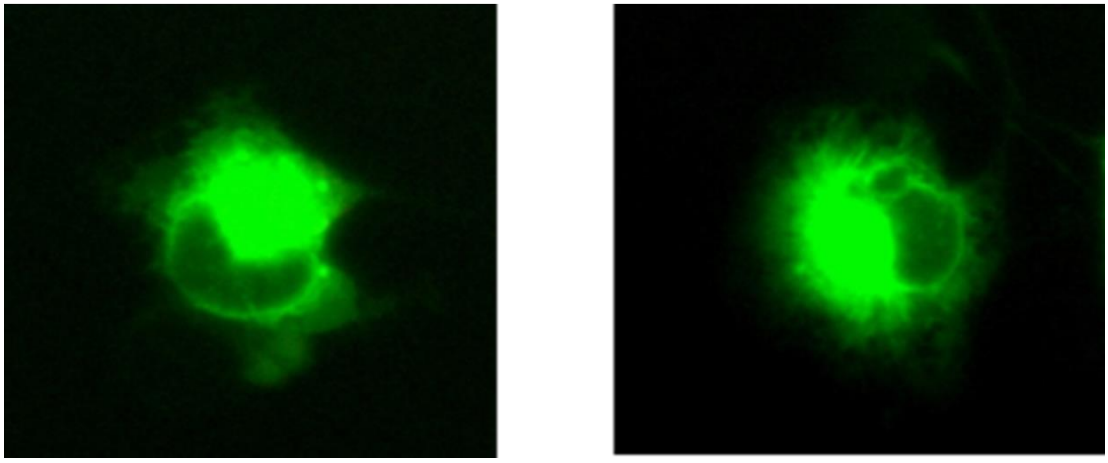


Figure 4.1 Transfections of sFRP4 and domains CRD and NLD on Mesothelioma Cells (48 hrs). JU77 and ONE58 cells expressing GFP-fusion protein fluorescence efficiency were imaged using a Olympus IX51 fluorescence inverted microscope.

A



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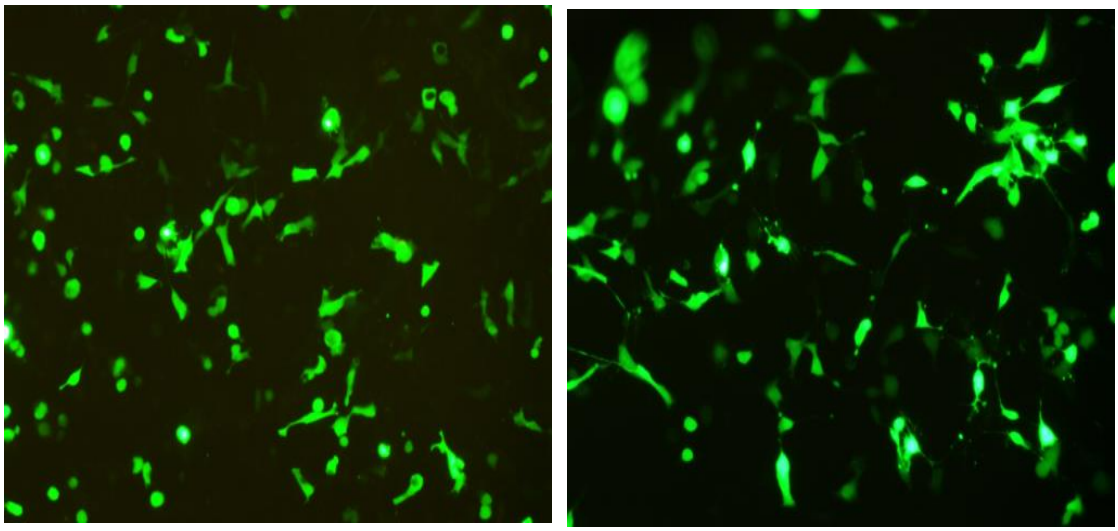


JU77 **ONE58**

Figure 4.2 Transfection, localisation and Efficiency

A Peri-nuclear localisation of sFRP4 CRD-GFP in ONE58 cells a representative figure of CRD-EGFP (Fig. 6.3A) overall CRD-EGFP was more broadly distributed throughout the cell. Imaging was performed using a Olympus IX51 fluorescence inverted microscope.

B Indicative images of transfection efficiency assessed at 48 hrs on mesothelioma cells with parental pEGFP-N1 vector expressing GFP was used as a control and determined by fluorescence and bright field counting using a Olympus IX51 fluorescence inverted microscope.

4.3 Effect of endogenous SFRP4 and domains upon mesothelioma cell proliferation and morphology.

Having established the conditions for transfection, the effects of over expression were investigated by microscopy and MTT assay. Following 48 hours of transfection the observed effects were quite small by microscopic examination (Fig 4.3A). However, following the cells for 6 days showed significant effects upon cell numbers and morphology by microscopy (Figure 4.3B) particularly for SFRP4 and NLD constructs. When cell viability was assayed at 6 days by MTT assay it was found that as with the exogenous protein SFRP4 over expression did down regulate proliferation. In both cell lines the NLD construct significantly down regulated proliferation/viability. The CRD had a small effect in ONE58 cells but little in JU77 cells (Figure 4.4). These results were consistent with the findings of chapter 3 and showed that the NLD alone was sufficient for the cell viability effects of SFRP4.

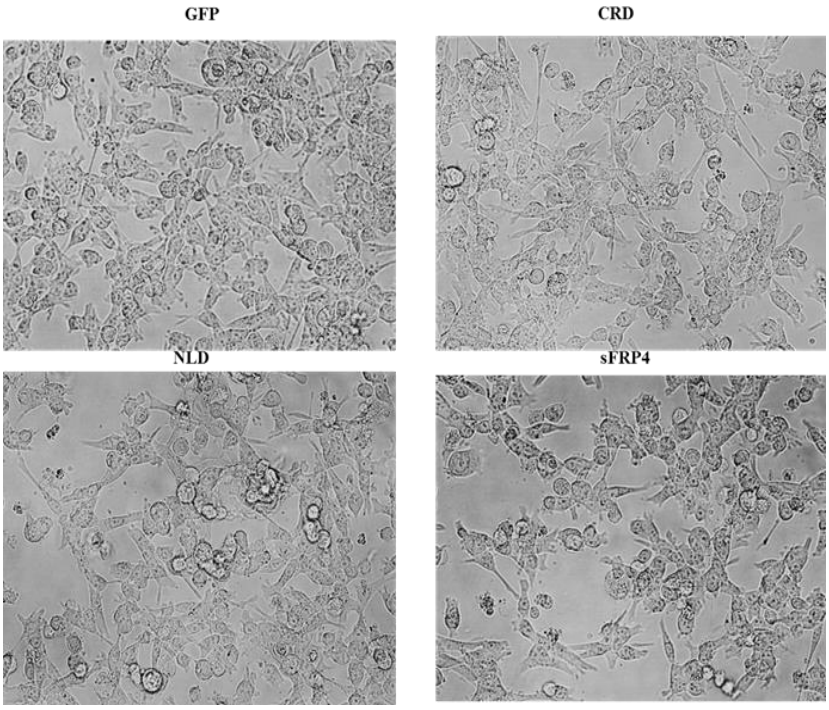


Figure 4.3 A Effect of endogenous sFRP4 and its domains upon transfection on JU77 cell morphology images at 48 hrs using Nikon inverted microscope

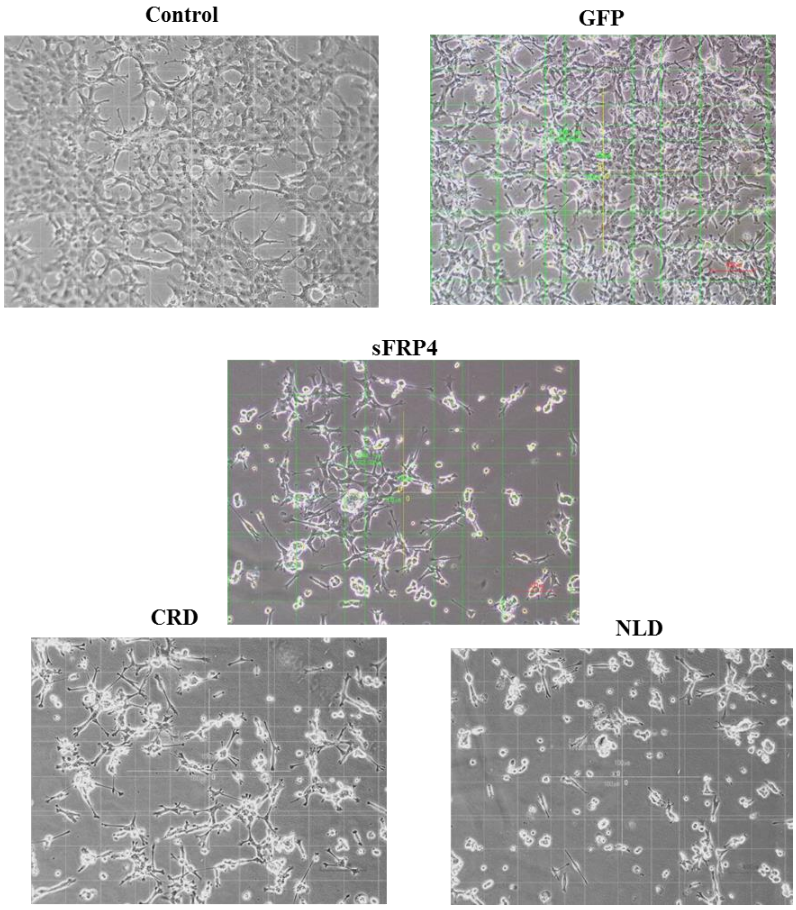
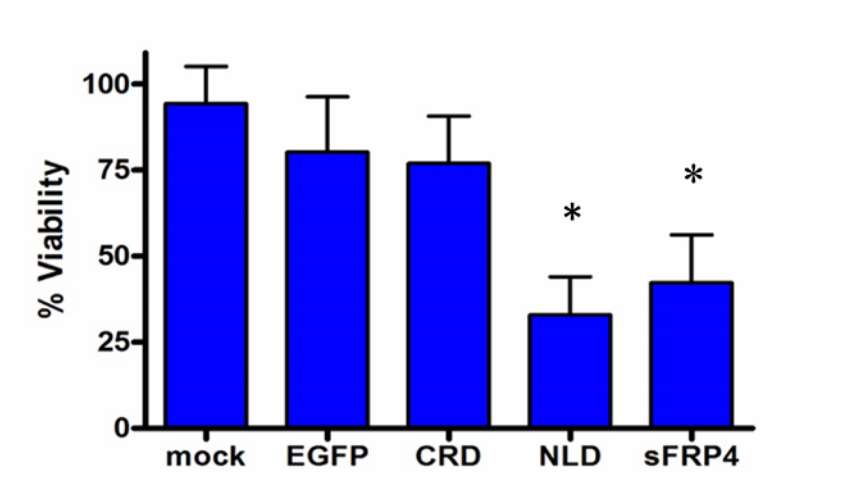


Figure 4.3 B Effect of endogenous sFRP4 and its domains upon transfection on JU77 cell morphology images after 6 days using Nikon inverted microscope



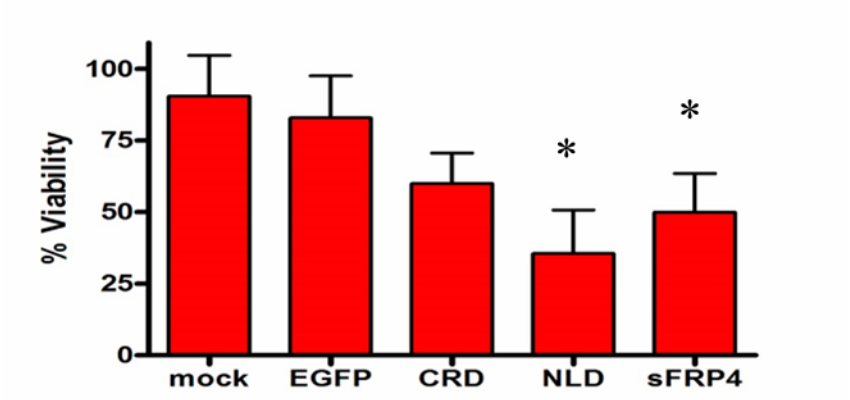


Figure 4.4 Effect of endogenous sFRP4 and its domains upon transfection on JU77 and ONE58 cell viability (48 hrs). Cells were transfected with sFRP4 and its domains cysteine rich domain (CRD) and netrin rich domain(NLD) for 48 hrs and viability determined by MTT assay.(A) JU77 (B) ONE58 and Data is mean (SD),*$p<0.05$ relative to control EGFP expressing cells. Statistical test used was one-way ANOVA with Tukey's post-hoc test.

4.4 Combination of endogenous SFRP4 and domains with cisplatin.

Having shown that endogenous SFRP4 expression could down regulate mesothelioma cell viability, the ability to sensitize cells to cisplatin was investigated. Cells were transfected with the various constructs and treated with 10 μM cisplatin for 48 hours then assayed by MTT assay. Results are shown in Figure 4.5. In ONE58 cells the effects of transfection appeared to be additive with 10 μM cisplatin resulting in approximately 50% cytotoxicity relative to the transfection alone (Figure 4.5B). In contrast in JU77 cells with SFRP4 and both domains effects appeared greater (Figure 4.5 A). As shown in Figure 4.5 sFRP4 did sensitise both JU77 and ONE58 cells to cisplatin and enhanced cytotoxicity. NLD domain facilitated 40% cytotoxic effect with 10uM cisplatin in JU77 cells compared to transfection. This data supports that the Wnt pathway inhibition by sFRP4 may sensitise mesothelioma cells to chemotherapeutic agents even at lower concentration.

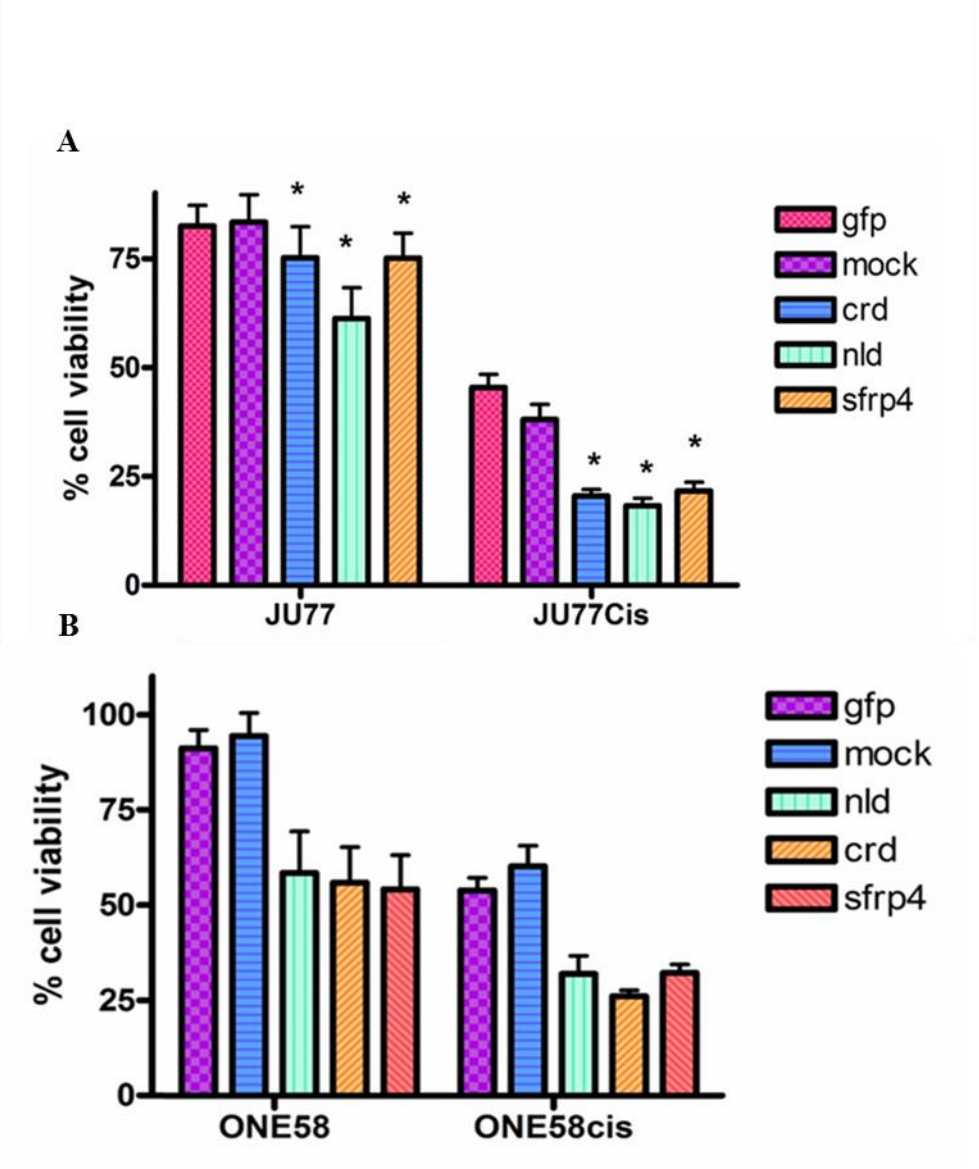


Figure 4.5 . Effect of endogenous sFRP4 and its domains upon transfection on mesothelioma cells and cisplatin 10µM treatment (48 hrs). Cells were transfected with sFRP4 and its domains cysteine rich domain (CRD) and netrin rich domain (NLD) with cisplatin 10µM for 48 hrs and viability was determined by MTT assay.(A) JU77 (B) ONE58. Results are expressed as % viability relative to control untreated cultures (100%). Statistical analysis is relative to EGFP expressing cells

4.5 Effect of endogenous SFRP4 and domain expression on mesothelioma cell migration.

In order to further investigate the effects of endogenous SFRP4 and domains upon mesothelioma cells transfectants were investigated using a wound healing assay. The effects of SFRP4 overexpression were not as great as that seen with the endogenous protein but it was still apparent in both cell lines (Figure 4.6). Quantitation of these effects showed that SFRP4 and the NLD did significantly inhibit wound closure while the CRD had no effect Figure 4.7. These results are consistent with experiments in Chapter 3 and also showed that as for proliferation the NLD plays an important role in the effects of SFRP4.

Overall both JU77 and ONE58 were quite rapidly able to close wounds in the monolayer in 6 hrs after transfection of sFRP4 and its domains for 48 hrs. While ONE58 required much longer incubations for wound closure (Figure 4.7). In both cell lines sFRP4 appeared to inhibit wound closure (Figure 4.6) and this result was significant as confirmed by open wound area image analysis (Fig.4.7). Interestingly, NLD domain and sFRP4 had a significant effect and CRD had little effect upon wound closure in JU77 and ONE58 cells (Figure 4.6). The NLD domain exhibited similar effect to sFRP4 migration whereas the CRD had no effect in 6 hrs after 48 hrs of post transfection.

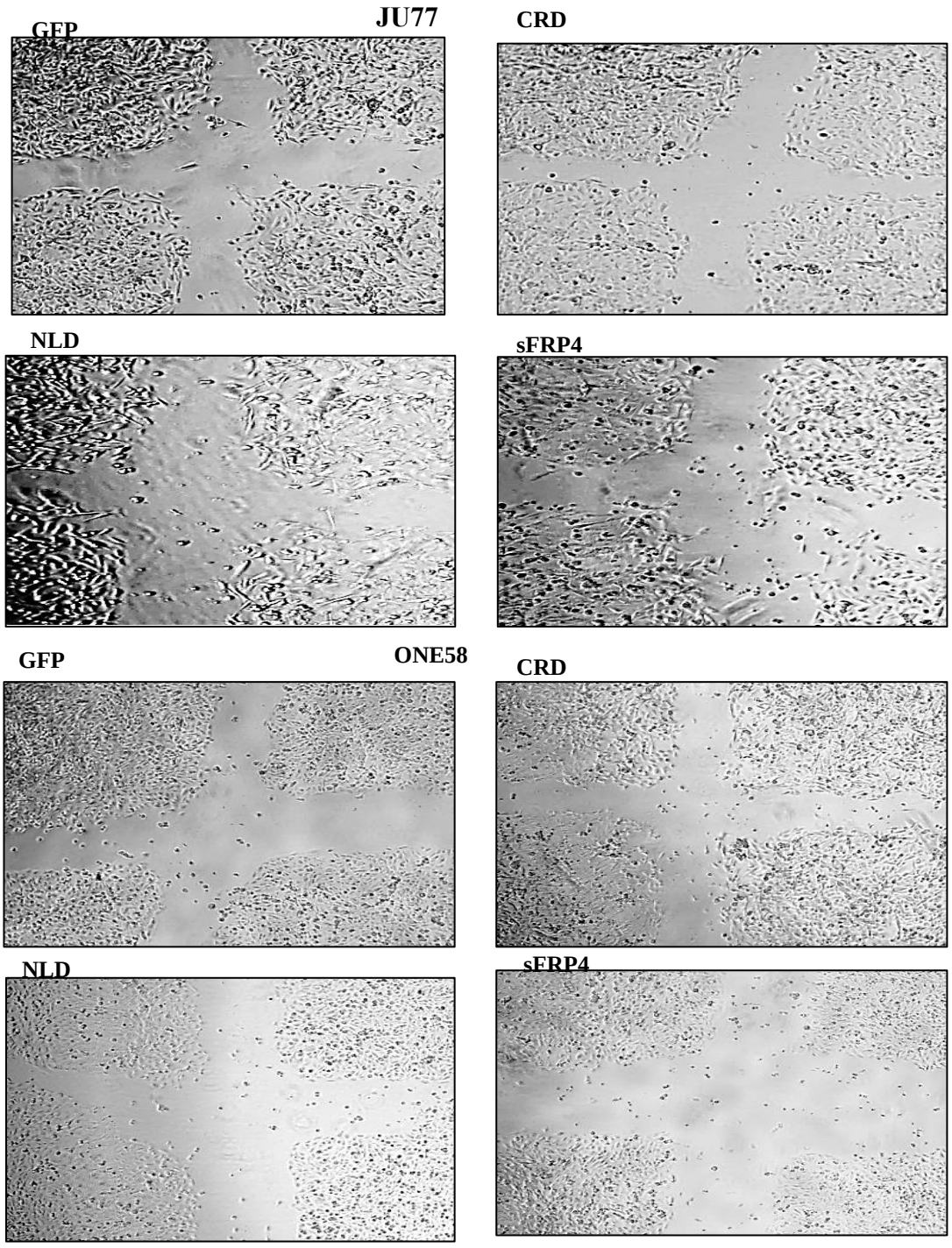


Figure 4.6 Effect of Endogenous sFRP4 and its domains upon mesothelioma cell migration. JU77, ONE58 cells were transfected with sFRP4 and its domains cysteine rich domain (CRD) and netrin rich domain(NLD) for 48 hrs and analysed for cell migration (bright field images) after 6 hrs using Nikon inverted microscope and analysis by Tscratch software. Mean (SD)*$p<0.05$

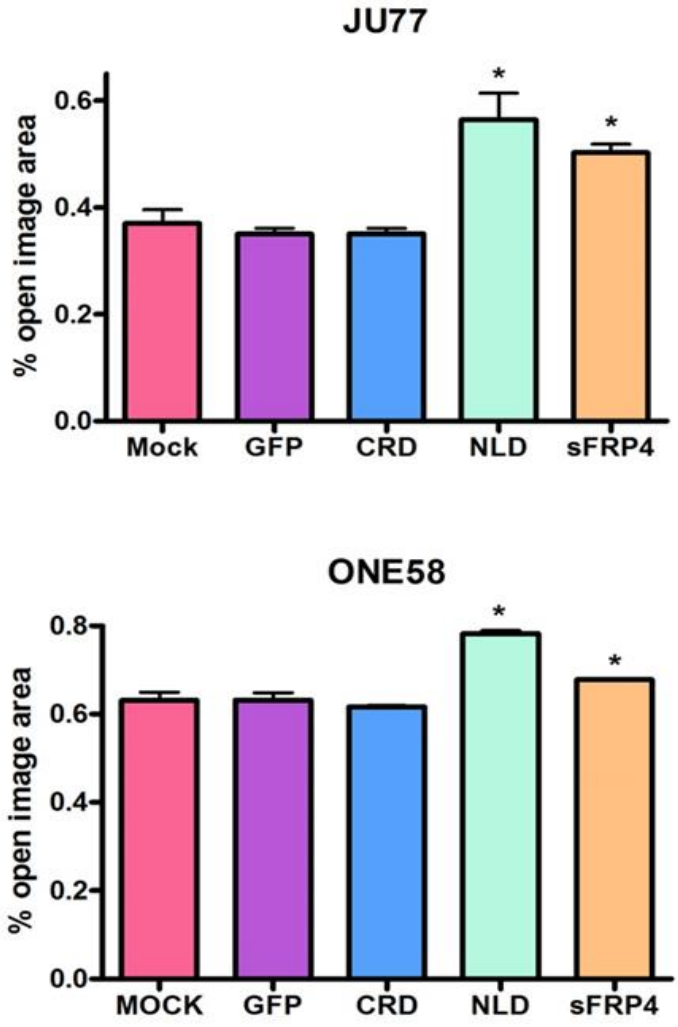


Figure 4.7 Effect of endogenous sFRP4 upon mesothelioma cell migration. (A) JU77, (B) ONE58 cells were analysed by scratch assay following Analysis by Tscratch software. Mean (SD)*p<0.05

4.6 Mechanisms of cell death induced by SFRP4 and domains.

Experiments described in Chapter 3 demonstrated a distinctive nuclear morphology in cells treated with SFRP4 that was suggestive of mitotic catastrophe. To determine whether endogenous SFRP4 had a similar effect and the role of the domains, various transfectants were assessed for nuclear morphology. It was found that in both JU77 and ONE58 cells expression of SFRP4 and the NLD induced a nuclear morphology characteristic of mitotic catastrophe which was consistent with that described in chapter 3 (Figure 4.8). Cells were characterised by an accumulation of multiple micronuclei which was greater only in JU77 cells. Cells expressing GFP alone or the CRD did not display this effect (Figure 4.8). The formation of nuclear envelopes around individual clusters misaggregated chromosomes is apparent by the appearance of nuclei undergoing mitotic catastrophe (Vakifahmetoglu et al., 2008). These morphological findings suggest that the effect of sFRP4 and its associated domains induced on mesothelioma cells more consistent to mitotic catastrophe than other forms of cell death. These observations are discussed in further detail in Chapter 7.

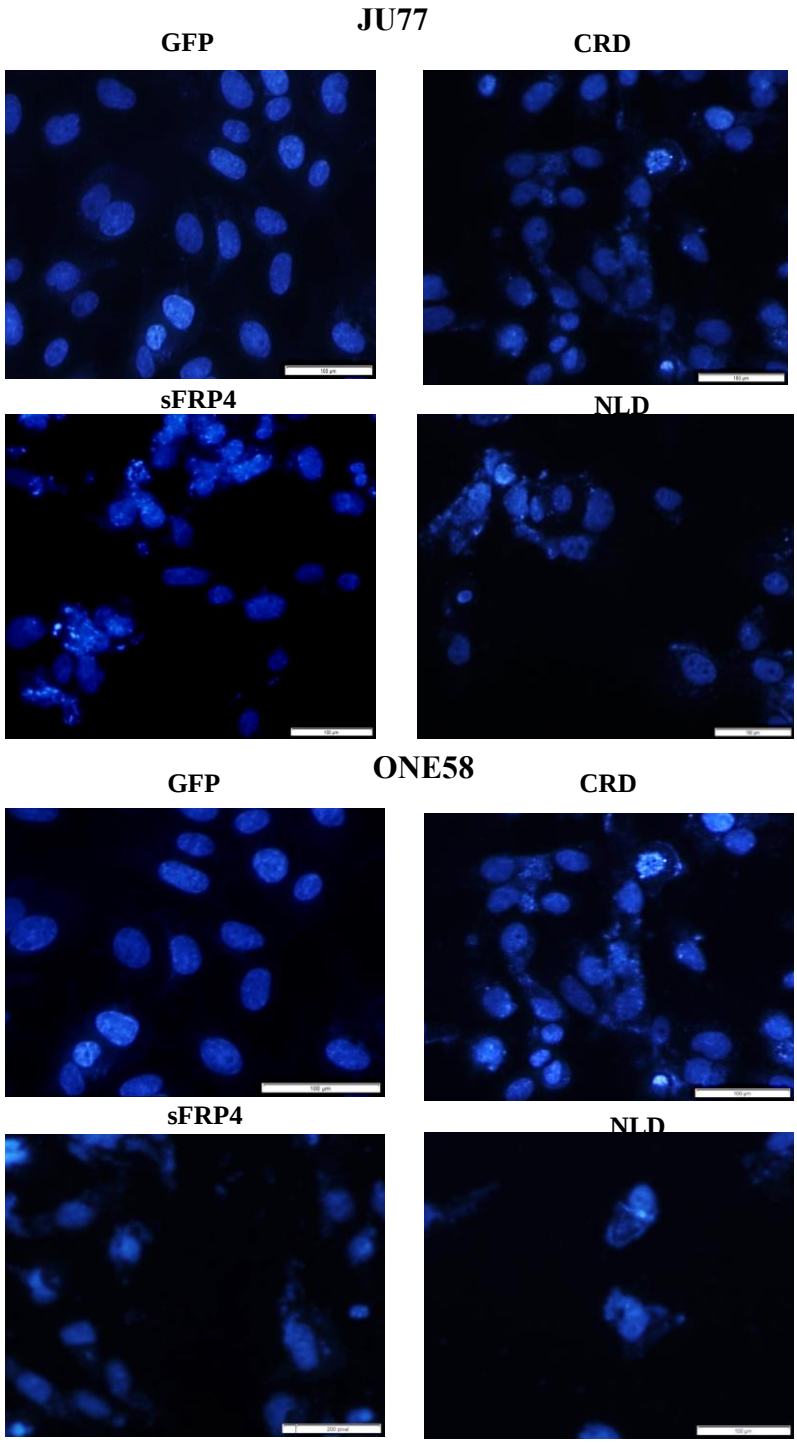


Figure 4.8 Effect of sFRP4 and its domains CRD and NLD on nuclear morphology by Hoechst staining on JU77 and ONE58 cells showing clusters misaggregated chromosomes. Nuclear morphology assessed by Hoechst 33258 staining after 48 hrs. Nuclei of cells treated with sFRP4 showed characteristics of mitotic catastrophe

4.7 Gene expression changes in response to SFRP4 and domain expression

To further investigate downstream Wnt signalling as studied by (Carmon and Loose. 2010) the effects of sFRP4 on mesothelioma cells gene expression of previously described, canonical Wnt target genes were assayed. The rationale for looking Axin2, APC, LEF1, cMYC and Cyclin D1 is because they are the target genes for canonical Wnt signalling.

Transfected cultures were assayed by real time RT-PCR for expression of target Wnt genes. Of the 5 genes assayed only Lef1 and cMYC showed substantial changes in gene expression in both cell lines. In JU77 expressing SFRP4 or the NLD but not the CRD there was an approximately 2 fold reduction on Lef1 expression and 1 fold reduction on cMYC expression. In ONE58 there was a 10 fold reduction in Lef1 for cells expressing SFRP4 and NLD relative to control cells (GFP) (Fig 4.9).

Based on our findings and others studies we know the canonical Wnt signalling plays an important role in mesothelioma cells. Based on our findings and others studies we know the canonical Wnt signalling plays an important role in mesothelioma cells. The effect may vary due to basal expression of endogenous sFPR4 in the cell and may be cell specific.

Gene expression data did not reveal many changes consistent with activation of canonical signalling. There are a number of possible reasons behind this including that these may not be the appropriate target genes in these cells and also that the timing of expression changes can vary.

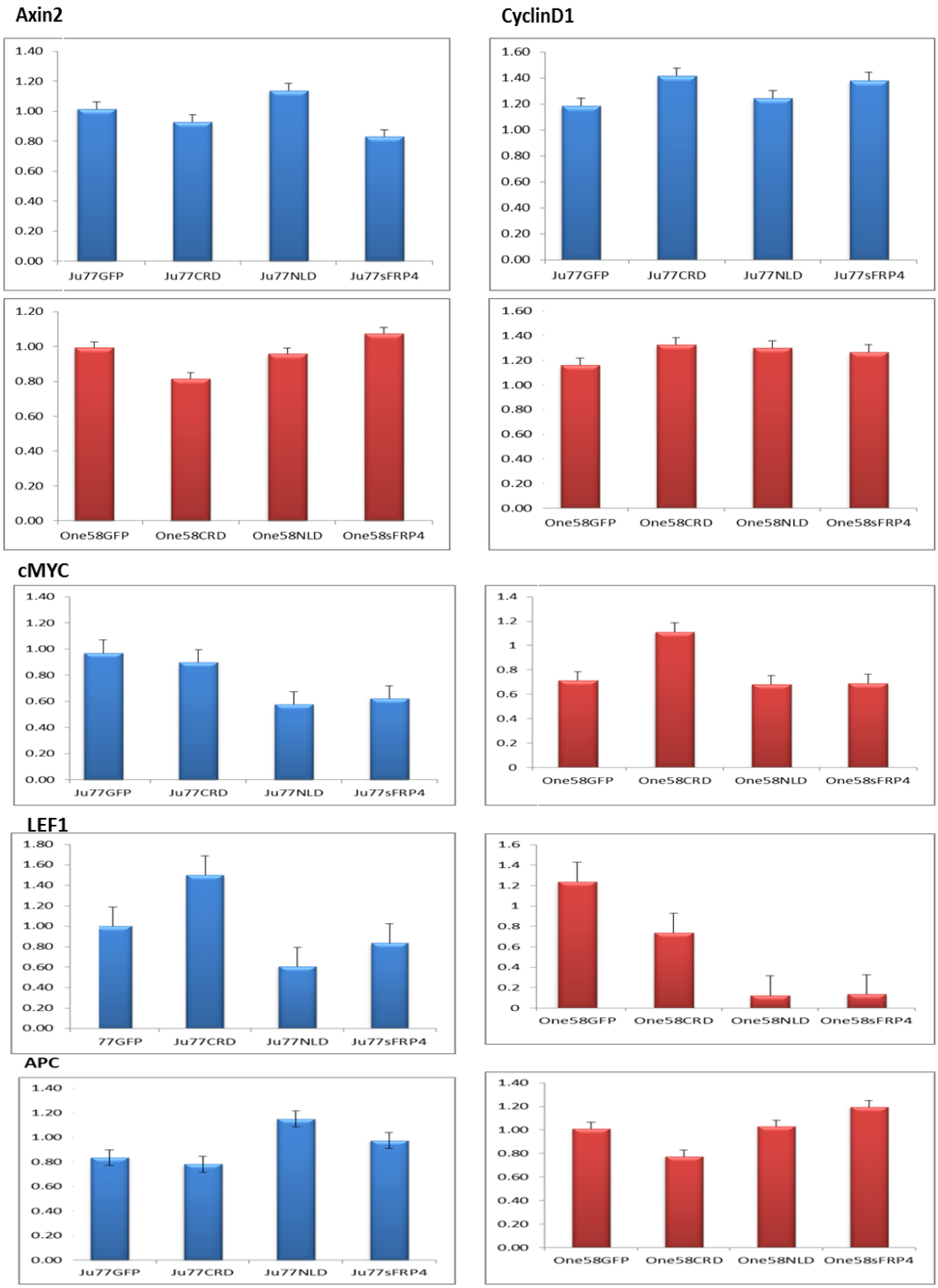


Fig 4.9 Effect of sFRP4 and its domains CRD and NLD on Wnt target gene expression Basal gene expression of Axin2, APC,cMYC, cyclinD1, LEF1 in JU77 and ONE58 cells. Total RNA isolated from cells were analysed by 2-step real time RT-PCR using gene specific primers. Basal gene expression is expressed as mRNA levels relative to the respective control cultures following normalisation by reference gene expression. Result are mean ± SD of three biological experiments.

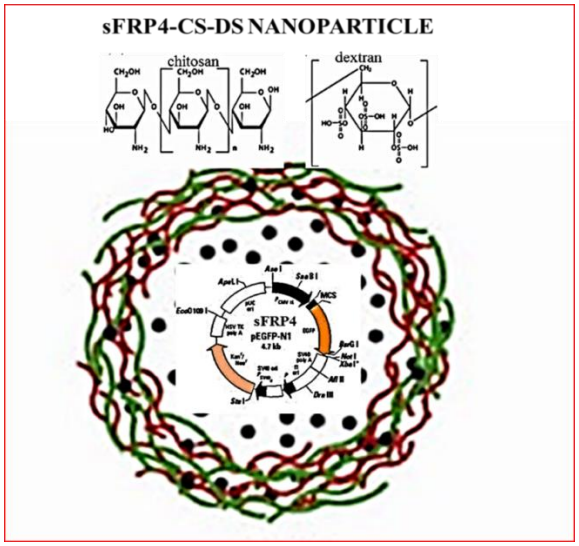
4.8 Conclusion

The results of this chapter experiments were confirming of those from Chapter 3 using a different experimental approach. Overexpression of sFRP4 showed similar biological effects upon proliferation, migration, chemoresistance and nuclear morphology as treatment with exogenous recombinant sFRP4. Importantly, the effect of sFRP4 in both experimental models in inducing mitotic catastrophe was the same. Notably, we found that it was the NLD domain which mediated these effects in mesothelioma cells. The CRD had only modest effects at the most. This has implication for our understanding of sFRPs in general, reinforcing reports which show the NLDs of other sFRPs interact directly with Wnts.

CHAPTER 5

Delivery of sFRP4 expression constructs by chitosan nanoparticles


their expectations were so high...
...the media hype...
..they wanted too much too soon...
Viral Trials
Lysosome Trials
gene therapy 2


sFRP4-CS-DS NANOPARTICLE
chitosan
dextran
sFRP4
pEGFP-N1

5.1 Introduction

The experiments which were performed in chapters 3 and 4 showed that SFRP4 could inhibit proliferation, induce cell death and inhibit migration in mesothelioma cells. These experiments gave some evidence for proof of principle that SFRP4 may provide a potential biological therapy. It was therefore decided to explore the potential of gene delivery of SFRP4 and its domains in mesothelioma cells.

Chitosan is a naturally derived biodegradable and compatible polysaccharide which has many useful properties for application in formulation of nanoparticles for delivery of therapeutic molecules. As well as being biodegradable, chitosan has good ability to attach to biological surfaces and has been shown to increase delivery of therapeutics (Lin et al., 2005). Because of its many amine groups and positive charge chitosan has an excellent ability to interact and complex with DNA (Tong et al., 2009). A number of studies have shown that chitosan nanoparticles can be used to deliver plasmid DNA both in vitro and in vivo (Dass, Tran, & Choong, 2007). Dextran sulphate (DS) is another biocompatible and biodegradable polymer which has been successfully used previously with chitosan for the formulation of nano particles (Yan Chen, Siddalingappa, Chan, & Benson, 2008).

In this chapter the feasibility of using chitosan nanoparticles to deliver SFRP4 expression plasmids is shown. Chitosan-DS nanoparticles loaded with different SFRP4 or domain constructs were synthesized and analysed for their physical properties. The ability of these nanoparticles to deliver plasmid DNA for gene expression was determined and the effect on cell proliferation assayed.

5.2 Nanoparticle physical characterization

The chitosan DS nanoparticles were synthesized by complex coacervation technique as described in the methods (Chapter 2). These nanoparticles loaded with plasmid constructs on control empty particles were kindly synthesized by Dr. Yan Chen (Curtin University) using plasmids purified and supplied by us. These nanoparticles were then characterized physically by Dr. Yan Chen using standard techniques (Table 5.1). The size of nanoparticles ranged from 300nm for empty particles to 337nm for NLD particles. Regarding the particle size distribution the polydispersity index was 0.25 for empty and 0.375 for loaded nanoparticles which indicates a relatively narrow size distribution (Yan Chen et al., 2008). Measurement of the zeta potential showed that all preparations were around 25 mV or above which suggests a stable formulation (Yan Chen et al., 2008). The polymers used had a good affinity for the DNA molecules.

Table5. 1 Characteristics of CS-DS nanoparticle formulations

Nanoparticle preparations	Active ingredient	Visual observation	Particle size±S.D (nm)	Poly-dispersity index ± S.D	Zeta potential ± S.D (mV)
Empty	none	Colloidal	300.2 ± 17.2	0.253 ± 0.099	47.2 ± 0.2
SFRP4	SFRP4	Colloidal	318.5 ± 6.6	0.375 ± 0.050	24.0 ± 0.9
CRD	CRD	Colloidal	337.0 ± 6.2		26.5 ± 1.4
NLD	NLD	Colloidal	325.0 ± 5.6		24.0 ± 1.1
pEGFP	pEGFP	Colloidal	318.5 ± 6.6	0.375 ± 0.050	26.9 ± 0.2

Number of replicates used to calculate standard deviation (SD), n = 3

Table 5.2 Entrapment Efficiency

NANO ENCAPSULATION	
GFP	74%
CRD	88%
NLD	76%
sFRP4	89%

5.3 DNA loading capacity, integrity and topological structure characterization

Gel retardation assay was performed on the CS-DS sFRP4 and its domain CRD and NLD DNA (2:1:1) nanoparticle. Encapsulation was evaluated on 1% TAE agarose gel electrophoresis containing gel red (Figure 5.1). Furthermore the topological structure of the encapsulated sFRP4 and its associated domains nanoparticle was confirmed by EcoR1 digest to confirm the size of inserts (Figure 5.2). The DNA entrapment efficiently was assessed and the overall effectiveness was high (74-89%) across all four DNA constructs suggesting that the polymers used had a high binding affinity for DNA molecules (Table 5.2). This indicated that almost all DNA was bound and maintained the integrity with CS DS nanoparticles.

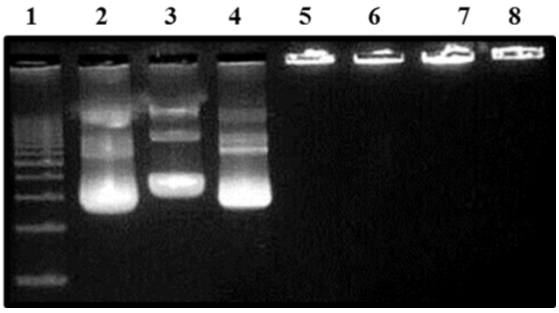


Figure 5.1 Electrophoretic mobility shift check of loaded nanoparticle

1% TAE Agarose gel panel showing. 1. Marker, 2. CRD, NLD same size 3. pEGFP 4. sFRP4 5. CRDNP 6. pEGFPNP 7. sFRP4NP 8. NLDNP

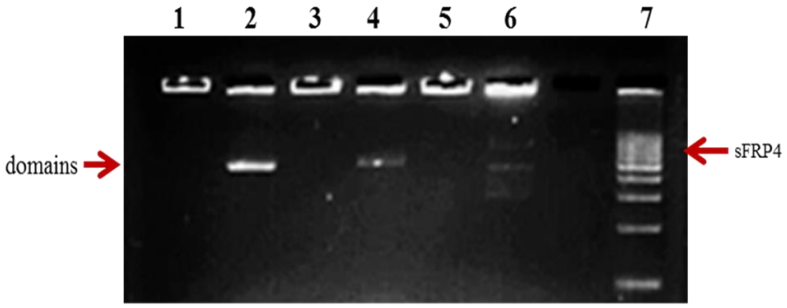


Figure 5.2 Topological structure of sFRP4 released from nanoparticle

1% TAE Agarose gel panel showing DNA fragment release from the nanoparticle-EcoR1 digest 1. CRDnp, 2. CRD digest, 3. NLD np, 4 NLD digest, 5. sFRP4 np, 6. sFRP4 digest, 7. Marker

5.4 Biological effect of Chitosan Dextrose-SFRP4 nanoparticles in vitro

Initially transfection efficiency of the various nano particle preparations was assessed by fluorescent microscopy for GFP expression. An equivalent concentration of 40ng/well plasmid DNA (96 Well plate) was used as higher nanoparticle concentrations displayed toxic effects. The efficiency of transfection was similar to that seen when 100ng/well Plasmid DNA was delivered by FUGENE® HD reagent However, the timing of GFP expression was much earlier with nanoparticle delivery than transfection reagent. In experiments described in chapter 4 maximum GFP expression was only seen after approximately 6 days. With nanoparticle gene delivery maximum gene expression occurred at 48 hours (Figure 5.3 and 5.4). This was consistent with both cell lines. The biological effects on cell proliferation and morphology were also seen much earlier.

The effect of nanoparticle mediated transfection of both JU77 and ONE58 at 48 hours. In both cell lines NLD and SFRP4 down regulated proliferation while no effect or slight stimulation occurred with CRD expression are shown in Figure 5.5. Morphologically there were some differences with the effects of nanoparticle gene. Delivery of SFRP4 and NLD in particular compared to FUGENE® HD reagent as seen in chapter 4 (Figure 5.6). Cells displayed distinctive vesicular membrane structures in cultures expressing Nano particle delivered SFRP4 and NLD. These membrane structures were associated with GFP fluorescence in the nucleus (Figure 5.7).

These crucial observations suggest that the CS: DS is strong which may be beneficial for improving the delivery of DNA to the target site. CS: DS/ EGFP DNA showed minimal effect on cell morphology and viability compared to CS: DS/sFRP4 and domains. As expected the encapsulated CS: DS/ sFRP4 NLD domain possessed greater effect on cell morphology and viability compared to CRD (Figure 5.5, 5.6)

These results suggest that the nanoparticle transfection of sFRP4 and its domains could be released from endosomes and quickly localised to the nucleus with higher efficacy of gene delivery than FUGENE® HD reagent.

JU77

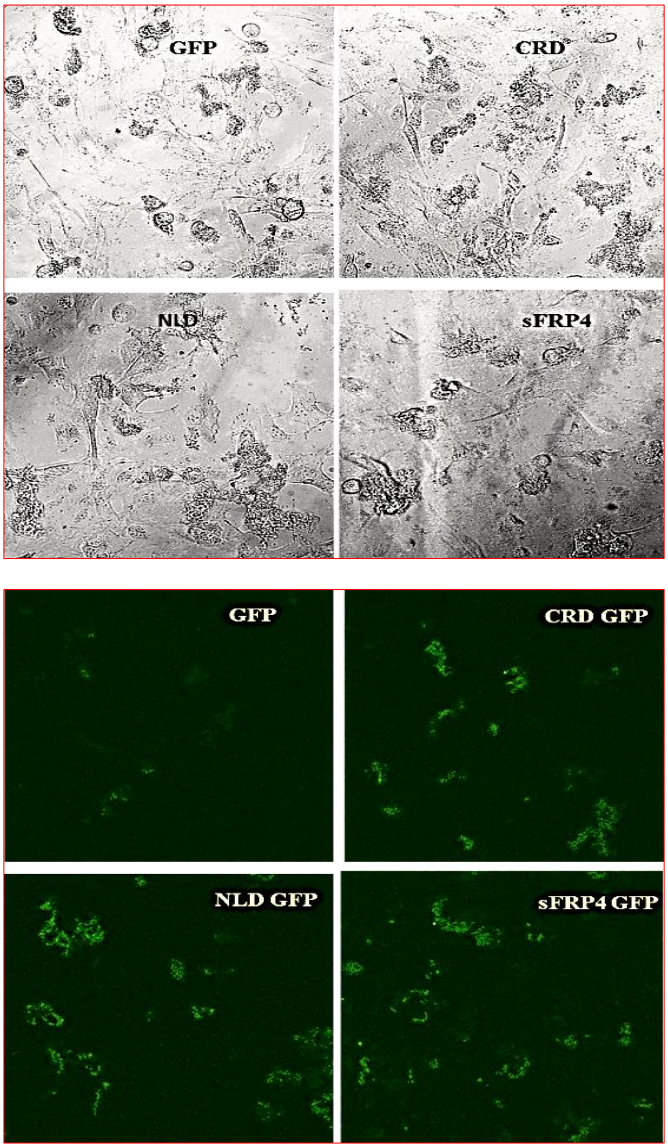


Figure 5.3 Effectiveness of chitosan sFRP4 and associated domains CRD and NLD nanoparticle gene delivery. Transfections of sFRP4 and domains CRD and NLD nanoparticles on JU77 cells expressing GFP-fusion protein. Fluorescence efficiency was assessed at 48 hrs using a Leica TCS SP2 AOBS confocal microscope (Leica, Mannheim, Germany)

ONE58

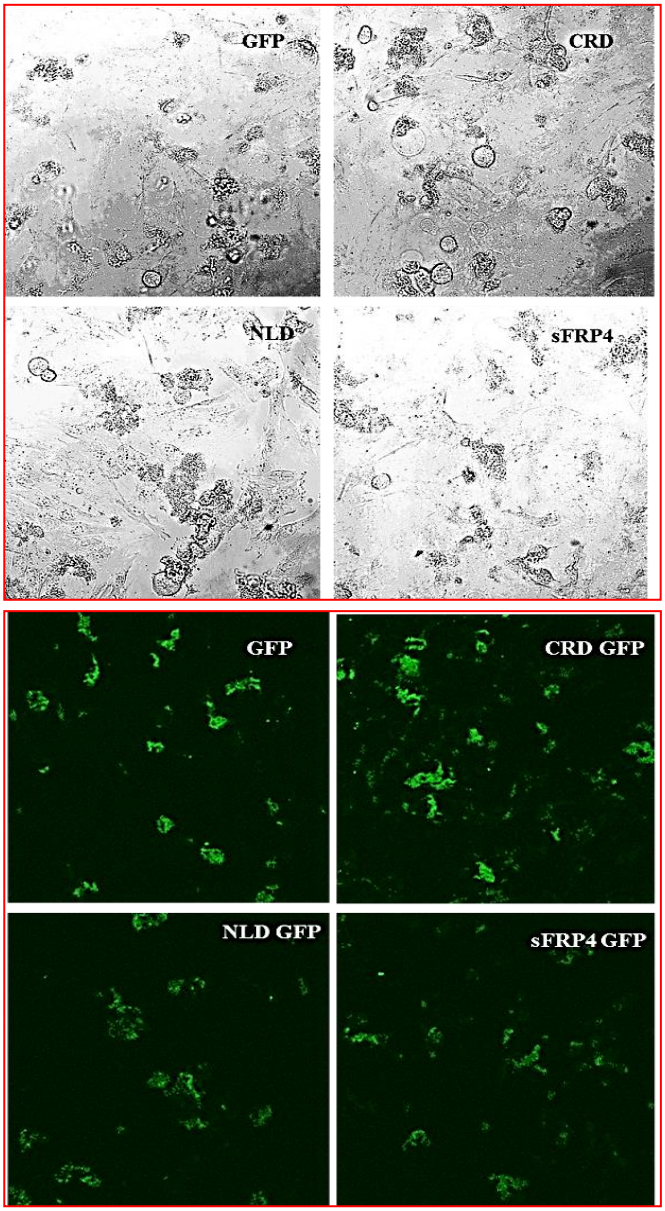


Figure 5.4 Effectiveness of Chitosan –DS sFRP4 and associated domains CRD and NLD nanoparticle gene delivery. Transfections of sFRP4 and domains CRD and NLD nanoparticles on ONE58 cells expressing GFP-fusion protein fluorescence efficiency were assessed at 48 hrs using a Leica TCS SP2 AOBS confocal microscope (Leica, Mannheim, Germany).

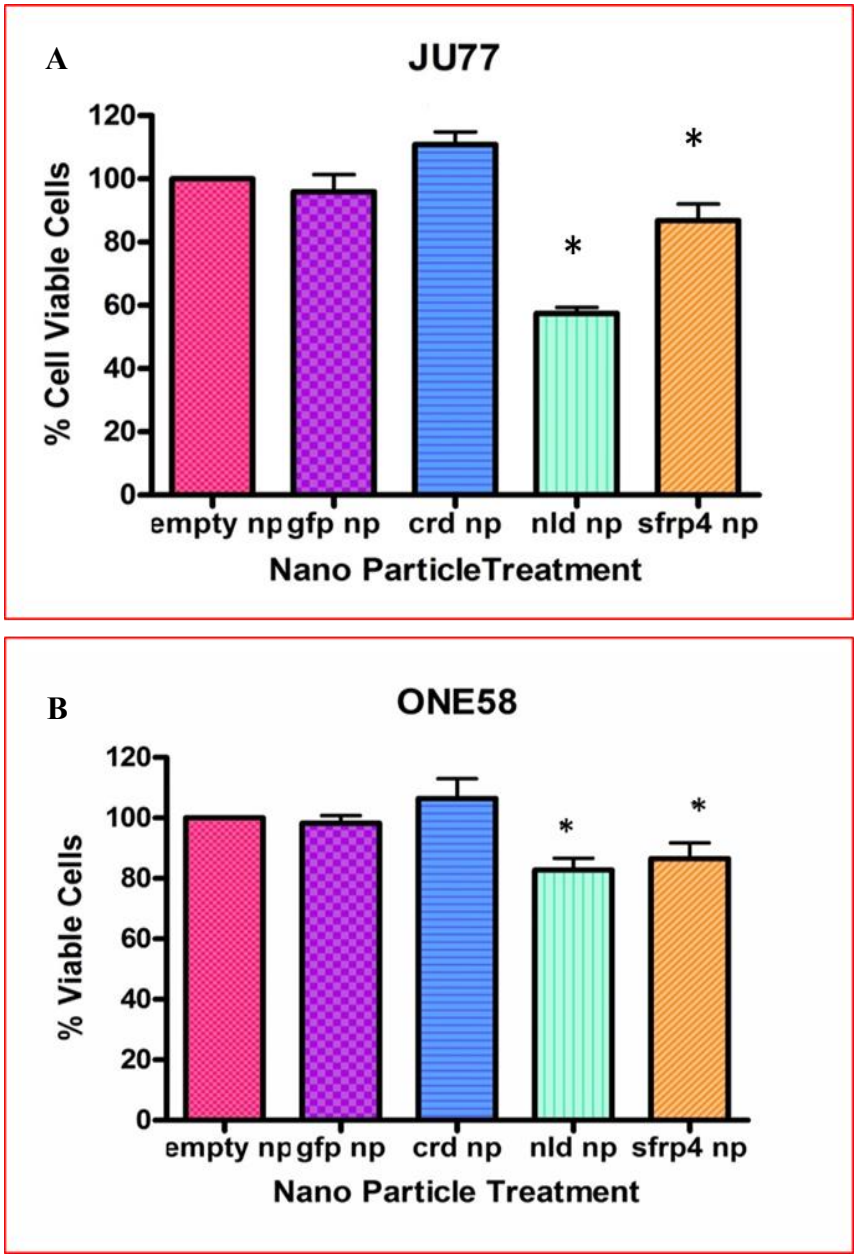


Figure 5.5 Effect of Chitosan-DS nanoparticle delivered genes on mesothelioma cell proliferation. (A) JU77 and (B) ONE58 cells were transfected with plasmid loaded nanoparticle preparations and assayed for cell viability after 48 hrs by MTT assay. n=3 mean (SD) *$p<0.05$, using one way ANOVA Tukey's post-hoc test

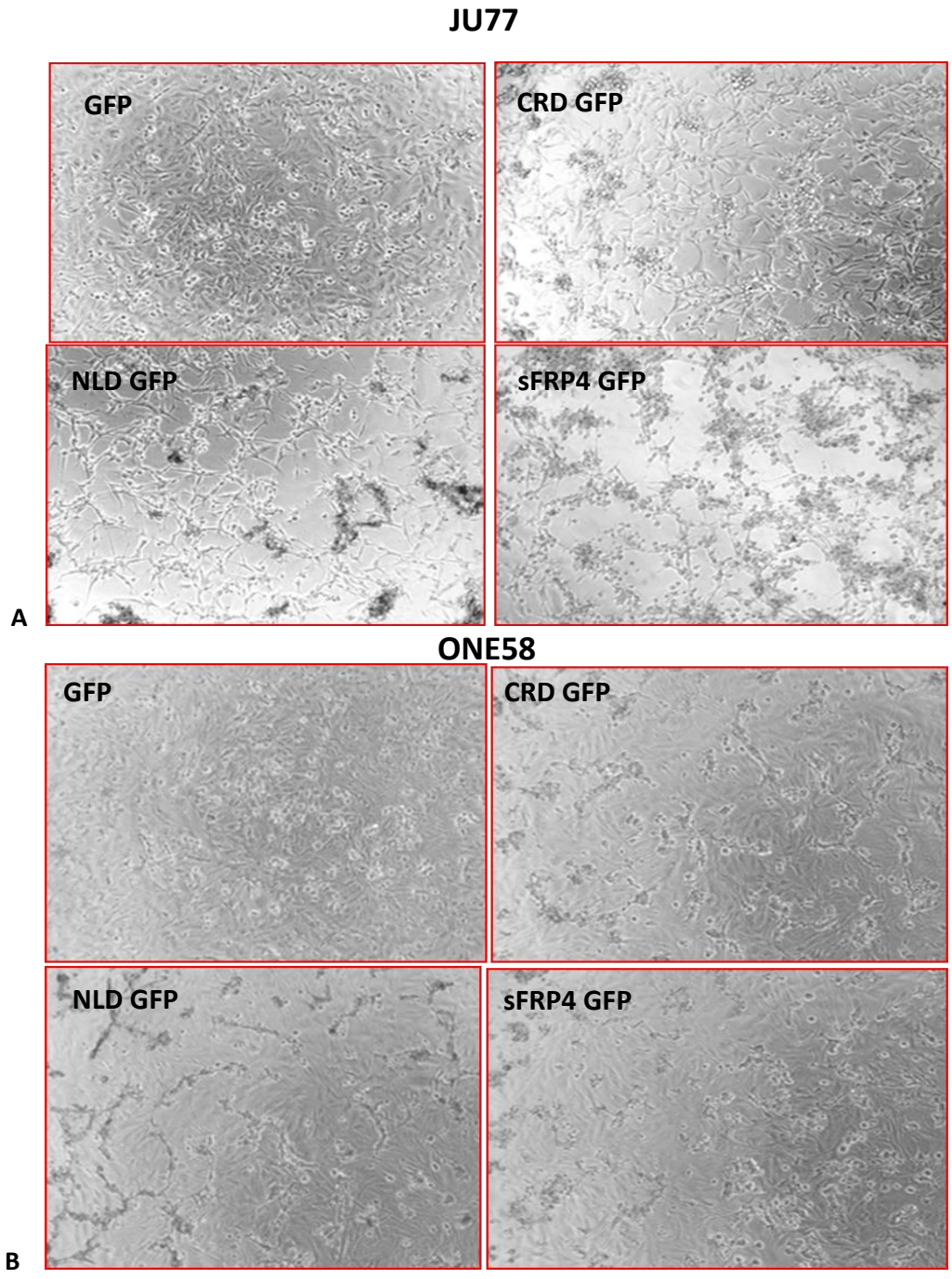


Figure 5.6 Effect of Chitosan-DS nanoparticle delivered gene on mesothelioma cell morphology. (A) JU77 and (B) ONE58 cells were transfected with plasmid loaded nanoparticle preparations imaged after 48 hrs

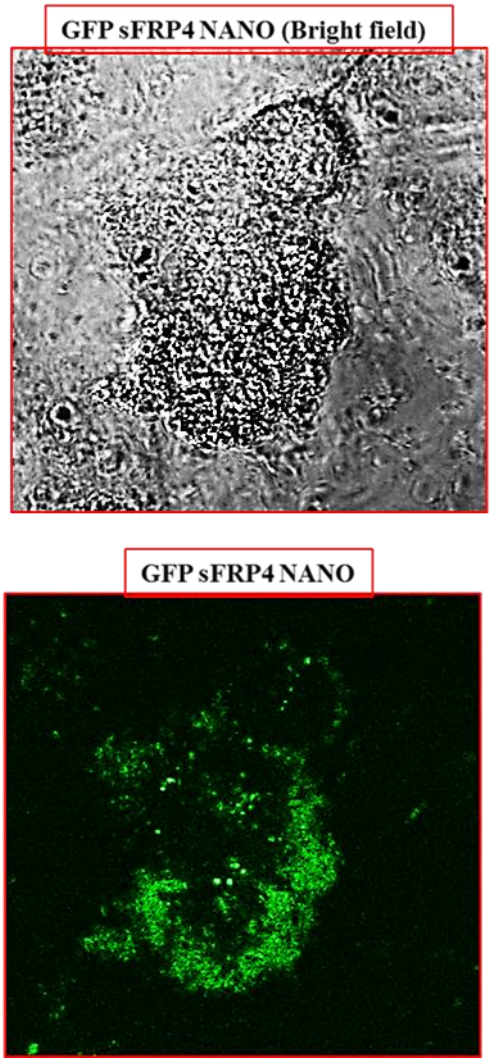


Figure 5.7 Imaging of JU77 cell treated with sFRP4 loaded nanoparticles showing characteristic membrane vesicular structures. (A) Bright field image (B) GFP fluorescence. GFP-fusion protein fluorescence efficiency were measured at 48 hrs using a Leica TCS SP2 AOBS confocal microscope (Leica, Mannheim, Germany) (magnification under 60X)

5.5 Conclusions

In conclusion we found that DNA chitosan nanoparticle formulation maintained high integrity and entrapment efficiency. The biological effects of the sFRP4/domain gene delivery were seen at an earlier time point and with lower DNA concentrations with chitosan nanoparticles than with conventional transfection reagent. Our results demonstrate the effectiveness of chitosan sFRP4 and NLD nanoparticle based gene delivery and shows promise as an effective alternative non-viral gene delivery system. This study also demonstrates the potential for targeting this pathway in mesothelioma.

CHAPTER 6

Live cell imaging of sFRP4 and domains in mesothelioma cells: number and brightness analysis

6.1 Introduction

The Wnts are secreted, lipidated glycoproteins (at least 19 in humans) that transduce signals by binding to specific frizzled (Fzd) receptor complexes (reviewed in Schulte, 2010) leading to activation of canonical or non-canonical pathways depending upon molecular context. Wnts are functionally integral to many processes during embryonic development and play an important role in homeostasis in adult tissues. The downstream pathways activated by Wnts play a role in a diverse array of cellular processes including differentiation, proliferation, migration, survival, polarity and stem cell self-renewal (Clevers & Nusse, 2012). Aberrant Wnt signalling is associated with several disorders, especially cancer (Anastas & Moon, 2012; Attanoos & Gibbs, 1997).

The mammalian secreted Frizzled-related proteins (sFRPs) are a family of five proteins which were first identified on the basis of their antagonistic effect upon Wnt signalling. Subsequent research has indicated more diverse functions for sFRPs, including potentiation of Wnt signalling in certain contexts (Kress, Rezza, Nadjar, Samarut, & Plateroti, 2009; Üren et al., 2000, Von Marschall & Fisher, 2010) and spacial diffusion of Wnts (Mii & Taira, 2009) or even Wnt independent effects (Martin-Manso et al., 2011). They contain two domains, a Fzd-like cysteine rich domain (CRD) and a netrin-like domain (NLD). The CRD of sFRPs was initially believed to play a role analogous to the CRD of Fzds as the principal mediators of Wnt binding (Lin et al., 1997). Although some studies have suggested that sFRPs interact with Wnts via their CRD domain (Lin et al., 1997). Subsequent experiments have indicated that the NLD may play a more prominent role in this interaction (Bhat et al., 2007; Lopez-Rios et al., 2008). In addition it has been demonstrated that SFRPs and Fzds can also interact via their CRD domains to form heterodimers and homodimers suggesting an alternative mechanistic basis for sFRPs to influence Wnt signalling (Bafico et al., 1999, Rodriguez et al., 2005).

The biological significance of sFRPs is evinced by studies in developmental models and the frequent observation of aberrant expression in many cancers. Despite this evidence many aspects of the biology of this family remains to be elucidated. In malignant mesothelioma (MM) sFRP4 has been shown to be downregulated by promoter methylation and to function as a tumour suppressor (Lee, He, You,

Dadfarmay, et al., 2004 ;Kohno et al., 2010). Nearly all studies of sFRPs to date have focussed upon their extracellular interactions and very little is known about the intracellular trafficking, localization and behaviour of sFRP4 and its domains. Using a mesothelioma cell model which showed little or no sFRP4 expression (Fox et al., 2013). We undertook to characterise the intracellular trafficking of sFRP4 domains, particularly in cells exposed to endogenous Wnt3a.

We used a recently described fluorescence microscopy technique (Ossato et al., 2010), Number and Brightness (N&B) fluctuation spectroscopy analysis, to study intracellular localisation and aggregation of sFRP4, sFRP4 CRD and sFRP4 NLD. The basis for the N&B method is the analysis of fluorescence intensity distributions and permits measurement of both the number (N) and brightness (B) at each pixel in a stack of images. Since the brightness is directly related to the molecular number observed, the method has been previously applied to live cell image analysis of intracellular protein aggregation (Ossato et al., 2010 ;Vetri et al., 2011). Our imaging and image analysis studies suggest that sFRP4 is localized in the perinuclear region and this localisation is likely to be a property of the CRD domain. We found that the NLD was likely to have a vesicle association signal involved in secretion of sFRP4. Notably we found that intracellular localisation and trafficking of sFRP4 domains could be modified in response to Wnt3a.

6.2 Establishment of B value for monomeric EGFP

In order to determine the brightness (B) of monomeric EGFP we transfected JU77 cells with the parental pEGFP-N1 vector with and without wnt3a and acquired images as described. This enabled us to establish background correction and subsequently determine the B value due to monomeric EGFP expressed in JU77 cells essentially as previously described (Plotegher, Gratton, & Bubacco, 2014). As a result of this analysis we were able to determine a mean value of B for monomeric EGFP of 1.25 with an upper limit of 1.5 (Fig 6.1A&B).

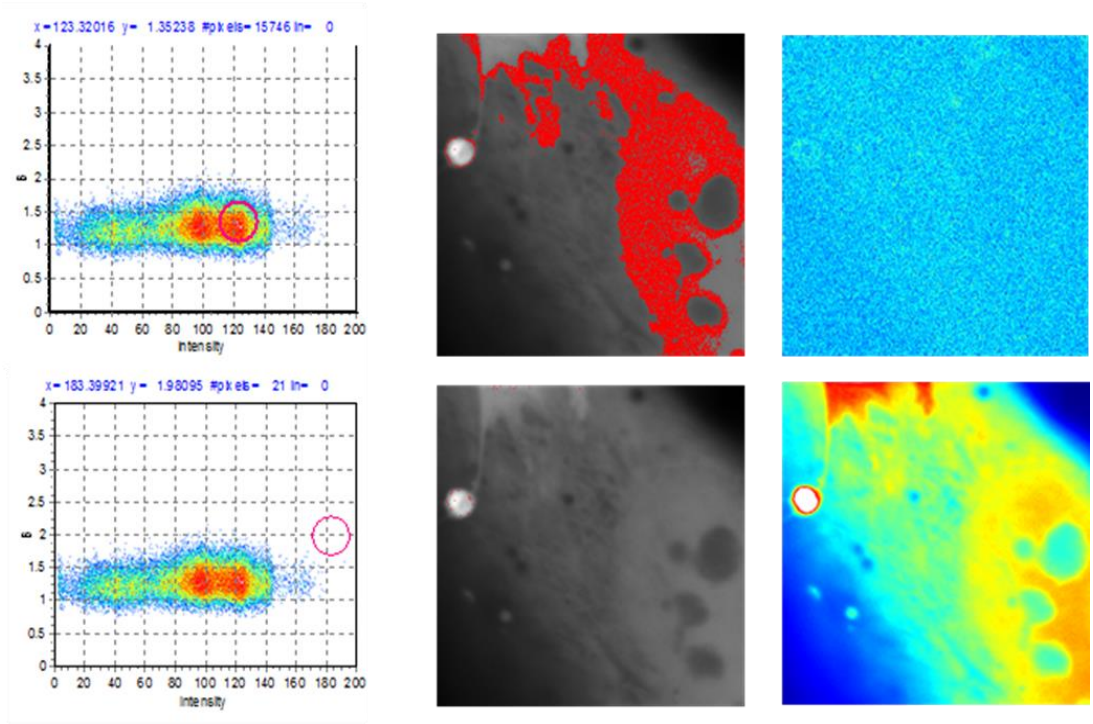


Figure 6.1A N&B analysis on JU77 cells expressing GFP without Wnt3a treatment. Establishment of B value for monomeric EGFP Establishment of B value for monomeric EGFP. No aggregation and change in intensities

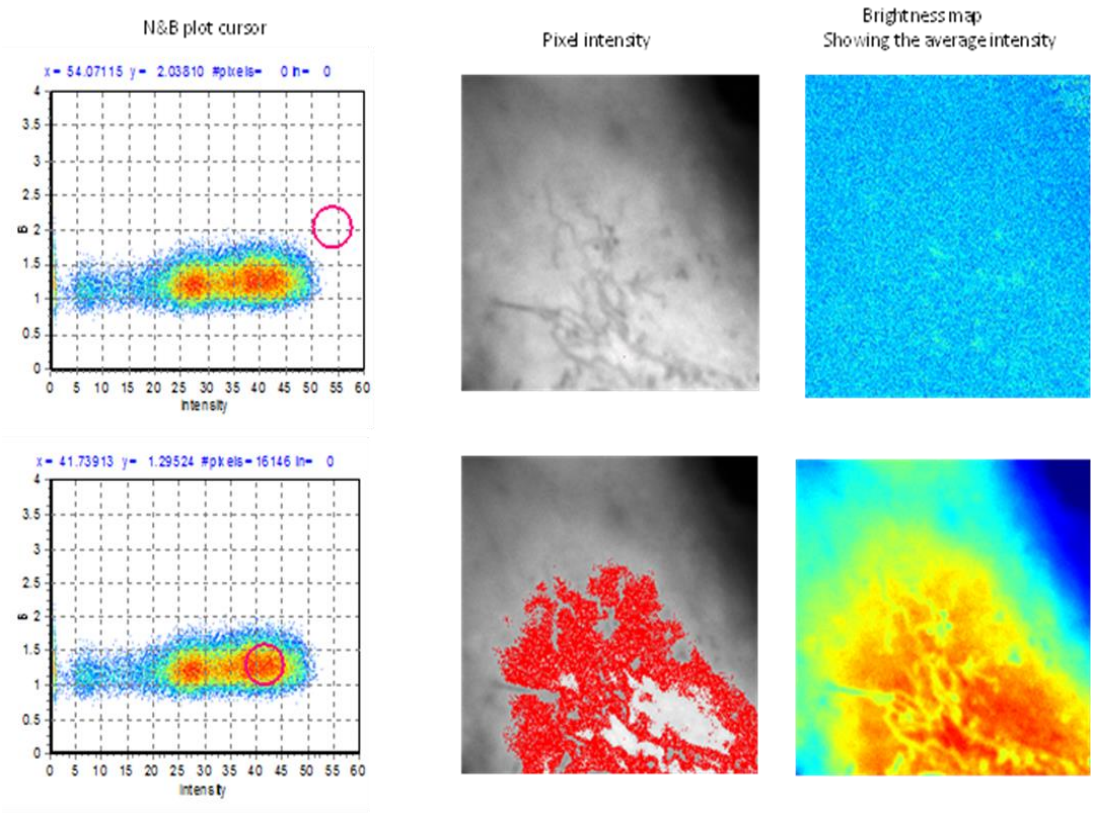


Figure 6.1B N&B analysis on JU77 cells expressing GFP without Wnt3a treatment. Establishment of B value for monomeric EGFP. No aggregation and change in intensities

6.3 Peri-nuclear localization and vesicular trafficking of sFRP4

The nature of the intracellular localisation of sFRP4 (or indeed sFRPs in general) and whether it forms oligomeric aggregates is not known. Therefore JU77 cells were transiently transfected with sFRP4-EGFP and observed by live cell confocal imaging 48 hours after transfection. The sFRP4-EGFP was localized predominantly in the peri-nuclear region (Fig 6.2) and to a lesser extent in the cytoplasm, whereas there was a very low intensity in the nucleus. The images demonstrated that there are small sFRP4-EGFP containing vesicles with a size ranging from 250 to 300 nm (essentially diffraction-limited spots) diffusing in the cytoplasm with a characteristic vesicle transport-like movement. These mobile vesicles observed in the cytosol are consistent with the secreted nature of this protein, where sFRP4 is synthesized, modified and accumulates in the endoplasmic reticulum in and around the peri-nuclear region, and then is transported via vesicles to the cell membrane from where they are secreted.

Interestingly, further analysis of these images using the number and brightness technique, did show that sFRP4-EGFP formed oligomeric aggregates and that these are particularly localised in the peri-nuclear region (Fig 6.2A). The fraction of relatively larger aggregates was determined by selecting pixels with B values larger than 1.5 (green pixels in figure 6.2, the cursor placement is shown in figure 6.2), while values of B less than 1.5 (red pixels) were classified as small aggregates or protein monomers. This indicated that overall the monomeric form is predominant within the cell and in particular this is so outside the perinuclear region. We did observe that the vesicular sFRP4-EGFP particle brightness was less than we would otherwise expect given the vesicle size and this can be indicative of quenching through interaction with other molecules. However, it is impossible to establish this with certainty based upon N&B analysis and further investigation is required to determine if this is the case.

We were interested to investigate the effect of the canonical ligand Wnt3a upon the intracellular behaviour of sFRP4 as we had previously found that sFRP4 can antagonize the effects of Wnt3a upon JU77 cells (Chapter 3). Therefore we treated sFRP4-EGFP expressing JU77 cells with Wnt3a and observed the effect by confocal fluorescent imaging. We did not observe significant changes in the area covered by

oligomeric sFRP4-EGFP (ie B>1.5) upon treatment with Wnt3a (green regions in Fig 6.2E and F). There was some indication that sFRP4-EGFP became more diffused intracellularly upon Wnt3a treatment, however, we did not observe a measurable effect upon intensity and brightness. There was little difference in the monomeric and oligomeric sFRP4-EGFP fractions as determined by the green and red selections (cursor positions shown in figure 6.2C and 6.2D) in response to Wnt3a (Figure 6.2 vs 6.2H).

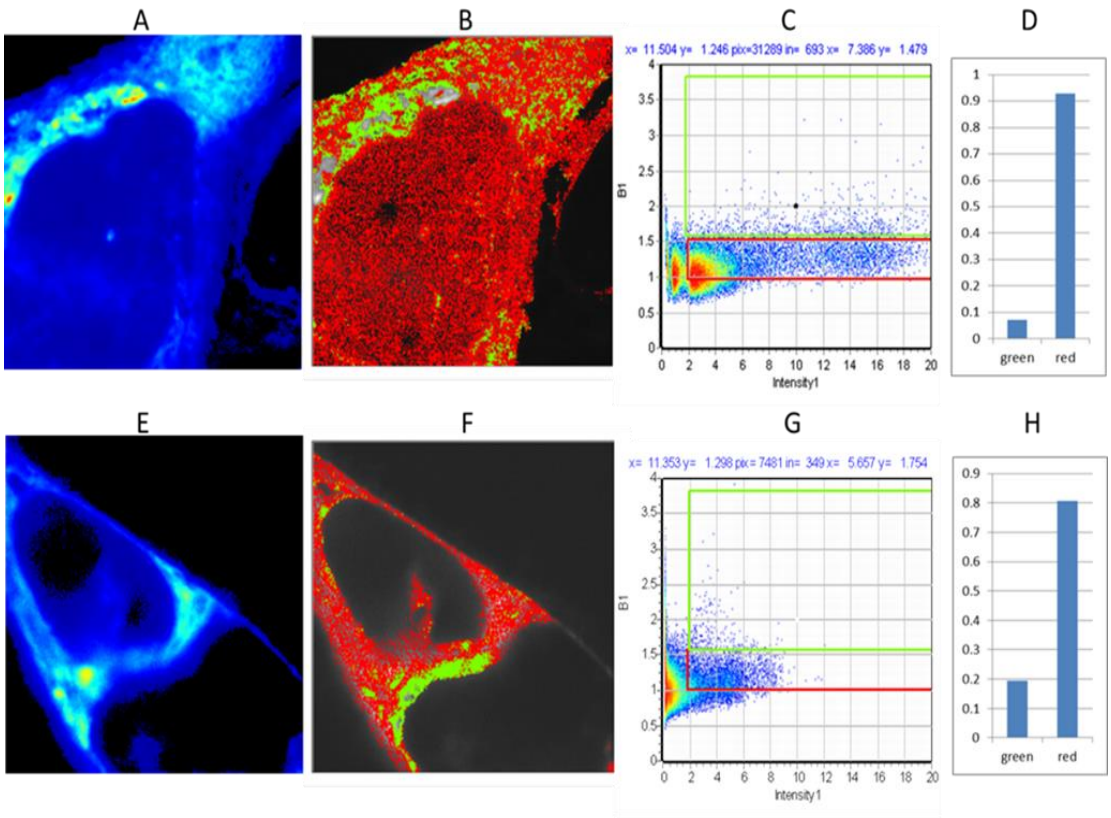


Figure 6. 2 N&B analysis on JU77 cells expressing sFRP4-GFP; A-D without Wnt3a treatment and E-H with Wnt3a treatment. A. Intensity plot. B. Selection of pixels with B<1.5 (red) and B>1.5 (green). C. B vs intensity plot. The red and green cursors were placed in the B range 1-1.5 (red) and >1.5 (green).
D. Fraction of pixel in the green and red selection cursors.

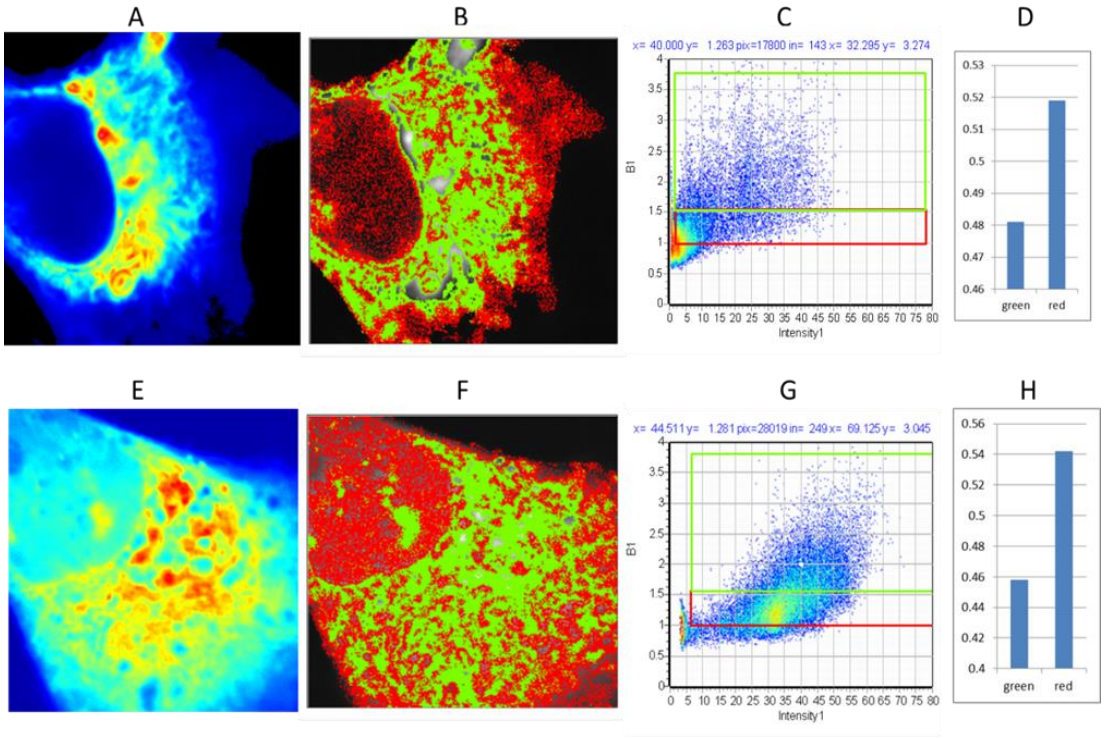
6.4 sFRP4 CRD localization, oligomerisation and response to Wnt3a

We next undertook to investigate the relative role of the CRD domain in the intracellular localisation and trafficking of sFRP4. The sFRP CRD domain is known to interact with other partners, specifically Wnts, Fzd receptors and may be capable of homodimerisation although most investigations have not identified the location of such interactions (Cruciat & Niehrs, 2013). We transfected JU77 cells with a CRD-EGFP expression construct and as before performed live cell confocal imaging after 72 hours. As with full length sFRP4 we found that CRD-EGFP was most concentrated in the peri-nuclear region, however, overall CRD-EGFP was more broadly distributed throughout the cell (Fig. 6.3A). In addition there are more pixels in the CRD-EGFP cells localized around the peri-nuclear region both with B<1.5 and B>1.5 (Fig. 6.3B) compared to sFRP4-EGFP (Fig. 6.2 A, B) which may reflect higher expression of this construct. Notably we did not observe evidence of vesicular trafficking of CRD-EGFP.

N & B analysis of these images demonstrated the CRD-EGFP behaved quite distinctly to sFRP4-EGFP with respect to aggregation properties and response to Wnt3a. The sizes of the oligomers (judged by the B value) are comparable to sFRP4 protein (Fig. 6.3B); however, the location and area where the oligomers are found are much larger in the CRD expressing cells (green area in Fig. 6.3B). In addition the distribution of aggregate sizes was different for CRD-EGFP with a much greater proportion of oligomers as shown in the graph (Fig. 6.3D).

When cells expressing CRD-GFP were exposed to Wnt3a, the distribution within the cell changed and fluorescence became more diffused (Fig. 6.3E). CRD-EGFP aggregates were not restricted to the peri-nuclear region; but seen in the cytosol as well as in the nucleus (Fig. 6.3E and F). Following Wnt3a treatment the actual area within the cell covered by the oligomers (green in Fig. 6.3F) remained constant although it became less localised (Fig. 6.3B and F). This is confirmed by the fact that the distribution of monomers/oligomers did not change (Fig. 6.3D and H). The CRD-EGFP protein must be bound to interacting partners for localisation to the peri-nuclear region and the effect of Wnt3a indicates that this interaction is affected through an undetermined mechanism. The nature and partners of this interaction with

CRD and their significance to Wnt signalling of requires further investigation since such direct effects by Wnts upon sFRPs have not previously been described.



Fgure 6. 3 N&B analysis on JU77 cells expressing the CRD . A-D without Wnt3a treatment and E-H with Wnt3a treatment. A. Intensity plot. B. Selection of pixels with B<1.5 (red) and B>1.5 (green). C. B vs intensity plot. The red and green cursors were placed in the B range 1-1.5 (red) and >1.5 (green). D. Fraction of pixels in the green and red selection cursors.

6.5 sFRP4 NLD has a vesicular association signal

In order to characterise the NLD domain we similarly transfected JU77 cells with a NLD-EGFP expression construct followed by live cell confocal imaging at 72 hours. Our observations for the localisation of NLD-EGFP were quite different to those of either the full length sFRP4 or the CRD construct. The NLD-EGFP protein was more or less uniformly distributed across the cell (Fig. 6.4). This indicates that the peri-nuclear localisation described above for the previous constructs is due to an inherent property of the CRD domain. It should be noted that all of the constructs used in the present study were engineered to contain the sFRP4 signal peptide and there is evidence that they are secreted (Longman et al., 2012).

In addition we observed bright particle-like aggregates formed by NLD-EGFP which demonstrate apparent associated with vesicles (Fig 6.5, Video Apendix 1). The size of these particles ranges from 250 nm to 300 nm and were similar in size to those seen in sFRP4-EGFP expressing cells. As can be seen in Fig 6.4B the larger oligomeric aggregates were distributed throughout the cell and significantly, there were a much higher proportion of oligomeric aggregates for NLD-EGFP (Fig. 6.4D) than for the other fusions used.

When cells expressing NLD-EGFP were treated with exogenous Wnt3a there were profound changes in the overall intracellular fluorescence intensity (Fig 6.4E) and in the relative proportion of oligomeric aggregates (Fig. 6.4H), Some particle-like aggregates remained in the perinuclear region but were strongly reduced as judged by the area in which they can be found (green areas in Fig. 6.4F, selection graph in Fig. 6.4G). This strong reduction in vesicular fluorescence in response to Wnt3a suggests that the NLD plays a role in secretion of sFRP4. Most significantly, it supports a role for Wnt3a in regulation of sFRP4 trafficking and secretion which has not previously been described in sFRPs generally. Confirmation of this observation and elucidation of the precise mechanisms and interacting partners will require further experimentation.

If we consider the relative changes of the number of pixels in the B values > 1.5, normalized to the total number of pixels, we found that activation of Wnt3a produces a larger change in the NLD expressing cells (Figure 6.6E). Therefore we concluded

that the NLD domain must have a strong vesicle associated signal since the changes in aggregation are much larger for this construct.

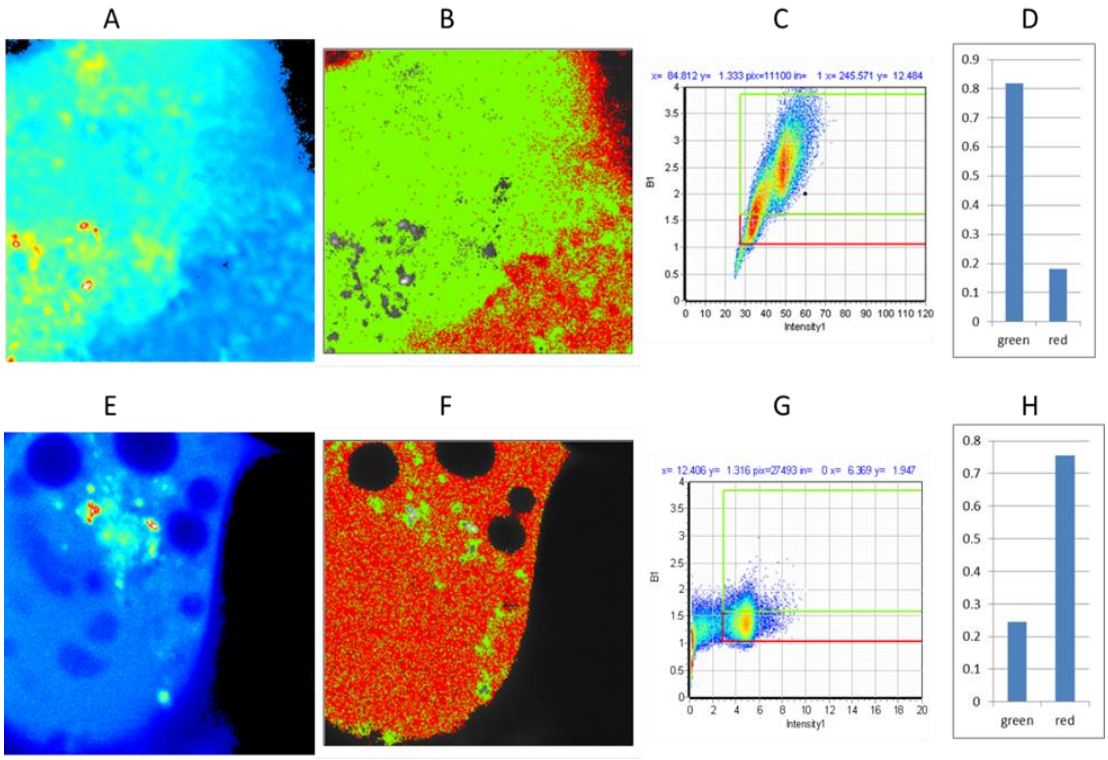


Figure 6. 4 N&B analysis on JU77 cells expressing the NLD . A-D without Wnt3a treatment and E-H with Wnt3a treatment. A. Intensity plot. B. Selection of pixels with B<1.5 (red) and B>1.5 (green). C. B vs intensity plot. The red and green cursors were placed in the B range 1-1.5 (red) and >1.5 (green).
D. Fraction of pixels in the green and red selection cursors.

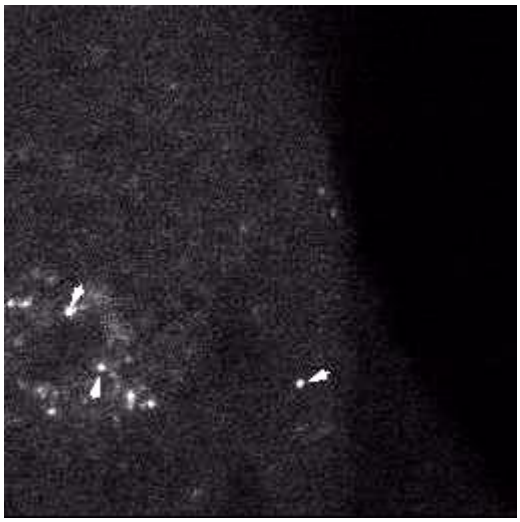


Figure 6.5 Spherical shaped vesicle like fast moving NLD domain is associated particles. White arrowheads point to these vesicle like particles.

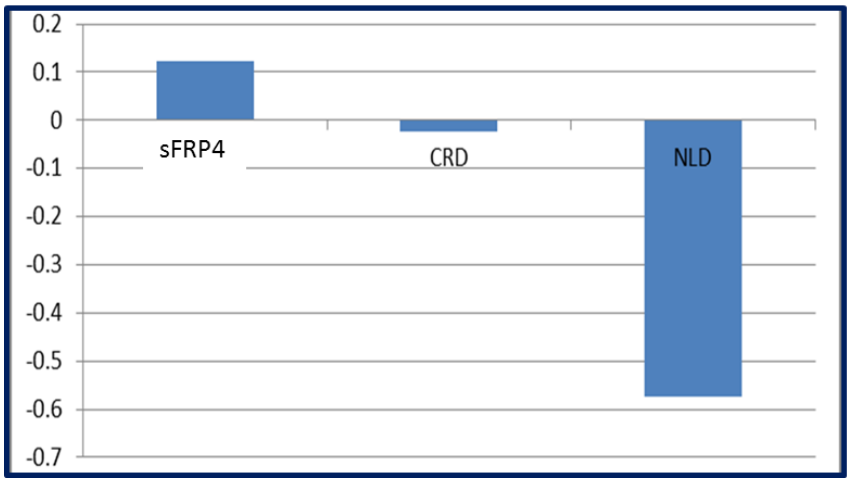


Figure 6.6 Relative change in number bright pixels with Wnt3a activation

Changes in the fraction (with respect to the total pixels) of green pixels (B>1.5) upon activation of Wnt3a.

6.6 Conclusions

Previous studies of sFRP interactions have focussed largely upon binding to relevant signalling partners such as Wnts or Fzd receptors and have not to any extent examined the intracellular behaviour trafficking and secretion of these molecules. Our results reveal a number of novel findings regarding sFRP4 which are likely to have relevance to this wider family. In particular we observed that the NLD domain has a vesicular association signal and most significantly our results suggest that Wnt3a can modulate intracellular localisation and secretion of sFRP4.

Discussion

This study reports new evidence regarding the biology of sFRP4 which is likely to have relevance to other members of this family and their role in regulation of Wnt signalling. While recent studies have improved our understanding of the intracellular production and trafficking of Wnts, very little is known regarding this aspect of sFRPs. We were able to identify vesicular trafficking of sFRP4 and we observed that the NLD domain has a vesicular association signal. Most notably our results suggest that Wnt3a can modulate intracellular localisation and secretion of sFRP4. Recent evidence has shown that the NLD domain of sFRPs interacts strongly with Wnts (Lopez-Rios et al., 2008) while the CRD most likely acts through interactions with Fzd receptors (Rodriguez et al., 2005). This is consistent with our unpublished observations that the NLD domain exerts the same biological effects as sFRP4 in mesothelioma cells. Interestingly, our results suggest that both the NLD and CRD exist in oligomeric form within the cell. Dimerisation of sFRP and Fzd CRDs has been widely reported (Dann et al., 2001; Bafico et al., 1999; Rodriguez et al., 2005), however, insofar as we are aware there is only one report of netrin dimerisation (Kochet al., 2000). How this influences the interaction of sFRP4 with other binding partners remains to be investigated, however, it has been suggested that the dimerisation of the CRD domain affects its activity (Xavier et al., 2014).The observation that Wnt3a may modulate intracellular localisation and secretion of sFRP4 is an unexpected finding of our study which requires further confirmatory investigation. Wnt signalling is tightly regulated through numerous feedback mechanisms (Logan and Nusse, 2004), primarily through gene expression of Wnt

pathway components. Negative regulators are often induced by the signals that they control including the secreted regulators Dkk (González-Sancho et al., 2005) and sFRP-1 (Gibb et al., 2013). In terms of protein trafficking, we know from recent evidence that Wnt signalling can induce ubiquitination and endocytosis of Fzd receptors as a mechanism of feedback regulation (Hao et al., 2012;Koo et al., 2012). Feedback by regulated secretion of sFRPs may provide additional fine adjustment of Wnt signalling activity at the cell membrane through interactions with ligands and receptors. In summary, our results provide evidence regarding the intracellular localisation, aggregation, trafficking and secretion of sFRP4 not previously reported. Further investigation of these findings are likely to provide insight into the function of sFRPs in Wnt regulation.

CHAPTER 7

General Discussion

Discussion

Malignant Mesothelioma continues to be a difficult and frustrating clinical problem. This aggressive type of tumor results in very poor survival in patients with most patients dying within 15 months of the first appearance of symptoms. While mesothelioma is a rare tumor it continues to have an increased incidence due to the widespread use of asbestos products until recently in industrial nations. In addition asbestos use has continued in some developing countries and so mesothelioma incidence is too likely to rise because of the long latency period (20-30years) before the disease occurs. Clinically the management of mesothelioma is difficult because of variability of the disease and lack of consistency in treatment protocols (Zucali et al., 2011). A major difficulty is the general late stage of the disease on diagnosis which limits the options for treatment in most patients. For this reason much research on mesothelioma has focussed on identifying biomarkers for earlier diagnosis. This is so far used especially in populations of asbestos exposed for ongoing monitoring. The mechanisms by which asbestos causes mesothelioma is not well understood although in recent years there have been some studies which suggest that asbestos may cause chronic inflammation (Zucali et al., 2011). The link between chronic inflammation is well organised (Elinav et al., 2013).

Understanding the alterations in molecular signalling that occur in mesotheliomas can help not just to develop suitable biomarkers but also lead to new targeted therapies. Much research is focussed on identifying these molecular changes which can drive tumorigenesis, but it is also important to follow up these studies to show the biological effects of these changes in experimental models. Then we can attain some kind of understanding of the importance of particular molecular changes to the tumour formation or progression. Similarly these changes may also be involved in the resistance of mesothelioma cells to standard chemotherapy because molecular changes which drive tumor growth can also increase anti apoptotic signalling.

The potential for particular molecules to be targeted in combination with standard chemotherapies therefore exists. The feasibility of these approaches needs to be tested in suitable cellular models to show whether a specific molecular change can affect resistance to therapy.

Many pathways have been identified as potential targets in mesothelioma by studying molecular changes in patient tumor samples (Zucali et al., 2011 ; Rascoe et al., 2012). A number of clinical trials have been carried out targeting some of these pathways with limited amount of preliminary experimentation. It is a feature of mesothelioma that because it is so deadly it is not uncommon for clinical trials to be started in this way. It would be ideal to gain a better understanding of the role of some of these molecular targets in the biology of mesothelioma which might lead to improvements in the design of these trials.

The experiments described in this thesis study were started based upon findings by other laboratories (Lee et al, 2004, Kohnoe et al 2010; He et al, 2005) and our own study (Fox et al, 2013). Showing differential down regulation of sFRP4 in mesothelioma cells and tissues. Evidence suggested that down regulation of sFRP4 by promoter methylation could result in Wnt pathway activation of concern to use what that the study that had been carried out investigating the function of sFRP4 in mesothelioma cells had used β-catenin deficient cells (He et al., 2005) and so were incapable of canonical Wnt signalling. The evidence shows that most mesotheliomas are β-catenin positive (Abutaily et al., 2003 , Orecchia et al., 2004; Uematsu et al., 2003). Therefore it was of great interest to us to investigate the biological effects and downstream signalling of SFRP4 in β-catenin expressing mesothelioma cell models (Elinav et al 2013 ; Zucali et al., 2011) cancer treatment reviews (Rascoe et al., 2012).

The effect of exogenous sFRP4 and Wnt3a on mesothelioma cells

The overall aim of this thesis study was to investigate the effects of exposure to sFRP4 in mesothelioma cells in which this gene has been down regulated. Therefore, establishing the contribution of this downregulation to mesothelioma tumorigenesis and progression. These studies could also give us information about the biology of sFRP4 in general as well as potential to inform about SFRPs. In the experiments described in Chapter 3 the effect of exogenous recombinant sFRP4 (and Wnt3a) was investigated in mesothelioma cells. These experiments were overall consistent with expectations based upon earlier studies of sFRP4 in other cancer types (Horvath et al., 2004). sFRP4 both down regulated mesothelioma proliferation and antogonised the effects of Wnt3a alone. Interestingly, the dose response study of Wnt3a revealed

a biphasic response in proliferation. Although it is difficult to find reports of biphasic Wnt activity in the literature, various researchers have commented on this to the supervisor of this project (K.Willert. personal communication). These effects may be due to feedback regulation of the pathway at high Wnt concentrations leading to downregulator of β-catenin levels (Lustig et al, 2002). An alternative explanation is that at high Wnt concentrations non-canonical signalling. While this observation is interesting based upon the literature it does not appear a common feature in other cells and most studies find the pathway is stimulated 10ng/mL Wnt3a. As this was not directly relevant to this thesis study it was not investigated further and 250pg/mL Wnt3a was used in all remaining experiments. The microscopic observation of sFRP4 treated mesothelioma cell lines showed cell rounding up morphology which caused by cytotoxic drug treatments other studies in this laboratory (Cregon et al., 2013). However, further investigation of caspase activation and mitochondrial membrane potential (MMP) failed to show clear evidence of apoptosis although there was moderate reduction in MMP. The findings of nuclear morphology were also not consistent with apoptosis. Actually they were most similar to the changes in the nuclear morphology seen in mitotic catastrophe. As recently reviewed by mechanism of cell death is less well defined but is seen as process which can later lead to necrosis or apoptosis (Vakifahmetoglu et al., 2008). Although the cell rounding up morphology is similar to Anoikis form of cell death induced by anchorage dependent cells detached from the surrounding extracellular matrix (ECM) (Frisch., et al 2001) the appearance of nuclei undergoing mitotic catastrophe is “formation of nuclear envelops around individual clusters misaggregated chromosomes” and cells are characterised by an accumulation of multiple micronuclei (Vakifahmetoglu et al., 2008). This is certainly similar to what was seen here in this study.

This mechanism has been featured an absence of caspase activation or MMP but not always (Vakifahmetoglu et al., 2008). Mitotic catastrophe would explain why most cells in sFRP4 treated cultures show rounding up morphology and abnormal nuclei but effects on MTT are not so great interms of the percentage of cells continuing to be viable by MTT assay. Mitotic catastrophe may not have as great effect on MTT assay results as seen by direct nuclear staining.

When the effect of sFRP4 on cisplatin sensitivity was determined, it was also found that sFRP4 increased sensitivity to the drug. These findings are consistent with

previous results from our lab (Fox et al., 2013) and from other cancer types (Saran., et al 2012). Our study is probably the only one that has used recombinant sFRP4 in addition to overexpression to study its role in cancer (Lustig & Behrens, 2003).

Further investigation of Wnt signalling was made in JU77 and ONE5858 using western blotting and quantitative gene expression. The results for western blotting were consistent with sFRP4 down regulating canonical signalling especially in JU77 which was generally the most responsive cell line in the experiments. Surprisingly, gene expression data did not reveal many changes consistent with activation of canonical signalling. There are a number of possible reasons behind this including that these may not be the appropriate target genes in these cells and also that the timing of expression changes can vary. It is notable that during this thesis study it was found that TCF/LEF to reporter assays did not function well in mesothelioma cell lines (data not shown). This work was also carried out by others in the lab and a collaborating laboratory and is part of ongoing research.

Most studies which have investigated the role of sFRP4 in cancer have studied proliferation or apoptosis but very few have studied migration even though canonical Wnt signalling is known to target genes involved in migration (Anastas and Moon 2012). Migration of mesothelioma cells was greatly inhibited by sFRP4 as demonstrated by the wound healing assay data. Interestingly, Wnt3a had little effect upon migration as a single agent. It did, however reduce the effect of sFRP4 on migration especially in JU77 cells. This suggests that what sFRP4 is interacting with to have its effect on migration may be competed by Wnt3a. We know from previous work that these mesothelioma cells express Wnt2b, Wnt3 and Wnt4 and Wnt5a although Wnt2b and Wnt4 are down regulated (Fox et al 2013). Hence, Wnt3 and Wnt5a in particular are possible interacting partners in this effect. Future studies could look at additional time points and wider range of genes than used here.

Interestingly, a recent report suggests that sFRP4 does not bind with Wnt3a or inhibit Wnt3a signalling as measured by reporter assay (Carmon and Loose. 2010) .This does not agree with our own results which suggest that there is some interaction between sFRP4 and Wnt3a and that sFRP4 can antagonise the effects of Wnt3a. However, the experiments were performed in different cellular context which could explain the different findings. They are not totally in disagreement with our own

results since sFRPs may also act by interactions with Fzd receptors and may not need to directly bind Wnts (Rodnguez et al, 2005).

The effect of endogenous overexpression of sFRP4 and its domains on mesothelioma cells

The objectives of the experiments described in Chapter 4 was to firstly confirm the results by Chapter 3 could be repeated by endogenous expression of sFRP4 and secondly to investigate the role of the domains of sFRP4 and its biological effects. Overall it was found that endogenous sFRP4 expression did show similar effects on proliferation, migration and drug sensitivity to those found using exogenous protein and reported in chapter 3. There were some differences in the size of the effects but this is not unexpected as endogenous over expression levels may vary. Of interest was the finding that the biological effects of overexpression needed longer to be seen in transfectants. Accumulation of EGFP protein within the cells took at least 48 hours and this may account for the delays. Importantly, in cells transfected with sFRP4 the same nuclear morphology features were seen indicating an induction of a mitotic catastrophe.

These experiments with overexpression of the CRD and NLD domains gave particularly interesting results which, while not consistent with the earliest studies of sFRPs, are completely in agreement with some recent reports of other sFRPs. It was clear from these experiments that the effect of sFRP4 on proliferation, migration and nuclear morphology were also seen when cells were transfected with netrin domain expression constructs. This was not the case for CRD domain which had only moderate effect on proliferation and now effect on migration or nuclear morphology.

Our results are consistent with recent studies that show that the NLD of other sFRPs bind with Wnts at high affinity and can antagonise Wnt signalling (Bhat et al., 2007; Rodriguez et al., 2005) Our findings suggest that the function of different domains in sFRP4 are similar to those described for other sFRPs atleast in the mesothelioma cell models used here. From previous studies in our laboratory (Fox et al, 2013) ONE58 expresses 7/10 Fzd receptors (1, 2, 4, 5, 6, 7, 8) while JU77 expresses 3/10 (2, 4, 6) and the significance of this for Wnt signalling and sFRP4 has not been investigated in these models.

Delivery of sFRP4 expression constructs by chitosan nanoparticles

Some studies have shown that sFRPs can potentiate Wnt signalling in some circumstances (Von Marschall & Fisher, 2010 ; Xavier et al, 2014). We did not see any evidence of this in the experiments performed in this thesis. In the experiments described in Chapter 5 the feasibility of using nanoparticle encapsulated sFRP4 expression constructs to deliver genes to mesothelioma cells was examined (Xavier et al., 2014). Chitosan nanoparticles represent an attractive method for gene delivery because of their biocompatibility and their affinity for DNA. Since chapter 3 and 4 showed the effects of sFRP4 and sFRP4 gene expression in mesothelioma models in Chapter 5 the delivery of these genes in a format suitable for possible future animals' studies was explored. Such studies were beyond the scope of this thesis but valuable preliminary data on formulation and ***invitro*** effect was obtained which could form a basis. The nanoparticles concentrations used were on the basis of observations of adverse effects on cultures of higher concentrations. This limited the delivery and such effects have been previously described (Dass et al., 2007).

Some studies have reported that transfection with chitosan nanoparticle DNA is less efficient than conventional transfection (Dass et al, 2007) and this is seen as a problem for future application (Mansouri et al., 2004). This was not the case in the mesothelioma cell lines used here where it was found that chitosan gene delivery was equally efficient to transfection as seen in chapter 5 studies. These contrasting findings may just be due to the well understood differences between efficiencies between cell lines (Mansouri et al., 2004). In addition it should be noted that for the nanoparticle transfections less plasmid DNA was used because of the nanoparticle toxicity and this means that effectively the nanoparticles were most likely more efficient.

Along with this similar efficiency those were some differences observed between the nanoparticle transfections and FUGENE® HD reagent (Promega, NSW, Australia). In the conventional transfections biological effects on the cells in terms of proliferation or morphological changes took up to 6 days to become apparent. In contrast in nanoparticle treated cells the biological effects were first seen sooner, after 48 hrs. The mechanism behind this effect is unknown and so far we have not found similar observations described in the literature. These nanoparticle studies

described in Chapter 5 can form a basis for future investigation and also can give ideas about the application and chitosan nanoparticles with sFRP4.

Live cell imaging of sFRP4 and domains in mesothelioma cells: number and brightness analysis

The experiments described in Chapter 6 were started to gain better understanding of sFRP4 in particular and sFRPs in general. In many ways they represent the major aim of the research which is not only to understand the basic biology of this molecule and its role in Wnt signalling. A recently developed method of fluorescence microscopy analysis the number and brightness method was applied to study sFRP4 trafficking, intracellular localisation and aggregation properties. This had not been previously done for any sFRP4 molecule. It is also important to admit that another motivation was the introduction and development of expertise in this technique in our laboratory. The microscope technology had only recently been installed in our facility and these experiments were carried out in collaboration with experts who were based in South Australia and California. As can be considered it was challenging to establish the techniques in these circumstances as only a few researchers worldwide use this technique although it has also been applied recently in another Wnt signalling study (Pate et al., 2014).

As a result we were able to identify some novel findings about sFRP4. The role of the NLD domain in vesicular association and trafficking of sFRP4 was established. Even so if there is evidence that the CRD domain is secreted from other studies of sFRP4 (Longman et al., 2012) and other sFRPs, (Xavier et al., 2014). Therefore the absence of vesicle association doesn't prevent sFRP-CRD secretion. The most surprising result of these experiments was the effect of Wnt3a on intracellular localisation and trafficking of sFRP4 and its domains. We had no expected outcomes from these experiments but were just exploring the biology involved. We considered that the Wnt3a might influence the aggregation of sFRP4. These results suggest that Wnt3a can induce secretion of sFRP4 through a mechanism involving the NLD and vesicular trafficking. Further experiments would be required to confirm these results but unfortunately time was not sufficient. It would be interesting to explore whether a similar mechanism exists for other sFRPs.

In conclusion, sFRP4 which is downregulated in mesothelioma is able to inhibit proliferation, migration and induce mitotic catastrophe in mesothelioma cells. These effects are mainly caused by the functions of the sFRP4 netrin like domain. sFRP4 is also able to inhibit signalling by Wnt3a in mesothelioma cells. Fluorescent microscopy studies showed that the sFRP4 netrin like domain possesses a vesicular localisation signal. The finding the Wnt3a can modulate the intracellular behaviour and likely secretion of sFRP4 is important for understanding sFRP biology. Overall this thesis has given supporting evidence for the targeting of the Wnt pathway in mesothelioma in future research. It has also given valuable information about sFRP4 biology and function.

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