School of Pharmacy

Wnt Signalling in Malignant Mesothelioma

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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 acknowledgement has been made.

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Date: 30th June 2016

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ABSTRACT:

An evolutionarily conserved signalling system with roles in development, disease and cancer, Wnt signalling is the subject of intense research in cancer biology for therapeutic targets. However, little is known about the role of this system in malignant mesothelioma, a cancer characterised by poor prognosis and resistance to chemotherapy. This thesis study explores a range of aspects of Wnt signalling in mesothelioma cell culture models.

The overall profile of Wnt signalling pathway molecules, the effect of directly targeting the canonical pathway using the tankyrase inhibitor XAV939 and the investigation of histone deacetylases using the inhibitor SAHA were performed *in vitro* using mesothelioma cell lines. We employed two and three-dimensional cell culture and a battery of functional assays to evaluate effect of these inhibitors. These included scratch assay, cell viability assays, three dimensional spheroid culture, colony formation / adhesion assays, Transwell® migration assay, reverse transcription PCR, quantitative PCR and RNA sequencing analysis.

The profile of Wnt signalling in three mesothelioma cell lines was established and a differential expression of a number of Wnt pathway genes identified including Wnt4 which was further explored although no phenotypic effects were observed. Inhibition of β catenin mediated Wnt signalling by tankyrase inhibition showed effects upon proliferation in two of four cell lines (JU77 and LO68) in monolayer but not in three-dimensional culture assays. Interestingly, in the two cell lines which did not show a proliferative inhibition by XAV939 (NO36 and ONE58), this compound did reduce cell adhesion and migration. Subsequent RNA sequencing experiments demonstrated downstream transcriptome profiles and patterns in JU77 and LO68 cells.

Inhibition of histone deacetylases by SAHA was cytotoxic to both ONE58 and JU77 cell lines although the latter was more resistant. This finding was consistent in both 2D and 3D culture models. Based upon these and other results from our laboratory, RNA sequencing was used to determine the effects of SAHA upon the profile of Wnt pathway genes in these cells and correlate this transcriptome data with other phenotypic data.

These studies characterise Wnt pathway signalling in mesothelioma models and indicate a role for this pathway in the biology of this cancer, suggesting that targeted strategies being explored in other cancers may have application here. Any further insight via the exploration of Wnt signalling in this disease would be of great value towards the discovery of therapeutic targets and treatment strategies.

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Lastly, I would like to dedicate this thesis to my late father, Dr. Benjamin G. Richards who encouraged my interest in the biological sciences and who taught me the value of education and perseverance.

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For simply being the very person I had always hoped to meet, at a time when I least expected it. Thank you for all your love and support, I could not possibly have asked for anything more.

Knowledge rests not upon truth alone,
but upon error also.

Carl Jung

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1.0. Introduction:

Malignant mesothelioma (mesothelioma) is an exclusively fatal malignancy that is characterised by three key attributes. These are a long latency from time of asbestos exposure to clinical presentation, a rapid and poor prognosis after diagnosis as well as a resistance to conventional cancer treatment methods. Despite the declining use of asbestos and mineral fibres in industry, the number of cases is still expected to increase with epidemiological predictions varying on the exact timing of peak incidence. It is hoped that investigations into this highly refractory malignancy will provide some insight towards its management and that research in this field will also contribute to the knowledge in other more common cancers.

The Wnt signalling system is an evolutionarily conserved set of morphogenetic proteins that govern tissue formation, function and turnover in development as well as in the developed organism. The complex interplay between the Wnt ligands, their receptors, associated biochemical pathways and functional outcomes is being avidly investigated by researchers in the fields of developmental biology and medicine. Pathological Wnt signalling has also been shown to be a contributing factor to malignancy and ongoing research has assisted in the discovery of several molecular targets with the aim of developing selective therapeutic strategies to augment the current methods employed.

The aetiology of mesothelioma, current clinical treatment modalities, pharmacological manipulation of Wnt signalling via inhibition of two classes of enzymes and the introduction of alternate culture techniques are discussed in this chapter. Brief overviews of Wnt signalling, the enzymes concerned and the pharmacological rationale for their inhibition is also included as background information upon which later experiments were designed.

1.1. Aetiology of malignant mesothelioma.

Malignant mesothelioma is characterised by the key traits of a prolonged latency between exposure to asbestos, late stage diagnosis followed by a short survival time and resistance to therapeutic modalities (Porpodis et al., 2013). The occurrence of a respiratory cancer associated with the mining and processing of this mineral fibre had been noted since the 19th century (*Wagner, E.*(1870) in McDonald and McDonald, 1996) but the definitive association of asbestos exposure as the principal causative factor was finally established in the seminal study by Wagner et al., (1960). Since then, a greater degree of awareness has been gained about this carcinogen and increased regulatory control has seen the decline of its use in industry. Similarly, an increase in research into its pathophysiology and management has also occurred over time. The estimated latency time between exposure to diagnosis is estimated to be between 20 to 50 years with a suggested mean of 32 years (Demirer et al., 2015; Porpodis et al., 2013) or in some cases, an even longer latency period is expected (Bianchi and Bianchi, 2006). The mining of asbestos and manufacture of asbestos products continues in isolated centres especially in the developing world and whilst the incidence rate has fallen in industrialised countries, regions such as the former Soviet Union, Brazil, and China may experience an increase in the incidence of mesothelioma (Becklake et al., 2007). The lack of data in areas that still produce asbestos outside of the developed world is acknowledged as a major limiting factor in the accurate prediction of epidemiological trends (Bianchi and Bianchi, 2006).

The term *"asbestos"* broadly describes at least five different fibrous silicate minerals classified as either serpentine or amphibole fibres based on their crystalline morphology. Comprising of mineral fibres with an essential $Si_2O_5(N)$ structure complexed with magnesium, iron, sodium, calcium and hydroxyl groups, the serpentine and amphibole fibres are the most widely used and investigated forms of asbestos The serpentine form consists of chrysotile ($Mg_3(Si_2O_5)(OH)_4$), otherwise known as 'white asbestos' which exists in a tangled, coiled structure. The amphibole fibres are needle-like in appearance and include crocidolite ($Na_2(Fe^{3+})_2(Fe^{2+})_3Si_8O_{22}(OH)_2$), amosite $Ca_2(MgFe)_5Si_8O_{22}(OH)_2$ known as 'blue asbestos' and 'brown asbestos' respectively. The less common forms of asbestos are tremolite, anthophyllite and actinolite which are also less widely used in industry (Weiner and Neragi-Miandoab, 2008; Roe and Stella, 2015).

Debate continues with a complex body of evidence that suggests greater carcinogenicity amongst the amphibole fibres, crocidolite in particular compared to the serpentine fibres represented mainly by chrysotile (Gibbs and Berry, 2007). However, the presence of tremolite together with chrysotile in nature remains a confounding factor and therefore, determination of carcinogenicity in the amphibole fibres remains unclear (McDonald and McDonald, 1996). Other mineral silicate fibres with asbestos-like properties have also been mined commercially and the carcinogenicity of erionite, an asbestos-like fibre has also been recently studied by meta analysis (Demirer et al., 2015). Analysis of data from the studies reviewed by Demirer and colleagues suggests that erionite possesses similar carcinogenic properties to asbestos in causing mesothelioma and the influence of other mineral fibres such as winchite, richterite, fluoroedenite and antigorite cannot be discounted.
Irrespective of chemical composition, the carcinogenic properties of mineral fibres cannot be ignored or their hazardous nature downgraded to a relative risk between members of a certain class. The deleterious consequences of exposure are apparent and thus every such substance should be considered a carcinogen (McDonald and McDonald, 1996).

1.2. Pathophysiology of malignant mesothelioma.

The mechanism(s) by which inhaled asbestos fibres are deposited to the pleural margin are not fully understood with crocidolite fibres being avidly investigated and the nature of the relationship between fibre type, length, diameter and carcinogenicity remains unclear. A range of dimension for crocidolite fibres of at least 8µm length and less than 0.25µm width has been suggested by Yang et al., (2008) who also discuss the difficulties in establishing a definitive sizing range. Several models have been proposed to explain the movement of fibres through the lung parenchyma and this includes possible movement along the paracellular pathway, following the movement of water as a result of the osmotic gradient created by Na^+/K^+/ATPase function (Miserocchi et al., 2008). These authors also suggests other mechanisms of fibre movement which include the loss of extracellular matrix integrity due to metalloproteinase activity associated with inflammation, migration along pulmonary lymph tracts and movement with the bulk flow of water into other body areas (Miserocchi et al., 2008).

The structure and function of the human mesothelium has been reviewed by Mutsaers (2002) who outlines its structure and function in the pleural, peritoneal and pericardial cavities with the obvious implications in metastasis. The ability of the mesothelium to secrete mediators and molecules as a biochemical response is of importance in leucocyte chemotaxis, tissue repair, particle transfer and tumour progression and it is this reactivity that underpins the pathogenesis of mesothelioma (for review, see Mutsaers, 2002).

Deposition of asbestos fibres into the pleural cavity initiates a series of biological consequences that give rise to mesothelioma. The exact order and nature of events is still not fully understood but cytotoxicity, DNA damage, impaired phagocytosis and chronic inflammation are known concurrent processes (Roe and Stella, 2015). Cytotoxicity is thought to be a result of an oxidative burst due to frustrated phagocytes, thereby generating reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Ramos-Nino et al., 2006) as well as the superoxide (O_2^-) and hydroxyl ($OH^\bullet$) radicals (Jaurand, 1997). The amphibole fibres crocidolite and amosite are considered the most toxic fibres due to their higher iron content (Ramos-Nino et al., 2006) and may produce a greater number of superoxide radicals by reaction with iron in the Fenton reaction mechanism (Jaurand, 1997). Similarly, crocidolite and amosite are also thought to have the greatest effect in direct interference of mitosis due to the needle-like morphology, although the tangled chrysotile fibres cannot be discounted as harmless either. (Yang et al., 2008; Roe and Stella, 2015). Direct entanglement with the mitotic spindle may also produce chromosomal abnormalities such as aneuploidy and polyploidy from deregulated cell division in addition to chromosomal breaks, deletions, translocations and inversions (Jaurand, 1997; Yang et al., 2008; Sekido, 2010). The profile of chromosomal abnormalities will vary greatly between patients and perhaps even between individual tumour sites when the random nature of chromosomal deregulation is considered. Certain trends however have been noted such as chromosomal gains in chromosomes 5,7,8 and 17 or deletions in chromosomes 1,3,6,9,13,14,15 and 22 (for review, see Musti et al., 2006).

The two best characterised genetic derangements in mesothelioma are the deletions of cyclin dependent kinase 2A (CDKN2A; also referred to as $p16I^{NK4A}$ or $p14^{ARF}$ depending on variant) and that of the tumour suppressor gene neurofibromatosis 2 (NF2) (de Assis et al. 2014). CDKN2A acts via its transcriptional variants to regulate the cell cycle through inhibition of the retinoblastoma (Rb) protein by $p16I^{NK4A}$ and stabilisation of p53 by $p14^{ARF}$ (Lee et al., 2007). The tumour suppressor protein NF2 is known to inhibit proliferation by suppressing cyclin D1 expression (Xiao et al., 2005) and interestingly, while p53 and Rb mutations are common in other cancers, these remain relatively rare in mesothelioma (Lee et al., 2007).

Other noteworthy tumour suppressor genes with potential roles in the pathogenesis of mesothelioma include BRCA1-associated protein (BRAP1) and Large Tumour Suppressor 2 (LATS2) (for review, see de Assis et al., 2014). Other entries include the FUS1/TUSC2 tumor suppressor gene which has been shown to be present in mesothelioma (Ivanova et al., 2009). It is interesting to note that both FUSC1/TUSC2 and BRAP1 are both located on chromosome 3 which is a known region of deletion in mesothelioma (Musti et al., 2006).

The influence of growth factors such as Transforming Growth Factor β (TGFβ), Platelet Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), Insulin-Like Growth Factor (IGF) and Interleukins 6 and 8 has been raised by Yang and colleagues (2008). These authors also highlight the ability of asbestos fibres to directly activate the Extracellular Signal Related Kinase pathway (ERK1, ERK2) by inducing dimerization of the Epidermal Growth Factor (EGF) receptor. The resultant autophosphorylation of dimerised EGF receptors is thought to provoke a pathologically active Wnt signal (Mossman et al., 2013). Additionally, the role of chronic inflammation must also be accounted for through the release of inflammatory cytokines such as Interleukin-1β and Tumour Necrosis Factor α (TNF-α), the latter of which stimulates activation of the NF-κB pathway which in turn favours the survival of genetically compromised cells with eventual transformation to the cancerous state (Carbone and Yang, 2012).

Gene methylation in mesothelioma has also been examined with a study of the methylation status of 28 genes in mesothelioma which stated that gene methylation in mesothelioma is very common but further research is required to determine the critical threshold in this disease. Several Wnt molecules were also examined and a statistically significant degree of methylation was noted for Secreted Frizzled Related Protein 4 (SFRP4), a tumour suppressing gene in the Wnt signalling system (Tsou et al., 2007).

The scientific debate continues on the influence of Simian Virus 40 (SV40) in the pathogenesis of mesothelioma either as a sole agent or as a co-carcinogen together with asbestos. Differing results between investigators and varying methodologies have produced evidence for and against the involvement of SV40. The ability of the SV40 Large T Antigen (Tag) to bind and inactivate p53 and Rb in cancer has been reviewed thoroughly by Testa and Giordano (2001) and follows a great degree of investigation following the administration of polio vaccines contaminated with SV40 during the period from 1953 to 1963 (Qi et al., 2011). The eventual transformation process from mesothelial cell to malignant cell has yet to be fully characterised as genetic studies are often conducted in tissue specimens, animal models and cell lines where observation of pathogenesis is not possible (Mossman et al., 2013).

1.3. Treatment and clinical aspects of malignant mesothelioma.

The long latency between asbestos exposure and overt disease often results in presentation at an advanced stage of malignancy where chest pain, dyspnoea, cough and pleural effusion are apparent. Pleural effusions in particular may not be present in all cases and therefore, despite being a notable characteristic, it is only one of several diagnostic criteria used for accurate diagnosis (Zhang et al., 2015). Differential diagnosis of mesothelioma involves the use of imaging methods, aspiration of pleural effusions and tissue biopsy together with the histological detection of tumour invasion into surrounding tissues (van Zandwijk et al., 2013). Tissue biopsy remains the principal method of investigation as tumour infiltration is observable as opposed to analysis of pleural effusions (van Zandwijk et al., 2013) but serum biomarkers such as mesothelin and osteopontin have been found to be of limited diagnostic value and are used to support immunohistochemical investigations which exclude other benign mesothelial hyperplasic disorders (van Zandwijk et al., 2013).

The coordinated treatment of malignant mesothelioma involves the application of a trimodal treatment strategy as developed by Sugarbaker and Garcia (1997) consisting of surgical tumour reduction, chemotherapy and radiotherapy which produced a median survival in that study of 21 months. A modified order of treatment has since been devised in which chemotherapy precedes surgery and post operative radiation treatment (van Schil et al., 2010). Currently there is no consensus on the order of modality utilisation due to the low numbers of eligible patients (due to co-morbidities), inability to complete treatment and the uncertainty surrounding the benefits of surgery (Zauderer and Krug, 2011). However, the regimen proposed by van Schil and colleagues (2010) has been favoured in the National Health and Medical Research Council guidelines developed by van Zandwijk et al., (2013) who also acknowledge the lack of controlled comparisons between treatment strategies.

Surgical interventions comprise of two major methods; pleurectomy / decortication (P/D) and extrapleural pneumonectomy (EPP). Pleurectomy/decortication involves the removal of the pleurae, pericardium and affected parts of the diaphragm and is thought to be associated with a slightly better prognosis (Cao et al., 2014). It is also thought that a reduction in tumour burden within the thoracic cavity will also allow a greater dose of post operative radiotherapy in order to improve treatment efficacy (Haas and Sterman, 2014).

Previous treatment regimes for mesothelioma were based on anthracycline DNA intercalating agents such as doxorubicin and epirubicin which were replaced by platinum agents due to cardiac toxicity. The platinum agents are namely cisplatin and carboplatin (which are considered to be therapeutically comparable) with relatively less known about the therapeutic value of oxaliplatin (Kindler, 2008). Topoisomerase inhibition in mesothelioma has also been relatively unexplored as studies in mesothelioma have not progressed beyond combined treatment consisting of topoisomerase I inhibition with irinotecan and cisplatin (Kindler, 2008).

The current chemotherapeutic rationale is the result of a pioneering study by Vogelzang and co-workers (2003) who used cisplatin and pemetrexed (an anti-folate) to produce a 12.1 month survival time and it is this regimen that forms the basis of the mesothelioma chemotherapy. The DNA alkylating actions of cisplatin were found to be augmented by the anti-folate effects of pemetrexed which inhibits not only dihydrofolate reductase but also

thymidylate synthase and glycinamide ribonucleotide formyl transferase (Kelly et al., 2011). The cisplatin/pemetrexed combination is currently the only FDA-approved treatment combination for mesothelioma (Zauderer et al., 2014) and has since become the regimen of choice replacing the previous combination of cisplatin and gemcitabine although this combination is still in use as a second-line treatment (Kelly et al., 2011). Despite being seen as a second-line adjunct to cisplatin, gemcitabine may still be of use in patients unable to tolerate other cisplatin / anti-folate drug combinations as the pharmacodynamics effects of gemcitabine appear to be independent of thymidylate synthase inhibition (for review, see Mini et al., 2006). Of the vinca alkaloids, vinorelbine has been investigated on its own and in combination with cisplatin or carboplatin in first and second-line therapy regimens. But proper evaluation has been hampered by a lack of scientific consistency and therefore its therapeutic value in mesothelioma is uncertain (for review, see Ceresoli and Zucali, 2015).

Other pharmacological approaches include blockage of growth factor signalling either by complexation of receptor binding sites with antibodies or small, synthetic kinase inhibitor molecules selective for various downstream cascades. Notable examples of receptor-level antibody-mediated signal inhibition include EGF blockade by cetuximab and VEGF pathway inhibition by bevacizumab (Favoni et al., 2015; Kotova et al., 2015). Downstream receptor tyrosine kinase inhibitor molecules have been synthesised to target growth factor pathways such as EGF (erlotinib, gefitinib), VEGF (sorafenib, sunitimib) and the mTOR (mammalian Target of Rapamycin) pathway by everolimus although their use in the treatment of mesothelioma has yielded indifferent results (Kotova et al., 2015).

The overall response to trimodal therapy is still poor with survival times measured in months rather than years and complication by profound drug resistance resulting in an exclusively fatal malignancy (Vogelzang et al., 2003; Kindler, 2008). Currently, the cisplatin/pemetrexed combination represents the mainstay of mesothelioma chemotherapy with marginal benefit noted with the use of other agents.

1.4. Molecular mechanisms of chemotherapy resistance in malignant mesothelioma.

The characteristic resistance of mesothelioma to chemotherapeutic agents has posed a significant challenge in both the research and clinical settings. The focus of this thesis was the role of Wnt signalling in malignant mesothelioma and there exists some evidence to suggest the involvement and potential manipulation of this system to attempt to resolve this issue. An early insight into Wnt signalling and drug resistance in mesothelioma was gained by the potentiation of the effects of pemetrexed with the abolition of the expression of the Wnt2 protein (Mazieres et al., 2005) and a subsequent study (Uematsu et al., 2007) increased the sensitivity of mesothelioma cells to cisplatin after downregulating the expression of the Dishevelled (Dvl) phosphoprotein. The induction of apoptosis with a monoclonal antibody against the Wnt1 protein also demonstrated the possibility of using the Wnt signalling system as an avenue for therapeutic intervention (He et al., 2004). The classification of the Secreted Frizzled Related Proteins (SFRPs; *see Section 1.7*) as tumour suppressor genes also provided another field of investigation (Shi et al., 2007) and survival data analysed by Hirata and co-workers (2015) also suggests the role of the Wnt7a protein as a potential predictor of prognosis and chemosensitivity. (*Further information on Wnt proteins, Dishevelled, and SFRPs is available in Section 1.10*)

Several *non-Wnt* mechanisms of cellular resistance have been described in the literature which encompasses a wide array of molecular targets and treatment methods. A panel of resistance mechanisms genes against chemotherapy and radiotherapy has been identified (Roe et al., 2010) which includes BIRC5 (Survivin), BRCA2, Fanconi Anaemia Complementation Group A (FANCA) and Checkpoint Kinase 1 (CHEK1). Another series of resistance proteins has also been described (Ting et al., 2013) that includes other candidate genes such as Endonuclease Excision Repair Cross-Complementing 1 (ERCC1), MutS Homologues 2,6 (MSH2, MSH6) and βIII Tubulin as possible biomarkers for optimising platinum compound therapy. A later review of resistance molecular drug targets (Bronte et al., 2016) suggested two novel resistance mechanisms; the development of resistance to small-molecule tyrosine kinase inhibitors (erlotinib, gefitinib) or the loss of function of the Phosphatase and Tensin Homolog (PTEN) tumour suppressor gene. Resistance to doxorubicin resistance has also been examined (Ogretmen et al., 1998), antibule agents (Catalano et al., 2004) and cisplatin (Cregan et al., 2013; Eguchi et al., 2015). Other mechanisms investigated so far include the role of cAMP Binding Element Response protein (CREB) (Shukla et al., 2009), Src Kinase (SFK) activation (Eguchi et al., 2015), and the PI3K/mTOR/RIP1 pathway (Echeverry et al., 2015).

The strategy of caspase-mediated apoptosis caused by DNA alkylating agents such as cisplatin has also been examined in the supervisor's laboratory (Cregan et al., 2013) in which RNAi silencing of the anti-apoptotic proteins Survivin and XIAP did not increase the sensitivity of mesothelioma cells to cisplatin which suggests that cisplatin resistance in the cell lines used may not involve caspase-mediated apoptosis. Interestingly, a subsequent study (Cortes-Derricks et al., 2014) demonstrated an association between increased drug detoxification by aldehyde dehydrogenase (ALDH) activity and cisplatin resistance as a potential mechanism. At the time of writing, this issue is still under investigation in our laboratory.

Similarly, research into the resistance of mesothelioma to antifolate agents has also been carried out and a series of seven possible biochemical pathways has been proposed (for review, see Assaraf, 2007). Confirmation that there is no cross resistance between cisplatin and pemetrexed has been shown *in vitro* (Kitazono-Saitoh et al., 2012) and a strategy of nucleotide depletion through antifolate treatment with gemcitabine before treatment with cisplatin has been suggested in order to optimise the current platimum compound/antifolate treatment regime (Zanellato et al., 2011).

To date, the identification of chemotherapy resistance pathways and molecules in mesothelioma has produced a unique set of therapeutic targets in addition to those already known in cancer. Investigations into the influence of Wnt signalling upon chemotherapy resistance have not progressed significantly since the publications discussed here and therefore, this aspect of mesothelioma research remains as challenging as it is unclear.

1.5. Wnt signalling: an overview.

Since the discovery of the murine *Int1* gene as the integration site for the Mouse Mammary Tumour Virus (Nusse and Varmus, 1982) and its subsequent identification as the mammalian homologue of the *Wingless* segment-formation gene in *Drosophila* (Rijsewijk et al., 1987), the study of Wnt signalling has arguably become a science of its own. (The acronym *Wnt* being a combination of 'Wingless' and 'Integrated'). The human Wnt signalling system comprises the essential components of Wnt ligands, receptors, co-receptors, the key mediator protein β catenin and a growing number of Wnt-specific regulatory molecules. Conserved elements have been detected in a diverse range of experimental models such as *Drosophila,* the *C. elegans* nematode, rodents, *Xenopus*,

Zebrafish and *Nemostella* sea anemones thereby suggesting a developmental role over a timespan of around 550 million years (Cadigan and Nusse, 1997; Clevers, 2006).

Numerous findings and advances have been made using developmental models of these organisms as well as other primitive species and the vast amount of information gained from these studies has contributed to the knowledge of Wnt signalling in humans. The discipline of Wnt signalling in development and its evolutionary phylogeny has been expertly reviewed by Cadigan and Nusse (1997), Wodarz and Nusse (1998), Logan and Nusse (2004) with more information on non-human models available in these publications.

The role of Wnt signalling in human development, tissue homeostasis and disease is avidly investigated due to its presence and action in cellular processes such as differentiation, mobility, morphogenesis, adhesion, survival, apoptosis and tumour generation (Li et al., 2006; Komiya and Habas, 2008, Clevers and Nusse, 2012). The notion that Wnt proteins act as morphogens in a concentration-dependent manner, along predefined lines has been questioned (Clevers and Nusse, 2012) with the suggestion that Wnt molecules act as close range signalling mediators rather than morphogens in the conventional sense.

1.6. The Wnt ligands.

The human Wnt ligand protein complement consists of 19 ligands with several key common features that include a molecular weight of between 36kDa to 46 kDa and a conserved region of 23-24 cysteine residues thought to be important in the folding of newly synthesised Wnt proteins (Miller, 2001; Hausmann et al., 2007). Two essential modifications are known to be associated with the maturation and secretion of a nascent Wnt molecule; firstly the addition of two lipid moieties followed by N-terminal glycosylation (Lorenowicz and Korswagen, 2009). Post-translational modification begins with the addition of a palmitate group to a cysteine residue towards the amino terminus and is then followed by the addition of a palmitoleic acid group at a conserved serine residue towards the carboxyl terminus (Lorenowicz and Korswagen, 2009).

The exact roles of the palmitate and palmitoylate groups are not understood but it is thought that the palmitate modification is require for either intracellular trafficking (Coudreuse and Korswagen, 2007) or possibly to assist with the binding of a Wnt ligand to the inner layer of the cell membrane prior to storage or further processing (Lorenowicz and

Korswagen, 2009). The palmitoylate group however may be required as an anchoring group for subsequent N-linked glycosylation or to bind a ligand to a Frizzled receptor (Mikels and Nusse, 2006). Glycosylation by the enzyme oligosaccharyl transferase has not been fully characterised in humans but a possible role has been suggested in export to the cell membrane and/or binding to extracellular heparan sulphate proteoglycans during extracellular trafficking (Haussman et al., 2007). The movement of a secreted Wnt ligand is also under investigation due to their hydrophobic nature (Mikels and Nusse, 2006) and two possible mechanisms have been proposed. Firstly, the migration of secreted Wnt ligands along the glycosaminoglycans of the extracellular matrix (Kikuchi and Yamamoto, 2007) or more interestingly, the formation of an aggregate via the palmitoyl moiety of a Wnt ligand and a carrier lipoprotein in an assembly referred to as an 'argosome' (Mikels and Nusse, 2006).

Functional classification of the Wnt ligands is based on information gained from studies that involve the formation of a second body axis in a *Xenopus* embryo or by the transformation of C57MG mouse mammary epithelial cells (Li et al., 2008; Chien et al., 2009). The involvement of the protein β catenin is another important criterion and has given rise to the broad classification of 'canonical' (β catenin mediated) and 'non canonical' (non β catenin mediated) Wnt signalling. In terms of the *Xenopus* model, the ability to duplicate a body axis has been seen with the actions of Wnts 1, 2, 3, 3a, 8a and 8b in a β catenin mediated manner whereas Wnts 4, 5a, 5b, 6, 7a and 11 do not. This has led to the formation of two divisions, the 'Class 1' Wnt and 'Class 5' Wnt ligand groups respectively, based on their prototypical members (Li et al., 2006). Evidence reviewed by Chien et al., (2009) however suggests a β catenin dependent mode for Wnts 7a, 9a, 9b, 10a, 10b, 11 and 16 based on findings from non *Xenopus* studies whilst the evidence from mouse C57MG cells is still somewhat incomplete.

More recently, the concept of context-dependent signalling is challenging this dichotomous 'canonical or non-canonical' nomenclature due to the the differing models from which information is gained. Models for investigating Wnt signalling vary greatly from developmental models in lower organisms to complex mammalian models where studies focus on homeostasis in the developed organism. Caution has been advised by van Amerongen and Nusse (2009) who suggest that the outcome of a Wnt signal may depend greatly upon the nature of the cells involved and the stimulus received. The degree of overlap of Wnt function between developmental models has been addressed in a

previous work by van Amerongen and co-workers (2008) with the proposal that the nature or presence of the receptor and co-receptor subset involved may be the determining factor between canonical or non-canonical signalling and not necessarily the ligand itself. The actions of a given Wnt ligand are therefore highly dependent on several factors. Primarily, the nature of the cell(s) involved, the temporal setting of either development or tissue homeostasis and the receptor subset receiving the signal may ultimately determine β catenin dependence. Any further derangements associated with disease must then also be accounted for in order for contextual guidelines to be established.

1.7. Frizzled receptors, LRP co-receptors and β catenin mediated signalling.

In humans, ten Wnt receptors are known to exist and are named the 'Frizzled' (Fzd) receptors after the disfigured phenotype produced in *Drosophila*. The Fzds receptors belong to a distinct family of seven transmembrane domain receptors and are thought to bind Wnt ligands through a conserved cysteine-rich domain (CRD) (Schulte, 2010). Despite structural similarities to other G protein coupled receptors, the involvement of G proteins in a Wnt/Fzd interaction remains unclear (Saito-Diaz et al., 2013).

The binding selectivities of Wnt ligands and Fzd receptors has yet to be fully mapped but the known associations have been reviewed by Dijkstorhuis et al., (2014) and these authors also emphasise the receptor context of a Wnt/Fzd binding event as a key determinant of downstream signal results as suggested by van Amerongen et al., (2009) who proposed that the receptor profile dictates physical outcome. Analysis of Fzd binding affinities has been limited by a lack of suitable compounds despite the development of other Wnt-mimetic compounds which interfere directly with Wnt/Fzd binding or disrupt the intracellular response (for review, see Schulte, 2010). The exploration and targeting of Fzd binding specificities represents an opportunity to intervene in many disease states with each of the ten Fzd members having an association with a particular pathology (for review, see Dijksterhuis et al., 2014). The involvement of Fzds in cancer is the subject of considerable interest due to discoveries such as the correlation between Fzd1 expression and that of the P-glycoprotein (MDR1) multi drug resistance gene (Zhang et al., 2012), the overexpression of Fzd8 in non-mesothelioma lung cancers (Wang et al., 2012) and the involvement of Fzd7 in colorectal, hepatocellular and triple negative breast cancers (for review, see King et al., 2012).

Binding of a Wnt ligand to a Fzd receptor also requires the presence of either of Lipoprotein Receptor Related Protein 5 or 6 (LRP5, LRP6) as co-receptors. Of the two, more is known about LRP6 (Macdonald et al., 2011) and together with a Wnt ligand bound to a Fzd receptor, the Wnt/Fzd/LRP trimeric complex forms the starting point of a β catenin mediated 'canonical' signal. (Niehrs, 2012, Saito-Diaz et al., 2013). The exact manner of LRP5/6 activation is unclear although the key molecular event involves the phosphorylation of the LRP intracellular domain which facilitates the binding of Axin (Niehrs, 2012,). Various mechanisms have been suggested, some of which involve (i) recruitment of glycogen synthase kinase 3β (GSK3β) in conjunction with Axin to the cell membrane, (ii) sequential phosphorylation of LRP6 by GSK3β and then by Casein Kinase I (CKI) or (iii) an unclear model involving the production of phosphatidylinositol bisphosphate (Maiese et al., 2008; Gao et al., 2014). Another alternative proposal involves the sequential phosphorylation of LRP by CKI first, then by GSK3β (Saito-Diaz et al., 2013) and serves to highlight the need for further investigation at this level. The structural differences between LRP5 and LRP6 were examined in detail by MacDonald and co-workers (2011) who also demonstrated that the LRP/Axin association requires phosphorylation by GSK3β but GSK3β itself is not a structural component in this process. As with the Fzd receptors, LRP co-receptor dysfunction is also associated with developmental disorders and cancer and the basis for pharmacological targeting has also been summarised (for review, see Joiner et al., 2013).

The activation of the phosphoprotein Dishevelled (Dvl) is a common and critical event following the formation of the Wnt/Fzd/LRP signalling complex. The primary function of Dvl is thought to be one that facilitates the phosphorylation of LRP5/6 by one of the mechanisms described above. The eventual outcome of the actions of Dvl is the binding of Axin to the phosphorylated intracellular tails of LRP5/6 thereby recruiting Axin to the cell membrane (Kafka et al., 2014). Together with the Wnt/FZD/LRP complex, Dvl facilitates binding of Axin to phosphorylated LRP5/6 which in turn prevents the breakdown of β catenin (see next section for details on β catenin degradation).

The role of the Frequently Rearranged in Advanced T Cell Lymphoma (FRAT1) protein is also unclear, its potential function being one of GSK3β inhibition either directly (Ilyas, 2005) or in conjunction with Dvl, thereby preventing LRP phosphorylation and subsequent recruitment of Axin to the cell membrane. The joint Dvl/FRAT model has been shown to require phosphorylation by CKI in an isoform-dependent manner whereby CKIε promotes Wnt signalling and CKIα inhibits it (Hino et al., 2003). The relative importance of FRAT in

Wnt signalling *in vivo* is also unknown as *in vivo* experiments with mice deficient in all three isoforms produced no gross abnormalities and no alterations of Wnt signalling *in vitro* (van Amerongen et al., 2005). FRAT, its isoforms and their influence on Wnt signalling remains an unanswered question in this field.

1.8. Role of β catenin and its regulation in Wnt signalling.

Further to the formation of the Wnt/Fzd signalling complex, activation of Dvl and phosphorylation of LRP5/6 and recruitment of Axin to the cell membrane, the result is one of β catenin stabilisation through the disassembly of its breakdown mechanism. The β catenin protein is the key effector molecule of canonical Wnt signalling and is unique as it serves as a component of the adherens junction in complex with ε-cadherin and α-catenin as well as a nuclear function by promoting the expression of target genes regulated by the TCF/LEF regulatory system (Willert and Nusse, 1998; MacDonald et al., 2009). Studies by Gottardi and Gumbiner (2004) have identified the carboxyl terminus as a conformational regulator of cadherin and nuclear transcription factor binding and proposed that Wnt signalling not only increases intracellular β catenin, but changes in the conformation of the carboxyl terminus may indeed determine balance between its structural and gene-expressing roles. The mobilisation of cadherin/catenin bound β catenin has been associated with the epithelial-mesenchymal transition (EMT) through the dissociation of the cadherin/catenin complex (Brembeck et al., 2006) as well as dysfunctional growth factor signalling and the resultant phosphorylation of critical tyrosine residues, thereby liberating β catenin from the adherens junction (Fang et al., 2007).

Control of the cytoplasmic pool of β catenin is mediated by a regulatory complex often referred to as the 'β catenin destruction complex' that consists of CKI, GSK3β, the adenomatous polyposis coli protein (APC) and an essential scaffold of Axin (Ikeda et al., 1998; Huang and He, 2008; Saito-Diaz et al., 2013). The interplay between Axin and APC is still the subject of much research, especially Axin which is thought to provide the molecular scaffold of the β catenin destruction complex (Ikeda et al., 1998). In humans, Axin exists as either Axin1 or Axin2 and despite being considered functionally equivalent, Axin2 is associated with the negative regulation of Wnt signalling and its implications in cancer (Mazzoni and Fearon, 2014). Regulation of intracellular β catenin levels requires the formation and stabilisation of the destruction complex and phosphorylation of Axin2 by GSK3β is critical to maintaining the stability of Axin (and therefore the complex as a whole)

in addition to increasing binding affinity for β catenin (Yamamoto et al., 1999). A role for CKI in Axin phosphorylation has also been suggested whereby the affinity of Axin for GSK3β is increased following phosphorylation by CKI in order to potentiate the breakdown of β catenin (Song and Li, 2014). The APC protein is also understood to be the other essential structural molecule of the breakdown complex yet however, its multiple roles in cytoskeletal organisation, chromosome segregation, cell adhesion and microtubule stabilisation may partially obscure its role in β catenin regulation (Yamamoto et al., 1999; Aoki and Taketo, 2007). Recently, an additional role has also been identified for APC as a co-factor in the transport of mitochondria to the cell membrane (Mills et al., 2016).
The presence of APC as a key component of the breakdown complex was confirmed by Hart et al., (1998) who demonstrated that phosphorylation of APC by GSK3β was enhanced in the presence of Axin. The ability of APC to bind to Axin via its SAMP domain and also to β catenin via a series of conserved 15 to 20 amino acid repeat sequences demonstrates a role in β catenin regulation although the precise mechanism remains unresolved (Xing et al., 2004). The structure and function of the β catenin breakdown complex is shown in schematic form in Figure 1.1 (*reproduced with permission from MacDonald et al.,(2009)).*

Several modes of action of APC have been proposed which include (i) facilitating the dissociation of phosphorylated β catenin from the Axin/APC complex, (ii) sequestration of free cytosolic β catenin, (iii) transport to the breakdown complex, (iv) recruitment of the proteasome or possibly the formation of an active site for β catenin phosphorylation and release (Wang et al., 2014). Of interest is the role of APC as a cofactor in the translocation of β catenin from the cytosol to the nucleus. Evidence reviewed by Henderson and Fagotto (2002) suggests that APC may not be essential for the entry of β catenin to the nucleus but rather that it assists with the export from the nucleus, possibly via the microtubule network. The nuclear role(s) of APC are still largely unknown and are the focus of ongoing studies.

In the absence of a Wnt signal, β catenin is subjected to sequential phosphorylation by CKIα and GSK3β within the Axin/APC framework. CKIα is thought to act as a "priming kinase" by phosphorylating the Serine 45 residue which then enables GSK3β to phosphorylate β catenin at Serine 33, 37 and Threonine 41. Binding of βTrCP (Beta-transducin repeat containing protein), a ubiquitin ligase then promotes proteosomal breakdown of phosphorylated β catenin. (Brembeck et al., 2006; Saito-Diaz et al., 2013; Feng and Gao, 2015). The phosphorylation of Axin by GSK3β results in its stabilisation within the β catenin

degradation scaffold (Yamamoto et al., 1999) but the destruction of Axin is mediated principally by the enzyme Tankyrase 1 through its action of polymerising ADP-ribose units to a target protein such as Axin2 which facilitates proteasomal breakdown (Huang et al., 2009; Bao et al., 2012; Haikarainen et al., 2014). Therefore, stabilisation of Axin2 by Tankyrase inhibition results in the attenuation of β catenin mediated Wnt signalling due to the preservation of the β catenin degradation complex and represents an important point of intervention for *in vitro* research.

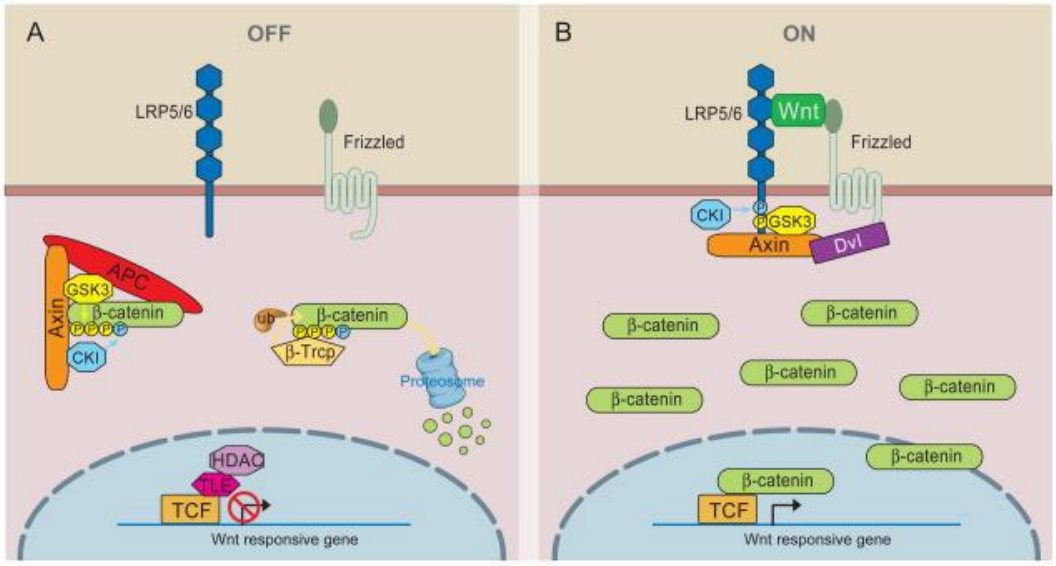

Figure 1.1. Schematic representation of canonical (β catenin mediated) Wnt signalling
Control of intracellular β catenin by the degradation complex results in proteosomal breakdown of free, intracellular β catenin by phosphorylation within the complex, leading to proteosomal destruction (A). Binding of a Wnt ligand to a Fzd receptor promotes dissociation of the breakdown complex by recruitment of Axin to the cell membrane. Dissociation of the degradation complex occurs, resulting in increased free β catenin which translocates to the nucleus, resulting in target gene expression (B).
Diagram from MacDonald et al., (2009), reproduced with permission from the authors.

1.9. Expression of Wnt target genes.

Translocation of β catenin to the nucleus has been shown to be independent of nuclear transport mechanisms such as the importin system and may instead require direct interaction with nucleoporin proteins on the nuclear envelope (Fagotto et al., 1998). Other components of the β catenin destruction complex such as Dvl, CKI, GSK3β and APC are also known to migrate to and from the nucleus and the β catenin binding properties of APC may also have a role in the β catenin mediated modulation of target gene expression (for review, see Willert and Jones, 2006).

The most widely studied Wnt-mediated nuclear interaction is that of β catenin and members of the T Cell Factor / Lymphoid Enhancer Factor (TCF/LEF) transcription factor family and its effects on Wnt target genes. The TCF group consists of TCF1, TCF3 and TCF4 (also previously known as TCF7, TCF7L1 and TCF7L2 respectively) and LEF separately. The TCF/LEF factors bind to DNA but do not exert any transcriptional activity until other repressor or activator molecules bind to them (Mao and Byers, 2011). A vital function of TCF/LEF factors is to produce bending of the DNA helix in order to facilitate access for other activator/repressor molecules to bind. In its simplest form, binding of β catenin to TCF/LEF proteins produces the release of repressor proteins such as Groucho / Transducin-like Enhancer of Slip (GRO/TLE) thereby allowing the expression of Wnt target genes (Mao and Byers, 2011; Cadigan and Waterman, 2012). Removal of co-repressor molecules also facilitates the binding of histone modifiers such as SWI/SNF, acetylation of histones by histone acetylases and transcript formation by RNA polymerase (Schuijers et al., 2014). According to Cadigan and Waterman (2012), histone acetylation is the most common chromatin modification in response to β catenin binding to TFC/LEF and that acetylation of histone H3 and H4 sub units promotes transcriptional activation.

The range of nuclear proteins that can bind to the TCF/LEF/β catenin complex is continually expanding (for review, see Shitashige et al., 2008) and a recent study in colorectal carcinoma cell lines has identified several Wnt components amongst many others modulated by a β catenin mediated Wnt signal (Herbst et al., 2014). This study also included the oncogenes cMyc and Cyclin D1 which are widely referred to in the literature as typical targets of β catenin mediated Wnt signalling (He, T-C. et al., 1998; Shtutman et al., 1999). Activation of genes by non β catenin mediated Wnt signalling has been reviewed and the interplay of growth factors, G protein coupled receptors and pathway crosstalk is

also discussed to remind investigators that TCF/LEF activation is common to other pathways, but β catenin solely requires TCF/LEF activation (Jin et al., 2008).

The outcome of a Wnt signal has been shown to be a highly context-dependent process as the target gene(s) involved will depend on the cell type and combination of Wnt ligand and Frizzled (Fzd) receptor (van Amerongen and Nusse, 2009; Dijksterhuis et al., 2014). A diverse array of Wnt targets has been elucidated from developmental models of mammalian and non-mammalian species (Cadigan and Nusse, 1997; Logan and Nusse, 2004; Li et al., 2006) which add to the two previously described (and often considered 'classical') Wnt targets of c-Myc and Cyclin D1 (He et al., 1998; Shtutman et al., 1999).

In terms of cancer research, other cancer related genes have since been identified and may be sub-classified as targets of β catenin mediated signalling (Herbst et al., 2014) and non β catenin mediated Wnt signalling (Wang, 2009). Using a colorectal cancer cell model, Herbst and co-workers identified several genes which were well characterised in cancer such as the ABC multidrug transporter (ABCB1), the anti-apoptotic protein BIRC5 (Survivin) and Vascular Endothelial Growth Factor (VEGF) as well as the Wnt pathway components Axin2 and TCF1 (Herbst et al., 2014). Interestingly, the mobilisation of β catenin to activate its target genes can also be produced by *non Wnt* stimuli from substances such as the mammalian Target of Rapamycin (mTOR) growth factor pathway through a form of cross talk between mTOR and Wnt receptors (for review, see Jin et al., 2008).

Comparatively less is known about target genes of the β catenin independent pathways such as the Planar Cell Polarity (PCP), Wnt/Ca^{2+} and mTOR pathways (described in Section 1.12). Although somewhat outside the scope of this thesis, target genes have been identified in the PCP pathway (Wang, 2009) and Wnt/Ca^{2+} pathway (Rao and Kuhl, 2010) while targets of the lesser known pathways such as RAP1, ROR2, RYK, Protein Kinase A and mTOR as reviewed by Semenov and colleagues (2008) remain poorly characterised.

As a result of the avid scientific interest in this field, the number of target genes identified is continually expanding and updated regularly with accompanying literature by Nusse and colleagues at the Wnt Homepage (http://web.stanford.edu/group/nusselab/cgi-bin/wnt) and the electronic processing of bioinformatic data greatly facilitated by pathway/gene maps such as the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (http://www.kegg.jp).

An interesting feature of the Wnt Homepage is the differentiation of genes as either functional effector targets of Wnt signalling or components of the Wnt system itself that are regulated by feedback from a Wnt signal. The inclusion of these two sub-categories of target genes in the KEGG Wnt pathway map has facilitated the Wnt-oriented transcriptome analysis in Sections 6.6 of this thesis in which the differential expression of Wnt target and Wnt pathway component genes was analysed following histone deacetylase inhibition with suberoylanilide hydroxamic acid (SAHA).

1.10. Secreted Frizzled Related Proteins and other Wnt-regulating molecules.

The regulation of a Wnt signal can be considered to be mediated by two differing families of regulatory molecules. The Secreted Frizzled Related Proteins (SFRPs) and two other distinct, secreted regulators; Wnt Inhibitory Factor 1 (WIF1) and the Dickkopfs (DKKs) (MacDonald et al., 2009). A unique feature of SFRPs is the preservation of a cysteine rich doman (CRD) of 10 cysteine residues in the N-terminal in a manner similar to that of Fzd receptors and a Netrin-like (NTR) domain which has yet to be fully characterised (Shi et al., 2007). An extensive study by Bovolenta and co-workers (2008) has also shown that there is a degree of selectivity in the binding of Wnt ligands to certain SFRPs. The authors propose that numerous Wnt/Fzd binding combinations are possible due to the large number of potential combinations, an unknown number of binding sites per SFRP molecule or the particular domain(s) concerned with binding. SFRPs exert their inhibitory actions by binding to their cognate Wnt ligands and preventing binding to the Fzd/LRP receptor complex (Bovolenta et al., 2008). The five known human SFRPs have been characterised as tumour suppressor genes with loss of function due to either chromosomal deletion or epigenetic silencing by promoter hypermethylation, especially SFRPs 1, 2, 4 and 5 (Shi et al., 2007). Antiproliferative effects of the SFRPs have been noted in several cancers and the ability of certain SFRPs to paradoxically activate Wnt signalling has also been demonstrated (for review, see Surana et al., 2014). Wnt Inhibitory Factor 1 shares a similar mechanism of

Wnt inhibition to that of the SFRPs but contains a unique WIF domain instead of a conserved CRD producing inhibition of Wnt signalling by sequestration of free Wnt ligands via its WIF domain instead of the CRD of the SFRPs (Kerekes et al., 2015). Additionally, the extracellular domain of the Receptor Tyrosine Kinase (Ryk) family of receptor-kinases have also been shown to contain a WIF domain, but the involvement (if any) of Fzd receptors in a Wnt/Ryk complex remains unknown (Kerekes et al., 2015). The nature of Wnt/Ryk signalling has been studied in developmental models of nervous system patterning and has been concisely reviewed elsewhere (Keeble and Cooper, 2006).

Inhibition of Wnt signalling by the DKKs is at the receptor level by the binding of DKKs to the LRP5/6 co-receptors and the Kremen transmembrane proteins (Krm1 and Krm2). A ternary complex of DKK/LRP/Krm produces the internalisation of the Fzd receptor, thus inhibiting Wnt signalling (Niehrs, 2006). Although the DKKs are considered as negative regulators, evidence from developmental *Xenopus* models shows a possible, context-specific role as a Wnt agonist, similar to the paradoxical activation of Wnt signalling by the SFRPs (Niehrs, 2006). Another Wnt antagonist with LRP5/6 affinity is Sclerostin (SOST), a gene product primarily associated with bone mass regulation (Li et al., 2005). The Wnt-inhibiting action of the evolutionarily conserved Notum enzyme has been recently explained as a deacylase capable of removing the palmitoyl moiety of Wnt ligands and rendering them inactive (Kakugawa et al., 2015).

Non-Wnt activators include the Norrie Disease Protein (Norrin) encoded by the NDP gene which has been shown to act as a Wnt agonist as a trimeric complex with Fzd4 and either of LRP5 or LRP6. A regulator of angiogenesis, Norrin differs from Wnt ligands by its lack of lipid modifications and binds to Fzd4 and LRP as a dimer in the presence of a co-factor, Tetraspanin12 (TSpan12) (Ke et al., 2013). The R Spondins (RSPO 1 to 4) were originally characterised in developmental models as multifunctional regulators of embryogenesis but are now known to bind to their cognate Lgr receptors (Lgr 4, 5 and 6). Potentiation of a Wnt signal by RSPO/Lgr interaction is produced by inactivation of two E3 ubiquitin ligases; RNF43 and ZNRF43 which ubiquitinate Fzd receptors for proteosomal degradation has been demonstrated by de Lau and colleagues (2014). Maintenance of the Fzd receptor complement on the cell surface in the absence of their removal mechanism therefore sustains a Wnt stimulus (de Lau et al., 2014). Also of interest is the ability of Norrin to bind to Lgr4 as seen in a HEK293 cell model, although its biological significance is unclear (Deng et al., 2013). An emerging picture is one of Wnt signal modulation by traditionally acknowledged regulatory molecules (such as the SFRPs) and newer candidate molecules in

a manner that once again depends greatly upon tissue role and developmental stage, each with its particular significance in disease.

1.11. Alternate Wnt signalling; the PCP and Ca^{2+} pathways.

At least two other non β catenin dependent pathways are known to exist although the two best characterised mechanisms are the Planar Cell Polarity (PCP) and Wnt/Ca^{2+} pathways. A common factor between these and the β catenin mediated pathways is the involvement of Dvl at the point of signal induction (Komiya and Habas, 2008).

The PCP pathway, originally characterised in *Drosophila* developmental models of convergent extension contains the essential elements of a Wnt ligand, Fzd receptor and Dvl with the activation of two sub-branches mediated by the GTPases Rho and Rac (Kikuchi et al., 2009). Activation of Rho GTPase produces activation of Rho Associated Kinase (ROCK) which in turn produces cytoskeletal events such as myosin phosphorylation and actin rearrangement. Activation of Rac GTPase stimulates the action of Jun Nuclear Kinase (JNK) with an unknown effect in humans and together with Rho GTPase cytoskeletal effects, may produce cell migration and directional polarisation (Semenov et al., 2007; Komiya and Habas, 2008).

Characterisation of the Wnt/Ca^{2+} pathway was also made possible from developmental models of *Xenopus* and Zebrafish which led to the prototypical model of Wnt5a binding to a Fzd receptor with the release of intracellular calcium as a result. (De, 2011; for review, see Kohn and Moon, 2005). In brief, Wnt/Fzd binding produces activation of phospholipase C and the production of inositol triphosphate and diacylglycerol from membrane-derived phosphatidylinositol-4,5-bisphosphate. Binding of diacylglycerol to calcium channels on the endoplasmic reticulum produces a temporary flux of calcium ions (De, 2011). Increased intracellular calcium activates several calcium dependent enzymes, notably Protein Kinase C (PKC), Calmodulin-Dependent Kinase II (CaMKII) and calcineurin (for review of PKC function, see Luna-Ulloa et al., 2011). Regulation of Nuclear Factor of Activated T Cells (NFAT) by calcineurin and actions of CaMKII on histone deacetylases 4 and 5 have been proposed as outcomes of Wnt/Ca^{2+} signalling (Rao and Kuhl, 2010).

Other mechanisms await further characterisation and these include the RAP1, ROR2, Protein Kinase A, Atypical Protein Kinase C, mTOR and RYK pathways (Semenov et al., 2008). Additionally, Wnt ligands previously shown to be β catenin dependent have also exerted effects in a β catenin independent manner and therefore a case-by-case approach has been recommended to any such investigations (Rao and Kuhl, 2010). The ability of Wnt5a to activate either arm of Wnt signalling, depending on the receptor profile has been demonstrated. Acting through the ROR2 pathway, Wnt5a produced a non β catenin dependent response, yet when bound to Fzd4, β catenin stabilisation was observed (Mikels and Nusse, 2006). The importance of receptor complex composition or receptor 'context' has been shown through the use of β catenin stabilising Wnt3a and non β catenin stabilising Wnt4 and Wnt5a. Both Wnts4 and 5a produced a β catenin mediated response when bound to LRP6 and either of Fzds 4, 5 or 10 with Wnt4 producing the greatest response with LRP6 and Fzd10 whereas a maximal Wnt5a response was seen with binding to LRP6 and Fzd4 (Ring et al., 2014). Further research is required to produce a clearer picture of the physical manifestations of alternate Wnt pathways and the receptor context behind such events.

1.12. The association between Wnt signalling with cancer.

There is ample evidence for aberrant activation of Wnt signalling driving cancer progression (Polakis, 2012; Anastas and Moon, 2013; Wang, 2009). The effects of mutations in the Adenomatous Polyposis Coli (APC) gene in familial adenomatous polyposis and spontaneous colorectal cancer provided insights into the complexities of Wnt signalling (Munemitsu et al., 1995). Mutant forms of APC and Axin2 were found to produce an ineffective breakdown complex for β catenin, therefore leading to prolonged, pathological activation of the TCF/LEF transcriptional complexes and mutations of β catenin were detected in other malignancies (Barker and Clevers, 2006). Other discoveries also included epigenetic silencing of tumour suppressing SFRPs (Bovolenta et al., 2008) yet interestingly, other vital components such as GSK3β, the LRP co-receptors and the nuclear β catenin-binding protein ICAT were not found to be associated with malignant mutations (Polakis, 2007). Genetic analysis of the ten Fzd receptors for association with cancer is however, still pending. Pharmacological intervention of the Wnt system is therefore possible at each point of the pathway from Wnt/Fzd/LRP binding to the three major pathways and at the nuclear level.

Research into modulatory substances selective for the Wnt pathways is in progress and the chemical properties of each class and their targets have been comprehensively reviewed elsewhere (see Voronkov and Krauss, 2013). Wnt signalling however, poses unique challenges at several levels of complexity; beginning with cell specific traits, the temporal stage of either development or homeostasis and receptor context. The potentially widespread effects of a Wnt signal and its modulation will need to be addressed. Lastly, the β catenin dependent and independent pathways described may appear to be discrete processes but a degree of crosstalk between these two divisions may exist (Kahn, 2014). Two notable examples are the Notch and Hedgehog pathways present in development and in cancer with the distinct possibility of 'escape' from pharmacological intervention by the upregulation of one in lieu of another (Takebe et al., 2015). Similarly, issues such as teratogenesis and the question of whether potentiation or antagonism is appropriate for a given condition also require further investigation. The current inventory of Wnt-targeting compounds may be categorised as follows; (i) nonspecific modulators, (ii) β catenin/TCF antagonists, (iii) Dvl PDZ domain binders, (iv) Porcupine and Tankyrase inhibitors, (v) biologicals (antibodies) and (vi) Wnt co-activator molecules (Kahn, 2014).

1.13. Research into Wnt signalling in malignant mesothelioma.

A relative paucity of information exists on the role of Wnt signalling in this particular cancer with a small number of studies on selected parts of the Wnt pathway carried out to date. An early study into lung cancer identified the homozygous deletion of β catenin in the H28 mesothelioma cell line (Shigemitsu et al., 2001): the applied significance of which is unclear given the considerable involvement of β catenin mediated signalling in cancer. A subsequent examination of the roles of N-cadherin, α catenin and β catenin in mesothelioma using fixed tissue samples reported the presence of membrane-bound and cytoplasmic pools of β catenin in all mesothelioma samples and a subset of 19% of samples also displaying nuclear β catenin staining (Abutaily et al., 2003). This finding was also supported by Dai et al., (2005) who found increased nuclear and cytoplasmic β catenin staining in 26 of 33 mesothelioma tissue sections with the further observation that the degree of staining was the greatest in the invasive section of the tumour. The relationship between β catenin and the E, N and P cadherins has also been investigated *in vitro* using nine mesothelioma cell lines with the finding that six of the nine lines showed a restoration of E cadherin and ɣ catenin expression (Orecchia et al., 2004) but no further advances have been made on this finding. Based on these findings, the presence of β catenin and in

particular, its localisation, appears to provide strong indirect evidence for canonical Wnt signalling in mesothelioma

The role of Dvl in mesothelioma was established by Uematsu and co-workers (2003) through the examination of tissue samples, pleural effusions and mesothelioma cell lines which illustrated the role and relationship between increased Dvl3 expression and increased β catenin-mediated gene expression. Reduction of Dvl activity by the use of an inactive mutant produced a decrease in β catenin dependent gene expression of targets such as c-Myc in the REN mesothelioma cell line and interestingly, a reduction of cyclooxygenase 2 (COX2) expression in the H513 line (Uematsu et al., 2003).

A tumour-promoting role for Wnt1 has been suggested through the induction of apoptosis in several cancer cell lines. The abolition of Wnt1 activity by complexation with a monoclonal antibody or RNA interference produced apoptosis in several lung cancer cell lines but the mesothelioma lines were not studied. (He et al., 2004). The reasons for the omission of the mesothelioma lines were not made clear but a subsequent study by the same group investigated the removal of Wnt1 function in the β catenin deficient cell lines H28 and MS1. A reduction in Dvl3 activity and increased c-Jun NH_2-terminal kinase (JNK) activity was seen in these cells with the end result of apoptosis (You et al., 2004). The reduction of Dvl3 activity was indicative of the inhibition of β catenin-dependent signalling but the association between Dvl3 and JNK-mediated apoptosis was unresolved (You et al., 2004). Further work involving suppression of Dvl3 by RNA interference also potentiated the response of mesothelioma cell lines to cisplatin, including that of the β catenin deficient H28 cell line (Uematsu et al., 2007).

A similar study to inactivate Wnt2 also provided insight to the survival advantages of Wnt2 expression and the potentiation of the effects of pemetrexed with the abolition of Wnt2 expression in β catenin expressing and non-expressing cell lines (Mazieres et al., 2005). Other experiments involving tissue samples demonstrated the upregulation of Wnt ligands (Wnts 1, 2 and 5), the Wnt target genes c-Myc, c-Jun and cyclin D1 and the downregulation of Wnt8a. Downregulation of the antagonist molecules DKK1, SFRP2 and SFRP4 was also noted and provided some insight into the deregulation of Wnt signalling *in vivo* (Mazieres et al., 2005). The increased expression of Wnt2b has also been determined to be a poor prognostic factor based on studies on patient tissues by Kobayashi and colleagues (2012) with immunohistochemical analysis showing the highest degree of expression being that of

and increased staining for Wnt1 and Wnt5a. Also noted was the increased expression of the anti-apoptotic factors Survivin and c-Myc.

Deregulation of Wnt regulatory molecules such as the SFRPs in mesothelioma has been reported in primary tissue and cell lines (Lee et al., 2004a) as well as β catenin deficient cell lines (He et al., 2005). The downregulation of SFRPs 1, 4 and 5 was described in primary tissue and absent in the mesothelioma lines tested with promoter hypermethylation proposed as the key mechanism behind the loss of SFRP function (Lee et al., 2004a). Another study by He and co-workers (2005) explored the loss of SFRP4 expression as a result of promoter hypermethylation in β catenin deficient lines H28 and MS1 . These authors also proposed the existence of β catenin independent mechanisms of SFRP-mediated apoptosis and also reported the downregulation of Dvl3 and Survivin upon restoration of SFRP4 function (He et al., 2005). Promoter hypermethylation has also been proposed as the genetic lesion responsible for WIF1 inhibition in primary tissue and cell lines (Batra et al., 2006). A broader study of WIF1, SFRP1, SFRP2 and SFRP4 promoter hypermethylation also revealed other widespread genetic alterations including effects on thirteen Wnt genes and a hypothesis that deregulation of β catenin independent pathways may in some manner contribute to the pathogenesis of mesothelioma (Kohno et al., 2010). The loss of DKK1 (Dickkopf 1) function remains poorly understood and the restoration of DKK1 activity in β catenin expressing and deficient cell lines has shown an association between DKK and the JNK pathway whereby growth suppression and apoptosis attributable to DKK1 may require the activation of the JNK pathway (Lee et al., 2004b).

To date, the function(s) of the Wnt components in mesothelioma have been only partially explored with several potential studies awaiting investigation using β catenin expressing and β catenin deficient cell lines. A number of studies have employed β catenin deficient cell lines but it is clear that most mesotheliomas express β catenin (Orecchia et al., 2004; Dai et al., 2005; Mazieres et al., 2005; Fox et al., 2013). Issues such as ligand/receptor combinations, LRP co-receptor associations and aberrations in the β catenin degradation complex all await further research. The expansion of knowledge of Wnt target genes in mesothelioma is also a priority and a model-by-model approach is required with the aim of establishing a generalised outline of Wnt signalling in mesothelioma to a degree of knowledge comparable to other cancers.

1.14. An overview of Tankyrase (TNK) function.

Human TRF1-interacting Ankyrin related ADP ribose polymerases (Tankyrases 1 and 2) are members of a larger family of 17 poly ADP-ribose polymerases (PARPs), sometimes referred to in other studies as ADP-ribosyltransferases, diphtheria toxin-like (ARTDs) (Kamal et al., 2014). Tankyrases 1 and 2 (or PARP5a/ARTD5 and PARP5b/ARTD6 respectively) as well as the other family members have a common purpose in catalysing the addition of ADP-ribose moieties to glutamate or lysine residues of their target substrates. However, in the case of TNKs 1 and 2, the addition of ADP-ribose groups is one of ADP-ribose polymerisation as chains whilst other PARP enzymes catalyse either mono or poly ADP-ribosylation in multi-directional branched groups (Lehtio et al., 2013). The polymerisation of several ADP-ribose groups in an appended chain is referred to as Poly ADP-ribosylation or PARylation. The physiological significance of protein PARylation is that of a rapid, reversible, post-translational modification that facilitates at least four known functions; (i) the inhibition of protein-to-protein interactions, (ii) inhibition of protein-to-nucleic acid binding, (iii) the formation of an intracellular scaffold for subcellular component organisation and (v) assistance with protein ubiquitination prior to proteasomal breakdown (Gibson and Kraus, 2012, Haikarainen et al., 2014). Such interactions are common within a cell and require stringent control, especially in processes such as DNA replication, mitotic separation and protein trafficking.

The physiological functions of PARylation have been extensively characterised through analysis of the function of PARP1 in triggering the apoptotic process in response to cellular stress from chemical insult and DNA damage as well as roles in the maintenance of chromatin structure, genome stability and transcriptional regulation (for review, see Kim et al., 2005). A small degree of functional overlap exists between PARP1 and the Tankyrases through their involvement in the regulation of mitosis, with a greater degree of information surrounding Tankyrase 1 (Kim et al., 2005). The prototypical role of Tankyrase 1 has been described through its actions in the maintenance of telomere length whereby PARylation of Telomeric Repeat binding Factor 1 (TRF1) produces its release from the telomere ends in order to allow telomere elongation by telomerase prior to mitotic division (Hsiao and Smith, 2008). Other key functions mediated by Tankyrase 1 include the regulation of the mitotic spindle via PARylation of Nuclear Mitotic Apparatus Protein 1 (NuMA), the resolution of sister telomeres during mitosis and translocation of the GLUT4 glucose uptake transporter to the cell membrane in association with PARylated IRAP (Insulin Responsive Amino Peptidase) (Hsiao and Smith, 2008).

1.15. Tankyrase inhibition and applications in Wnt signalling.

A pioneering study by Huang and co-workers identified the Wnt-inhibiting molecule XAV939 which was validated in SW480 colon cancer cells which exhibit high β catenin activity. The pharmacodynamic action of XAV939 was deduced to be one of Axin stabilisation through inhibition of TNKS1 and 2 whereby the inhibition of Axin PARsylation prolonged its presence in the cytosol. (Huang et al., 2009). The currently proposed model for Axin degradation suggests that PARsylation of Axin by TNKs1 and 2 recruits the RNF146 ubiquitin ligase leading to degradation in the 26S proteasome (Haikarainen et al.,2014). Stabilisation of Axin therefore prolongs the activity of the β catenin degradation scaffold, thereby reducing the fraction of free β catenin with consequent reduction in the expression of β catenin dependent target genes – a schematic version is shown in Figure 1.2

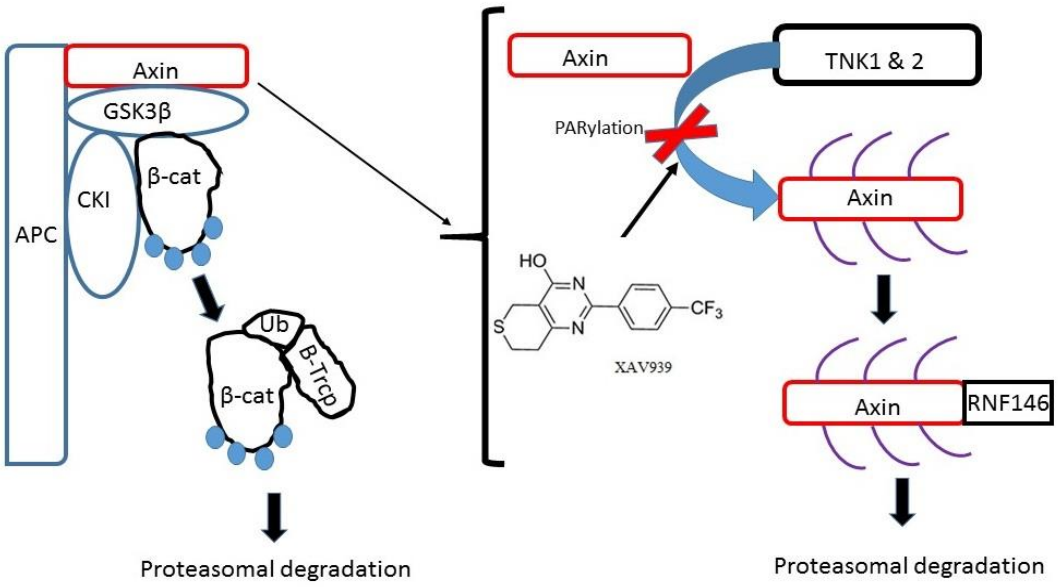

Figure 1.2. Tankyrase inhibition by XAV939 in Wnt signalling. The β catenin degradation complex consists of the key components APC, Axin and the two kinases GSK3β and CKI. Sequential phosphorylation of β catenin (blue dots) results in the binding of β-Trcp and ubiquitination of β catenin and subsequent proteosomal degradation. Addition of poly-ADP ribose chains (purple lines) to Axin by Tankyrases 1 and 2 results in RNF146 attachment and proteosomal degradation. Stabilisation of Axin via Tankyrase inhibition with XAV939 sustains the activity of the β catenin degradation complex, thereby attenuating β catenin mediated Wnt signalling.

Of the component proteins that comprise the β catenin degradation complex, the scaffolding protein Axin has been identified as the rate-limiting component through mathematical analysis of a *Xenopus* oocyte extract model (Lee et al., 2003). Mathematically estimated to exist at a concentration of 0.02nM in this model, the abundance of Axin is much less than that of the other degradation complex components existing in a range of 1 to 100nM. Therefore, any modulation of Axin content may indeed alter the degree and outcome of a Wnt signal. The authors suggest that the low abundance of Axin may be related to its cross-involvement with other pathways or that it may serve to isolate the Wnt pathway from other systems that share common components such as APC and GSK3β (Lee et al., 2003).

Further work by another group (Chen et al., 2009) produced two further series of compounds; the Inhibitor of Wnt Processing (IWP) and Inhibitor of Wnt Response (IWR) molecules. Five IWP and four IWR molecules were identified and attempts were made to explain their actions. The IWP compounds were found to inhibit the Porcupine membrane bound O-acyltransferase responsible for addition of the palmitoyl lipid moiety to newly synthesised Wnt proteins, the loss of which renders the Wnt ligand inactive (see section 1.4). The IWR compounds were thought at the time to stabilise Axin by direct binding (Chen et al., 2009) but this has since been attributed to the inhibition of TNKs1 and 2 in a parallel study with XAV939 (Busch et al., 2013). Through the use of two IWR diastereoisomers, TNK1 and TNK2 gene silencing and comparison with XAV939, Busch and co-workers demonstrated the selectivity of IWR1 for TNKs1 and 2 but also acknowledged the possibility that inhibition of other PARP/ARTD members may also occur (Busch et al., 2013).

Other TNK selective inhibitors include JW67 and JW74 (Waaler et al., 2011), JW55 (Waaler et al., 2012), G244-LM (an XAV939-like derivative) and the novel compound G007-LK (Lau et al., 2013). Recent investigations of IWR1, 3, 6 and 8 also showed TNK1 binding as their mechanism of action but with an additional non-Wnt effect of producing telomere degradation. The proposed mechanism being that prolonged exposure produces excessive binding of TRF1 to telomeric DNA and subsequent loss of telomere replication by telomerase blockade with the eventual result of telomere shortening (Kulak et al., 2015) but no suggestions were made as to the nature of the resulting cell death. It also remains to be shown whether or not this is the case with prolonged XAV939 treatment. Lastly, an association has recently been made between TNK inhibition by XAV939 and the reduction

of 26S proteasomal activity which, despite being outside the scope of this study, illustrates a possible additional role for XAV939 beyond its current role of Axin stabilisation (Cho-Park and Steller, 2013).

1.16. An overview of histone deacetylase function.

The higher order structure of chromatin comprises of 147 base pairs of DNA and a histone protein core containing eight histones as two each of histones H2A, H2B, H3 and H4. The degree of DNA/histone and histone/histone interaction is determined by histone post-translational modifications at the amino termini such as acetylation, methylation and phosphorylation (Ropero and Esteller, 2007; Barneda-Zahonero and Parra, 2012).

Of interest in these studies is deacetylation of lysine residues by histone deacetylase (HDAC) which regulates the conformation of chromatin. Acetylation produces a relaxed chromatin structure and the reverse upon deactylation. A less condensed state of chromatin is associated with gene transcription whereas the condensed, deacetylated form prevents gene expression through hindering access to DNA by transcriptional molecules (Ropero and Esteller, 2007; Ververis et al., 2013). The molecular aspects of histone post-translational modification have been reviewed elsewhere by Bowman and Poirier (2014) and a schematic version is shown in Figure 1.3.

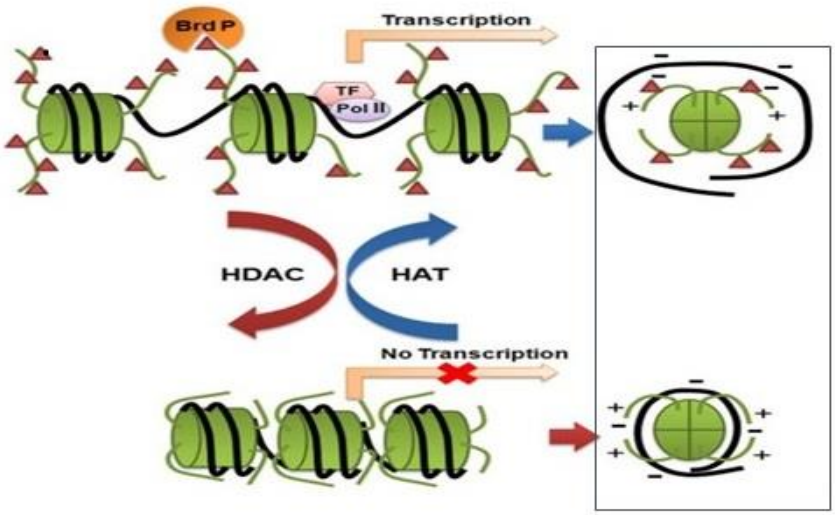

Figure 1.3. Schematic representation of histone acetylation and deacetylation. Acetylation of histone proteins (red triangles) by histone acetyltransferases (HATs) produces a relaxed, open chromatin conformation thereby allowing gene transcription. Deacetylation results in the condensation of chromatin and inhibition of transcription. Histone nucleosomes are shown in green with DNA represented as a black line. Figures within inset illustrate the electrostatic forces producing changes in chromatin structure. *Diagram from Parbin et al., (2013). Reproduced with permission from authors.*

The mammalian HDAC complement comprises 18 members classified into four groups according to structure, enzymatic function, intracellular location and bodily distribution. Class I consists of HDACs 1, 2, 3 and 8 while Class II includes HDACs 4, 5, 6, 7, 9 and 10). Class III comprises of the Sirtuin family of proteins (Sirtuins 1 to 7) and Class IV contains HDAC11 as the sole, poorly characterised member (Haberland et al., 2009). Pharmacologically, the eleven HDACs across Classes I, IIa, IIb and IV are characterised by the presence of a zinc-containing catalytic site, unlike the Sirtuins (Class III) which are NAD^+ dependent (Dokmanovic et al., 2007). Although the nomenclature has been derived from the known function on histones, several other non-histone substrates have been identified. These include structural proteins, nuclear receptors, DNA repair enzymes, high mobility group (HMG) proteins and transcription factors (Glozak et al., 2005; Marks 2007).

Separate to effects of acetylation and deacetylation on histone-DNA binding and regulation of gene transcription, the stability, binding, interactions, localisation and function of other cytoplasmic proteins have also been described as being dependent on the given lysine acetylation status (Glozak et al., 2005; Spange et al., 2009). Notable examples being the stabilisation of transcription factors (p53 in particular) against ubiquitination and subsequent proteosomal breakdown with a similar mechanism proposed to explain the stabilisation of c-Myc (Glozak et al., 2005). Interestingly, the control of NFκB signalling and that of Hypoxia Inducible Factor 1α (HIF1α) have also been noted as being HDAC mediated (Glozak et al., 2005).

Other associations include that of increased HDAC expression and positive regulation of Vascular Endothelial Growth Factor in tumour angiogenesis and deregulated cell-to-cell adhesion during epithelial-mesenchymal transition and metastasis (for review, see Parbin et al., 2013). Deacetylation of other non-histone targets such as α-tubulin, viral proteins and effects on the function of Heat Shock Protein 90 have also been described (Bolden et al., 2006; Singh et al., 2010; Parbin et al., 2013). Taken together, histone deacetylation is a widespread and complex topic with consequences dependent on the target molecule and HDAC isozyme involved and any interpretation of the pharmacological effects of a HDAC inhibitor will also require investigation into the non-nuclear effects as well.

1.17. Pharmacological inhibition of histone deacetylases.

The discovery of hexamethylene bisacetamide and its *in vitro* effects on murine leukaemia led to further clinical investigation in human leukaemia with the eventual characterisation of suberoylanilide hydroxamic acid (SAHA) from a series of candidate hydroxamic acid compounds (Marks, 2007). The mechanism of action of SAHA was deduced to be that of histone hyperacetylation based on the known effects of Trichostatin A (Marks, 2007) and research into its application in cancer chemotherapy has continued in both its epigenetic potential and non-nuclear effects (Marks, 2007). HDAC inhibition produces cytotoxicity through two key mechanisms, (i) transcriptional deregulation and/or DNA damage and (ii) oxidative stress with the end result of apoptosis. Other concurrent mechanisms such as HDACi-mediated necrosis and autophagy have also been noted (Bolden et al., 2006).

The agents employed in research and clinical therapy targeting are broadly classified into four main categories; hydroxamic acids, short chain fatty/alkyl acids, cyclic tetrapeptides (or

epoxides) and benzamides (de Ruijter et al., 2003). Of the short chain fatty acids, butyric and valproic acids have been examined in characterisation studies but are thought to be of limited therapeutic use with effective doses in the millimolar range compared to other compounds effective at nanomolar concentrations (de Ruijter et al., 2003). These studies are focused on HDAC inhibition by SAHA as a representative of the hydroxamic class of histone deacetylase inhibitors (HDACi), based on its use in cutaneous T-cell lymphoma treatment. The mechanism of inhibition by hydroxamic acids involves reversible chelation of the Zn^{2+} ion in the catalytic site of all members in Classes I, IIa, IIb and IV. The Class III Sirtuins being unaffected due to their NAD^{+} dependence (de Ruijter et al., 2003, Witt et al., 2009).

An early attempt at determining the actions of SAHA discussed the nuclear effects of the acetylation of histones and transcription factors resulting in the altered expression of a small number of genes. Induction of growth arresting genes such as p21 and repression of proliferation-related genes such as cyclin D1 were proposed as key transcriptional effects (Richon, 2006). The involvement of the DNA Damage Response (DDR) pathway has since been discovered with activation through direct effect on chromatin structure and the formation of double stranded breaks (DSBs), the possible inhibition of DNA repair mechanisms through either transcriptional repression or direct acetylation have all been attributed to HDAC inhibition (Bose et al., 2014).

Another unique aspect of HDAC inhibitors (including SAHA) is that of reactive oxygen species (ROS) accumulation by increasing expression of thioredoxin binding protein 2 (TBP2), thereby depleting intracellular stores of ROS-quenching thioredoxin (Li and Zhou, 2014). Eventually, a degree of oxidative stress is reached where mitochondrial disruption occurs, followed by apoptosis via the intrinsic cell death pathways (Zhang and Zhong, 2014). Cell death by either apoptosis or mitotic cell death is the result of excessive histone and non-histone acetylation and the formation of aberrant chromatin structure all of which contribute to dysfunctional mitosis, deregulated segregation and cell death through mitotic catastrophe (Marks and Xu. 2009). Other pharmacodynamics effects of HDAC inhibitors such as Vorinostat include involvement of JNK and inhibition of the signal transducer and activator of transcription (STAT) pathways STAT3 and STAT5 (Bose et al., 2014).

Identification of the particular process(es) responsible for cell death by HDAC blockade may not be altogether possible as the mechanisms involved may depend on the genetic basis of the cancer and the particular HDAC enzymes involved (West and Johnstone, 2014) as well as the transcriptional inducer/repressor complex to which a given HDAC may bind (Dokmanovic et al., 2007). The current state of novel HDAC research and clinical trial progress has been reviewed by West and Johnstone (2014) and is a useful resource for further reading.

1.18. An overview of three dimensional (3D) cell culture methods.

Conventional two dimensional (2D) monolayer culture is a simple, reliable method for assessing biological activity of a substance *in vitro* and is often used to empirically determine basic parameters such as toxicity, proliferation and an effective concentration range. Whilst useful as a basic research tool, the field of 3D cell culture may provide a more physiologically relevant model with which to study subjects such as Wnt signalling and its pharmacological modulation. A three dimensional microenvironment provides the important factor of cell-to-matrix interaction in addition to cell-to-cell interactions beyond the normal basolateral contact experienced in monolayer culture.

The term “spheroid” given to the microtissue masses employed in 3D culture has often produced confusion with the expectation of a perfec sphere despite the fact that some cell lines and tissue masses may form irregularly shaped aggregates which are still useable in certain experimental models (Mueller-Klieser, 2000). A clearer system of nomenclature has been proposed (Weiswald et al., 2015) that demarcates four groups of spherical cancer models. These authors suggest the term ‘multicellular tumour spheroids’ for microtissue masses generated in non-adherent conditions from a single cell suspension. Other categories proposed are tumourospheres, tissue derived tumour spheres and organic multicellular spheroids. In these studies, the term ‘spheroid’ is used to describe multicellular tumour spheroids in discussion and as a model.

The history of 3D culture has been summarised by Mueller-Klieser (2000) in addition to establishing some key features of a spheroid, most notably a heterogeneous cell population that can be localised to a relative position within the spherical mass of cells and that cell shape and microenvironment of a spheroid resembles that of an *in vivo* tumour. The

essential, schematic structure of a spheroid can be considered to be that of a viable outer layer of proliferating cells, a middle layer of quiescent cells and a core of cells undergoing either apoptosis or necrosis (Mueller-Klieser, 2000). Given the vast array of cell lines that can be cultured into a spheroid, there are few guidelines on the optimal size of a spheroid with some models growing as large as 3mm, although a diameter of 500µm is generally considered as a 'large' spheroid. Spheroids within this size range also differ in growth rates to those in monolayer culture by having an early stage of exponential growth which slows to a plateau whilst monolayer cultures continue growing exponentially (Weiswald et al., 2015). The speed of formation and resolution of a spheroid is an important factor in determining experimental issues such as seeding population, formation time and experiment duration, all of which are dependent on the duration of exponential growth and onset of the growth rate plateau.

The tumour microenvironment is a mixed setting of a stroma and parenchyma made of non cancerous cells, vasculature, tumour associated fibroblasts and macrophages as well as chemical gradients for gases, nutrients, wastes and growth factors (Thoma et al., 2014). The presence of cell-cell and cell-matrix interaction provides points of attachment in three dimensions onto a variable substrate producing markedly different cellular responses to those seen in monolayer culture (Cukierman et al., 2002). Of interest is the role of the Integrin-mediated signalling in response to the composition of the extracellular matrix resulting in the activation of pathways such as the Mitogen Activated Protein Kinase (MAPK) and Focal Adhesion Kinase (FAK) pathways (for review, see Tai et al., 2015 and Juliano, 2002).

Extracellular proteins such as collagens, glycosaminoglycans and adhesion receptors such as integrins and cadherins are also known to influence cellular processes as a result of the molecular landscape. The relationship between the extracellular terrain and cytoskeletal conformation is thought to determine the nature of the signalling cascade(s) such as Rho-activated Kinase (ROCK), the Rac GTPase and the inositol triphosphate / diacylglycerol (IP3/DAG) pathways (Rubashkin et al., 2014).

Wnt signalling has also been shown to be subject to regulation by cadherin-mediated cell adhesion which is a process that serves two purposes; the maintenance of cell-to-cell adhesion as well as regulation of the free pool of β catenin by complexation with ε cadherin (Barth et al., 1997). Dissociation of the catenin/cadherin complex has been shown to be

part of the EMT (Brembeck et al., 2006) and tyrosine phosphorylation at specific residues as a result of pathological growth factor signalling is known to produce the mobilisation of β catenin from its bound state to participate in Wnt signalling. Examples of such deregulated pathways include EGF, HGF (Hepatocyte Growth Factor), TGF-β (Transforming Growth Factor β) and IGF II (Insulin-like Growth Factor II) (Barth et al., 1997; Heuberger and Birchmeier, 2010). Whilst cadherin-mediated adhesion is present in monolayer culture, the added complexity of three dimensional adhesion and the influence of deregulated growth factor signals upon Wnt signalling cannot be accounted for in simple 2D culture and is indeed, more representative of the *in vivo* tumour setting. Experimentally, the attenuation of Wnt signalling by the restoration of β catenin homeostasis via cadherin overexpression has been demonstrated and provides a corollary for the adhesion/mobilisation model (Stockinger et al., 2001).

Additionally, sequestration and gradient formation of intercellular signalling molecules such as Wnt ligands and morphogens by heparan sulphate proteoglycans (HSPGs) are known to add further complexity to the 3D microenvironment in development (Hacker et al., 2005) and a similar effect may be expected in malignancy as well. As a function of spheroid diameter, the oxygen tension towards the core is also of interest due to its effects on cellular metabolism and viability. Debate continues on the nature of the central, oxygen-poor portion of a spheroid on whether cells in that region are either quiescent or dying by either apoptosis or necrosis (Mehta et al., 2012). As a 3D model of a solid, non-vascularised tumour, the hypoxic core of a spheroid is a useful *in vitro* representation of the *in vivo* situation, albeit one that is subject to cell line specific characteristics.

Several models of 3D culture are currently employed and range from simple self-assembling methods to mechanised, template based formats suited to either high-throughput screening or micro-organoid formation and are broadly classified as as either scaffold based or scaffold free (Rimann and Graf-Hausner, 2012). Scaffold free methods include protocols such as pellet culture from centrifuging, the hanging drop method, liquid overlay (self assembly) and suspension/microgravity (Achilli et al., 2012). Methods involving natural and artificial matrices and hydrogel culture form the scaffold based protocols which include the widely used extracellular matrix derived from Engelbreth-Holm-Swarm (EHS) murine tumour cells (Matrigel®) as well as hydrogel substrates such as alginates and collagen (Breslin and O'Driscoll, 2012) and either of agarose or polyacrylamide (Achilli et al., 2012). An extension of matrix based culture is soft lithography involving a base of extracellular matrix extracts or a hydrogel embossed with a template for wells or customised outlines

(for review of proprietary products, see Rimann and Graf-Hausner, 2012). The most sophisticated method to date is that of microfluidics which involves a cell suspension being slowly percolated through a microchannel tree within a culture chamber to form spheroids that can be subjected to fluid flow variations and exposure to various substances in a controlled manner (for review, see Fennema et al., 2013). A recent model developed by Patra and colleagues (2013) allows the reproducible formation of small spheroids in a relatively simple microfluidic chamber that is practical, robust and suited to studies in basic spheroid research.

In its simplest form, 3D spheroid formation can be achieved by the liquid overlay (also referred to as 'forced floating' method) involving the coating of a culture vessel such as a 96 well plate with a low adhesive substance such as poly (2-hydroxethyl methacrylate) (poly-HEMA) or agarose. Spheroids of relatively uniform size are produced and array studies on individual spheroids are possible. The Hanging Drop method relies on the formation of a spheroid from a small portion of cell suspension seeded in an inverted position so that a spheroid forms within the droplet. Although popular, some technical issues such as sensitivity to vibration, difficulties in medium change / reagent addition and culture duration times have been reported (Breslin and O'Driscoll, 2012; Fennema et al., 2013). The relative advantages, disadvantages and considerations associated with choice of method have been expertly reviewed by Mehta and colleagues (2012).

1.9 Scope of this thesis.

The aims of this thesis were to characterise the profile of Wnt signalling in three locally derived mesothelioma cell lines (JU77, LO68 and ONE58) in order to determine the presence and expression of the various Wnt signal pathway components so that a Wnt profile of these cell lines could be established. Attempts were also made to modulate Wnt signalling through the use of recombinant Wnt proteins in order to observe any effects on cell activity via the use of functional assays such as wound scratch healing and proliferation assays.

The effect of Tankyrase inhibition with XAV939 was also evaluated in terms of its effects on cell viability, migration, morphology and gene expression as this aspect of pharmacological inhibition in mesothelioma research had not been previously explored. At the time of experiments, the effects of XAV939 were unknown in mesothelioma and this also led to the

development of functional assays optimised to suit the cell lines used. Furthermore, the introduction of three dimensional culture allowed examination of this compound in a setting other than conventional monolayer culture. A similar method of study was also employed to assess the viability of histone deacetylase inhibition by SAHA *in vitro* through the use of functional assays such as viability assays (in two and three dimensions) and morphology studies.

The use of RNA sequencing technology was then applied to both compounds in order to investigate the nature of their effects on gene expression, considering their different pharmacodynamic mechanisms of action. Particular attention was paid to genes of the Wnt signalling system and its associated genes so that a transcriptional profile of these mesothelioma cells could be established after treatment with either a Tankyrase or histone deacetylase inhibitor as has been the case for other cancers.

Taken together, this thesis aims to add to the knowledge of Wnt signalling in mesothelioma with an emphasis on the epigenetic sequalae caused by Tankyrase inhibition with XAV939 and histone deacetylase inhibition by SAHA. Further research into mesothelioma and its clinical treatment is also essential to identity potentially useful points of experimental and therapeutic intervention.

Chapter 2.

Materials and methods.

Table of contents.

The experiments described in this thesis were performed by the candidate unless explicitly stated otherwise.

2.1. Chemicals and reagents.

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (New South Wales, Australia) except for XAV939 which was purchased from Selleckchem (Houston, TX USA) and suberoylanilide hydroxamic acid (SAHA) from Tocris Bioscience (Bristol, United Kingdom).

2.2. Cell culture, counting and periodical mycoplasma testing.

The malignant mesothelioma cell lines JU77, LO68, NO36 and ONE58 were derived from pleural effusions of patients with malignant mesothelioma as described by Manning et al., (1991) and propagated from stocks in the supervisor's laboratory. Primary mesothelial cells were isolated and cultured as previously described by Lansley et al., (2011). The NO36 cells used in later studies were a kind gift from Dr. Steve Mutsaers (Institute For Respiratory Research, Western Australia). All lines were cultured in RPMI 1640 (with glutamine) medium (Hyclone®, GE Health Sciences. USA) supplemented with 5 % foetal bovine serum purchased from either Bovogen Biologicals (Victoria, Australia) or Serana Australia (Bunbury, Australia) as well as penicillin 100 IU/mL and streptomycin 100µg/mL (Penstrep ®, Life Technologies, Australia). Cells were incubated at 37^{o}C in a humidified incubator with an atmosphere of 5% CO_2.

Routine mycoplasma tests were conducted regularly at six month intervals using a protocol based on that of Harasawa et al., (2005). DNA was extracted from a 50µL portion of pelleted cells using a EDNA Hi-Spex ® DNA extraction kit (Fisher Biotec, Perth, Western Australia). Ribosomal RNA sequences unique to *Mycoplasma spp* were amplified by PCR using the primers MycoF1 -5'-ACA CCA TGG GAG YTG GTA AT-3' and MycoR1-5'-CTT CWT CGA CTT YCA GAC CCA AGG CAT-3' with thermal cycling conditions as described in Section 2.12.

2.3. Scratch test assay.

Cells were seeded at a density of 3x10^4 cells per well in a 24 well tissue culture plate coated with collagen. For collagen coating, 500 µL of a 60µg/mL solution of Type II collagen (Sigma, New South Wales, Australia) was used. The plate was incubated at room temperature for one hour, the excess solution aspirated and the wells rinsed twice with

sterile phosphate buffered saline (PBS). Following seeding, the cells were allowed to attach and incubate overnight before the medium was removed, the monolayer rinsed once with sterile PBS and a second portion of PBS added immediately before wounding with a 200µL micropipette tip. The PBS was aspirated to remove cellular debris and fresh control medium or medium containing the test reagent was added. Recombinant murine Wnt4 (R&D Systems, Minneapolis, USA) was used and photographs were taken using a Canon EOS digital camera mounted on an Olympus CK2 microscope.

2.4. Trypan blue viability assay.

Cells were seeded overnight at $3x10^4$ cells per well in a 24-well culture plate in 500µL of complete medium and then rinsed once with sterile PBS before the addition of control or treatments followed by incubation for 48 to 72 hours. Cells were rinsed once with 200µL sterile PBS and detached with trypsin. A 20µL aliquot of the cell suspension was taken and mixed with an equal volume of Invitrogen® 0.4% w/v Trypan blue (Thermo Scientific, Victoria, Australia) and the sample analysed using an Invitrogen Countess® automated cell counter, according to the manufacturer's instructions.

2.5. Spheroid propagation.

Spheroid experiments were conducted based on studies by Vinci et al., (2012). For initial formation studies, $5x10^3$ cells were seeded in 200µL complete medium per well in a 96 well Corning Ultra Low Adhesion (ULA) plate (Corning, NY, USA). For viability studies, cells were seeded at $1x10^4$ cells per well in 200 µL complete medium using the same ULA plates. Cells were allowed to aggregate by overnight incubation and photographs were taken using a Nikon DS-L3 monitor display unit attached to a Nikon Eclipse TS-100 microscrope (Nikon, NY, USA). Spheroid circumference was measured by using the on-screen diameter estimation tool in the same Nikon DS-L3 display unit. Replenishment of medium was done on alternate days with the addition of a fresh portion of 100µL complete medium. In all cases, a 24-hour formation period was allowed; morphology studies were conducted over 0 to 10 days and experiments involving inhibitors were between 24h to 48h duration.

2.6. Acid phosphatase viability assay.

Based on a method devised by Friedrich et al., (2007), cells were cultured either as spheroids or a monolayer by overnight incubation as a formation period in 200µL complete medium. A 100µL portion of medium was removed and reagents added prior to incubation for 24 or 48 hours. After incubation, the supernatant was removed, 100µL of acid phosphatase assay buffer (see recipe below) added and the samples incubated for a further 1 ½ hours (monolayer culture) or 2 ½ hours (spheroids) before the addition of 10µL of 1M sodium hydroxide. The supernatant was transferred to a conventional flat-bottomed 96 well plate and the absorption at 405nm read using an EnSpire plate reader (Perkin Elmer, Victoria, Australia).

The acid phosphatase assay buffer itself comprised of 100mM sodium acetate; pH 5.2, 0.1% v/v Triton X-100 and 2mg/mL p-Nitrophenyl phosphate (p-NPP) in Milli-Q grade water. All reagents for this buffer were purchased from Sigma-Aldrich and the assay buffer prepared immediately prior to use by addition of p-NPP to the sodium acetate / Triton X-100 mixture.

2.7. Transwell® migration chamber assay.

The method described by Green et al., (2009) formed the basis for these studies using Corning Transwell® migration chamber inserts (Corning, USA, Cat. No 3422). Inserts were coated for two hours at 37^{o}C with a 50µL aliquot of 10µg/mL fibronectin (Sigma-Aldrich) the excess was aspirated and the membrane rinsed twice with sterile PBS before addition of the cell suspension. Due to the depth of the Transwell insert, the corresponding Corning Costar® 24 well plates were used as carriers during incubation and processing.

Cells were harvested with trypsin and counted in serum-free medium so that a 100µL volume of suspension would yield 3x10^{4} NO36 and 1x10^{4} ONE58 cells respectively. In order to account for dilution effects from the bottom chamber, individual cell suspensions were prepared for each treatment concentration and agitated frequently to prevent cell sedimentation. A migration negative control (serum free medium) and positive migration control (5% v/v serum and DMSO) were included. Transwells were then incubated overnight with the appropriate concentration of treatment in the lower chamber in 500µL medium supplemented with freshly added serum from a frozen aliquot.

Following incubation, the supernatant was aspirated and a cotton bud was used to remove cells that remained on the upper membrane surface. Cells were then fixed and stained for 30 minutes in a staining solution comprising of crystal violet 0.25% w/v and methanol 10%v/v in normal saline. The inserts were rinsed with normal saline and allowed to air dry overnight before images were taken.

2.8. Transwell® invasion assay.

The same inserts (Corning, USA. Cat. No 3422) were used for these studies with a coat of basement membrane extract (BME) derived from murine Engelbreth-Holm-Swarm (EHS) tumour cultures. The BME (Cultrex PathClear®, Trevigen, Maryland, USA) was supplied at 1.551mg/mL and diluted to 0.5X, 0.25X and 0.125X with ice-cold, serum free RPMI 1640 medium. A 100µL portion was applied to the Transwell membrane and incubated at for one hour at 37^{o}C prior to addition of the cells. Excess BME was carefully aspirated away and 3x10^{4} of both NO36 and ONE58 cells were added before incubation overnight for at least 18 hours. Following incubation, the excess BME and cells on the upper side of the membrane were removed with a cotton bud and stained as per the protocol for the Transwell migration assay. Images were also taken using the same method.

2.9. Colony formation assay development and assay with XAV939 treatment.

In order to assess colony forming ability of the cell line, starting populations of 100 JU77, LO68, NO36 and ONE58 cells were seeded into 24 well tissue culture plates using complete medium. Cells were observed for colony formation on alternate days for seven days without medium replenishment. Colonies were then fixed and stained as for the Transwell migration assay (see 2.7). Colonies of 30 cells or more were counted manually. Experiments involving XAV939 were conducted as above with the addition of either XAV939 or DMSO (vehicle) to medium at time of seeding.

2.10. Adhesion assay.

A 24 well tissue culture plate was coated with either Type II collagen (30µg/mL) or fibronectin (10µg/mL) for one hour at 37^{o}C and the excess removed by rinsing with sterile PBS. A population of 250 cells was seeded in complete medium and incubated for a further 30 or 60 minutes. The medium was then aspirated and the well washed once with 1mL of sterile PBS. Cells were fixed as for the Transwell migration assay (see 2.7). Cells were then manually counted under a light microscope. Experiments with XAV939 involved overnight pre-treatment of cells with 1µM, 10µM XAV939 or vehicle control prior to harvesting and experimentation.

2.11. RNA extraction and cDNA synthesis.

RNA extraction from monolayer cell cultures was carried out using either Ultraspec® RNA isolation reagent (Fisher Biotec, Australia) or the MoBio Ultraclean® RNA Isolation Kit (MoBio Laboratories, California, USA) according to the manufacturer's instructions and quantitated using a Shimadzu BioSpec Nano® spectrophotometer (Shimadzu Medical Systems, New South Wales, Australia). RNA integrity was assessed visually by denaturing agarose gel electrophoresis essentially as described by Masek et al., (2005). A 2µL aliquot of RNA was mixed with 3µL formamide and 1µL of Type II 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll 400 in water) and denatured at 65^{o}C for five minutes. The sample was then resolved at 80 V for 90 minutes in a tris-acetate edetate buffer (Tris base 40mM, acetic acid 40mM, EDTA 1mM) before staining with Gel Red ®nucleic acid stain (Fisher Biotec, Perth, Australia). The gel was visualised using a Vilber Lourmat Gel Doc ® UV illumination system (Fisher Biotec, Perth, Australia).

Prior to cDNA synthesis, DNA was removed by DNAse digestion using the RQ1 DNAse system (Promega, Wisconsin, USA) according to the manufacturer's instructions. DNAse treated RNA was either used directly or stored at -80^{o}C. For reverse-transcription PCR, synthesis of cDNA was done by use of the AMV-RT® reverse transcription system (Promega) according to the manufacturer's instructions. Synthesis of cDNA for Real Time (Quantitative) PCR was carried out by use of either the Superscript® III first strand synthesis kit (Life Technologies, Victoria, Australia) or ProtoScript® II first strand cDNA synthesis kit (New England BioLabs, MA, USA) with 1µg input RNA and oligo dT(VN) priming.

2.12. Reverse Transcription PCR (RT-PCR) and agarose gel electrophoresis.

Gene specific PCR primers were designed using the Primer 3 software system and RT-PCR was performed essentially as previously described (Rozen and Skaletsky, 2000) in a 25µL reaction comprising 2mM $MgCl_2$, DNA polymerase buffer (Fisher-Biotec, Perth, Western Australia), 200µM dNTP, 100nM each primer, 1U Taq DNA polymerase (Fisher-Biotec) and 2µL cDNA. Amplification reactions were run in MJ Research PTC-100 thermal cyclers (MJ Research, Quebec, Canada). The thermal cycling for amplification involved denaturation at 95^{o}C for three minutes, followed by either of two cycling programmes. Target genes were amplified by denaturation at 95°C for 20 s, and then by either annealing at 55°C for 20s followed by elongation at 72°C for 60s (40 cycles) or by annealing at 60°C for 20s followed by elongation at 72°C for 60s (35 cycles). Both cycling patterns were followed by a terminal extension at 72°C for a further five minutes before cooling to 10°C. The PCR amplification products were resolved on a 2% agarose gel, in TAE buffer (Tris base 40mM, acetic acid 40mM, EDTA 1mM) at 80V for 45 minutes and stained with Gel Red® nucleic acid stain for at least 30 minutes. Visualisation was done using the Vilber Lourmat Gel Doc® imaging system. Primer sequences are as listed in Tables 2.1 AND 2.2 (*see over*).

Table 2.1. Oligonucleotide primers for Wnts and Frizzleds.

Gene	Primer Sequence 5'-3'	Genbank Accn.
Wnt 1	CGGCGTTTATCTTCGCTATC GCCTCGTTGTTGTGAAGGTT	NM_005430
Wnt2	TGATCCAAAGAAGATGGGAAG TGTAGCGGTTGTCCAGTCAG	NM_003391
Wnt2b	CACCCGGACTGATCTTGTCT CACAGCACCAGTGGAATTTG	NM_004185
Wnt3	TGTGAGGTGAAGACCTGCTG AAAGTTGGGGGAGTTCTCGT	NM_030753
Wnt3a	CCTCGGAGATGGTGGTGGAG TCTCCCGGCGCCGCTCCGCT	NM_033131
Wnt4	TGCCACTGAGGTGGAGCCAC TCAGCCAGCTCCACCTGCGC	NM_030761
Wnt5a	CTGCAGCTGGCAGACTTCCG TCCGTCTGCACGGTCTTGAA	NM_003392
Wnt6	GTCACGCAGGCCTGTTCTAT CGTCCATAAAGAGCCTCGAC	NM_006522
Wnt7a	GTGCGTGCCAGCCGCAACAA CTGGCCTGGGGAGCCGTCTT	NM_004625
Wnt8a	TGCAAGTTCCAGTTTGCTTG ATCCTTTCCCCAAATTCCAC	NM_058244
Wnt10a	AAGCTGCACCGCTTACAACT ATTCTCGCGTGGATGTCTCT	NM_025216
Wnt10b	TGGGCCGGGCCATCTTCATT GGCTGCCACAGCCATCCAAC	NM_003394
Wnt16	TGCTCGTGCTGTTCCCCTAC ATCATGCAGTTCCATCTCTC	NM_057168
Fzd1	GTGAGCCGACCAAGGTGTAT AGCCGGACAAGAAGATGATG	NM_003505
Fzd2	GTCCTCAAGGTGCCATCCTA CAGCCCGACAGAAAAATGAT	NM_001466
Fzd3	CTCTCTTTGGCCCTTGACTG ACAAAGAAAAGGCCGGAAAT	NM_017412
Fzd4	CCTGGCCAGAGAGTCTGAAC AGGCTCCTTTTCACCCAGAT	NM_012193
Fzd5	TTCTGGATAGGCCTGTGGTC CGTAGTGGATGTGGTTGTGC	NM_003468
Fzd6	TTGTTGGCATCTCTGCTGTC CCATGGATTTGGAAATGACC	NM_003506
Fzd7	CGACGCTCTTTACCGTTCTC GCCATGCCGAAGAAGTAGAG	NM_003507
Fzd8	TCTTGTCGCTCACATGGTTC GGTGCCGATGAAGAGGTAGA	NM_031866
Fzd9	AGACCATCGTCATCCTGACC CCATGAGCTTCTCCAGCTTC	NM_003508
Fzd10	CCTCCAAGACTCTGCAGTCC GACTGGGCAGGGATCTCATA	NM_007197

Table 2.2. Oligonucleotide primers for other genes.

Gene	Primer Sequence 5'-3'	Genbank Accn.
LRP6	TGGATGGTTCTGACCGTGTA TCCCTCTCTGCACTTCGTTT	NM_002336
LRP5	GCAGCCTTTCTTCCACACTC TCTAGCGGGTCGTAGTCGAT	NM_002335
Cyclin D1	AACTACCTGGACCGCTTCCT CCACTTGAGCTTGTTCACCA	NM_053056
cMyc	CCTACCCTCTCAACGACAGC CTCTGACCTTTTGCCAGGAG	NM_002467
β-catenin	CCCACTAATGTCCAGCGTTT AACGCATGATAGCGTGTCTG	NM_001904
LEF1	GACGAGATGATCCCCTTCAA AGGGCTCCTGAGAGGTTTGT	NM_016269
APC	TGCGAGAAGTTGGAAGTGTG GATTTGCCTGTGGTCCTCAT	NM_000038
Axin2	CTCCCCACCTTGAATGAAGA TGGCTGGTGCAAAGACATAG	NM_004655
SFRP1	ATCTCTGTGCCAGCGAGTTT AAGTGGTGGCTGAGGTTGTC	NM_003012
SFRP2	AGGACAACGACCTTTGCATC TTGCTCTTGGTCTCCAGGAT	NM_003013
SFRP3	AAACTGTAGAGGGGCAAGCA GGCAGCCAGAGCTGGTATAG	NM_001463
SFRP4	CGATCGGTGCAAGTGTAA ACCACCGTTGTGACCTCATT	NM_003014
SFRP5	TGGAGCCCAGAAAAAGAAGA GCAGGGGTAGGAGAACATGA	NM_003015
G3PDH	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA	NM_002046
HPRT1	TGACACTGGCAAAACAATGCA GGTCCTTTTCACCAGCAAGCT	NM_000194
UBC	ATTTGGGTCGCGGTTCTTG TGCCTTGACATTCTCGATGGT	NM_021009

2.13. Real Time (Quantitative) PCR (Q-PCR).

Quantitative PCR analysis was performed in the manner essentially as described by Fox et al., (2012). In brief, cDNA generated in section 2.11 was used as a template for amplification using a SensiFAST™ SYBR® No-ROX Kit (Bioline, NSW, Australia) using the primer sequences outlined in Table 2.1. Real-time PCR was performed using a RotorGene 2000 real-time amplification instrument (Corbett Research, N.S.W., Australia) with 10µl reaction volumes. Fluorescence data was acquired at 72°C and 80-87°C (optimised for each assay). To confirm amplification specificity a melt curve analysis was performed at the end of each run.

Standard curves were generated using serially diluted cDNA. Real-time PCR assays were conducted in duplicate for each sample and control. The threshold cycle (CT) was determined automatically by the RotorGene software (v6) using the dynamic tube normalisation setting. In order to allow for sample-to-sample variability, gene expression data was normalised to levels of expression of reference (housekeeping) genes. The most stable reference genes were determined using the geNorm software (v3.3) and used to generate a normalisation factor for each sample essentially as described by Vandesompele et al., (2002). Relative expression of the target gene was normalised using this factor and expressed as mean ± standard deviation relative to a control or calibrator sample.

2.14. Immunoblotting.

For analysis of proteins by immunoblotting in response to treatments, $3x10^5$ cells per well were seeded in 6-well tissue culture plates and allowed to attach overnight for at least 18 hours before treatment. Cell monolayers were washed once with PBS and lysed by addition of 1x SDS loading buffer (BioRad) containing 5% mercaptoethanol (Sigma-Aldrich). The lysate was disrupted by pipetting and transferred to a microfuge tube on ice. Samples were sonicated (QSonica, Q125, CT, USA) at amplitude 40 for 3x 15 seconds at 30 second intervals then centrifuged at 10,000 x g for 5min and the supernatants recovered and stored at -20^{o}C. Proteins were resolved on 4-12% Mini Protean TGX gels (BioRad) and transferred to a nitrocellulose membrane (BioRad) then probed with the following antibodies: monoclonal rabbit anti-human β-Catenin (Cell Signalling Technology (CST), MA, USA) and monoclonal mouse anti- actin (Sigma-Aldrich). After incubation with horse radish peroxidase conjugated secondary antibody (Jackson Immunosearch, PA, USA)

chemiluminescent detection was performed with Clarity ECL substrate (BioRad) and images captured using a GelDoc XR imaging system (BioRad).

2.15. RNA sequence analysis.

Expression analysis was performed using an AmpliSeq Transcriptome kit (Life Technologies) on an Ion Proton semiconductor sequencer (Life Technologies, Victoria, Australia) at the Lotterywest State Biomedical Facility – Genomics node at the University of Western Australia. For each sample, 10 ng of total RNA was reverse transcribed using the Ion AmpliSeq Transcriptome Human Gene Expression kit according to the manufacturer's protocol (Life Technologies). The cDNA was amplified using Ion AmpliSeq Transcriptome Human Gene Expression core panel (Life Technologies). Primer sequences were then partially digested and barcoded sequencing adaptors ligated. Final libraries were amplified for 5 cycles and quantified on a high-sense chip using a 2100 Bioanalyser (Agilent). Then 8 samples were pooled at a final concentration of 175pM and emulsion PCR and P1 chip loading was performed using a Ion PI HiQ templating kit on an Ion Chef (Life Technologies). Samples were sequenced on an Ion Proton System using Ion P1 Hi-Q sequencing kit for 520 cycles (>200 bp read length; Life Technologies). Signal-processing, basecalling and quality trimming were performed using Torrent Suite v4.4 and reads were aligned to a custom cDNA reference sequence (containing only the known exons being sequenced) using TMAP (Life Technologies) .

Analysis of sequence reads and differential gene expression

Aligned reads were analysed using the AmpliSeqRNA analysis plugin, v 4.4, in the Torrent Suite Software (Life Technologies). This program counts and normalises the number of sequences obtained for all cDNA amplicons. The resulting counts represent the gene expression levels for over 20,800 different genes present in the AmpliSeq Human Gene Expression panel. The expression level counts for all of samples were then merged into a single table which was then used for differential gene expression analysis with the R/Bioconductor packages DESeq2 (v 1.10.1), edgeR (v2.4.6) and VOOM/limma (v 3.16.6) (http://www.bioconductor.org/) as Galaxy implementations (http://galaxy-qld.genome.edu.au/). Analysis with these packages were performed using standard parameters. Adjusted p-values (padj) for multiple testing, using Benjamini-Hochberg

analysis to estimate the false discovery rate (FDR), were calculated for final estimation of DE significance. Generation of Venn diagrams and analysis of gene lists was performed using BioVenn (http://www.cmbi.ru.nl/cdd/biovenn/index.php) (Hulsen et al, 2008). Functional annotation of gene lists was performed using the DAVID Bioinformatics Resources 6.7 (https://david.ncifcrf.gov/) (Huang et al, 2009). Pathway analysis and gene ontology analysis were conducted using FIDEA (Functional Interpretation of Differential Expression Analysis) (D'Andrea et al., 2013) (http://circe.med.uniroma1.it/fidea/index.php).

2.16 Data analysis and presentation

With the exception of RNA sequence analysis data which was processed as described in section 2.15, data is expressed as mean +/- standard deviation (or standard error of means where stated) of at least three independent experiments. Statistical significance was calculated by Student's T-test using GraphPad Prism 4.0 (GraphPad Software, California, USA). A p value of ≤ 0.05 was considered significant.

Chapter 3.

Expression of Wnt signalling molecules and functional investigations of Wnt4 activity in malignant mesothelioma.

Table of contents.

(The results presented in this chapter formed part of the publication: **Fox, S. A., Richards, A. K., Kusumah, I., Perumal, V., Bolitho, E. M., Mutsaers, S. E., & Dharmarajan, A. M. (2013). Expression profile and function of Wnt signaling mechanisms in malignant mesothelioma cells. *Biochem Biophys Res Commun, 440*(1), 82-87. doi:10.1016/j.bbrc.2013.0).**

3.0. Introduction.

Malignant mesothelioma (mesothelioma) is characterised by rapid progression, late metastases and poor prognosis as well as resistance to conventional therapy methods which makes it one of the exclusively fatal malignancies. The identification of molecular targets in mesothelioma is a priority in order to develop better treatment strategies and the presence of Wnt signalling elements in other cancers provides a basis for such studies in this disease. Considerable evidence exists to demonstrate the association between Wnt signalling and cancer via the canonical, β-catenin mediated expression of target genes associated with proliferation, invasion and metastasis. As previously discussed in chaper 1, in the field of mesothelioma research, the deletion of the β-catenin gene has been noted (Shigemitsu et al., 2001), as has the overexpression of dishevelled (Dvl) (Uematsu et al., 2003) as well as the identification of Wnt1 and Wnt2b as potential therapeutic targets (He et al., 2004; Mazieres et al., 2005). Additionally, the regulatory role of the secreted frizzled-related proteins (SFRPs) has also been explored with studies showing the downregulation of SFRPs as a result of promoter hypermethylation (Lee et al., 2004) and a similar situation exists for Wnt inhibitory factor 1 (WIF1) (Kohno et al., 2010). However, a wider investigation of the Wnt signalling pathway has not been previously undertaken.

This study undertook a comprehensive analysis of Wnt signalling molecules in order to establish the profile of Wnt signalling in three mesothelioma lines and three non-malignant mesothelial control lines. The expression of Wnt ligands, Fzd receptors, associated signalling molecules and the SFRPs was analysed by reverse transcription PCR (RT-PCR) to begin with and further analysis of selected genes carried out by quantitative real-time PCR (Q-PCR). Based upon the results of these investigations, functional assays were employed to determine the role of Wnt4 in the modulation of cell migration and viability in mesothelioma.

3.1. Mesothelioma cells express both canonical and non canonical Wnt proteins.

The presence or absence of the Wnt ligands was analysed using RT-PCR with mRNA transcripts of the malignant lines LO68, ONE58, JU77 and three primary mesothelial cultures: MC-A, B and C. The expression of Wnt proteins in these lines is shown in Figure 3.1 and shows Wnt2b to be present in all lines tested, suggesting a relatively lower expression in LO68 and ONE58 cells compared to the other mesothelioma line JU77 and mesothelial cells. Wnt3 was consistently detected in both sets of mesothelial and malignant lines and interestingly, Wnt4 expression in malignant lines was apparently lower than that of the mesothelial controls. Wnt5a was shown to be present in all cell cultures, whereas Wnt6 appears to be unique to ONE58. Wnt10b (formerly Wnt12) was found to be present across all samples although the PCR band intensity was weak. Wnt ligands 1, 2, 3a, 7a, 8a, 10a and 16 were not detected and the Wnt genes 5b, 7b, 8b, 9a (formerly Wnt14), 9b (formerly Wnt15) and Wnt11 were not examined in this study.

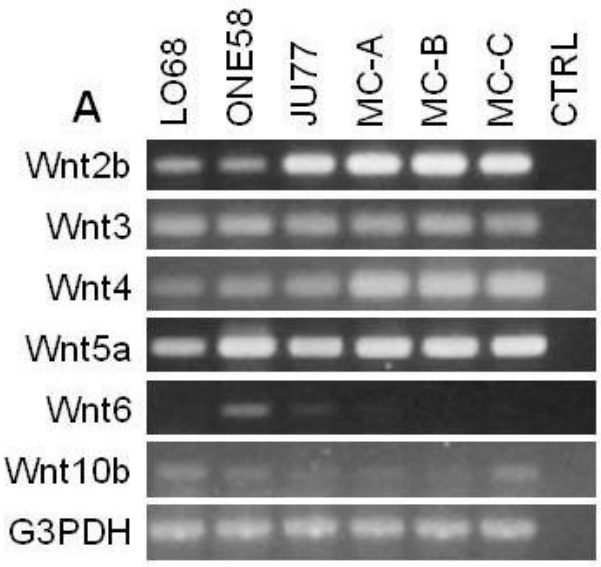

Figure 3.1. Expression profile of Wnt ligand molecules in mesothelioma cell lines and mesothelial cell cultures. The expression of Wnt ligands in control and malignant lines was analysed by RT-PCR. Total RNA isolated from cells was analysed by 2-step RT-PCR using gene-specific primers. Wnts 1, 2, 3a, 7a, 8a, 10a, and 16 were not detected.

3.2. Mesothelioma cells express Fzd receptors.

The pattern of Fzd receptors expression in both malignant and mesothelial lines was also assayed and is shown in Figure 3.2. Fzds 2, 3, 4 and 6 were ubiquitously expressed in all the cells tested and Fzd3 was present to a lesser extent, particularly in the malignant lines. The expression of Fzd1 was variable with ONE58 being the only malignant line to express it whereas its presence was noted in all three mesothelial controls. Similarly, Fzd5 was detected in two of the three malignant lines (LO68 and ONE58) and also in two of the three mesothelial controls (MC-A and B). The expression of Fzd7 observed in ONE58 and poorly expressed in all other samples, possibly being absent in LO68. The expression of Fzd8 and Fzd10 was also poor with weak band intensity of Fzd8 in ONE58 and MC-B and similarly weak expression of Fzd10 in MC-A. Fzd9 was not detected at all.

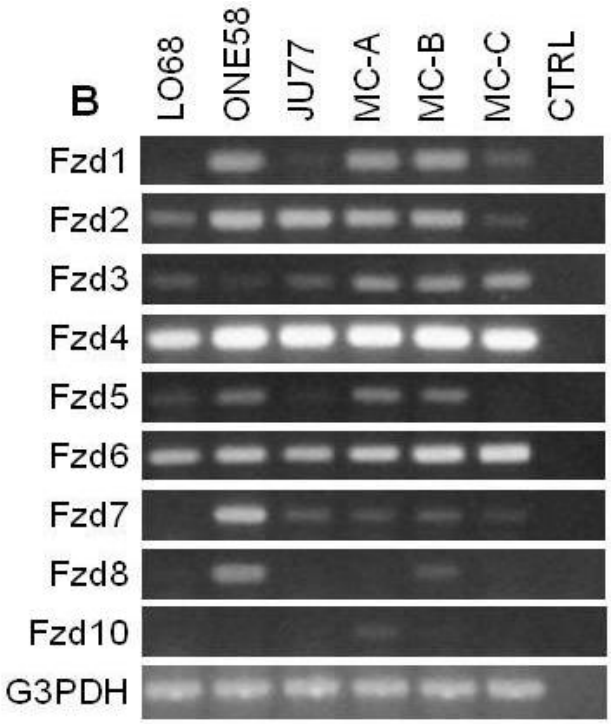

Figure 3.2. Expression profile of Frizzled receptors in mesothelioma cell lines and mesothelial cell cultures. The expression of the Frizzled receptors in control and mesothelioma cultures was analysed by RT-PCR. Total RNA isolated from cells was analysed by 2-step RT-PCR using gene-specific primers, Fzd 9 was not detected.

3.3. Mesothelioma cells express key Wnt pathway components and targets.

In order to further determine the nature and components of Wnt signalling in mesothelioma, the key molecules involved in the transduction of a canonical Wnt signal were also analysed by RT-PCR (Figure 3.3). The presence of the key LRP5 and LRP6 co-receptors for β catenin mediated (canonical) signalling was demonstrated in all cells. The key mediator of Wnt signalling, β catenin, was expressed in all cells, although there was no indication of overexpression at the mRNA level between the malignant and control cell lines. Similarly, the scaffold molecule APC involved in the formation of the β catenin degradation complex was ubiquitously expressed. The widely described nuclear targets of canonical signalling such as c-Myc, cyclin D1 and Axin2 were also expressed. Interestingly, expression of Lymphoid Enhancer-Binding Factor 1 (LEF1) was present in the malignant mesothelioma cells and absent in the control mesothelial cells.

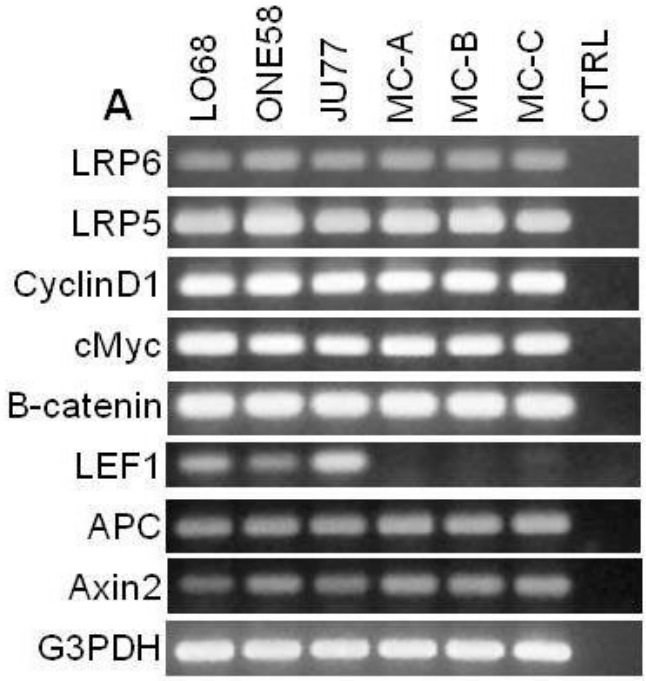

Figure 3.3. Expression of Wnt signalling molecules and nuclear targets in mesothelioma cell lines and mesothelial control cell cultures. The expression of key Wnt signalling molecules was analysed in mesothelial and mesothelioma cell cultures by RT-PCR. Total RNA isolated from cells was analysed by 2-step RT-PCR using gene specific primers.

3.4. Secreted regulators of Wnt signalling are differentially expressed in malignant mesothelioma.

The expression of this key family of secreted regulators that are often downregulated in cancer was determined next. An interesting pattern was noted as demonstrated in Figure 3.4 amongst the SFRP molecules with SFRP1 being expressed in all lines except for LO68 and similarly for SFRP3 that was expressed in all others except for JU77. The presence of SFRP5 was observed in all cell lines and SFRP2 was not detected. Strikingly, SFRP4 appeared downregulated in malignant mesothelioma lines compared to mesothelial controls, being weakly detected in JU77 and absent in LO68 and ONE58.

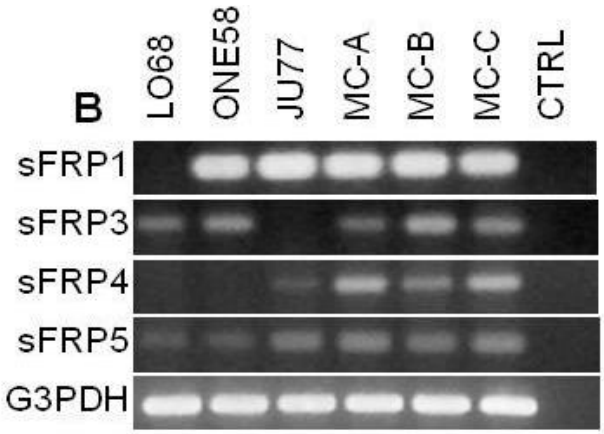

Figure 3.4. Expression profile of SFRP proteins in mesothelioma cell lines and mesothelial cell cultures. Total RNA isolated from cells was analysed by 2-step RT-PCR using gene specific primers. SFRP2 was not detected.

3.5. Quantitative analysis of Wnt pathway genes.

The conventional RT-PCR assays shown in Figures 3.1 – 3.4 provided evidence for different gene expression patterns. In order to gain more definitive differential expression data for these genes, quantitative PCR (Q-PCR) was used. The apparent difference seen by RT-PCR in the expression of Wnt2b and Wnt4 (Figure 3.1) between the malignant and mesothelial control lines warranted further analysis by Q-PCR. Wnt2b was found by RT-PCR to be strongly expressed in mesothelial control lines A, B and C and the malignant line JU77 with a likely downregulation in LO68 and ONE58. Q-PCR analysis confirmed the relative expression pattern of Wnt2b as being very low in LO68 and ONE58 cells with JU77 showing an expression ratio of at least half that of mesothelial controls B and C (Figure 3.5A). Interestingly, one of the mesothelial cell cultures (derived from different individuals), MC-C also showed low Wnt2b expression. The apparent downregulation of Wnt4 seen during RT-PCR analysis (Figure 3.1) was also corroborated by Q-PCR analysis which showed expression of Wnt4 being at least one thousand fold less in the malignant cell lines than the mesothelial cells (Figure 3.5B). It was this marked downregulation which served as a basis for further investigation described in this chapter (sections 3.6 and 3.7).

The important roles of APC and Axin2 in the transduction of a canonical Wnt signal provided the reason to investigate potential differences in the expression of these two genes between mesothelioma and mesothelial cultures. Analysis by Q-PCR showed that in both cases, expression of APC and Axin2 was at least one hundred fold downregulated relative to the lowest expressing mesothelial culture lines (Figures 3.5C and 3.5D respectively). The genes c-Myc and cyclin D1 are well known targets of canonical Wnt signalling in cancer due to their roles in controlling cell proliferation and Q-PCR analysis showed a rather uniform degree of expression in mesothelioma and mesothelial cells. A notable exception being the high level of c-Myc expression in LO68 cells (Figures 3.5E and 3.5F respectively).

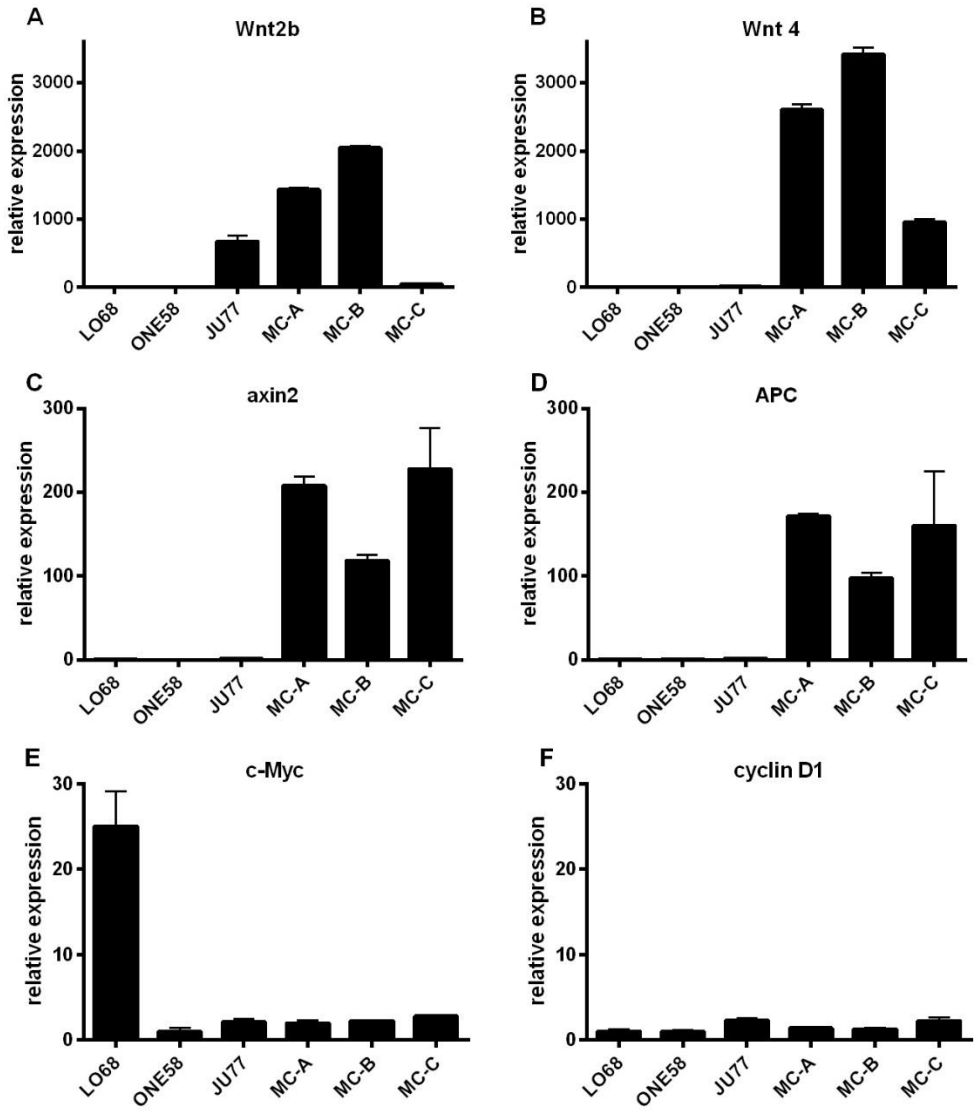

Figure 3.5. Basal gene expression of (A) Wnt2b, (B) Wnt4, (C) Axin2, (D) APC, (E) c-Myc and (F) cyclin D1 in malignant mesothelioma cells and mesothelial control cell cultures. Total RNA isolated from cells was analysed by 2-step real-time RT-PCR using gene specific primers. Basal gene expression is expressed as mRNA levels relative to the lowest expressing culture following normalisation by G3PDH expression. Results are mean +/- SD for three cultures.

3.6. Effect of recombinant Wnt4 on viability of JU77 and ONE58 mesothelioma cells.

Based upon differential expression shown in Figure 3.5B, the biological effects of Wnt4 were investigated to determine the possible consequences of the downregulation. Therefore, recombinant Wnt4 was used to investigate a possible effect on cell viability in JU77 and ONE58 mesothelioma cells. Previous experiments with recombinant Wnt proteins in our laboratory demonstrated biological effects in the dose range 0 to 10ng/mL and this was applied to experiments using Wnt4. JU77 cells were cultured for 48 hours and 72 hours (Figures 3.6A and 3.6B) and similarly for ONE58 cells (Figures 3.6B and 3.6D) with cell viability assessed by Trypan Blue exclusion counting using a Countess automated haemocytometer as described in section 2.4. No significant effect on viability was seen at this dose range in both cell lines at both time points. At this dose range, it is unclear whether Wnt4 is either ineffective due to a sub-optimal dose or that it may not exert an affect in these mesothelioma cell lines.

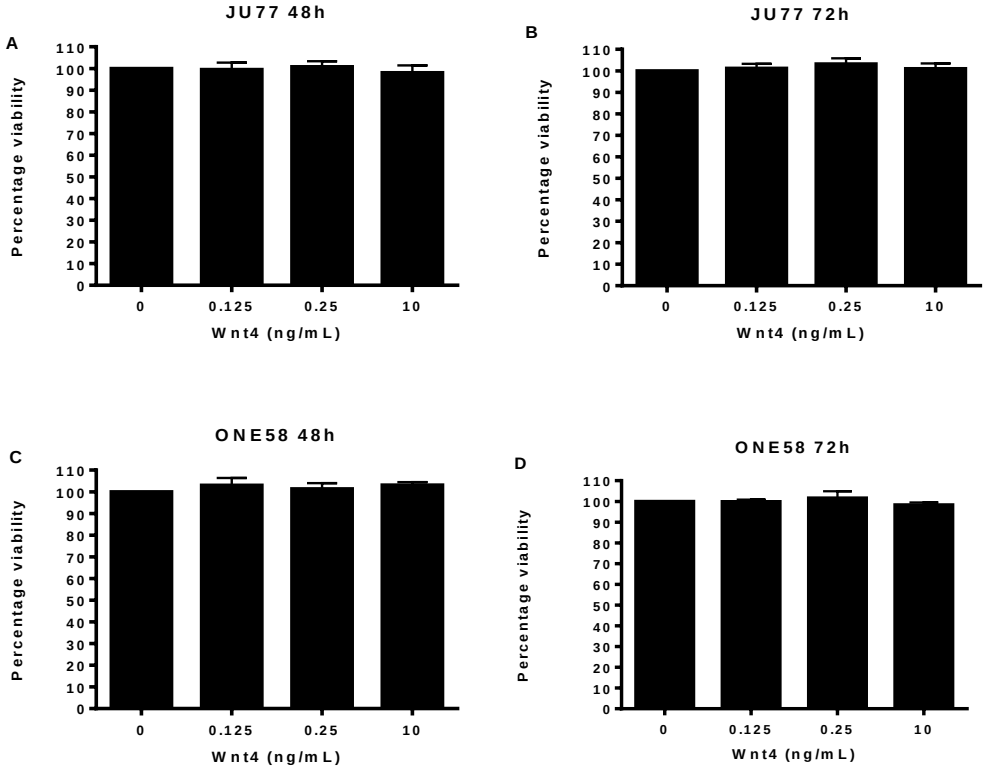

Figure 3.6. Recombinant Wnt4 does not affect the viability of JU77 and ONE58 cells. Cells were incubated with recombinant Wnt4 for 48h (3.6A and C) or 72h (3.6B and D) and viability assessed by trypan blue exclusion counting. Results are shown as mean percentage viability +/- SEM from three independent experiments. (A) JU77 48h, (B) JU77 72h, (C) ONE58 48h and (D) ONE58 72h.

3.7. Activity of Wnt4 in malignant mesothelioma cells by wound-scratch assay.

In addition to reported effects of Wnt4 in development (Bernard and Harley, 2007), the possible role of Wnt4 in cellular migration was investigated using the same dose range as the viability studies in section 3.6. Having found no observable effect on cell viability, the effect of recombinant Wnt4 upon mesothelioma cell migration was examined using a scratch assay. The method described by Liang et al., (2007) was used to perform the assay so that the T-Scratch software could be used to analyse the effect of Wnt4 on wound scratch closure. Since a scratch assay using mesothelioma cells had not previously been used in our laboratory, preliminary experiment were performed to establish the conditions.

Trials were conducted to determine the optimal seeding populations and a suitable recovery time post-seeding in order to form a useable monolayer. A differential seeding of 50,000 JU77 and 30,000 ONE58 cells was decided upon with JU77 cells seeded 12 hours prior to ONE58 cells so that both cell lines were allowed at least 24 hours recovery time. The requirement for collagen coating arose due to disruption and peeling of the ONE58 monolayer at the time of wounding, the JU77 monolayer being more robust. Coating of the culture surface with Type I collagen produced a useable monolayer in both lines.

In neither cell line was an effect upon cell migration observed following treatment with recombinant Wnt4. Even at the highest concentration of Wnt4 of 10ng/mL, there was no discernible change in the rate of JU77 and ONE58 wound closure compared to control (Figures 3.7 and 3.8). Due to the absence of any apparent effect, quantitative analysis using T-Scratch software was not performed. It was therefore concluded that at this dose range, Wnt4 did not exert any effect on cell migration.

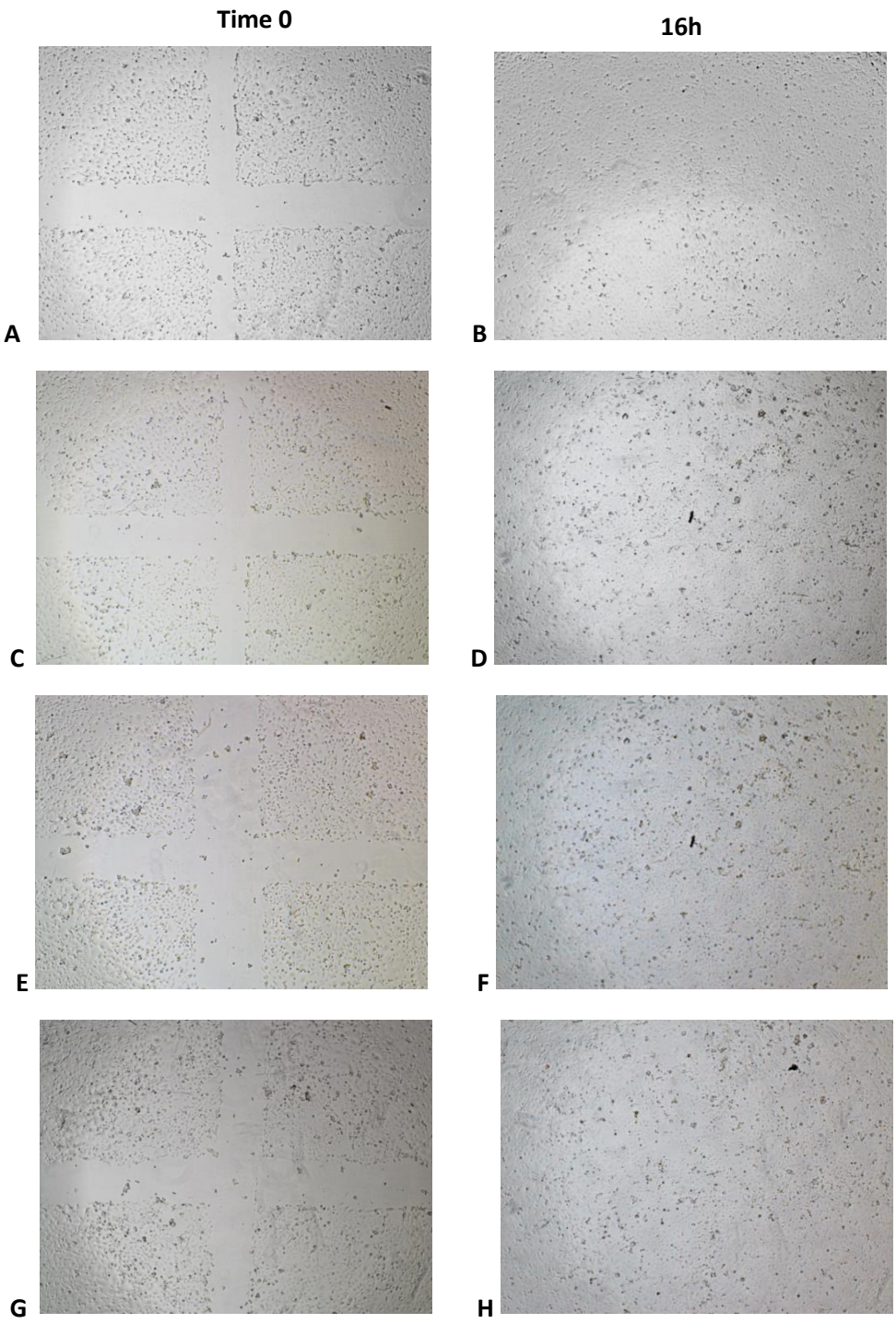

Figure 3.7. Wound closure assay of JU77 cells at time 0 and 16h. (A) non-treatment control time 0, (B) non-treatment control 16h, (C) Wnt4 125pg/mL time 0, (D) Wnt4 125pg/mL 16h, (E) Wnt4 250pg/mL time 0, (F) Wnt4 250pg/mL 16h, (G) Wnt4 10ng/mL time 0 and (H) Wnt4 10ng/mL 16h. Photographs are representative samples taken from at least three independent experiments. (Magnification x40)

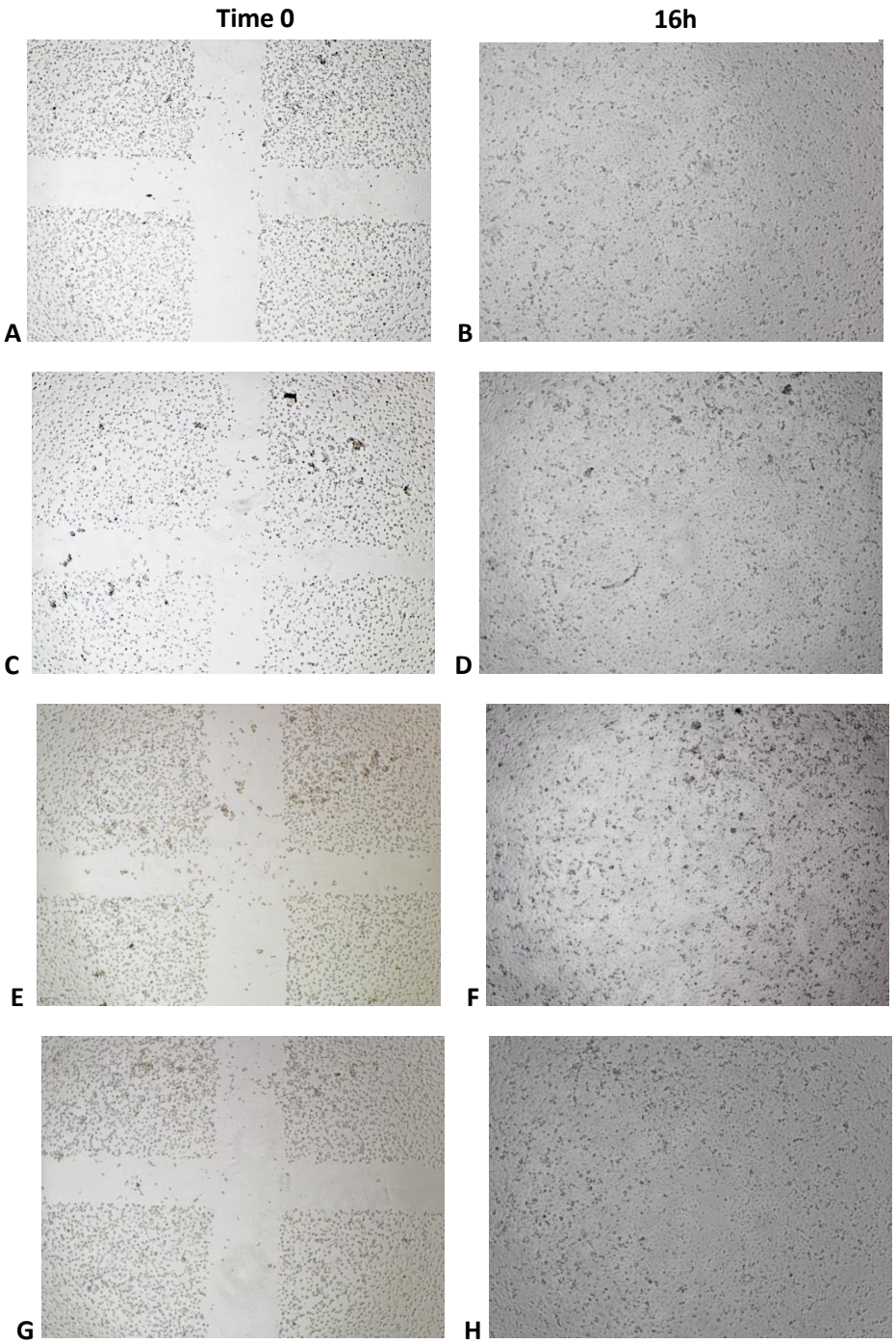

Figure 3.8. Wound closure assay of ONE58 cells at time 0 and 16h. (A) non-treatment control time 0, (B) non-treatment control 16h, (C) Wnt4 125pg/mL time 0, (D) Wnt4 125pg/mL 16h, (E) Wnt4 250pg/mL time 0, (F) Wnt4 250pg/mL 16h, (G) Wnt4 10ng/mL time 0 and (H) Wnt4 10ng/mL 16h. Photographs shown are representative examples taken from at least three independent experiments. (Magnification x40).

3.8 DISCUSSION.

Studies on Wnt signalling to date have focused on only a few Wnt ligands and selected inhibitory regulators. Research interest to date has examined the Wnt ligands Wnt1 (He et al., 2004), Wnt2 (Mazieres et al., 2005), Wnt2b (Kobayashi et al., 2012) and Wnt inhibitors such as the SFRPS and WIF1 (Lee et al., 2004a; He et al., 2005; Kohno et al., 2010). In this chapter, the expression of all Wnt ligands and Frizzled receptors was examined as well as several other key Wnt signalling molecules. Five Wnt ligands (Wnts 2b, 3, 4, 5a and 10b) and three Frizzled receptors (Fzd 2, 4 and 6) were detected. Other essential elements of the Wnt signalling pathway such as the co-receptors LRP5, LRP6, the key mediator protein β catenin and nuclear targets c-Myc, cyclin D1 and LEF1 were also detected.

In terms of classification as either canonical or non-canonical Wnts based on the models of C57 mouse mammary gland transformation and *Xenopus* axis duplication assay (Li et al., 2008; Chien et al., 2009), it may be said that the canonical Wnt ligands (Wnts 2b, 3 and 10b) and the non-canonical Wnt ligands (Wnts 4, 5a and 6) have been detected in these mesothelioma cell lines. A case-by-case approach to assigning classification to Wnt ligands in mesothelioma is required as the Fzd receptor profile in these lines may indeed produce an unexpected result. It is known that certain Wnt ligands (eg: Wnt5a) may activate either the canonical or non-canonical Wnt pathway depending on the molecular context (Mikels and Nusse, 2006). Similarly, a potential duality of Wnt4 action may exist in mesothelioma despite it being traditionally considered as a non-canonical Wnt ligand. Studies in myoblast differentiation and nephron tubule formation support the non-canonical actions of Wnt4 (Tanaka et al., 2011; Tanigawa et al., 2011) but an earlier study in another nephron module provided evidence for activation of canonical Wnt signalling instead (Lyons et al., 2004). Possible explanations include the presence or absence of a Wnt4-cognate Fzd receptor in addition to any possible cell type differences (Lyons et al., 2004) and therefore, the nature of Wnt4 signalling in mesothelioma remains poorly understood. Considering the varied responses across differing models, the actions of each of the five Wnt ligands detected in this study will all require further investigation.

The role of Wnt2b is considered to be that of a tumour promoter and therefore, a poor prognostic marker in mesothelioma (Kobayashi et al., 2012) as well as other cancers such as colorectal carcinoma (Ma et al., 2008), pancreatic cancer (Jiang et al., 2014) and head and neck squamous cell carcinoma (Li, S-J et al., 2015). It is therefore interesting to note the expression of Wnt2b in the malignant JU77 cell line and in all the mesothelial control cell lines MC-A, B and C but a lower degree of expression in the malignant lines LO68 and ONE58. Overall the mesothelioma lines showed a lower expression of Wnt2b and this difference warrants further investigation. Further analysis of other mesothelioma cell lines will yield more information on the relative influence of Wnt2b in mesothelioma as any assumptions to its redundancy may be indeed incorrect.

The proliferative and oncogenic effects of β catenin mediated Wnt signalling are attributable to the β catenin/TCF/LEF transcription complex as a whole with LEF1 receiving considerably less scientific interest than the TCF group. The oncogenic potential of LEF by itself has been described in a chicken fibroblast model (Aoki et al., 1999) and the association of LEF1 with cancer has since expanded to include breast cancer (Gebeschuber et al., 2007), prostate cancer (Li, Y. et al., 2009), colorectal cancer (Kriegl et al., 2010) and glioblastoma (Gao et al., 2014). An additional role of LEF1 in the promotion of motility and invasiveness has been described in studied of breast cancer (Nguyen et al., 2005), prostate cancer (Li, Y. et al., 2009) and glioblastoma (Gao et al., 2014). The role of LEF1 in mesothelioma will also be a worthwhile topic for further study in these mesothelioma lines and others as well. The pattern of expression in the cell lines studied is noteworthy as an apparent upregulation of LEF1 appears in mesothelioma cell lines compared to mesothelial control cells. Further investigation of LEF1 in other mesothelioma cell lines will assist greatly in understanding any potential association with mesothelioma.

Axin2 has been studied extensively as a structural component of the β catenin degradation complex but the control of its expression in a negative feedback loop has revealed that it is also a target of Wnt signalling (Lustig et al., 2002). These authors propose that the upregulation of Axin2 is an early and inducible event subsequent to the initiation of a Wnt signal in order to maintain physiological levels of β catenin and that aberrant β catenin expression overwhelms this process, leading to tumourigenesis (Lustig et al., 2002). The further differentiation of control between Axin1 and Axin2 was also investigated with the

suggestion that Axin1 exists as a constitutively expressed form whereas Axin2 is the inducible form, activated upon Wnt signalling (Leung et al., 2002). A possible mechanism for Axin2 induction may involve the regulation of gene transcription by the retinoblastoma/E2F1 transcriptional complex whereby the relative proportions of the three known mRNA transcripts determines the degree of upregulation and transcript stability (Hughes and Brady, 2005). Furthermore, regulation of Axin2 by *caudal*-related homeobox transcription factor 2 (CDX2) has also been described in a colorectal cancer model (Olsen et al., 2013).

The downregulation of Axin2 in the malignant lines is of interest as the reduction or loss of Axin 2 function is expected to produce upregulated β catenin mediated Wnt signalling, thereby driving malignancy. Axin2 expression, despite being somewhat variable is still considerably greater in in the mesothelial control cells compared to the malignant lines and the mechanism(s) behind this downregulation remain unclear. Currently, no information exists regarding Axin2 inactivation in mesothelioma, but studies in large cell lung carcinoma lines have revealed epigenetic silencing of Axin2 in this model (Yang et al., 2013) with similar findings in neuroendocrine tumours (Kim et al., 2013). An interesting suggestion based on studies in mouse pancreatic islet tumour cells involves regulation of Axin by Menin (Multiple Endocrine Neoplasia Type 1 protein), a DNA binding protein with transcription regulating activity (Chen et al., 2008). Similarly, there have been no studies on the deregulation of APC in mesothelioma in order to explain the lower expression seen in this study (Figure 3.5). Deregulation of APC function has been well characterised in studies on colorectal cancer where germline mutations such as frameshifts and nonsense mutations lead to truncated, dysfunctional mutant protein in the majority of cases (Munemitsu et al., 1995; Fearon, 2011). Promoter hypermethylation of the APC gene has been described as part of a small number of colorectal cancers but appears to be more common in gastric cancers, with mutations being more common in colorectal cancers (Esteller et al., 2000). Both mechanisms have yet to be investigated and characterised in mesothelioma.

The Fzd receptors are slowly being understood through a variety of models and the field of mesothelioma research is in need of further information on this topic. Individual cell line-specific differences are to be expected and have been demonstrated here with various

receptor complement profiles seen in Figure 3.2. Varying degrees of Fzd expression are noted with the ubiquitous expression of Fzds 2 and 4, the absence of Fzd9 and weak expression of Fzd in one mesothelial control cell line only. The ONE58 cell line is also of interest, showing the expression of the most number of Fzds including Fzd7 (along with JU77 to a lesser extent). Of the Fzds, considerable interest has been shown in Fzd7 due to its promotion of proliferation and invasiveness in several malignancies. Examples include colorectal cancer (Ueno et al., 2009), triple negative breast cancer (Yang et al., 2011), ovarian cancer (Asad et al., 2014) and cervical cancer (Deng et al., 2015). In view of the characteristic invasiveness that is one of the hallmarks of mesothelioma, any investigation into the role of Fzd7 in mesothelioma would be highly warranted.

Information on the actions of the SFRP molecules in mesothelioma is also scarce with three studies to date in this field. The downregulation of SFRPs 1, 4 and in primary mesothelial tissue and their absence in two β catenin deficient cell lines has been reported (Lee et al., 2004a). The silencing of SFRP4 by promoter hypermethylation and restoration of SFRP4 activity in a β catenin deficient cell line and another β catenin expressing cell line suggested that β catenin-independent pathway may indeed contribute to SFRP-mediated growth arrest (He et al., 2005). Promoter hypermethylation of SFRPs 1, 2 and 4 in primary tissue has also been described along with insights into other genetic deregulations in mesothelioma (Kohno et al., 2010). Previous work in our laboratory has shown a similar profile of SFRP downregulation in mouse mesothelioma models (Fox and Dharmarajan, 2006) and recent work has shown inhibition of mesothelioma cell line growth by SFRP4 consistent with other studies as well as the downregulation of SFRP4 in mesothelioma cell lines reported in this study (Fox et al., 2013). Further investigations into the role of SFRP4 in mesothelioma are under way in our laboratory (Fox, S. – pers. comm).

The role and mechanisms of Wnt4-mediated events in cancer are poorly understood but considerable information exists on its roles in development, particularly gender assignment through Mullerian duct formation, gonadal vascularisation and steroidogenesis (Bernard and Harley, 2007). In the developed state, Wnt4 mediates the formation of a sufficient reserve of healthy ovarian follicles and regulates steroidogenesis in both granulosa cells and the corpus luteum (Boyer et al., 2010). A human autosomal recessive disorder termed SERKAL (sex reversal, kidney, adrenal and lung dysgenesis) syndrome is known to be a loss-

of-function mutation in Wnt4 with gender reversal from female to male and kidney malformations being the most obvious defects (Mandel et al., 2008).

Normally considered a non-canonical Wnt ligand, Wnt4 has been shown to activate β catenin in a mouse myoblast model (Tanaka et al., 2011), formation of the decidua from the endometrial stroma (Li, Q. et al., 2013), thymocyte generation (Louis et al., 2008) and kidney tubule formation in a canine cell model (Lyons et al., 2004) although a later mouse model study showed involvement of the non-canonical Ca^{2+}/CAMKII pathway (Tanigawa et al., 2011). Therefore, having shown effects in β catenin dependent and independent manners, cell line specificity must be a key consideration in any examination of Wnt4 activity. It is currently difficult to determine the nature of a Wnt4 signal in these mesothelioma lines as no significant effects on viability or migration were seen and the discovery of the mechanism(s) behind its downregulation also remains unexplored.

A growing body of knowledge is developing with regard to Wnt4 in malignancy and this includes the discovery of two response elements for p63 and p73 in the Wnt4 gene promoter in Saos2 osteosarcoma cells and the hypothesis that both sites may need to be involved together in modulating expression of Wnt4 (Osada et al., 2006). Downregulation of Wnt4 has been reported in human malignant breast tissue but upregulated in benign breast adenocarcinomas (Huguet et al., 1994), similarly downregulated in human endometrial carcinoma cell lines (Bui et al., 1997) and an opposing report of Wnt4 upregulation in other breast cell cancer lines (Benhaj et al., 2006). An interesting, inverse relationship between Wnt4 and Wnt5a expression has also been described in oral squamous cell carcinoma lines whereby increased Wnt5a expression correlated with a downregulation of Wnt4 (Taki et al., 2005). A correlation between Wnt4 downregulation and cancerous motility induced by Ras in rat thyroid epithelial cells has been reported by de Menna and co-workers (2013). Overexpression of Wnt4 opposed Ras-mediated motility in a β catenin independent manner and furthermore, the differential regulation of Wnt4 expression was described whereby inhibition by the MAPK cascade and activation by the PI3K pathway was demonstrated. Additionally, the regulation of Wnt4 mRNA by micro RNA 24 (miR24) was also noted (de Menna et al., 2013). Studies in leukaemia-derived cell lines showed growth arrest with the addition of recombinant Wnt4 at a considerably higher dose of 200ng/mL (Garcia-Castro et al., 2013) compared to the dose range used in this study.

This same study also demonstrated growth arrest at the G1 phase of the cell cycle with the overexpression of Wnt4 (Garcia-Castro et al., 2013). Lastly, an alternate mechanism has been described in HEK293 cells by Bernard and colleagues (2008) in which the attenuation of Wnt signalling by Wnt4 involves relocation of free cytoplasmic β catenin to the membrane bound β catenin / E cadherin junction complex. Three potential mechanisms were suggested; (i) a phosphorylation state dependent regulation of β catenin binding associations, (ii) the involvement of B-cell CLL/Lymphoma 9-Like Protein (BCL9L) or possibly (iii) a β catenin independent pathway involving mitogen activated protein kinase 8 (MAPK8) (Bernard et al., 2008). No further information has come to light since this study.

The results in this chapter provided the basis for the use of global gene expression analysis through the introduction of RNA sequencing technologies as employed in later chapters. Sufficient evidence was gathered to suggest that canonical β catenin mediated Wnt signalling was present in both mesothelial and mesothelioma cell lines and that modulation of its activity by pharmacological agents would produce changes in expression of the various components.

With only a small number of studies in this area, further insight into the actions of Wnt signalling in mesothelioma is a priority. Basic characterisation studies such as these have yet to be applied to other commercially available mesothelial and mesothelioma cell lines in order to produce a generalised view of the Wnt component profile in this disease. In particular, the growth modulating properties of Wnt4 and SFRP4 deserve further study, especially when cell line specific differences may influence experimental findings. Our results demonstrate the expression of key Wnt components and other associated molecules not previously explored using three mesothelioma cell lines that are different to those already used in previous publications by other research groups. Work is continuing in our laboratory to identify potential targets in the Wnt signalling system in order to facilitate the development of treatment strategies that may enhance the effects of clinically employed chemotherapeutic agents.

Chapter 4.

Three dimensional culture and functional assay development in four malignant mesothelioma lines

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4.0. Introduction.

Previously in the supervisor's laboratory, the cell biology of mesothelioma cell lines in response to therapeutic agents and biological modifiers had been performed using conventional 2D culture models (Cregan et al., 2013). Furthermore, studies of other aspects of cell biology related to Wnt signalling such as migration and colony formation, had been limited to the wound healing assays described in chapter 3. In order to determine the suitability of the mesothelioma cell lines used in our laboratory to other experimental methods, a series of assays was carried out to assess their capacity for growth in three dimensional (3D) culture as well as assays for colony formation, migration, adhesion and invasion.

The conventional method of cell culture as a monolayer forms the basis of numerous types of studies across many fields of research and has its own set of benefits and limitations. The use of monolayer-based models is rapid, reproducible and economical but lacks the complexity of a three dimensional microenvironment such as that of a tumour. Recent advances in three dimensional cell culture range from reliable methods for producing cell spheroids suitable for high-throughput screening to sophisticated co-culture models to approximate the *in vivo* setting (Vinci et al., 2012).

Using locally derived Western Australian cell lines isolated by Manning and co-workers (1991), we investigated their ability to form spheroids, their physical characteristics and application in a cell viability assay adapted from an existing protocol in colon carcinoma cell spheroids (Friedrich et al., 2007). A previous study employing several cancerous lines was used as an empirical basis to develop a culture method for these four cell lines (Vinci et al., 2012). Additionally, four functional assays were adapted or optimised to produce a set of reliable methods with which to investigate the responses of these four cell lines under various experimental conditions.

4.1. Features of malignant mesothelioma cell lines in culture.

In order to assess if any changes had occurred in these cell lines over time, fresh stocks of the four mesothelioma lines were examined in standard culture conditions for changes in morphology and growth characteristics. Four of the five lines isolated and propagated by Manning and co-workers (1991) were used in this study (the DeH128 line was not available). The morphology observed during routine propagation (Figure 4.1) was consistent with that noted by Manning et al., (1991) who described JU77, LO68 and ONE58 as being spindle-shaped and NO36 as thick and stellate. Confluent monolayers were easily formed under our standardised culture conditions (section 2.2) and in all cases, the doubling time was approximately 18 to 24 hours which was also in keeping with their original characterisation. Post-subculture recovery times in LO68, NO36 and ONE58 was rapid with cells ready for experimentation after 16 hours. The JU77 line however, required an equilibration period of nearly 24 hours and thus all four lines were seeded at least 24 hours prior to experimentation.

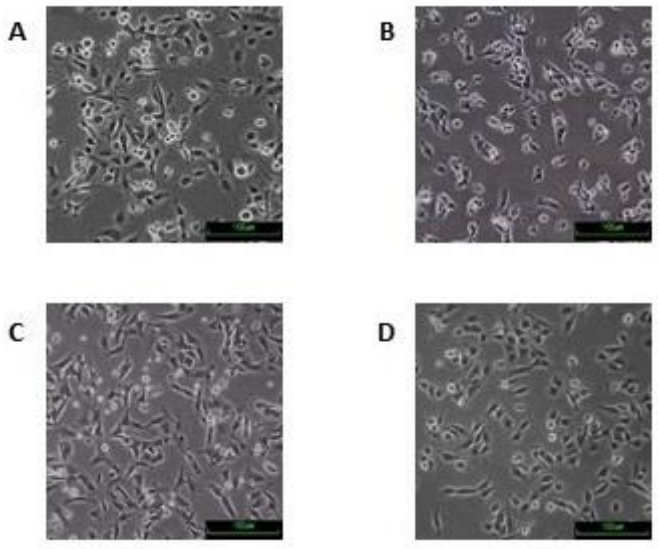

Figure 4.1. Representative images of the four malignant mesothelioma lines. Pictures taken under normal culture condition: (A) JU77, (B) LO68, (C) NO36 and (D) ONE58. (Scale bar 100µm).

4.2. Establishment of mesothelioma 3D spheroid model.

Prior to these experiments, these four mesothelioma cell lines had not been grown in a 3D environment in our laboratory and therefore, guidelines were sought for a simple, practical method with which to evaluate their usefulness in a 3D model. A detailed study by Vinci et al., (2012) provided an empirical basis for determining whether or not a particular cell line would form spheroids and if they were useable for experiments. The liquid overlay method was employed whereby a cell suspension was seeded into Corning® ultra-low adhesion plates (Corning 7007) and a starting population of $5x10^3$ cells was allowed to self-associate over the course of four days into a spheroid with observations taken on alternate days, following the method employed by Vinci and co-workers (2012). A range of seeding densities was examined using populations of $5x10^3$, $1x10^4$, $2x10^4$, $3x10^4$ and $5x10^4$cells, the highest seeding population of $5x10^4$ cells producing large, disorganised spheroids unsuited to experimental work. These initial, qualitative experiments showed that a seeding of as little as $5x10^3$ cells reliably produced discrete, manageable spheroids and this enabled further development. Having established that these four lines were capable of spheroid formation, a seeding population of $1x10^4$ cells was used for further investigation of the growth characteristics of the spheroids over time. In addition, the percentage of serum supplementation was also explored using medium supplemented with 5, 10 or 15% serum over a course of seven to ten days. Interestingly, 15% serum actually produced spheroids of irregular appearance and a distinct brownish discolouration of the culture medium. Subsequent experiments therefore used 5% serum as employed in 2D culture models of these mesothelioma cell lines.

The formation of mesothelioma cell spheroids generated from a starting population of $1x10^4$ cells was observed over four days by microscopy (Figure 4.2) and all four lines initially formed a loosely packed mass after 24 hours. By 48 hours, a discrete spheroid was noted in LO68, NO36 and ONE58 cells but less so in JU77 cells which had a slightly more disorganised structure. The self-association of cells into a three dimensional mass was accompanied by a condensation of cells, the subsequent reduction of spheroid circumference and darkening of the mass as a core developed. Spheroid formation was essentially complete by 72 hours with the most discrete spheroids arising from the NO36 and ONE58 lines whilst some edge irregularities were noted with JU77 spheroids. Spheroid circumference was measured over time (Figure 4.3A) and it was noted that JU77 spheroids showed the greatest degree of consolidation with a reduction in circumference from approximately 800 µm as a loose

aggregate at 24h to a discrete structure of approximately 400µm by 96h. A unique aspect of LO68 spheroids was the resumption of expansion at the periphery from 72 hours onwards after an initial condensation to approximately 500µm circumference at 48h. After 96 hours incubation, JU77 spheroids were considered fully formed with a distinct, dark core region but still displaying a peripheral corona of cells and LO68 spheroids continued to expand whereas both NO36 and ONE58 cells formed dense, stable masses with little or no change in size or shape except for the continued formation of a darker, condensed core. Circumference in both NO36 and ONE58 spheroids remained relatively constant at around 400µm.

In addition to the above experiments, an assessment of longer term (96h onwards) spheroid culture was made (Figure 4.3B). This corresponded to the day of experimentation used in the reference study (Vinci et al., 2012). Spheroids were examined for circumference and observations as indicators of progress. Some interesting properties unique to each cell line were noted in a longer culture duration such as the condensation of JU77 spheroids into a tightly packed mass, similar to that of the NO36 and ONE58 spheroids at the same time point. The slightly irregular margin of a JU77 spheroid at 72h and 96h had regressed to a darker, more compact structure. LO68 spheroids continued to grow in diameter with the formation of small cell patches separate to the spheroid mass, most likely a consequence of detachment and proliferation. However, NO36 and ONE58 retained their near-spherical morphology, condensing slightly further with dark, dense cores becoming more prominent. In all cases, morphology was lost by 10 to 12 days in culture through the disruption of spheroid superstructure into irregular, discoloured fragments. Spheroid circumference was also measured during the medium term culture trial (Figure 4.3) during which, the condensation of JU77 spheroids continued as well as the gradual increase to a near-plateau of LO68 spheroid size of about 800µm. The diameter of spheroids of NO36 and ONE58 cells remained relatively unchanged until days 10 to 12 when spheroid disintegration was noted.

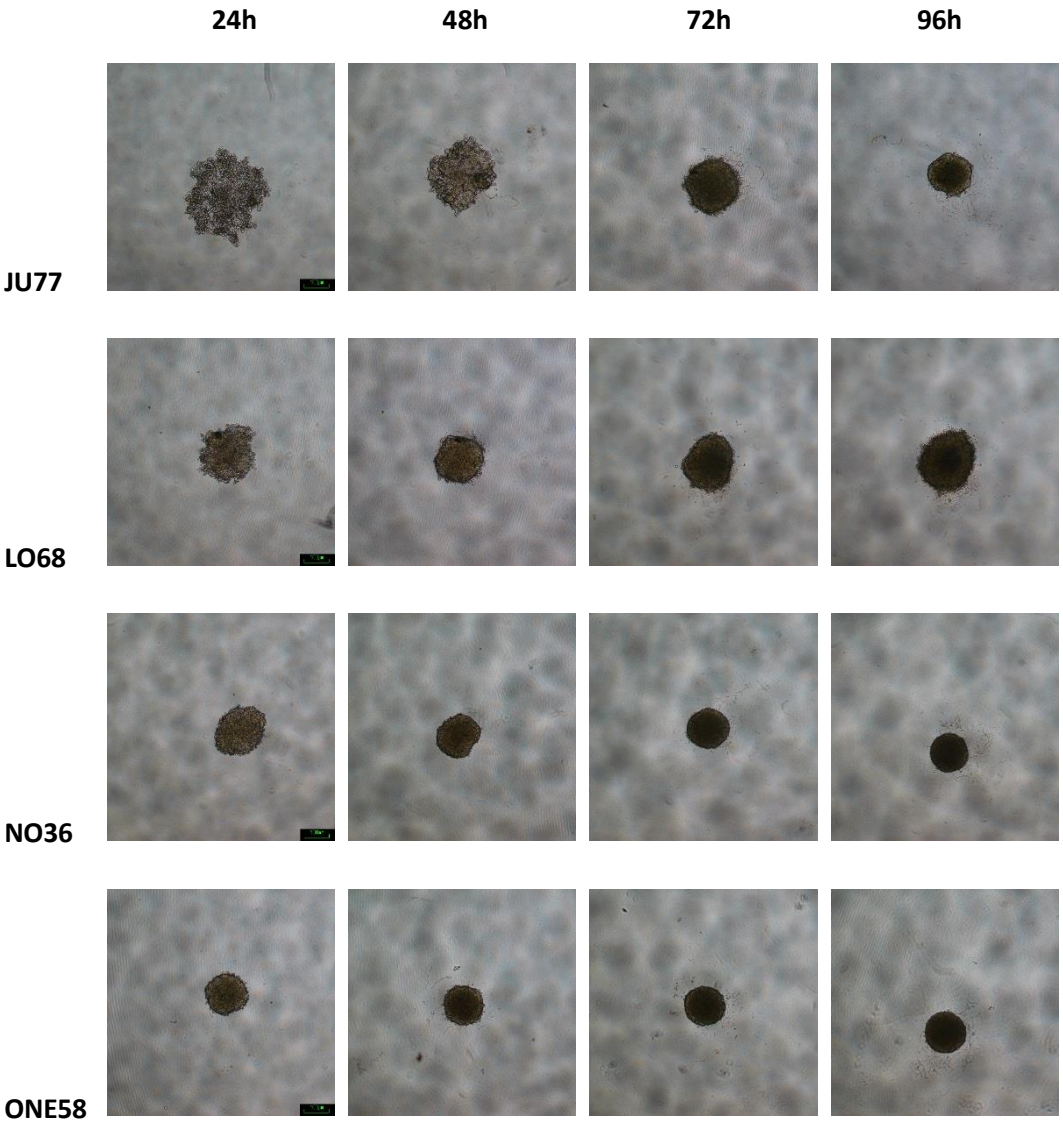

Figure 4.2. Formation of mesothelioma cell spheroids. Representative images of the formation of malignant mesothelioma spheroids. An initial population of $1x10^4$ cells was seeded and allowed to form over 96 hours in Corning® ultra-low adhesion 96 well microplates and observed daily (Scale bar 100 µm).

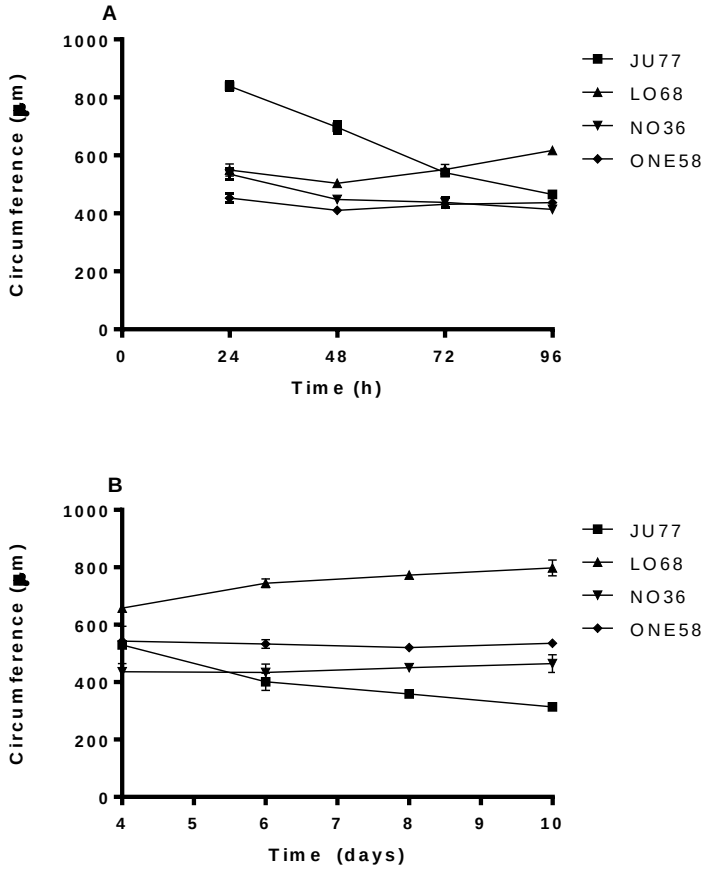

Figure 4.3. Growth of malignant mesothelioma spheroids during initial spheroid formation (A) and longer term culture (B). Cells were cultured under normal conditions and allowed to associate into spheroids. Circumferences were measured daily using the method described in section 2.5 to investigate growth during experimental periods from 24h post-seeding to a maximum of 96h (A) and a separate trial for 4 to 10 days (B). Data shown as mean +/- SD derived from three independent experiments. Measurements of spheroid circumference were found to be highly consistent and error bars are shown but not entirely visible in some instances.

4.3. Establishment of acid phosphatase assay for assessment of spheroid cell viability.

Having established a spheroid model for four mesothelioma lines, a method to quantitatively assess the viable cell population in mesothelioma cell spheroids was developed by use of an acid phosphatase assay (APH assay). With the exception of LO68 spheroids which showed initial consolidation followed by moderate expansion, spheroids formed by JU77, NO36 and ONE58 cells instead showed a contraction and consolidation of size over time rather than an outward expansion of size and processes. Therefore, an assay was required which enabled determination of viability in a manner independent of changes in either diameter or circumference. The APH assay had been previously shown to be most appropriate for application to spheroid cultures (Friedrich et al., 2007). A range of different seeding populations was explored to apply this assay to mesothelioma 3D culture. Optimisation of seeding populations was investigated so that a linear response to the assay could be obtained for the duration of the experiment to a maximum of 96h. Spheroids of $5x10^3$, $1x10^4$, $2x10^4$ and $3x10^4$ cells were generated without treatment and assayed at 24, 48 and 96 hours (Figures 4.4A to D). The circumference of spheroids formed from a starting population of $1x10^4$ cells was also measured (Figures 4.4E to H) and was found to be consistent with the measurements described in section 4.2 and Figure 4.3 (A).

The optical absorbance of the APH assay was also measured over time (Figures 4.4A to D) using the seeding populations described above and a set of individual, cell-line specific trends was observed. It was noted that JU77 spheroids followed a similar trend to that of their growth as the condensation of JU77 spheroids also produced a reduction in absorbance reading up to 48 hours and remaining relatively unchanged thereafter. The resumption of growth in LO68 spheroids after a formation period of approximately 48h also produced an increase in absorbance over time and a similar but less marked trend was also seen in NO36 spheroids. The absorption of ONE58 spheroids however, remained relatively consistent following an initial reduction in absorption after 24h in the higher populations of $2x10^4$ and $3x10^4$ cells. The initial reduction in absorbance seen in spheroids of JU77, NO36 cells and spheroids comprising of more than $1x10^4$ ONE58 cells between 24h to 48h was unique to these lines and may the result of the intercellular organisation during the initial stages of spheroid formation.

It was found using the APH assay that a spheroid formed from an initial seeding of $1x10^4$ cells produced an absorbance reading which could be reliably obtained and within the limits of the assay. The spheroids formed from a $1x10^4$ cell seeding were also sufficiently robust to withstand minute movements such as those experienced during medium changes. Spheroids generated from $5x10^3$ cells often produced poor and often variable results and their use was discontinued.

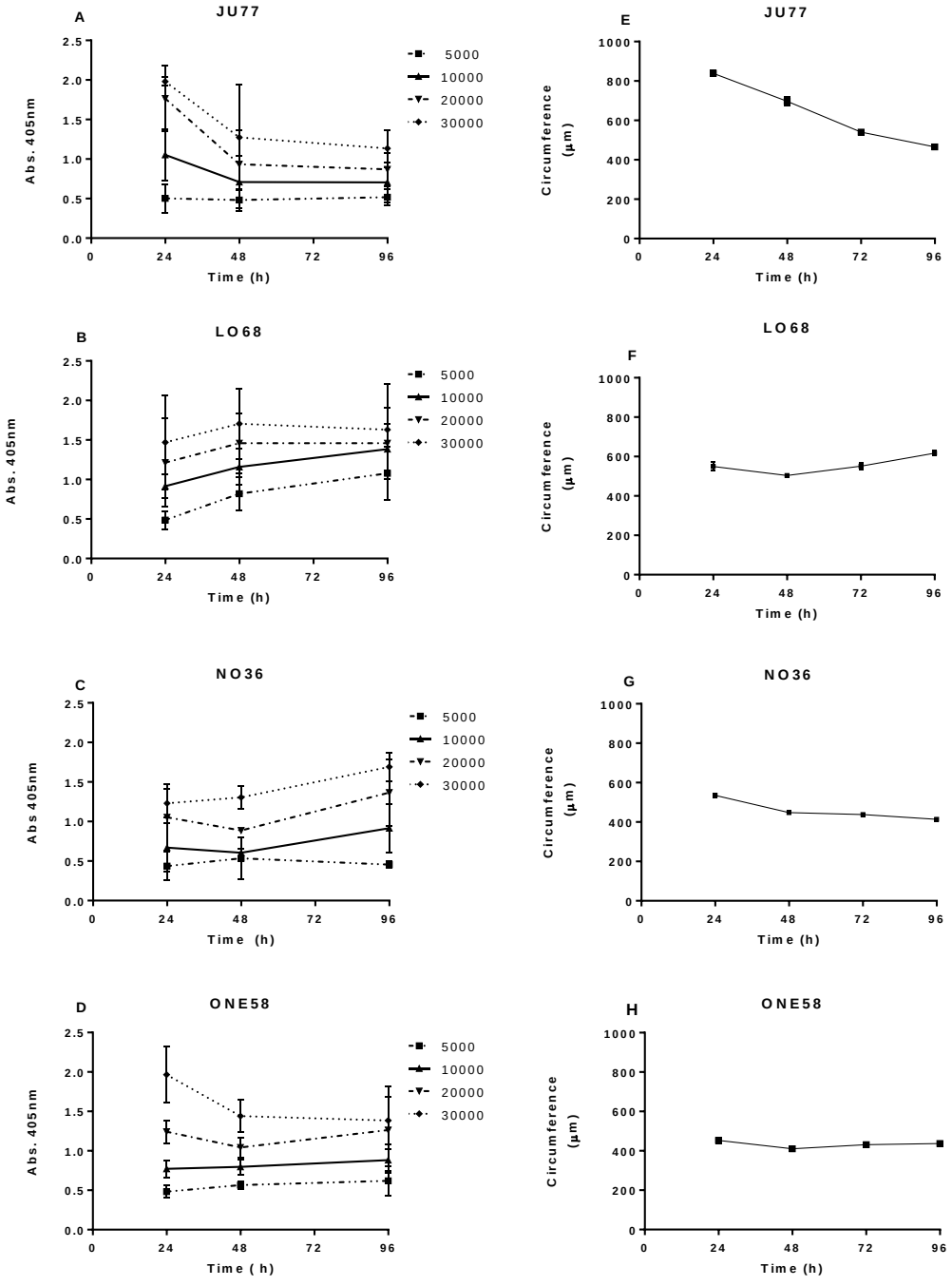

Figure 4.4. Assessment of spheroid growth by APH assay and circumference.

Assay absorbance was measured over time using four different seeding densities for each cell line (A-D) and the circumference of a $1x10^4$ cell spheroid measured over the same incubation time (E-H). Data expressed as mean +/- SD from three independent experiments. It was generally observed that the circumference measurements of spheroids between replicates was highly consistent within cell lines with a coefficient of variation in the range of between 1 – 5 %.

4.4. Colony formation in malignant mesothelioma lines.

Colony formation of these four mesothelioma lines had not been previously determined in our laboratory and therefore, the ability of the four mesothelioma lines to form colonies under low seeding density was not known. A colony formation assay was performed to assess which lines were capable of doing so. These experiments showed that only NO36 and ONE58 cells were able to form colonies when seeded at low density and that NO36 cells were able to do so more readily than ONE58 cells (Figure 4.5).

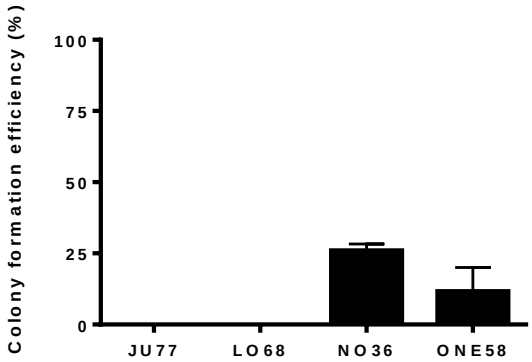

Figure 4.5. Colony formation in malignant mesothelioma cell lines. The ability to form colonies was assessed by low density seeding of 100 cells in a 24 well plate followed by incubation for seven days. Results are expressed as a mean colony forming efficiency +/- SD (percentage of starting population) with data derived from three independent experiments.

4.5 Adhesion assay development.

In order to investigate the effects of Wnt signalling upon mesothelioma cell adhesion, it was first necessary to establish the conditions for these assays since adhesion assays had not been previously performed on these cells. Therefore, a method (see section 2.10) based on that of Humphries (2001) was developed by firstly evaluating two culture surface coating substrates at two time points using collagen and fibronectin as adhesion substrates. Type I collagen (30µg/mL) and fibronectin (10µg/mL) were tested over 30 and 60 minute attachment times with fibronectin producing slightly more adhesion at both time points in all four cell lines (Figures 4.6A – D) although this difference was not statistically significant. As the degree of adhesion after 60 minutes was comparable to a 30 minute incubation, the method was standardised to a 30 minute incubation with the use of fibronectin coating.

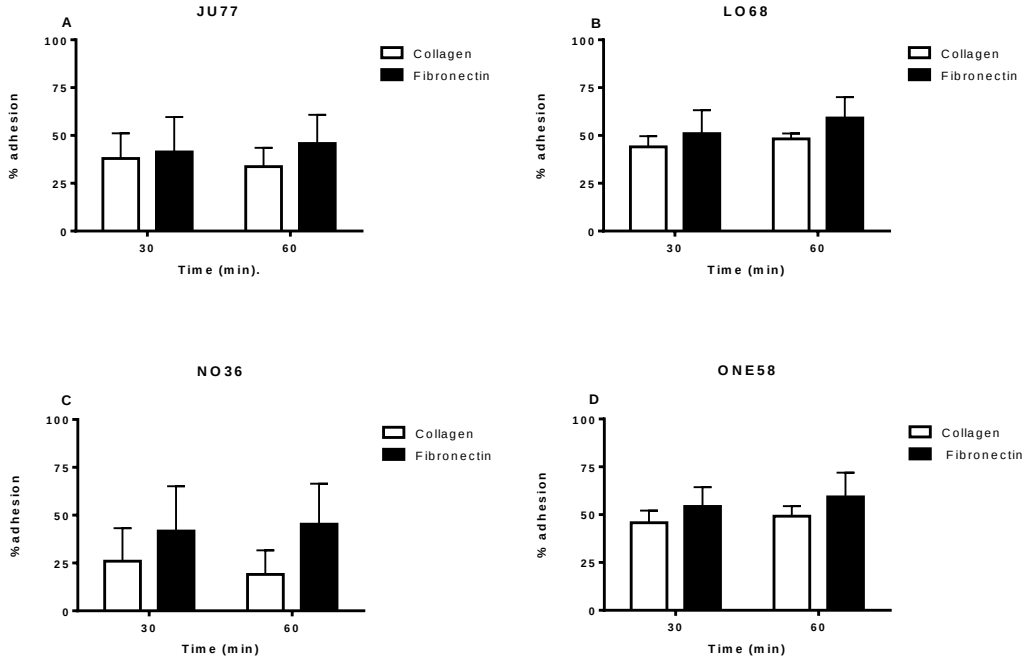

Figure 4.6. Effect of substrate upon mesothelioma cell adhesion. A seeding population of 250 cells (JU77 (A), LO68 (B), NO36 (C) and ONE58 (D)) was incubated in a 24 well plate with either Type I collagen or fibronectin for 30 and 60 minutes and adhesion measured by counting the entire culture surface following staining with crystal violet. Results are expressed as a mean percentage +/- SD of seeding population derived from three independent experiments.

4.6. Development of Transwell® migration model.

Transwell® assays are used to measure the regulation of cellular migration in response to a particular stimulus and a migration assay was employed to assess the ability of the four mesothelioma cell lines to migrate through a Transwell® membrane. Based upon experience using the scratch assay (section 3.7), the use of the potentially more biologically informative Transwell® migration assay was explored. Such experiments had not been previously conducted in our laboratory and therefore, the method described in section 2.7 was devised based upon that of Green et al., (2009). Preliminary experiments were undertaken using a range of serum concentrations as a chemoattractant to assess which of the four lines were capable of active migration across a permeable filter. These involved optimisation of serum supplementation using 2.5%, 5% and 10% serum (v/v) which produced a graded response between 2.5% and 5% with no significant difference seen between 5% and 10% serum supplementation. Care was also taken to use fresh, frozen aliquots of serum added at the time of seeding in order to maintain serum potency. In addition, the Transwell® membranes were coated with 10µg/mL fibronectin as per the method in our reference study (Green et al., 2009). In these experiments, migration was seen only in NO36 and ONE58 cells and it was found that 5% serum supplementation was sufficient to induce migration in these two lines over a period of at least 18 hours (overnight). Cell seeding was also titrated due to the highly migratory nature of ONE58 cells whereas NO36 cells migrated less actively (*data not shown*). The optimised seeding populations of $3x10^4$ NO36 and $1x10^4$ ONE58 cells per Transwell® insert produced a reliable pattern of migration which allowed consistent field counting of both cell lines.

4.7. Invasion assay development in malignant mesothelioma cell lines.

In addition to migration, the invasive ability of mesothelioma cell lines NO36 and ONE58 was assessed with a Transwell® based invasion assay by adding a basement membrane extract (BME) barrier to the Transwell model. A range of BME concentrations suggested by the manufacturer (½ , ¼ , $^1/_8$) was used to explore the effect of matrix density on invasive ability in both cell lines. Overall, neither NO36 nor ONE58 cells showed invasive ability in this assay with very few cells traversing the membrane (*results not shown*). This limited and irregular response was considered too inadequate and variable for use as a method to investigate any potential effect of XAV939 upon invasiveness as the effect of the drug would be difficult to discern.

4.8. Discussion.

The four cell lines used in these experiments have served as the platform for recent studies in mesothelioma undertaken in the supervisor's laboratory which have investigated apoptotic mechanisms in response to cisplatin treatment (Cregan et al., 2013) and most recently, the description of Wnt signalling in these cell lines (Fox et al., 2013). Under light microscopy, these cells retained their epithelioid morphology and growth characteristics described by Manning and co-workers (1991). The heterogenous nature of chromosomal aberrations described by these authors is also consistent with the diverse nature of other reported chromosomal defects reported in later studies using other cell lines (Jaurand, 1997; Yang et al., 2008; Sekido, 2010). Since their propagation, the JU77, LO68, NO36 and ONE58 cell lines have been used in other investigations including panel screening of cytotoxic agents and cytokines (Bowman et al., 1991), topoisomerase expression in drug resistance (McLaren et al., 2001), matrix metalloproteinase expression (Zhong et al., 2006), Hedgehog pathway signalling (Lim et al., 2013; Lim et al., 2015) and the control of growth and mobility by stathmin expression (Birnie et al., 2015). These studies, taken together with those mentioned above in the supervisor's laboratory (Cregan et al., 2013; Fox et al., 2013) lend support to the continued use of these cell lines in both conventional monolayer and three dimensional culture methods.

The studies conducted in this chapter represent the first such investigations into aspects of 3D culture growth in these four mesothelioma cell lines and the development of three reliable methods for investigating spheroid viability, adhesion and migration. Points of interest include the unique formation of JU77 spheroids after an initial condensation of the cell aggregate, the assembly and expansion of LO68 spheroids in culture over time compared to the relatively uniform self-assembly of NO36 and ONE58 spheroids. The relative ease of spheroid formation using simple liquid overlay in an ultra low adhesion 96-well plate has also been noted to a maximum of 12 days culture time. A previous study in the formation of breast cancer cell spheroids (Ivescu and Kubbies, 2007) suggested three categories of spheroids based on gross morphology; compact spheroids, tight aggregates and loose associations. Based on these classifications, the four cell lines examined here may be considered as compact spheroids in view of their physical appearance and resolution of size over time. A three-dimensional model such as a spheroid aims to recreate the *in vivo* microenvironment through the formation of diffusion gradients, the

presence of additional extracellular matrix not present in 2 D culture, three dimensional attachments and signalling as well as a heterogenous cell population of proliferating, quiescent and/or dead cells (Mueller-Klieser, 2000; Ivescu and Kubbies, 2007). This approximation of the *in vivo* situation will provide a more physiologically relevant model to simulate avascular micrometastases for basic research into the actions of an agent in malignant mesothelioma.

Given the relatively rapid overnight self-assembly of these four cell lines into useable, compact spheroids, the issue of spheroid size (measured as circumference) required consideration in terms of metabolite diffusion gradients, oxygen in particular. Guidelines on spheroid size are scarce due to the inherent variability of cell line growth properties and the unknown composition of the internal matrix structure produced. This is evident in the markedly different properties of JU77 and LO68 spheroids compared to NO36 and ONE58 spheroids discussed above. Therefore, the notion of a 'small' or 'large' spheroid will only be a model-specific categorisation although some empirical guidelines exist based on the ability of oxygen to diffuse across a tissue thickness (spheroid diameter) of between 100µm to 200µm with the formation of chemical gradients in masses larger than this range (Asthana and Kisaalita, 2012). A similar estimate also includes spheroids between 200µm to 500µm (depending on cell line) with a cautionary note on the possible development of necrosis in spheroids exceeding 500µm (Hirschauser et al., 2010). Despite cell line specific differences, spheroids within this size range are considered a reasonably faithful model of avascular tumours and micrometastases (Mueller-Kleiser, 2000; Hirschauser et al., 2010). The spheroids cultivated in these studies grew to an average circumference of approximately 500µm, translating to a diameter of around 160µm which suggests that their size range is one where gross, necrotic hypoxia would be less likely.

The APH assay applied here to mesothelioma cell spheroids was based on a method developed by Friedrich et al., (2007) in human colon carcinoma spheroids which displayed a linear colourimetric response for spheroids of up to $8x10^4$ cells at a diameter of up to 900µm. The mesothelioma cell seeding population was therefore titrated as described (section 4.3) in order to establish the relationship between starting population and spheroid size over time (measured as circumference) so that a linear response from the APH assay could be measured over the course of an experiment to a maximum of 96h.

A mesothelioma cell spheroid formed from a starting population of $1x10^4$ cells produced a linear response within the saturation limits of the APH assay. These spheroids were also within the currently accepted range that minimises the risk of hypoxia. Most importantly, the spheroids formed in this manner were suitably robust to withstand handling without any loss of structural integrity and this method formed the basis for further experiments in our laboratory as described in chapters 5 and 6.

Clonogenic survival assays to test the effects of compound require the development of a colony formation assay prior to the evaluation of reagents or any other experimental manipulation. Therefore, the ability of the four mesothelioma lines to form colonies was assessed with only the NO36 and ONE58 lines found to be capable of doing so. Initial studies of low density seeding in these cell lines required consideration of the need for medium changeover intervals for a ten-day experiment. However, preliminary experiments with medium changeover on alternate days did not produce any difference in results to those without medium replenishment (*data not shown*). Therefore, a seven-day incubation without medium changeover was employed as a method. Clonogenic survival assays have been utilised in mesothelioma research to investigate the role of Colony Stimulating Factor-1 Receptor (CSF-1R) in the induction of β catenin mediated resistance of primary cultures and cell lines to pemetrexed (Cioce et al., 2014). A similar method to that used in these experiments demonstrated increased clonogenicity and resistance to pemetrexed through a simple plate-based assay followed by crystal violet staining (Cioce et al., 2014). These preliminary experiments also serve to highlight the fact that not all mesothelioma cell lines are capable of colony formation under low density seeding conditions.

Adhesion assays are less frequently performed than clonogenic assays and migration assays but they do provide information about another aspect of cell biology which may be regulated by Wnt signalling. The influence of Wnt signalling upon cell adhesion, motility and patterning has been described in detail through studies in developmental models (Logan and Nusse, 2004) and the interplay between Wnt signalling, cellular adhesion and migration therefore cannot be discounted. Events such as the β catenin-mediated downregulation of ε-cadherin expression, matrix degradation by expressed proteases (e.g. matrix metalloproteinases and urokinase) together with the concomitant loss of adhesion have been reported in malignancies (Amin and Vincan, 2012). A role for β

cateninindependent (non-canonical) Wnt signalling has also been identified in cytoskeletal rearrangement and deregulation of cell adhesion together with the involvement of other transmembrane proteins such as Integrins and Syndecans (Amin and Vincan, 2012). Syndecan 4 has been shown to promote β catenin independent signalling in *Xenopus* developmental models and these models have also provided insight into the roles of fibronectin in the regulation of cell adhesion and movement – also in a non-canonical manner (Astudillo and Larain, 2014). Additionally, the role of Integrins and their binding partners in the modulation of β catenin-mediated Wnt signalling has been described through evidence of the binding of various Integrin heterodimers with fibronectin, laminin and Type I collagen (for review, see Astudillo and Larain, 2014).

Coating of the culture surface with Type I collagen has been previously used in our laboratory (see section 3.7) and was compared against fibronectin coating in order to determine a suitable substrate for adhesion assays. A slightly higher degree of cell adhesion was observed in all four cell lines with use of fibronectin coating and little difference noted between 30 minute and 60 minute incubations, hence the method devised in this chapter (see section 4.5). The adhesive properties of fibronectin have been examined closely and binding sites for collagen, gelatin, heparin and integrins have been described (for review, see Magnusson and Mosher, 1998). Given the importance of integrin proteins in adhesion, migration and cell-to-matrix signalling, fibronectin coating represents a physiologically appropriate method to assess the effects of a given compound upon cellular adhesion. Despite the seemingly simple nature of an adhesion assay, it can nonetheless be used as a worthwhile method to investigate the cellular mechanisms responsible for a change in adhesion caused by a particular treatment. A small cohort of candidate molecules associated with adhesion has been identified in the literature as well as the β catenin dependent / independent nature of their associated signalling cascades. Any information gained from the manipulation of Wnt signalling by any experimental means will therefore be complemented by findings in the area of cellular adhesion.

The chemotaxis of these four mesothelioma cell lines was also investigated and cell migration was seen only in the NO36 and ONE58 cell lines under the conditions used with ONE58 cells showing greater migratory behaviour than NO36 cells. The lack of migration of JU77 and ONE58 cells under these conditions does not preclude them from other

investigations as there may be a potential requirement for another chemoattractant in order to induce this phenomenon in these cells. A modification to the original method devised by Green et al., (2009) involved the use of fresh frozen serum aliquots so that serum potency was maintained by minimisation of freeze/thaw cycling. Using this method as a basis for further refinement, adjustments were made during inhibitor studies to reduce dilution effects between the upper and lower chambers (see section 2.7). We have therefore successfully established a model to investigate the chemotactic response of mesothelioma cells using Transwell® membrane inserts which may also be used to assess the effects of pharmacological agents targeting the Wnt signalling system. However, further investigation is required to refine the invasion assay method in order to produce a reliable response that is capable of further experimentation.

Taken together, the description and characterisation of these malignant mesothelioma cell lines in both 2D and 3D culture has been made with observations on morphology, growth properties and suitability for functional assays. An existing colourimetric assay has been optimised to measure the viability of spheroids formed from these lines and three functional assays have been applied to examine the effects of pharmacological treatment by the use of simple, reliable laboratory methods. Further refinement and expansion will allow for larger future studies using these experiments as a basis for improvement.

Three dimensional culture more closely mimicking the *in vivo* microenvironment is a method that is rapidly gaining popularity in cancer research and it is hoped that further work using this format will allow greater insight into the pathobiology of mesothelioma. Factors such as multi-directional attachment (and concomitant signalling sequalae), exposure to growth factors / morphogens, diffusional gradients and physical barriers are at least partially emulated in either a tumour fragment or a spheroid. Additional consideration therefore must be given to topics such as novel compound synthesis and drug dosage formulation. Conventional monolayer culture may not be replaced altogether by 3D methods as it still has a role in basic characterisation studies and cost considerations as a prelude to more sophisticated experimental strategies.

Chapter 5.
Tankyrase inhibition by XAV939 and its effects on functional assays in mesothelioma cells.

Table of contents.

5.0. Introduction.

Inhibition of Wnt signalling by tankyrase blockade has been shown to reduce cell proliferation by the stabilisation of Axin, thereby prolonging the effect of the β catenin degradation complex and in turn, reducing the fraction of free β catenin (Huang et al., 2009). This mechanism has been investigated in other notable cancer models including triple negative breast cancer (Bilir et al., 2013) and neuroblastoma (Tian et al., 2014) amongst several others.

At the time of these experiments, the effects of XAV939 have yet to be investigated in a 3D culture setting. Studies have been conducted using mesothelioma cell spheroids in other aspects of mesothelioma research such as the mammalian target of rapamycin (mTOR) pathway (Barbone et al., 2008) and the process of apoptosis (Kim et al., 2005). XAV939 was chosen to investigate tankyrase inhibition in these four cell lines initially by conventional monolayer culture and subsequently by application of the functional assays developed in the previous chapter. Further to exploring the nature of mesothelioma cell responses to XAV939, investigations then focused upon the downstream targets of XAV939 on gene expression in the susceptible lines. The interplay between cell-to-cell and cell-to-matrix adhesion with signalling phenomena has also been shown to have an effect on mesothelioma cell spheroids (Kim et al., 2012) but the influence of XAV939 at the level of gene expression in mesothelioma cells has yet to be undertaken.

5.1. Inhibition of tankyrases downregulates mesothelioma cell proliferation.

In order to determine the susceptibility of the four mesothelioma cell lines to tankyrase inhibition, the Wnt inhibitor XAV939 was tested using the APH assay in a 2D culture setting to determine any potential effect on proliferation. A dose range of 0.001µM to 10µM was used over 48 hours (Figure 5.1) and a dose dependent decrease in proliferation was seen in JU77 and LO68 cells, with JU77 being more susceptible. Conversely, NO36 and ONE58 cells were not affected under these conditions.

Cells from all four lines were also treated with 10µM XAV939 to investigate any changes in morphology. Interestingly, some JU77 cells were noted to have formed elongated processes after XAV939 treatment (Figures 5.2A and B). Whilst noticeable, this elongation was not accompanied by cellular detachment and other obvious signs of toxicity such as the formation of apoptotic bodies. LO68 cells showed signs of toxicity in the form of cells that were either rounded up, detached from the monolayer or forming apoptotic bodies (Figures 5.2C and D). No changes were seen in NO36 and ONE58 cells (data not shown).

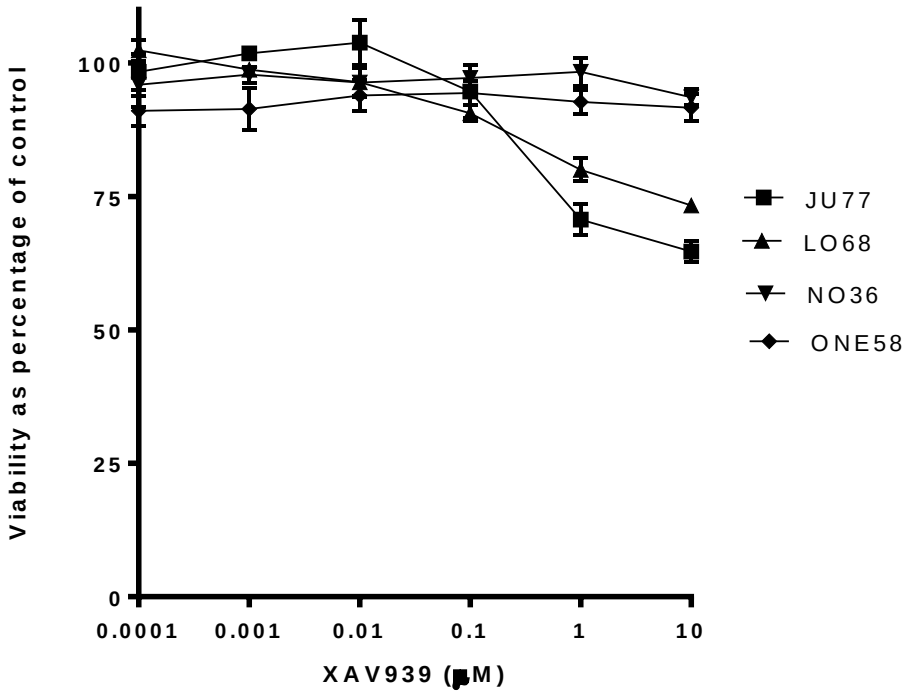

Figure 5.1. Effect of XAV939 upon mesothelioma cell proliferation: 2D culture. Dose response of malignant mesothelioma cell proliferation after 48 hours incubation with XAV939 measured by acid phosphatase assay absorbance and expressed as percentage relative to control. Data expressed as mean +/- SD and derived from three independent experiments.

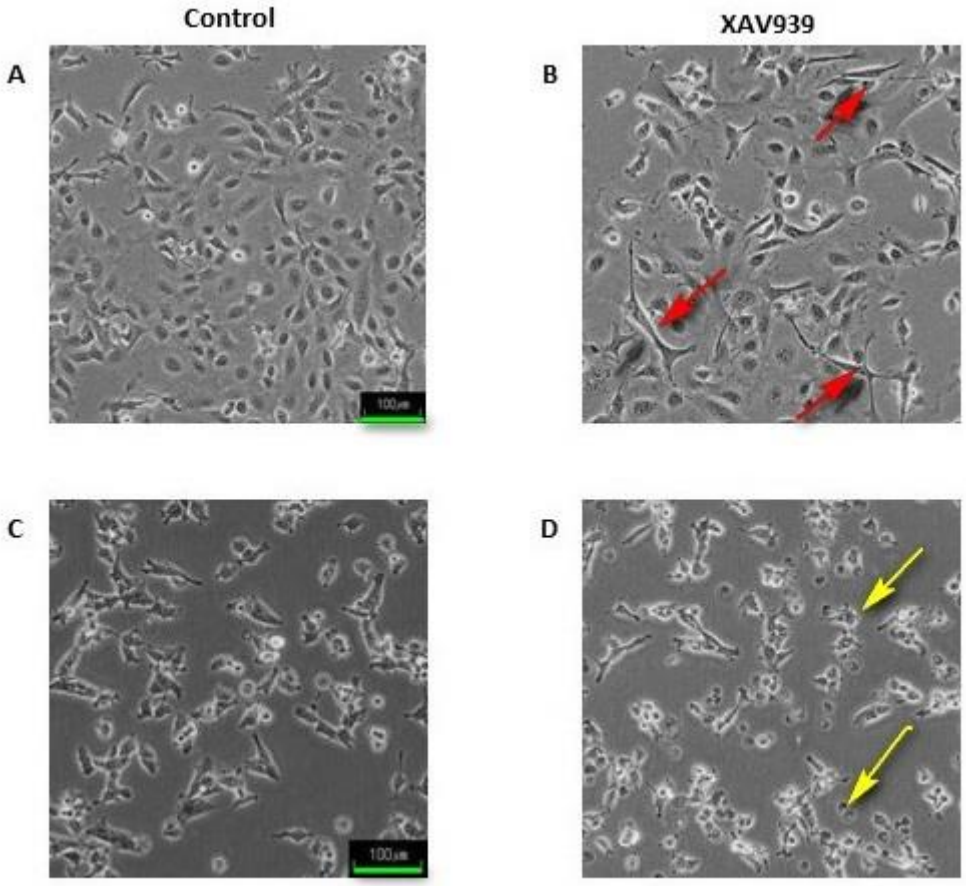

Figure 5.2. Morphology of JU77 and LO68 malignant mesothelioma cells treated with XAV939. Cells were seeded overnight and treated with 10µM XAV939 for 24 hours; (A) JU77 Control, (B) JU77 XAV939, (C) LO68 Control, (D) LO68 XAV939. Images are representative samples taken from three independent experiments. Red arrows denote unusually elongated JU77 cells and yellow arrows denote apoptotic bodies of LO68 cells. (Scale bar 100µm).

5.2. XAV939 treatment promotes degradation of β catenin in mesothelioma cells.

Attenuation of a β catenin mediated Wnt signal by XAV939 has been established in several other cell models with the mechanism of action characterised as the stabilisation of Axin through inhibition of PARylation by tankyrases which results in the inhibition of proteosomal degradation of Axin (Huang et al., 2009). The presence and actions of the β catenin degradation complex are therefore sustained and intracellular β catenin levels are reduced with consequences on gene expression. In order to demonstrate this effect in mesothelioma cells, the four mesothelioma lines were incubated overnight (18h) with 10μM XAV939 and analysed by immunoblotting for β catenin protein. All four mesothelioma cell lines showed a reduction in β catenin content relative to control, consistent with a prolongation of β catenin destruction.

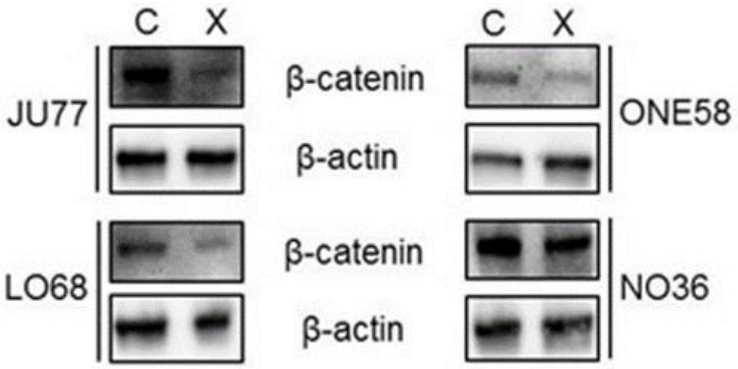

Figure 5.3. Reduction in cellular β catenin in response to XAV939. Cells were treated for 18 hours with 10μM XAV939 and analysed by immunoblotting for total β catenin. All four lines showed a reduction in β catenin (upper panel) compared to vehicle (DMSO) control ('C') following treatment with 10μM XAV939 ('X'). Lower panels show corresponding β actin loading controls. Results are representative of three independent experiments.

5.3. Effect of XAV939 treatment on mesothelioma cell spheroids.

There is ample evidence in the literature for drug sensitivity differences in responses between traditional 2D culture models and spheroids. Therefore, we examined the effect of XAV939 upon mesothelioma spheroid culture. In general, the pattern of responsiveness in 3D spheroids after 48h incubation with XAV939 was similar to that of monolayer cultures with a response seen in JU77 and LO68 spheroids and not in NO36 or ONE58 spheroids (Figure 5.4). A modest effect was seen in JU77 spheroids at both 24h and 48h (Figure 5.4A) but a delayed response was observed in LO68 cells only at 48h (Figure 5.4B) with NO36 and ONE58 spheroids remaining relatively unaffected (Figures 5.4C and D).

Given the differences in the three dimensional microenvironment, the morphology of the spheroids was investigated by microscopy following treatment (Figure 5.5). In order to assess whether tankyrase inhibition had any effect on spheroid integrity, observations were made over 48 hours beginning at 24 hours post-seeding when spheroids were formed (experimental time 0). Despite the proliferation data, JU77 and LO68 spheroids showed no noticeable change in either size or shape between treatment and control during the study with the condensation and resolution of structure continuing unaffected. Similarly, the formation of NO36 and ONE58 spheroids was unaffected by XAV939 with the same consolidation and darkened core formation as observed in the formation studies.

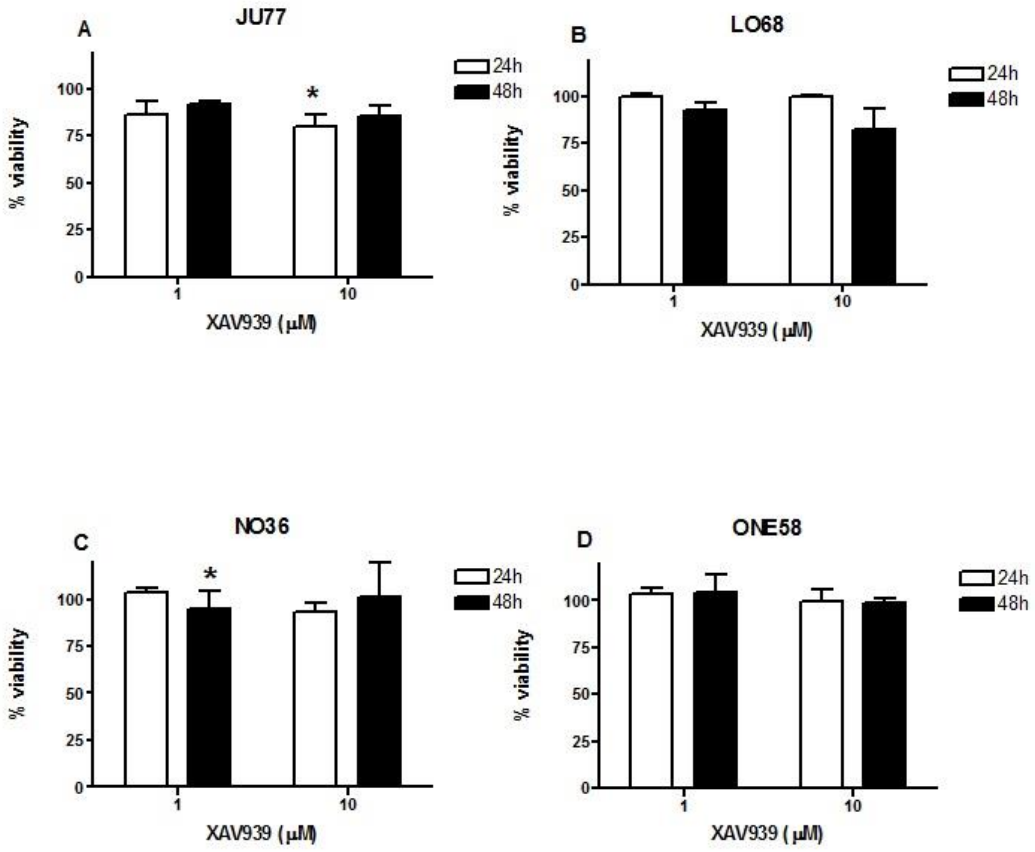

Figure 5.4. Effect of XAV939 upon mesothelioma cell proliferation: 3D spheroids.
Spheroids were incubated for 24 or 48 hours with viability measured using acid phosphatase assay and expressed as percentage relative to control. Data shown as mean +/- SD from three independent experiments using JU77 (A), LO68 (B), NO36 (C) and ONE58 (D) cells, (* $p < 0.05$).

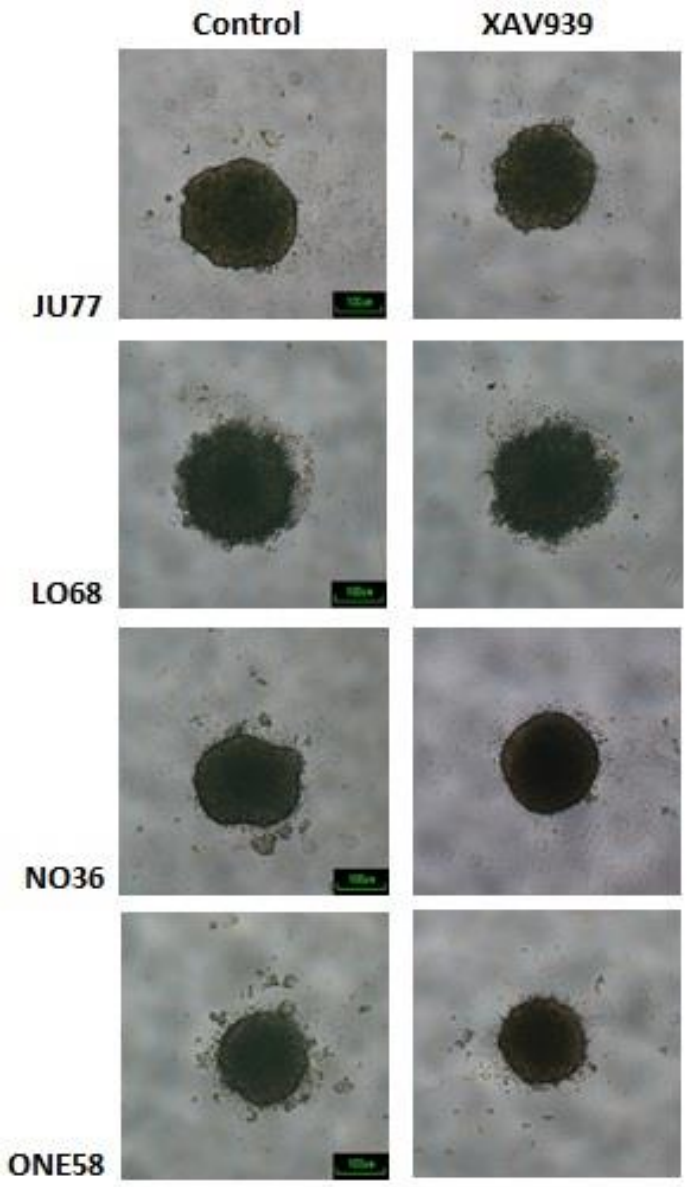

Figure 5.5. Effect of XAV939 on mesothelioma cell spheroid morphology. Representative images of spheroids from three independent experiments are shown for untreated vehicle control and 10µM at 48 hours. (Scale bar 100µm)

5.4. XAV939 reduces colony formation in mesothelioma cells.

Having established, as described in chapter 4 (Section 4.4), that only NO36 and ONE58 cells were suitable for colony formation, the effect of XAV939 to modulate the colony forming ability of these cells was investigated. A dose-dependent reduction in colony formation was seen in both cell lines with ONE58 cells showing greater susceptibility to XAV939 treatment than NO36 cells (Figure 5.6). A statistically significant reduction in colony formation was observed in ONE58 cells at 10µM and these results suggest that Wnt inhibition in malignant mesothelioma cell lines can influence colony formation.

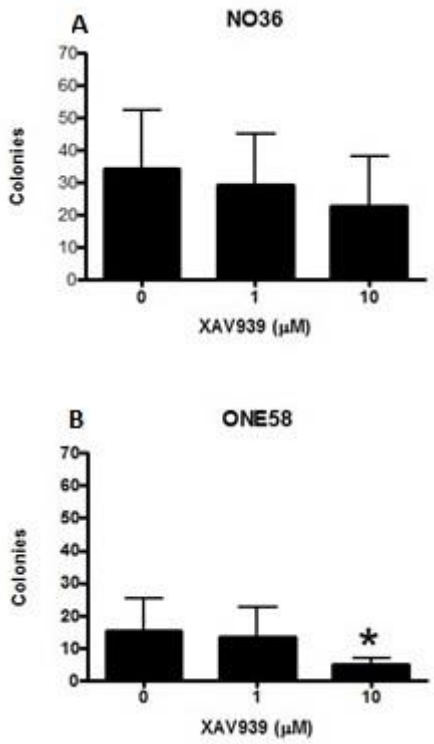

Figure 5.6. Colony formation in malignant mesothelioma cell lines treated with XAV939. The effect of XAV939 on colony forming cell lines NO36 (A) and ONE58 (B) was investigated. A starting population of 250 cells was seeded in a 24 well plate and treated with 1µM and 10µM XAV939 for seven days and the well surface counted for colonies. Data shown is derived from five independent experiments and expressed as mean number of colonies per well +/- SD, (* $p < 0.05$).

5.5. Effect of XAV939 on adhesion of malignant mesothelioma cells.

Wnt signalling not only modulates proliferation but other cellular processes such as migration, patterning, invasion, differentiation and adhesion are also influenced at least in part by this pathway. Considering the effect of Wnt signalling on the cytoskeleton, focal adhesions and cell surface adhesion molecules, the adhesion assay method developed previously (Section 4.5) was applied to investigate the effect of XAV939 on all four mesothelioma cell lines. Overnight pre-treatment with 10µM XAV939 produced an effect in LO68, NO36 and ONE58. Although not statistically significant, a trend was detected showing a reduction in adhesion which was most noticeable for LO68 and ONE58 cells (Figure 5.7). A slight increase in adhesion of JU77 cells was seen and a modest reduction of adhesion was observed in NO36 cells which together with the lowered adhesion observed in LO68 and ONE58.

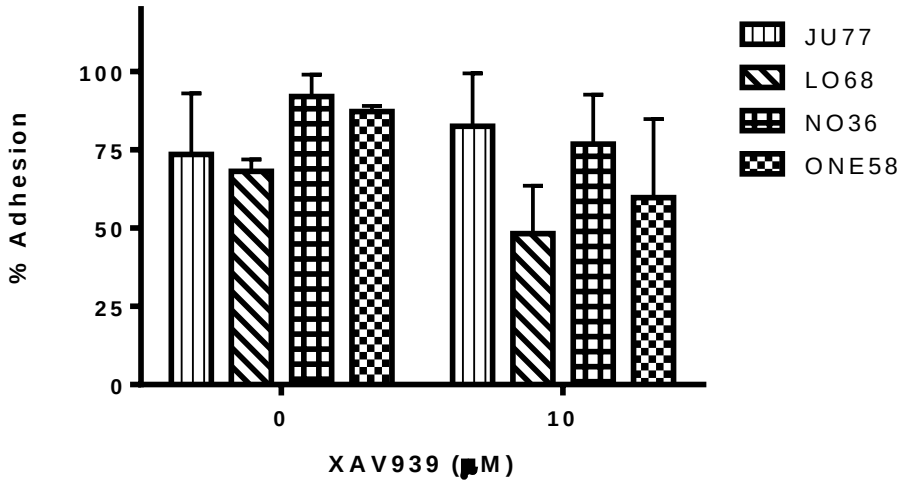

Figure 5.7. Effect of XAV939 pre-treatment on malignant mesothelioma cell adhesion. Cells were pretreated overnight with DMSO (vehicle control) or 10µM XAV939 and then seeded at 250 cells per well in 24-well plates pre-coated with fibronectin for 30 minutes. Results are expressed as mean percentage +/- SD of seeding population. Data shown is from three independent experiments.

5.6. Effect of XAV939 on migration of malignant mesothelioma cells.

The effect of XAV939 upon mesothelioma cell migration was examined next. The Transwell® migration method optimised for ascertaining migratory activity in these four lines (Section 4.6) was used to evaluate the effect of XAV939 on the migratory NO36 and ONE58 cell lines, measured by random field counting (Figures 5.8A and B). Both cell lines showed a dose-dependent reduction in migration: the NO36 line displaying more of a reduction than the modest reduction seen in ONE58 cells. Representative images of control, 1µM and 10µM XAV939 counting fields are also shown (Figure 5.9).

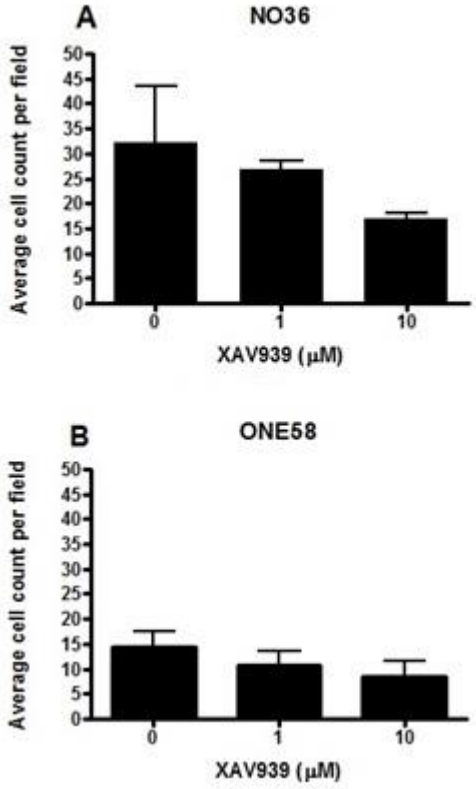

Figure 5.8. Tankyrase inhibition by XAV939 reduces migration of mesothelioma cells. Migration of NO36 (A) and ONE58 (B) cells through Transwell® migration chamber. Cells were incubated for at least 18h in the presence of 1µM or 10µM XAV939. Cells were fixed, stained with crystal violet and counted by counting of five random fields. Data derived from three independent experiments and expressed as mean cell count per field +/-SD

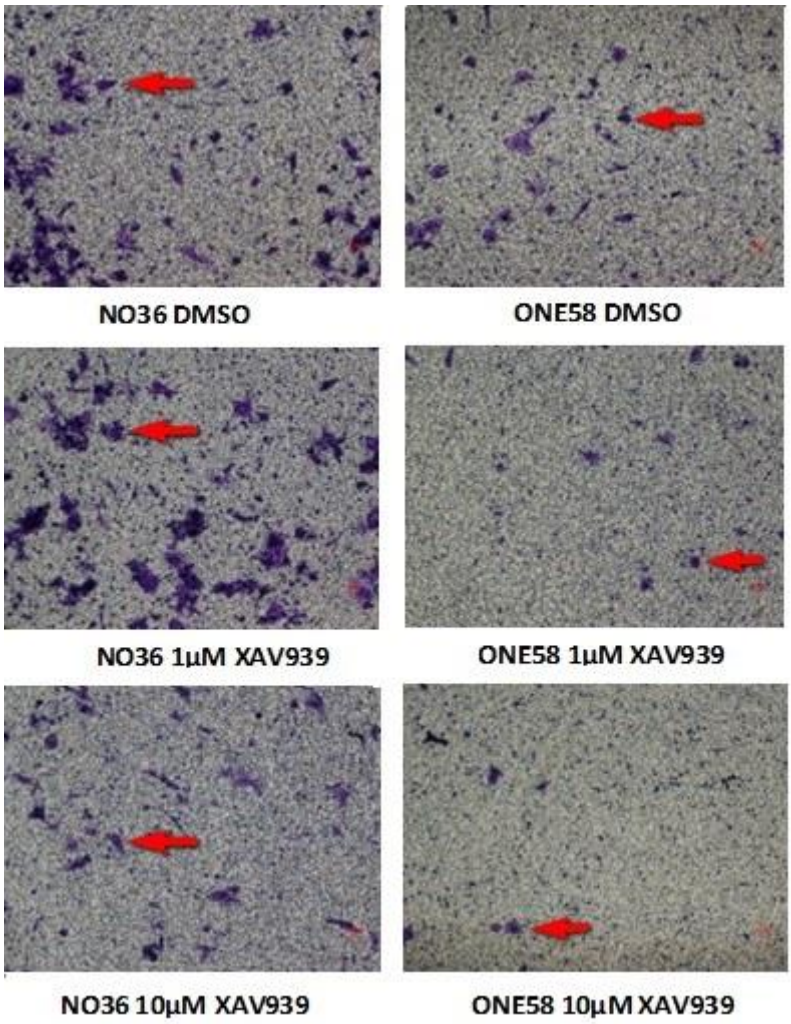

Figure 5.9. Representative field views of NO36 and ONE58 mesothelioma cell migration upon treatment with XAV939. Cells were incubated overnight with either vehicle control (DMSO) or XAV939, then fixed and stained on Transwell® membrane, red arrows indicate cells that have migrated (magnification x100).

5.7. Expression of Wnt target genes in response to XAV939 treatment.

The expression of c-Myc and Cyclin D1 is considered a key characteristic of β catenin mediated canonical Wnt signalling and therefore, analysis by Q-PCR was employed to assess the effect of overnight 10μM XAV939 treatment on these two key Wnt target genes in JU77 and LO68 cells which showed the most effect in viability assays. No significant effects were observed in the expression of these two Wnt targets with a large degree of variability observed with the expression of c-Myc (Figure 5.10).

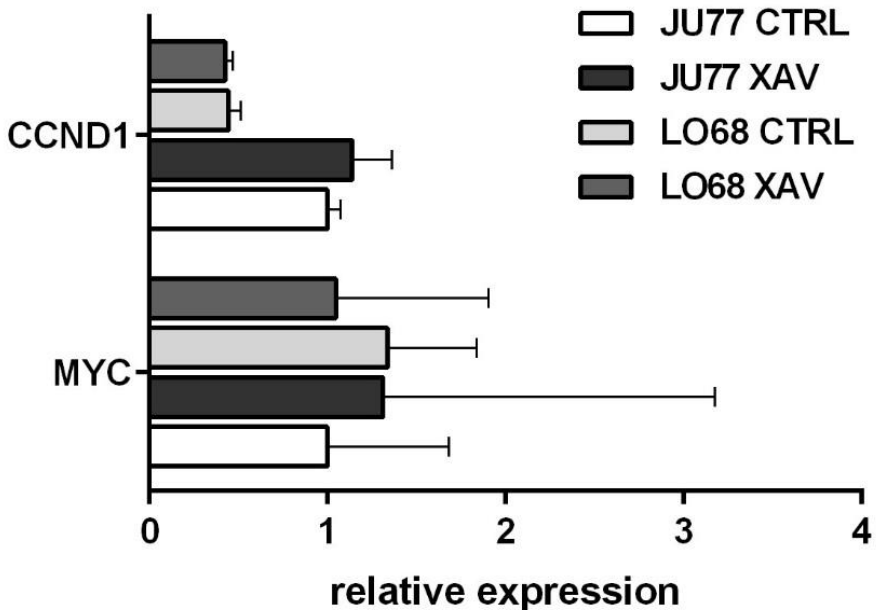

Figure 5.10. Expression of Wnt target genes in response to XAV939 treatment. Expression of c-Myc (MYC) and cyclin D1 (CCND1) in JU77 and LO68 mesothelioma cells. Total RNA isolated from cells was analysed by 2-step real-time RT-PCR using gene specific primers. Gene expression is presented as mRNA levels relative to the JU77 control following normalisation by reference gene expression (HPRT1 and UBC). Results are mean ± SD for three independent experiments.

5.8. RNA sequence analysis of mesothelioma cell lines susceptible to XAV939.

Given the lack of effect upon known Wnt target genes, the wider transcriptional consequences of XAV939 treatment was then investigated by RNA sequence analysis (as described in Section 2.15) in order to investigate the downstream pathways involved in the effects of XAV939 in JU77 and LO68 cells. Depletion of intracellular β catenin has been shown to diminish canonical Wnt signalling in numerous studies throughout the literature and therefore, the effects on differential gene expression in responsive mesothelioma cell lines was carried out using a paired analysis of two replicate sets. The four samples used were subjected to analysis against the 20800 genes of the AmpliSeq Gene Expression Panel producing a final library of 95% useable reads with 89% sequence alignment. Sequencing depth was calculated to be at an average of $9x10^6$ reads per sample with a mean read length of 118 base pairs.

Three different software packages (EdgeR, VOOM and DESeq2) evaluated for the determination of upregulated and downregulated genes (the data for which is shown in Figure 5.11) as detection by the software programs on their own and in comparison with each other. In these studies, a 1.5-fold difference (0.58 log2) was used to determine significant upregulation or downregulation. Three software analysis packages were employed in order to determine any biologically significant changes in gene expression as a result of XAV939 treatment. The DESeq2 package was found to be the most stringent due to its process of compensating for artificially higher fold-changes in low expressing samples by logarithmic compression of outlier results, thereby producing a lower false discovery rate. A different method is used by VOOM to minimise false discoveries in which the degree of variability between the mean count of a sample and its variance determined the relative degree of modulation of expression. Conversely, EdgeR did not filter or adjust for low expressing genes which are therefore prone to disproportionately large variations in relative fold-change. Together, EdgeR and VOOM detected nearly all the differentially expressed genes detected by DESeq2 but DESeq2 was shown to be more discerning than VOOM and EdgeR, with EdgeR yielding a larger dataset due to the influence of low expressing samples (Figure 5.11).

A scatter plot for both cell lines (Figure 5.12; A and B) shows the relative degree of expression (as normalised transcript count) and relative log2 fold change which is greater in LO68 cells than JU77 cells. A sample-distance matrix for these two lines also shows the degree of similarity between samples within each replicate as well as the difference between the individually dated experiments of the paired comparison (Figure 5.12; C and D).

The magnitude of XAV939 action upon gene expression determined by DESeq2 analysis was modest in JU77 cells (Figure 5.12 (A)) with 25 genes upregulated to a maximum of 1.2 log2 (equivalent to 2.29 fold; Table 5.1) and 21 genes downregulated to a minimum of -1.65log2 (equivalent to downregulation to 0.318 fold; Table 5.2). A slightly larger gene cohort was derived from LO68 cells in which the top 40 upregulated genes to a maximum of 1.96 log2 (equivalent to 3.89 fold; Table 5.3) and the top 40 downregulated genes to a maximum reduction of -1.75 log2 (equivalent to downregulation to 0.297 fold; Table 5.4) was observed. These results reflect the overall greater global gene expression changes in LO68 cells as shown in Figure 5.12 (B). The genes that have been upregulated or downregulated the most that are common between the two lines have been summarised in Table 5.5 and represent the genes that have undergone the greatest degree of differential expression across both cell lines.

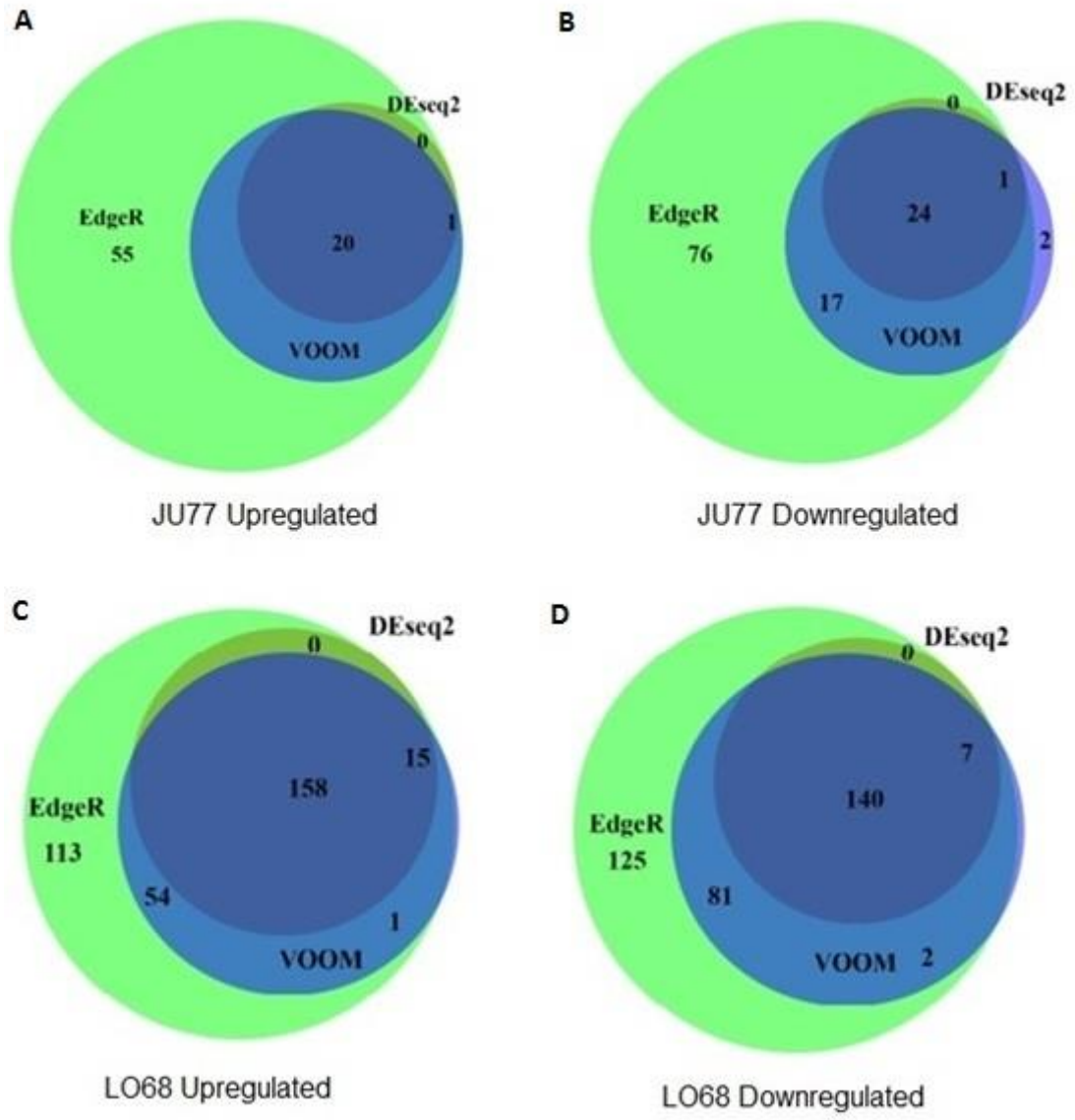

Figure 5.11. Detection of upregulated and downregulated genes by EdgeR, VOOM and DEseq2 expression software packages. JU77 and LO68 cells were treated overnight with 10µM XAV939 and differential expression analysed for upregulation or downregulation by more than 1.5-fold (0.58 log2). The number of genes detected is shown for the individual programs and for those detected by more than one program in conjunction with another for JU77 upregulated (A), JU77 downregulated (B), LO68 upregulated (C) and LO68 downregulated (D).

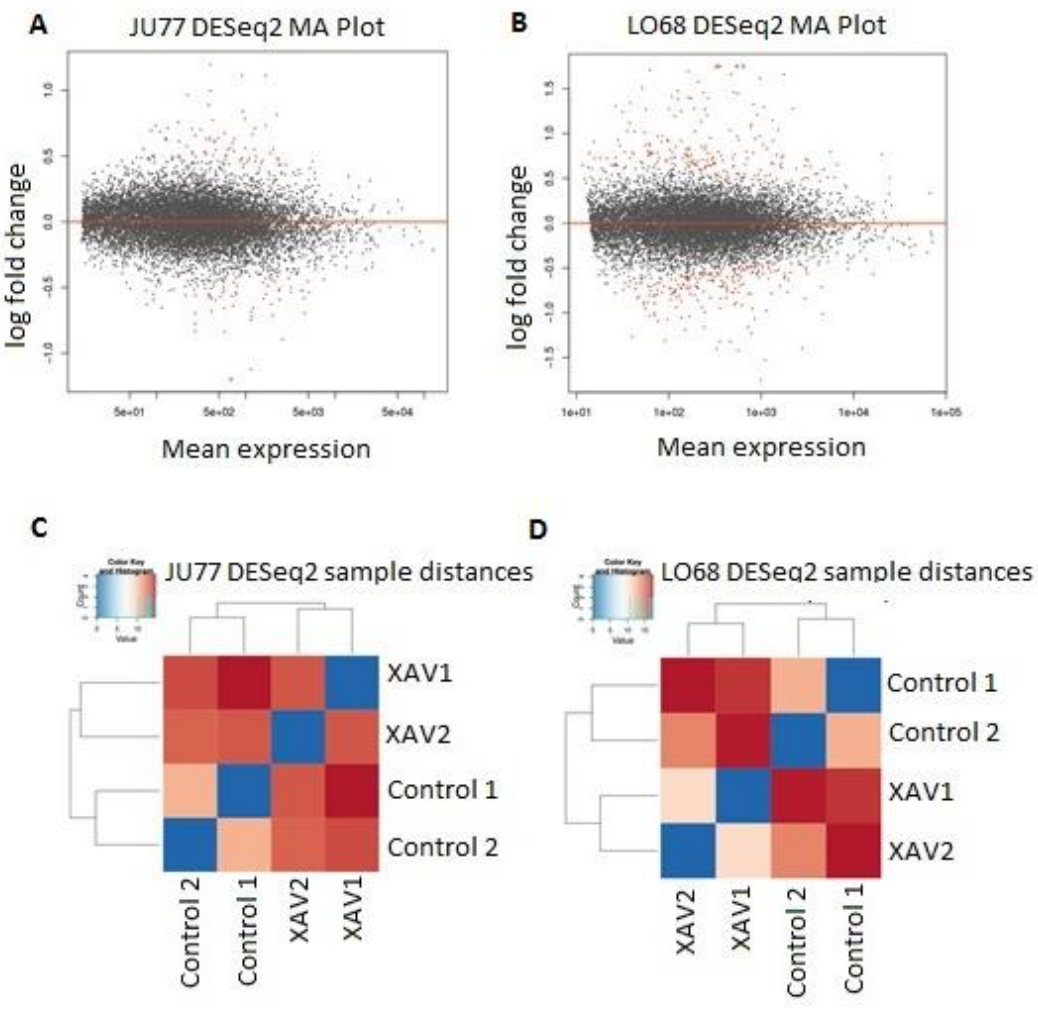

Figure 5.12. DEseq2 Analysis of relative fold change in gene expression and sample similarity. DESeq2 MA plots for JU77 (A) and LO68 (B) cells are shown as log2 fold changes produced by treatment against mean normalised counts and red dots indicate statistically significant differential expression. A greater degree of log2 fold change in expression is noted in LO68 cells. The corresponding sample distance matrices (C) JU77 and (D) LO58 are shown for the individual replicates shows the degrees of similarity between replicates and differences between treatments.

Table 5.1. mRNAs upregulated by more than 1.5 fold (0.58 log2) in JU77 cells treated with XAV939.

Gene Symbol	Gene Name	log2FC	Padj
PLAU	plasminogen activator, urokinase	1.20	3.84E-12
ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	1.11	1.22E-09
PHLDA1	pleckstrin homology-like domain, family A, member 1	1.11	9.18E-11
ESM1	endothelial cell-specific molecule 1	1.00	4.87E-07
DUSP6	dual specificity phosphatase 6	0.97	1.80E-06
HMOX1	heme oxygenase (decycling) 1	0.89	6.22E-06
ALDH1A3	aldehyde dehydrogenase 1 family, member A3	0.84	6.22E-06
SLC20A1	solute carrier family 20 (phosphate transporter), member 1	0.82	1.21E-07
HIF1A	hypoxia inducible factor 1, alpha subunit	0.78	4.58E-06
ANTXR2	anthrax toxin receptor 2	0.78	1.43E-04
NRP1	neuropilin 1	0.73	5.24E-04
PLAT	plasminogen activator, tissue	0.71	5.95E-04
KCNN4	potassium intermediate/small conductance calcium-activated channel N4	0.70	3.75E-03
IL1B	interleukin 1, beta	0.70	1.40E-03
POLD4	polymerase (DNA-directed), delta 4	0.67	1.40E-03
PODXL	podocalyxin-like	0.67	5.10E-04
SOX9	SRY (sex determining region Y)-box 9	0.66	5.18E-03
SYNJ2	synaptojanin 2	0.65	3.41E-03
CDCP1	CUB domain containing protein 1	0.63	4.73E-03
CSF1	colony stimulating factor 1 (macrophage)	0.63	1.47E-03
PITPNA	phosphatidylinositol transfer protein, alpha	0.63	1.08E-02
MLPH	melanophilin	0.62	8.30E-03
FMNL2	formin-like 2	0.62	1.12E-02
MTSS1	metastasis suppressor 1	0.61	5.34E-03
DHRS3	dehydrogenase/reductase (SDR family) member 3	0.60	1.31E-02

FC = fold change; Padj=adjusted p-value

Table 5.2. mRNAs downregulated by more than 1.5 fold (0.58 log2) in JU77 cells treated with XAV939.

Gene Symbol	Gene Name	log2FC	Padj
NPPB	natriuretic peptide precursor B	-1.65	3.29E-20
SDPR	serum deprivation response (phosphatidylserine binding protein)	-1.13	7.23E-14
IGFBP3	insulin-like growth factor binding protein 3	-0.89	3.36E-08
TUFT1	tuftelin 1	-0.84	1.63E-05
STXBP6	syntaxin binding protein 6 (amisyn)	-0.83	6.55E-05
CDC45	CDC45 cell division cycle 45-like (S. cerevisiae)	-0.77	1.03E-03
HIST1H2BF	histone cluster 1, H2bi; H2bg; H2be; H2bf;H2bc	-0.75	5.37E-04
SKP2	S-phase kinase-associated protein 2 (p45)	-0.72	1.11E-04
TRIM58	tripartite motif-containing 58	-0.71	9.74E-06
BMP4	bone morphogenetic protein 4	-0.70	2.74E-03
HIST1H2BC	histone cluster 1, H2bi; H2bg; H2be; H2bf; H2bc	-0.69	1.31E-03
HIST1H2AE	histone cluster 1, H2ae; histone cluster 1, H2ab	-0.68	1.11E-04
PKP2	plakophilin 2	-0.68	1.19E-03
PELP1	proline, glutamate and leucine rich protein 1	-0.66	1.23E-03
HIST3H2A	histone cluster 3, H2a	-0.66	1.60E-03
MBIP	MAP3K12 binding inhibitory protein 1	-0.64	2.79E-03
DIO2	deiodinase, iodothyronine, type II	-0.63	5.34E-03
RAB11FIP1	RAB11 family interacting protein 1 (class I)	-0.59	8.96E-03
FASN	fatty acid synthase	-0.59	5.18E-03
ATP8B1	ATPase, class I, type 8B, member 1	-0.58	1.40E-03
B4GALT4	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 4	-0.58	1.52E-02

FC = fold change; Padj=adjusted p-value

Table 5.3. Forty (40) most upregulated mRNAs in LO68 cells upon treatment with XAV939.

Gene Symbol	Gene Name	log2FC	Padj
AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	1.96	1.33E-19
TGM2	transglutaminase 2	1.87	3.14E-19
NT5E	5'-nucleotidase, ecto (CD73)	1.86	1.17E-18
ARL4C	ADP-ribosylation factor-like 4C	1.77	1.25E-19
IL1B	interleukin 1, beta	1.71	1.00E-11
ETV1	ets variant 1	1.69	2.69E-14
ANXA10	annexin A10	1.66	1.81E-14
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	1.66	1.17E-24
STC2	stanniocalcin 2	1.61	1.27E-17
DIRAS3	DIRAS family, GTP-binding RAS-like 3	1.58	6.15E-12
IGFBP4	insulin-like growth factor binding protein 4	1.51	4.50E-09
PTGS2	prostaglandin-endoperoxide synthase 2	1.48	6.36E-09
ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	1.46	2.78E-11
ANPEP	alanyl (membrane) aminopeptidase	1.45	2.37E-10
PAPPA	PAPPA antisense RNA (non-protein coding)	1.44	6.04E-14
TGFA	transforming growth factor, alpha	1.41	1.32E-08
ETV4	ets variant 4	1.40	2.22E-09
SMIM3	Small Integral Membrane Protein 3	1.40	1.24E-10
DUSP6	dual specificity phosphatase 6	1.39	1.64E-08
MLPH	melanophilin	1.38	1.36E-08
AREG	amphiregulin; amphiregulin B	1.34	2.15E-07
ANTXR2	anthrax toxin receptor 2	1.32	1.01E-09
SAMD3	sterile alpha motif domain containing 3	1.31	3.83E-07
IER3	immediate early response 3	1.29	1.47E-12
ALDH1A3	aldehyde dehydrogenase 1 family, member A3	1.28	2.81E-15
VCAN	versican	1.28	2.86E-07
CXCL2	chemokine (C-X-C motif) ligand 2	1.27	6.11E-07
VEGFA	vascular endothelial growth factor A	1.27	1.32E-08
TSPAN18	tetraspanin 18	1.27	2.45E-06
TMEM158	transmembrane protein 158	1.25	5.95E-07
HAS2	hyaluronan synthase 2	1.24	2.57E-06
HPCAL1	hippocalcin-like 1	1.21	3.30E-10
TIPARP	TCDD-inducible poly(ADP-ribose) polymerase	1.20	5.92E-09
OTUB2	OTU domain, ubiquitin aldehyde binding 2	1.19	1.19E-06
MMP14	matrix metallopeptidase 14 (membrane-inserted)	1.15	3.70E-10
SMOX	spermine oxidase	1.14	1.74E-07
ESM1	endothelial cell-specific molecule 1	1.12	3.56E-05
SPHK1	sphingosine kinase 1	1.10	8.86E-08
ADORA2B	hypothetical LOC100131909; adenosine A2b receptor	1.08	6.31E-10
NRP1	neuropilin 1	1.04	4.42E-07

FC = fold change; Padj=adjusted p-value

Table 5.4. Forty (40) most downregulated mRNAs in LO68 cells upon treatment with XAV939.

Gene Symbol	Gene Name	log2FC	Padj
DAB2	disabled homolog 2, mitogen-responsive phosphoprotein	-1.75	1.17E-24
PLK2	polo-like kinase 2 (Drosophila)	-1.53	8.81E-20
ZNF323	zinc finger protein 323	-1.42	1.78E-15
MATN2	matrilin 2	-1.40	2.73E-11
TLE4	transducin-like enhancer of split 4	-1.40	8.44E-17
PSG4	pregnancy specific beta-1-glycoprotein 4	-1.32	4.22E-12
CYR61	cysteine-rich, angiogenic inducer, 61	-1.32	1.49E-10
IGFBP3	insulin-like growth factor binding protein 3	-1.31	5.46E-07
LRRC7	leucine rich repeat containing 7	-1.26	3.10E-06
AMOTL2	angiomotin like 2	-1.22	7.11E-12
CABLES1	Cdk5 and Abl enzyme substrate 1	-1.19	2.08E-07
EVI2A	ecotropic viral integration site 2A	-1.16	7.20E-08
FAM198B	chromosome 4 open reading frame 18	-1.16	2.03E-07
PLAC8	placenta-specific 8	-1.09	8.30E-05
PCDH18	protocadherin 18	-1.07	4.76E-05
SH2D4A	SH2 domain containing 4A	-1.07	1.91E-09
RBMS3	RNA binding motif, single stranded interacting protein	-1.05	1.56E-05
ANKRD37	ankyrin repeat domain 37	-1.04	3.19E-04
TMEM52B	transmembrane protein 52B	-1.04	4.50E-05
C11orf87	chromosome 11 open reading frame 87	-1.03	3.42E-04
COL3A1	collagen, type III, alpha 1	-1.02	2.72E-08
GFRA1	GDNF family receptor alpha 1	-1.00	6.16E-04
SAMD11	sterile alpha motif domain containing 11	-0.99	2.90E-05
LUM	lumican	-0.98	2.83E-08
TRPC4	transient receptor potential cation channel C4	-0.95	1.65E-03
SH3BP5	SH3-domain binding protein 5 (BTK-associated)	-0.95	1.43E-07
TNS3	tensin 3	-0.94	1.33E-04
ADCYAP1	adenylate cyclase activating polypeptide 1 (pituitary)	-0.94	5.12E-04
CTGF	connective tissue growth factor	-0.94	9.90E-04
PRSS23	protease, serine, 23	-0.91	1.43E-05
KLHL4	kelch-like 4 (Drosophila)	-0.91	3.53E-03
SDPR	serum deprivation response (phosphatidylserine binding protein)	-0.90	7.72E-05
CPA4	carboxypeptidase A4	-0.89	3.97E-03
MXD4	MAX dimerization protein 4	-0.88	1.53E-05
PRKG1	protein kinase, cGMP-dependent, type I	-0.88	4.64E-03
PDE5A	phosphodiesterase 5A, cGMP-specific	-0.87	5.59E-03
PTPRQ	protein tyrosine phosphatase, receptor type, Q	-0.87	1.88E-03
C1orf226	chromosome 1 open reading frame 226	-0.86	4.66E-04
GPR87	G protein-coupled receptor 87	-0.85	4.05E-03
FZD2	frizzled homolog 2 (Drosophila)	-0.84	1.11E-04

FC = fold change; Padj=adjusted p-value

Table 5.5. mRNAs commonly differentially expressed* in JU77 and LO68 upon treatment with XAV939.

Gene Symbol	Gene Name
Upregulated	
PLAU	plasminogen activator, urokinase
ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)
PHLDA1	pleckstrin homology-like domain, family A, member 1
ESM1	endothelial cell-specific molecule 1
DUSP6	dual specificity phosphatase 6
ALDH1A3	aldehyde dehydrogenase 1 family, member A3
ANTXR2	anthrax toxin receptor 2
NRP1	neuropilin 1
IL1B	interleukin 1, beta
CDCP1	CUB domain containing protein 1
MLPH	melanophilin
MTSS1	metastasis suppressor 1
Downregulated	
SDPR	serum deprivation response (phosphatidylserine binding protein)
IGFBP3	insulin-like growth factor binding protein 3
PKP2	plakophilin 2
ATP8B1	ATPase, class I, type 8B, member 1

* criteria fold change >1.5 and adjusted p-value <0.1

5.9. Discussion.

These studies represent the first investigations into the inhibition of Wnt signalling by XAV939 in mesothelioma using the 2D and 3D methods developed in this thesis study. To date, no other studies have been published at the time of writing using tankyrase inhibition in mesothelioma cells. The susceptibility of four mesothelioma lines to XAV939 was assessed in both 2D and 3D culture as well as the effects of this agent on migration and adhesion in the two migratory lines (NO36 and ONE58) which were also most resistant to proliferation. Changes in gene expression were investigated using the two susceptible lines (JU77 and LO68) and in particular, the effects on targets of β catenin-mediated Wnt signalling. A wider approach was then taken to investigating the transcriptional effects of tankyrase inhibition by XAV939 in mesothelioma cells as there was no significant effect observed in the expression of the Wnt targets c-Myc and Cyclin D1. Therefore, RNA sequence analysis was used to examine downstream targets of the pharmacological actions of XAV939 in mesothelioma cell lines. The list of commonly differentially expressed genes (Table 5.5) identifies genes which encompass a broad range of biological roles in cancer. These investigations using XAV939 in mesothelioma are, to our knowledge, the first to determine such a list following tankyrase inhibition with this agent.

The effects of Wnt signalling have been widely described in cell type-specific processes such as proliferation, migration, invasion, adhesion and differentiation during development, tissue homeostasis and disease, particularly cancer (Logan and Nusse. 2004; Brembeck et al., 2006; Neth et al., 2007; Polakis 2012; Kahn 2014). Therefore, targeted intervention at the level of tankyrase inhibition with compounds such as XAV939 allows for the investigation of these cellular phenomena. A similar pattern of mesothelioma cell spheroid sensitivity to XAV939 was seen in 3D culture after 48 hours incubation, resembling the susceptibility observed in 2D culture over the same period. A reduction in viability was observed in JU77 and LO68 spheroids and only a slight effect in NO36 and ONE58 spheroids. Inhibition of growth by XAV939 and siRNA knockdown has been demonstrated in breast cancer cell lines (Bao et al., 2012) and apoptosis of lung adenocarcinoma cells has also been induced solely by knockdown of both telomerase and tankyrase expression (Lu et al., 2013).

Relative resistance to XAV939 by NO36 and ONE58 cells was also corroborated by the colony formation assay with a dose dependent reduction in colony formation seen in both lines. Migration of NO36 and ONE58 cells through a Transwell® membrane was also reduced by XAV939 treatment (more so in NO36 cells). Similar studies using XAV939 treatment together with RNAi gene silencing have also demonstrated reductions in growth, colony formation and migration in breast cancer cell lines (Bao et al., 2012) and also in two studies by Tian and co-workers in neuroblastoma cell lines. Tankyrase inhibition in neuroblastoma cells also produced growth arrest, sensitisation to apoptosis together with a reduction in colony formation (Tian et al., 2013) and a subsequent study also demonstrated a reduction of cell invasion (Tian et al., 2014). A reduction in adhesion following XAV939 treatment was observed in three of the four lines; especially in ONE58 and a slight, unexpected increase in adhesion of JU77 cells.

Inhibition of Wnt signalling by XAV939 has been shown to augment the effects of cisplatin in colon cancer (Wu et al., 2016) and to produce growth arrest and apoptosis in mantle cell lymphoma (He et al., 2015). Recently, growth arrest and a reduction in colony formation have demonstrated in hepatocellular carcinoma using XAV939 and WXL-8 (a novel XAV939 derivative) (Ma et al., 2015). The results presented in this chapter are the first reports, to our knowledge, of the inhibition of Wnt signalling by XAV939 in mesothelioma cells producing reductions in cell viability, colony formation, cellular adhesion and migration.

It has been reported in the literature that interference with PARylation by inhibitors or dysfunctional PARylation as a result of cellular injury will result in cell death by apoptosis. However, there appears to be a division in the pathophysiology between cases of apoptosis resulting from PARP1 activation (often as a result of DNA damage) and those of selective inhibition of tankyrases 1 and 2 (PARP5a and PARP5b). Activation of PARP1 and subsequent induction of apoptosis has been the most intently studied pathway and apoptosis is now thought to occur as a result of metabolic dysfunction due to NAD+ depletion (Morales et al., 2013) or a cell-type and stimulus specific phosphorylation cascade (for review, see Virag et al., 2013). XAV939 is however selective for tankyrase 1 (Riffell et al., 2012) and therefore, its action is unlikely to involve PARP1.

Stabilisation of Axin by the inhibition of its PARsylation via tankyrase blockade enhances the stability of the β catenin degradation complex and therefore promotes the breakdown of phosphorylated β catenin by the proteosomal complex (Section 1.6). Evidence for this pharmacological effect has been demonstrated through the reduction of cellular β catenin in all four mesothelioma cell lines as a result of treatment with XAV939. The assembly of β catenin with members of the TCF/LEF transcription factor group to initiate target gene transcription has been well characterised as a critical event in Wnt-mediated gene expression (Mao and Byers, 2011; Cadigan and Waterman, 2012) and therefore the consequences of β catenin depletion upon Wnt target expression provided the rationale for investigating this mechanism in malignant mesothelioma.

The effect of XAV939 treatment upon gene expression in the susceptible JU77 and LO68 cell lines was investigated by RNA sequence analysis (RNASeq) using the methods outlined in Section 2.15. Three software packages were used to determine the magnitude of fold change for differential expression (DE) significance; EdgeR (V2.4.6), DESeq2 (V1.10.1) and VOOM/Limma (V3.16.6). A Venn diagram format (Hulsen et al., 2008) was used to illustrate the detection of upregulated and downregulated genes according to the settings of each program. The varying detection rates between these programs is apparent with EdgeR showing the greatest number of differentially expressed genes (DEGs), followed by VOOM and DESeq2; this difference being attributable to the mathematical basis used to calculate differential expression significance in these programs.

EdgeR has been briefly described by its authors (Robinson et al., 2010) and its mathematical principles reviewed by Chen et al., (2014) who suggest that modelling by EdgeR is suited to experimentation with small numbers of replicates due to its use of Bayesian (probability based) statistics which may suit experiments with small numbers of replicates. The statistical mechanisms of DESeq2 have also been explained by its authors (Love et al., 2014) who state that DESeq2 is also suited to low replicate numbers with high variability as the software subjects the raw count data to a form of adaptive shrinking of log fold changes, based on the overall variability of the dataset. Furthermore, shrinkage of log fold estimates by DESeq2 has been calculated in a manner that exerts greater shrinkage for low count or highly variable datasets in addition to the probability-based estimates like those calculated by EdgeR (Love et al., 2014). This additional stringency explains the considerably lower

detection rate of upregulated and downregulated genes by DESeq2 compared to EdgeR as the adjusted data is again weighted and ranked in DESeq2 by the Benjamini-Hochberg method in order to minimise the risk of false-positive results – consequently resulting in a smaller list of differentially expressed genes (Love et al., 2014). The stringency of the DESeq2 software over that of EdgeR was observed in the upregulation and downregulation of genes in the susceptible JU77 and ONE58 cells through consistently lower numbers of genes being marked as differentially expressed by DESeq2 as opposed to considerably higher numbers of genes detected by EdgeR.

The VOOM software package however, employs a different approach by analysing the relationship between the mean and variance of each gene and the fitting of the standard deviation to a sample size dependent trend and calculation of a predicted count (Law et al., 2014). According to the authors, the mean-variance relationship makes fewer assumptions and has an inherently lower risk of false positive detection (Law et al., 2014). The results from VOOM analysis are an intermediate result between the somewhat general nature of EdgeR analysis and the more stringent methods of DESeq2. This is corroborated by the numbers of significantly upregulated or downregulated genes detected by VOOM being less than those detected by EdgeR whilst still considerably more than those detected by DESeq2. According to Seyednesrollah and colleagues (2013), EdgeR appears to be more susceptible to false positive detection of low expression genes that may seem to be upregulated or downregulated, albeit from a very low expression baseline – a problem which has been minimised by the methods used in DESeq2. The results from VOOM analysis are of interest due to the slightly different approach and may represent a suitable compromise, especially in libraries which contain large numbers of low-expressing genes and/or where modulation of expression is less obvious.

The choice of software is a key consideration with small sample numbers as analysis of broad patterns of gene expression may still be performed with small sample numbers (Khang and Lau, 2015). A cross-comparison of several software packages by Seyednesrollah et al., (2013) confirmed the notion that no single program was superior to the others although the vulnerability of EdgeR to false positive results was again noted. DESeq2 was recommended for studies of limited size (Seyednesrollah et al., 2013) and similarly so by Khang and Lau (2015) in the case of strong biological responses. Both groups still

emphasised the importance of sample size for statistical power and VOOM was not evaluated in either discussion.

The inhibition of Wnt signalling by XAV939 in the JU77 and LO68 mesothelioma cell lines produced a modest biological effect which is consistent with an equally modest degree of differential gene expression. However, a small but unique set of commonly differentially expressed genes has emerged and represents targets downstream of the β catenin/TCF/LEF transcriptional complex. The upregulated genes have a significant association with the progression of cancer and in some cases are influenced at least in part by β catenin-mediated Wnt signalling. Comparatively less is known about the commonly downregulated genes with only Plakophilin 2 being associated with Wnt signalling via evolutionary conservation.

Upregulation of Plasminogen Activator, Urokinase (PLAU), was observed in both cell lines and provides an interesting topic for further investigation. A serine protease characterised by its role in converting plasminogen to plasmin, PLAU indirectly promotes proliferation, migration, adhesion, matrix degradation, angiogenesis and invasion in addition to its self-activation (for review, see Duffy (2004)) and a mutual relationship between Wnt7a expression and that of PLAU has been described in a medulloblastoma model (Asuthkar et al., 2012). Considerable evidence supports the roles of integrins in cancer and this includes regulation of the PLAU system in addition to the integrin-mediated events such as migration and proliferation dependent upon the particular integrin heterodimer combination (Desgrosellier and Cheresh, 2010). However, comparatively little is known about integrin α_2, its binding partner(s) and biological roles and therefore, its upregulation in both lines by XAV939 warrants further examination.

The Pleckstrin Homology-like Domain Family A, member 1 (PHLDA1) protein was also upregulated in both cell lines and its role appears to be a cell context specific one. Originally identified in platelet-bound pleckstrin and further characterised through its roles in phosphoinositide metabolism (Lemmon, 2007), PHLDA1 has been reported as acting either as a tumour suppressor in a melanoma cells and breast cancer primary tissues (Neef et al., 2002; Nagai et al., 2007) or as a promoter of proliferation in oral cancers (Murata et al.,

2014) and colorectal cancer model by Sakthianandeswaren and colleagues (2011) with the suggestion that PHLDA1 expression may be controlled by an alternate (β catenin-independent) Wnt signalling pathway. Increased PHLDA1 expression has also been described in a human intestinal epithelial crypt cells (Guezguez et al., 2014) and immunohistochemical analysis of pilomatricomas (hair matrix tumours) also showed increased PHLDA1 expression together with that of β catenin (Batistella et al., 2014).

The upregulation in JU77 and LO68 cells of Endothelial Cell Specific Molecule 1 (ESM1) or endocan has also been described in other cancers such as breast, kidney, uterine and lung cancers (Sarrazin et al., 2006) and more recently in gastric cancer (Zhao et al., 2014). ESM1 is a proteoglycan expressed by the vascular endothelium with roles in growth factor regulation, matrix binding, cytokine binding and control of coagulation (Sarrazin et al., 2006) but its role in mesothelioma remains unclear. An association between increased Neuropilin 1 (NRP1) expression, the depletion of cellular β catenin and the progression of medulloblastoma has been described by Yogi et al., (2015) and further investigation in mesothelioma cell lines may yield additional information on this topic.

Of the downregulated genes, neither of Insulin-like Growth Factor Binding Protein 3 (IGFBP3) and Plakophilin 2 (PKP2) have been investigated in mesothelioma. The tumour suppressing action of IGFBP3 have been attributed to its sequestration of insulin-like growth factor 1 (IGF1) which restricts access of IGF to its receptor (Kim, M.S. et al., 2015) and more recently, by its epigenetic silencing by promoter hypermethylation in hepatocellular carcinoma (Han et al., 2014). However, any association between the modulation of IGFBP3 expression and inhibition of Wnt signalling by tankyrase inhibition remains to be discovered.

Characterisation of the role of Plakophilin 2 (PKP2) has been made through its role in Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) (for review, see Awad et al., (2008) and the nature of PKP2 deregulation appears to be cell type-specific. Contrasting results have been reported with progression of gastric cancer due to reduced PKP2 expression (Demirag et al., 2011) or progression of bladder cancer as a result of its increased expression (Takahashi et al., 2012). The evolutionary relationship between PKP2

and β catenin through the conservation of their Armadillo repeat motifs has been reviewed by Hatzfeld (2007) and a role in the regulation of β catenin levels by the complexation of PKP2 with ε cadherin has been proposed by Chen and colleagues (2002). The reduction of PKP2 expression subsequent to XAV939 treatment may therefore represent a negative feedback mechanism triggered by the depletion of β catenin.

The aim of these experiments was to investigate the effects of tankyrase inhibition by XAV939 in four mesothelioma cell lines and in particular, the modulation of adhesive and migratory activity in the resistant cell lines (NO36 and ONE58) and transcriptional phenomena in the two susceptible cell lines (JU77 and LO68). Biological consequences such as a reduction of viability, loss of adhesion and inhibition of migration were measured *in vitro* as well as the differential regulation of a small set of genes common to both lines. The effects of XAV939 were modest in the functional assays and the depletion of intracellular β catenin provided evidence of its action in sustaining the activity of the β catenin breakdown complex. Consistent with this, the transcriptional response was also modest and a marked effect was not seen in the prototypical Wnt target genes c-Myc and cyclin D1. Instead, a unique set of differentially expressed genes was identified and reinforces the concept that Wnt target genes may vary greatly depending upon cellular context.

Wnt target genes have been identified in almost every field of cell biology research ranging from development (Logan and Nusse, 2004; Hoppler and Nakamura, 2014), stem cell biology, metabolic diseases and cancer (Reya and Clevers, 2005; Shitashige et al., 2008; Clevers and Nusse; 2012; Polakis, 2012). Moreover, Wnt target genes have also been identified in the control of cellular events such as invasion (Neth et al., 2007), cell-to-cell adhesion (Heuberger and Birchmeier, 2010) and cell migration (including control and crosstalk with non β catenin dependent Wnt signalling pathways (Amin and Vincan, 2012). The most current listings are available at the excellent '*Wnt Homepage'* which is administered by the Nusse group (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes). An alternate view has been proposed by Nakamura and colleagues (2016) following studies of Wnt8a in a *Xenopus* developmental model which suggested the possibility that β catenin may bind to several other genes beyond those already described and that their activation may occur in a controlled, time and tissue context-specific manner. Such suggestions challenge the conventional hypothesis that β catenin activates a specific

set of genes with immediate modulation of their expression. Therefore, a wider scope of investigation is advisable when investigating Wnt signal inhibition by tankyrase blockade as changes in gene expression may still occur, albeit at locations beyond those already described.

Several sets of genes have been identified in these experiments; these being genes that are significantly upregulated or downregulated in JU77 and LO68 cells as well as those that have been significantly differentially expressed in both lines. A certain subset of these genes will in fact be responsible for the phenotypic effects observed although the relative paucity of information on Wnt signalling in mesothelioma prevent further inferences being made. The use of RNA sequencing together with inhibitory methods such as RNAi gene silencing, oligonucleotide knockdown or other epigenetic methods will allow further investigation into the relative role(s) and importance of the genes identified in these studies.

Chapter 6.

Modulation of the Wnt pathway through histone deacetylase inhibition in mesothelioma cells.

Table of contents.

6.0. Introduction.

Regulation of histone deacetylase function in cancer by HDACi (histone deacetylase inhibitors) is a topic actively investigated in several malignancies and other disease states such as diabetes mellitus and psychiatric conditions. Efforts are under way to utilise the disruption of transcriptional control caused by HDACis as an adjunct therapy method to increase the efficacy of existing treatments such as cytotoxic drugs and radiotherapy with the aim of provoking apoptotic pathways either as a sole agent or in conjunction with other treatment methods. The actions of HDACi have been previously investigated in mesothelioma but their effects on the Wnt signalling systems have not been examined. The aim of these experiments was to explore changes in the expression of Wnt component genes, Wnt target genes and associated gene products in order to characterise the response to SAHA (**Suberoylanilide hydroxamic acid)** in the JU77 and ONE58 mesothelioma cell lines.

Histone deacetylase inhibition has been shown to both activate and inactivate Wnt signalling in different cellular contexts and therefore challenges the notion of Wnt signal attenuation being the classical response to histone deacetylase inhibition. Activation of Wnt signalling in primary tissue and cell lines by valproic acid and SAHA has been shown to dedifferentiate cancerous cells towards a stem cell-like state and to also promote their expansion in the undifferentiated phenotype (Debeb et al., 2012) whilst a more typical scenario of Wnt signal inhibition through histone deacetylase blockade has been described in a developmental Zebrafish heart valve model (Kim et al., 2012). The activation of Wnt signalling through loss of histone deacetylase function has also been demonstrated in various models by non-pharmacological methods such as genetic deletion of HDAC1 and HDAC2 in mouse oligodendrocytes (Ye et al., 2009) and RNA knockdown of HDAC3 in colon cancer cells (Godman et al., 2009). Conversely, genetic deletion of HDAC1 and HDAC2 has also been shown to abolish β catenin mediated Wnt signalling in a mouse kidney developmental model (Chen et al., 2015). Taken together, the inhibition of histone deacetylases is an intervention that will require careful consideration in terms of cell type, disease state, choice of agent and developmental context as the biological sequalae may indeed extend beyond therapeutically targeted cell death, with potentially unexpected effects upon Wnt signalling.

Using SAHA as an example of a clinically utilised HDACi, experiments were conducted to assess its effects upon the Wnt signalling system in the JU77 and ONE58 mesothelioma 3D spheroid models. These lines were chosen as a result of previous work by another student in the supervisor's laboratory that demonstrated the susceptibility of JU77 and ONE58 to SAHA in 2D culture. An optimised concentration of SAHA was determined and evaluated for cytotoxicity in conventional monolayer culture and as a spheroid. Morphological changes were also assessed. Since there are conflicting reports regarding the effect of histone deacetylase inhibition upon the Wnt pathway, RNA transcriptome sequencing was used to gain insight into effects upon pathway components and target genes.

6.1. Determination of SAHA dose range in JU77 and ONE58 cells.

Initial exploratory studies were carried out by Ms. J. Huang, to determine response to SAHA in these two lines. There was a dose dependent reduction in cell viability in both cell lines as measured by MTT assay with ONE58 cells more sensitive than JU77 cells (Figure 6.1A). These results were further confirmed using a trypan blue assay although both cell lines displayed similar sensitivity (Figure 6.1B).

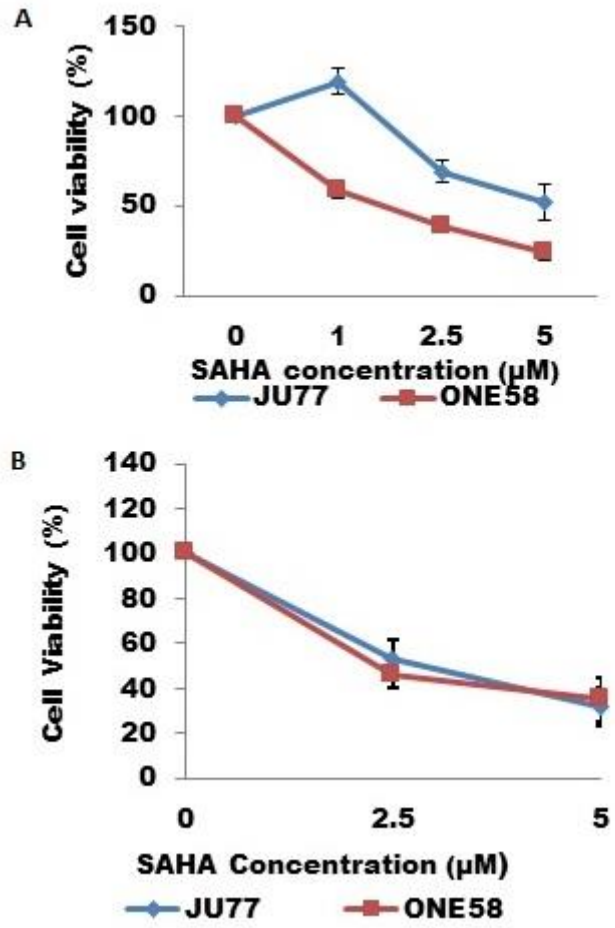

Figure 6.1. Effect of SAHA treatment on viability of JU77 and ONE58 cells.
Cells were cultured for 48h in the presence of SAHA (0 - 5 µM) with a 0.01% DMSO vehicle control. Cell viability is expressed as a percentage relative to control using MTT proliferation assay (A) and by trypan blue exclusion counting using an Invitrogen Countess automated cell counter (B). Data kindly provided by Ms. J. Huang, from the supervisor's laboratory.

6.2. Morphological observations of SAHA-treated JU77 and ONE58 cells.

The morphological effects of SAHA treatment in these cell lines had not been previously recorded in 2D culture. Therefore, cells treated with 5µM SAHA were examined over time with both lines showing signs of toxicity after 48 hours. Treated wells displayed the signs of cytotoxicity such as a sparser monolayer and the hallmarks of apoptosis such as shrinkage, rounding up and detachment from the surface. Significant quantities of cellular debris in the medium of SAHA-treated cultures was also observed.

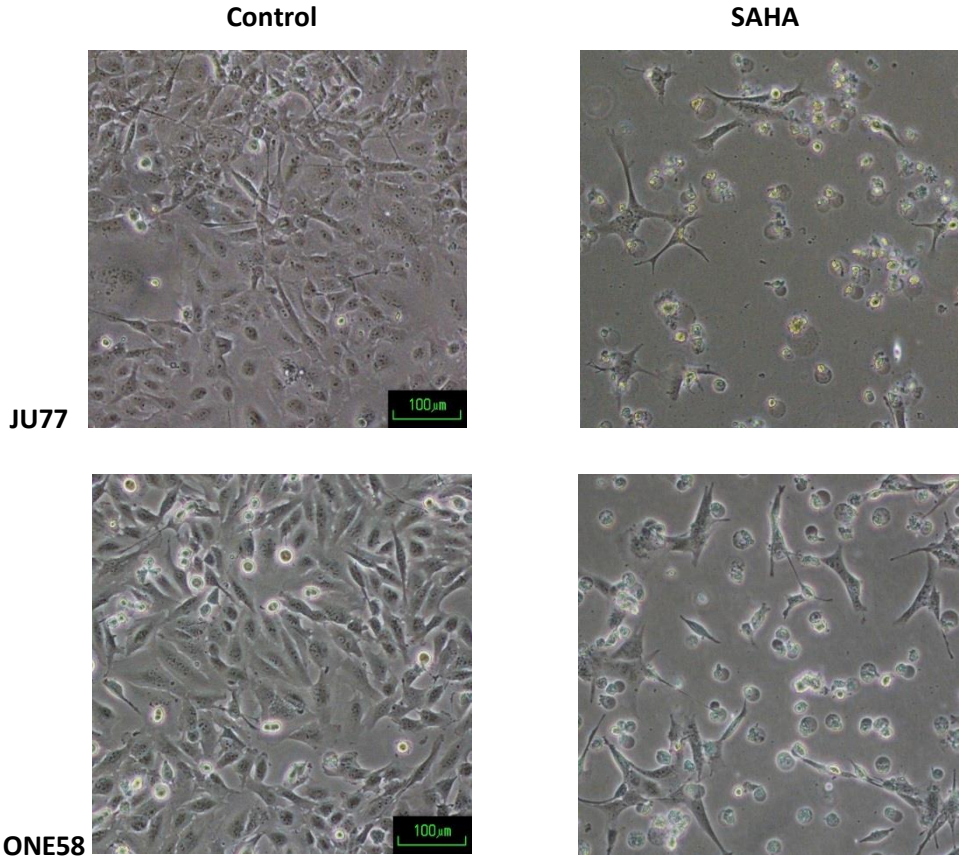

Figure 6.2. Morphologial effects of 5µM SAHA on JU77 and ONE58 cells after 48h incubation. Cells were incubated with 5µM SAHA for 48h and assessed by microscropy. Representative images shown from three independent experiments (Scale bar 100µm).

6.3. Effect of SAHA on viability of JU77 and ONE58 spheroids.

Further to the results seen 2D culture (Figure 6.1), the effect of SAHA on JU77 and ONE58 cell viability in spheroid cultures was investigated. A statistically significant reduction in spheroid viability relative to untreated control was observed in both cell lines. The reduction in viability was time and dose dependent with ONE58 spheroids showing greater susceptibility than JU77 spheroids, consistent with the trend seen in 2D culture.

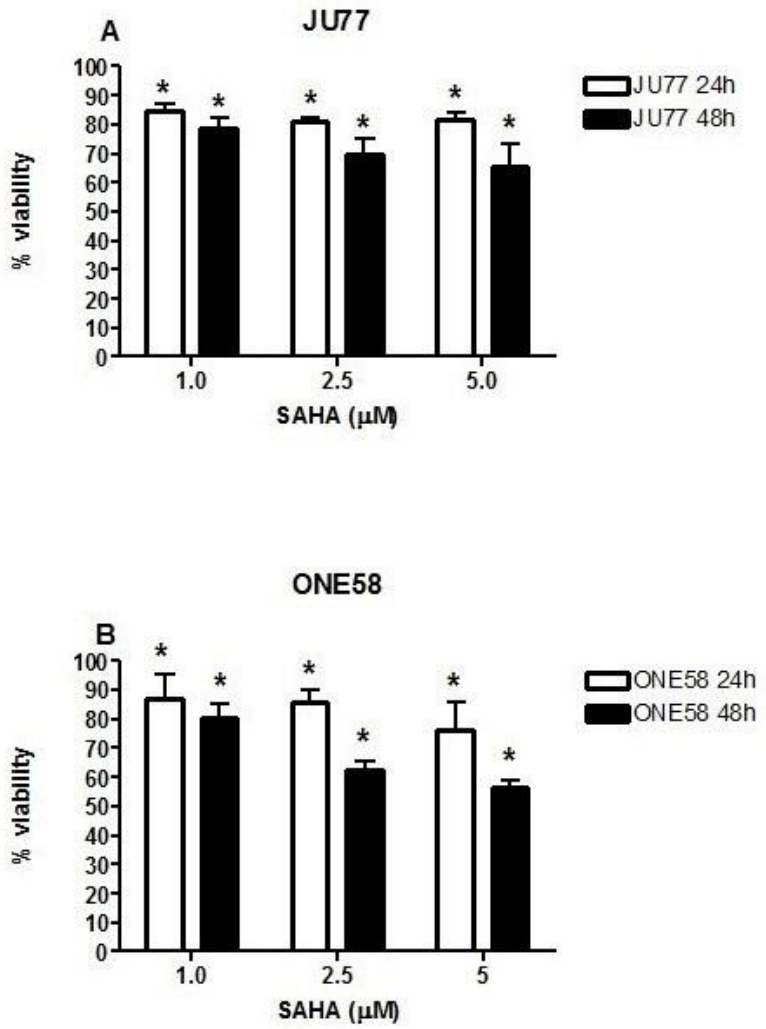

Figure 6.3. Effects of SAHA on JU77 and ONE58 mesothelioma cell spheroids.
Viability of JU77 spheroids (A) and ONE58 spheroids (B) after incubation with SAHA determined by acid phosphatase viability assay. Data expressed as mean +/- SD relative to untreated control and derived from four independent experiments, ($p < 0.05$)

6.4. Morphological observations of SAHA-treated JU77 and ONE58 spheroids.

The effect of SAHA on JU77 and ONE58 spheroids was also investigated to assess if SAHA treatment interfered with spheroid morphology. Unlike the monolayer-based study where significant morphological effects were seen, treatment with 5µM SAHA did not produce any obvious morphological changes in spheroids (Figure 6.4). Despite the reduction in viability over time measured by the APH assay, spheroids from both lines retained their structural integrity and did not show significant differences in morphology from untreated wells.

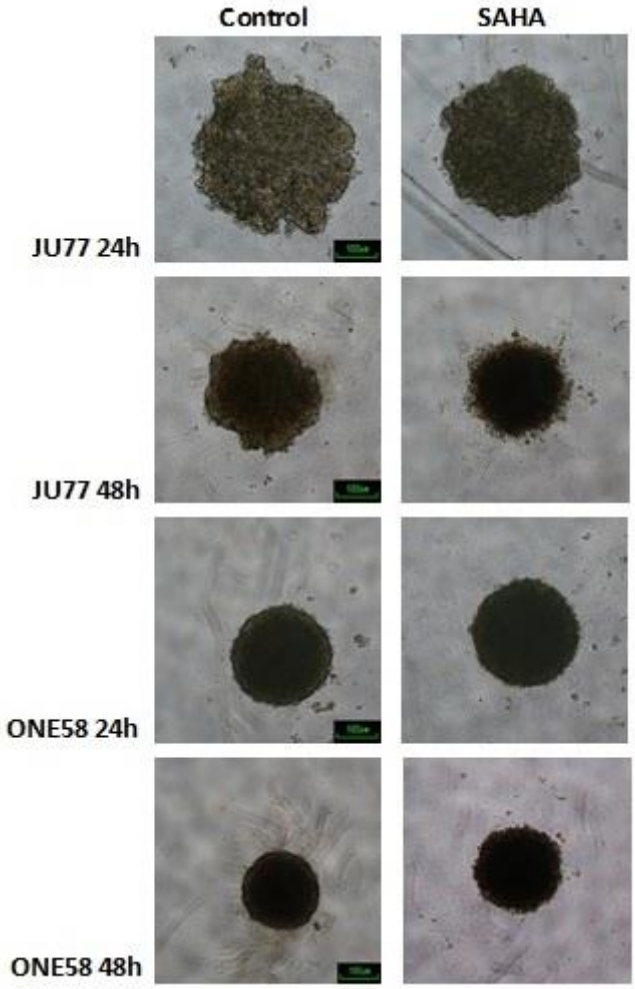

Figure 6.4. SAHA does not produce morphological changes in JU77 and ONE58 mesothelioma cell spheroids. JU77 and ONE58 mesothelioma cell spheroids were seeded to form overnight then incubated for 24h and 48h using either a untreated vehicle control or 5µM SAHA. Images shown are representative examples from three independent experiments. (Scale bar 100µm).

6.5. Analysis of differential gene expression data in SAHA-treated JU77 and ONE58 cells.

The effect upon the transcription of Wnt genes and Wnt target genes caused by histone deacetylase inhibition in mesothelioma had not been previously examined and therefore the aim of these experiments was to gain some insight into the transcriptional effects of SAHA in these two cell lines. Following incubation with 5μM SAHA for six hours, RNA was extracted and analysed in the same manner described in Section 2.17 and the data processed similarly as per section 5.8. In brief, six samples were used and subjected to analysis against the 20800 genes of the AmpliSeq Gene Expression Panel producing a final library of 85% useable reads with 92% sequence alignment. The average number of reads per sample was approximately $1x10^7$ reads per sample at a mean read length of 118 base pairs per read.

Treatment with SAHA produced a broader and more biologically profound response compared to XAV939 as a larger number of genes were affected and a greater degree of fold variation was detected using the DESeq2 software system. The DESeq2 MA plot (Figure 6.5, upper panels) which measures the degree of expression (as normalised transcript count) against relative log2-fold change shows a greater degree of variability in log2-fold change following SAHA treatment compared to XAV939 exposure (c.f. Figure 5.11), indicative of altered transcription across a larger number of genes. A sample distance matrix (Figure 6.5, lower panels) shows the similarity between replicates and the degree of difference between individually dated experiments.

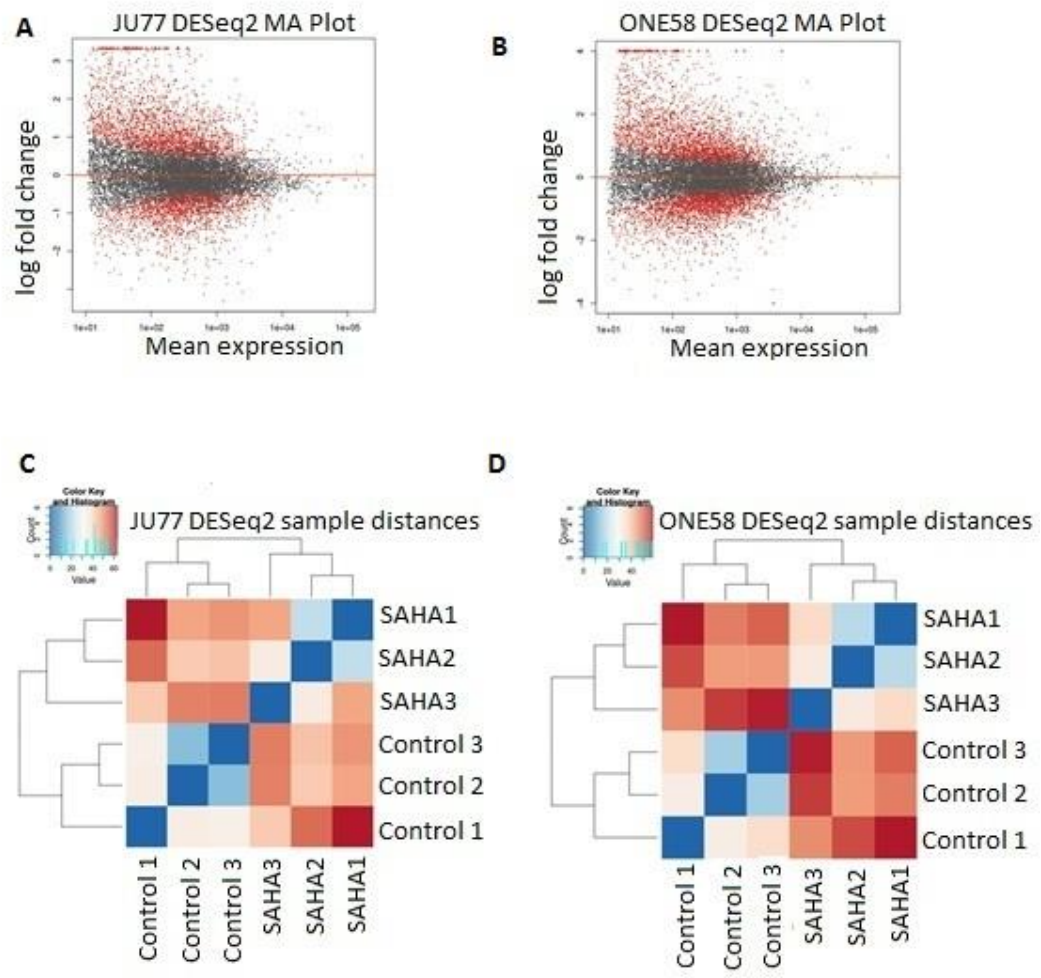

Figure 6.5. DESeq2 Analysis of relative fold change in gene expression and sample similarity. DEseq2 MA plots for JU77 and ONE58 cells are shown as log2 fold changes produced by treatment against mean normalised counts for (A) JU77 and (B) ONE58 cells where statistically significant differential expression is indicated by red dots. The corresponding sample distance matrices are shown for (C) JU77 and (D) ONE58 cells for the individual replicates to indicate the degrees of similarity between replicates and differences between treatments.

Particular attention was paid to elements of the Wnt signalling system and a cohort of 182 Wnt and Wnt-associated genes from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/pathway.html; ref: map04310) was used to determine the effect of SAHA on the expression of this subset of genes due to the widespread effects of this compound compared to the relatively modest effect of XAV939. Furthermore, a supplementary group of genes was added to those listed by KEGG in order to widen the investigation into the effects of SAHA upon Wnt signalling. Based on our reading of literature, we concluded that a number of recently described Wnt pathway genes and targets were absent. Therefore, another 25 genes were curated and included in the analysis of differential expression. These were BIRC5, CDX1 / 2, CXCR4, DKK3, FN1, Hras, KRM1, MMP (1, 2, 9, 13), NRTRKR1 / 2, RNF43, ROR1 / 2, RSPO1, RYK, SFRP3 / 4, SP5, TBX1 and TRABD2A.

Overall, a set of 31 genes from within the 183 genes evaluated were either upregulated or downregulated by more than 1.5 fold (0.58 log2) in both JU77 and ONE58 cells, representing 17% of the genes examined in this experiment. A total of 22 Wnt-associated genes were found to have been upregulated by 1.5 fold (0.58 log2) or more in JU77 cells treated with SAHA (Table 6.1). The range of gene upregulation in JU77 ranged from a relatively small increase in MAP3K7 expression of 0.61 log2 (equivalent to 1.52 fold) to the 2.15 log2 upregulation (equivalent to 4.4 fold) of DAAM1. Also observed in JU77 cells, was the downregulation of 20 Wnt and Wnt-associated genes (Table 6.2). Downregulation of genes ranged from the modest -0.61 log2 reduction of CSNK2A1 expression (equivalent to a reduction down to 0.65 fold) to a marked -3.13 log2 inhibition of the expression of the Wnt inhibitor DKK1 (equivalent to a reduction down to 0.11 fold). Notably, the Wnt target genes c-Myc, cyclin D1, and LEF1 were all downregulated in JU77 cells.

A similar number of genes were also found to be upregulated by more than 1.5 fold (0.58 log2) or more in ONE58 cells treated with SAHA as 23 such genes were detected by DESeq2 analysis (Table 6.3). A slight increase of 0.6 log2 expression of LRP6 (equivalent to 1.5 fold) was observed and a significant 4.84 log2 (equivalent to 29 fold) upregulation of CXCR4. A set of 22 genes downregulated by 1.5 fold (0.58 log 2) or more was also detected in ONE58 cells (Table 6.4) which ranged from a slight -0.58 log2 decrease in PLCB4 expression (equivalent to a reduction down to 0.6 fold) to a significant downregulation of -3.30 log2

(equivalent to a reduction down to 0.1 fold) of Fos-like antigen (FOSL1). The differentially expressed genes that are common to both JU77 and ONE58 cells are listed in Table 6.5. Upregulation of Wnt components such as cyclin D3, Fzd3, Fzd4, Wnt9a, LRP6 and APC2 was observed in both cell lines. Genes commonly downregulated included, Fzd2, Wnt3, Wnt5b, Axin1 and the known Wnt target, cyclin D1.

Table 6.1. mRNAs upregulated by more than 1.5 fold (0.58 log2) in JU77 by SAHA.

Gene Symbol	Gene Name	log2FC	Padj
DAAM1	dishevelled associated activator of morphogenesis 1	2.15	7.45E-18
NKD1	naked cuticle homolog 1 (Drosophila)	2.09	9.65E-04
PPP2R5B	protein phosphatase 2, regulatory subunit B', beta isoform	2.00	1.54E-08
CCND2	cyclin D2	1.81	7.83E-04
AXIN2	axin 2	1.51	1.83E-02
WNT9A	wingless-type MMTV integration site family, member 9A	1.29	1.67E-02
CAMK2G	calcium/calmodulin-dependent protein kinase II gamma	1.26	1.10E-02
FZD4	frizzled homolog 4 (Drosophila)	1.17	5.39E-03
APC2	adenomatosis polyposis coli 2	1.10	6.25E-02
PPP2R5C	protein phosphatase 2, regulatory subunit B', gamma isoform	1.04	2.11E-02
RAC2	ras-related C3 botulinum toxin substrate 2	1.04	1.20E-05
PORCN	porcupine homolog (Drosophila)	1.01	9.58E-03
PRKACA	protein kinase, cAMP-dependent, catalytic, alpha	0.96	2.70E-04
LGR4	leucine-rich repeat-containing G protein-coupled receptor 4	0.96	1.92E-03
PRICKLE2	prickle homolog 2 (Drosophila)	0.92	2.89E-02
LRP6	low density lipoprotein receptor-related protein 6	0.90	1.48E-03
FZD3	frizzled homolog 3 (Drosophila)	0.88	2.75E-02
CHP1	Calcineurin-Like EF-Hand Protein 1	0.83	9.58E-03
CCND3	cyclin D3	0.82	4.04E-02
PRKACB	protein kinase, cAMP-dependent, catalytic, beta	0.77	4.41E-02
PPP2R5D	protein phosphatase 2, regulatory subunit B', delta isoform	0.74	6.52E-02
MAP3K7	mitogen-activated protein kinase kinase kinase 7	0.61	4.41E-02

FC = fold change; Padj=adjusted p-value

Table 6.2. mRNAs downregulated by more than 1.5 fold (0.58 log2) in JU77 cells upon SAHA treatment.

Gene Symbol	Gene Name	log2FC	Padj
DKK1	dickkopf homolog 1 (Xenopus laevis)	-3.13	4.13E-34
CCND1	cyclin D1	-1.55	4.56E-06
EP300	E1A binding protein p300	-1.52	5.60E-09
WNT5B	wingless-type MMTV integration site family, member 5B	-1.48	7.30E-03
FZD2	frizzled homolog 2 (Drosophila)	-1.44	3.18E-04
WNT3	wingless-type MMTV integration site family, member 3	-1.33	1.18E-02
CUL1	cullin 1	-1.30	2.70E-04
CREBBP	CREB binding protein	-1.27	3.34E-08
FZD8	frizzled homolog 8 (Drosophila)	-1.13	4.41E-02
CTBP2	C-terminal binding protein 2	-1.03	1.10E-02
PLCB4	phospholipase C, beta 4	-1.00	3.25E-05
SMAD3	SMAD family member 3	-0.90	6.53E-05
AXIN1	axin 1	-0.87	8.73E-04
LEF1	lymphoid enhancer-binding factor 1	-0.78	4.41E-02
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	-0.78	4.41E-02
CTBP1	C-terminal binding protein 1	-0.77	8.21E-05
WNT5A	wingless-type MMTV integration site family, member 5A	-0.74	8.25E-02
TBL1XR1	transducin (beta)-like 1 X-linked receptor 1	-0.66	9.49E-03
SMAD4	SMAD family member 4	-0.62	9.77E-02
CSNK2A1	casein kinase 2, alpha 1 polypeptide	-0.61	8.30E-03

FC = fold change; Padj=adjusted p-value

Table 6.3. mRNAs upregulated by more than 1.5 fold (0.58 log2) in ONE58 cells upon SAHA treatment.

Gene Symbol	Gene Name	log2FC	Padj
CXCR4	chemokine (C-X-C motif) receptor 4	4.84	7.40E-13
APC2	adenomatosis polyposis coli 2	2.70	1.81E-06
FZD3	frizzled homolog 3 (Drosophila)	2.28	5.84E-06
FZD4	frizzled homolog 4 (Drosophila)	1.99	2.48E-08
DAAM1	dishevelled associated activator of morphogenesis 1	1.77	1.56E-08
FZD8	frizzled homolog 8 (Drosophila)	1.44	2.24E-03
PRICKLE2	prickle homolog 2 (Drosophila)	1.44	3.29E-06
WNT9A	wingless-type MMTV integration site family, member 9A	1.33	1.07E-03
PPP2R5B	protein phosphatase 2, regulatory subunit B', beta isoform	1.32	1.07E-03
PPP2R5C	protein phosphatase 2, regulatory subunit B', gamma isoform	1.25	4.38E-04
SFRP1	secreted frizzled-related protein 1	1.23	1.02E-03
KREMEN1	kringle containing transmembrane protein 1	1.15	1.58E-04
LGR4	leucine-rich repeat-containing G protein-coupled receptor 4	1.10	6.05E-04
WNT7B	wingless-type MMTV integration site family, member 7B	1.03	3.55E-02
CAMK2G	calcium/calmodulin-dependent protein kinase II gamma	0.95	3.81E-02
FRAT2	frequently rearranged in advanced T-cell lymphomas 2	0.87	4.40E-03
CHP1	Calcineurin-Like EF-Hand Protein 1	0.86	1.15E-02
CCND3	cyclin D3	0.86	3.70E-03
MAP3K7	mitogen-activated protein kinase kinase kinase 7	0.85	1.67E-02
PRKACA	protein kinase, cAMP-dependent, catalytic, alpha	0.65	1.27E-02
LRP6	low density lipoprotein receptor-related protein 6	0.60	1.50E-02
SENP2	SUMO1/sentrin/SMT3 specific peptidase 2	0.59	3.80E-02
NLK	nemo-like kinase	0.58	3.63E-02

FC = fold change; Padj=adjusted p-value

Table 6.4. mRNAs downregulated by more than 1.5 fold (0.58 log2) in ONE58 cells upon SAHA treatment.

Gene Symbol	Gene Name	log2FC	Padj
FOSL1	FOS-like antigen 1	-3.30	1.50E-16
WNT5B	wingless-type MMTV integration site family, member 5B	-2.49	6.92E-09
DKK1	dickkopf homolog 1 (Xenopus laevis)	-2.45	8.30E-10
CCND1	cyclin D1	-2.01	1.56E-08
WNT3	wingless-type MMTV integration site family, member 3	-1.87	1.42E-07
CUL1	cullin 1	-1.70	1.56E-08
SMAD3	SMAD family member 3	-1.63	8.68E-12
EP300	E1A binding protein p300	-1.53	4.97E-14
CREBBP	CREB binding protein	-1.52	1.88E-14
SP5	Sp5 transcription factor	-1.45	1.72E-04
FZD2	frizzled homolog 2 (Drosophila)	-1.28	3.29E-06
CTBP1	C-terminal binding protein 1	-1.18	7.72E-10
CSNK2A1	casein kinase 2, alpha 1 polypeptide	-1.03	5.50E-05
AXIN1	axin 1	-0.96	1.35E-04
SMAD4	SMAD family member 4	-0.91	6.52E-03
RBX1	ring-box 1	-0.90	2.73E-04
PLCB2	phospholipase C, beta 2	-0.88	2.40E-02
CTBP2	C-terminal binding protein 2	-0.88	9.33E-03
BIRC5	baculoviral IAP repeat-containing 5	-0.83	1.69E-02
TP53	tumor protein p53	-0.73	2.65E-02
TBL1XR1	transducin (beta)-like 1 X-linked receptor 1	-0.62	3.65E-03
PLCB4	phospholipase C, beta 4	-0.59	2.02E-02

FC = fold change; Padj=adjusted p-value

Table 6.5. Common* Wnt Pathway mRNAs differentially expressed in JU77 and ONE58 by SAHA

Gene Symbol	Gene Name
Upregulated	
APC2	adenomatosis polyposis coli 2
CAMK2G	calcium/calmodulin-dependent protein kinase II gamma
CCND3	cyclin D3
CHP1	Calcineurin-Like EF-Hand Protein 1
DAAM1	dishevelled associated activator of morphogenesis 1
FZD3	frizzled homolog 3 (Drosophila)
FZD4	frizzled homolog 4 (Drosophila)
LGR4	leucine-rich repeat-containing G protein-coupled receptor 4
LRP6	low density lipoprotein receptor-related protein 6
MAP3K7	mitogen-activated protein kinase kinase kinase 7
PPP2R5B	protein phosphatase 2, regulatory subunit B', beta isoform
PPP2R5C	protein phosphatase 2, regulatory subunit B', gamma isoform
PRICKLE2	prickle homolog 2 (Drosophila)
PRKACA	protein kinase, cAMP-dependent, catalytic, alpha
WNT9A	wingless-type MMTV integration site family, member 9A
Downregulated	
AXIN1	axin 1
CCND1	cyclin D1
CREBBP	CREB binding protein
CSNK2A1	casein kinase 2, alpha 1 polypeptide
CTBP1	C-terminal binding protein 1
CTBP2	C-terminal binding protein 2
CUL1	cullin 1
DKK1	dickkopf homolog 1 (Xenopus laevis)
EP300	E1A binding protein p300
FZD2	frizzled homolog 2 (Drosophila)
PLCB4	phospholipase C, beta 4
SMAD3	SMAD family member 3
SMAD4	SMAD family member 4
TBL1XR1	transducin (beta)-like 1 X-linked receptor 1
WNT3	wingless-type MMTV integration site family, member 3
WNT5B	wingless-type MMTV integration site family, member 5B

* criteria: fold change >1.5 and adjusted p-value <0.1

6.6. Pathway analysis of differentially expressed genes following SAHA treatment in JU77 and ONE58 cells.

In order to assess the functional significance of SAHA-mediated differential gene expression upon Wnt signalling, the normalised expression counts for the genes listed in the KEGG pathway analysis were mapped using the Pathview software package. The significance of the various differential expression counts was determined according to the relative degree of upregulation or downregulation in the β catenin dependent and independent Wnt pathways for JU77 cells (Figure 6.6) and ONE58 cells (Figure 6.7).

In JU77 cells (Figure 6.6), an expression profile suggestive of the inhibition of β catenin-mediated Wnt signalling was observed through a relative reduction in expression of key signalling genes such as the Fzds, casein kinases 1ε and 2, TCF/LEF and c-Myc together with increased expression of the negative regulators Axin and APC which comprise the β catenin destruction complex. A reduction in Fzd gene expression was also seen in the planar cell polarity (PCP) pathway but increased expression of DAAM1 and Rac may indicate an overall activation of the PCP pathway. Similarly, an increased expression of Wnt/Ca^{2+} pathway components CaMKII and Protein Kinase C, together with those of the PCP pathway may indicate the parallel activation of β catenin independent Wnt signalling. Interestingly, the expression of β catenin was not significantly altered at the mRNA level.

A response profile consistent with the attenuation of β catenin-mediated Wnt signalling was also observed in ONE58 cells (Figure 6.7) with the increased expression of APC and a reduction in the expression of Wnt nuclear targets TCF/LEF, c-Myc and Cyclins. However, ONE58 cells also displayed the increased expression of regulatory molecules such as the SFRPs (referred to in diagram as FRPs) and a reduction in the expression of the Wnt ligands. An increased expression of PCP pathway components such as DAAM1 and JNK (Jun-Nuclear Kinase) was also observed as well as a similar degree of upregulation in the Wnt/$Ca2^{2+}$ pathway as that seen in JU77 cells.

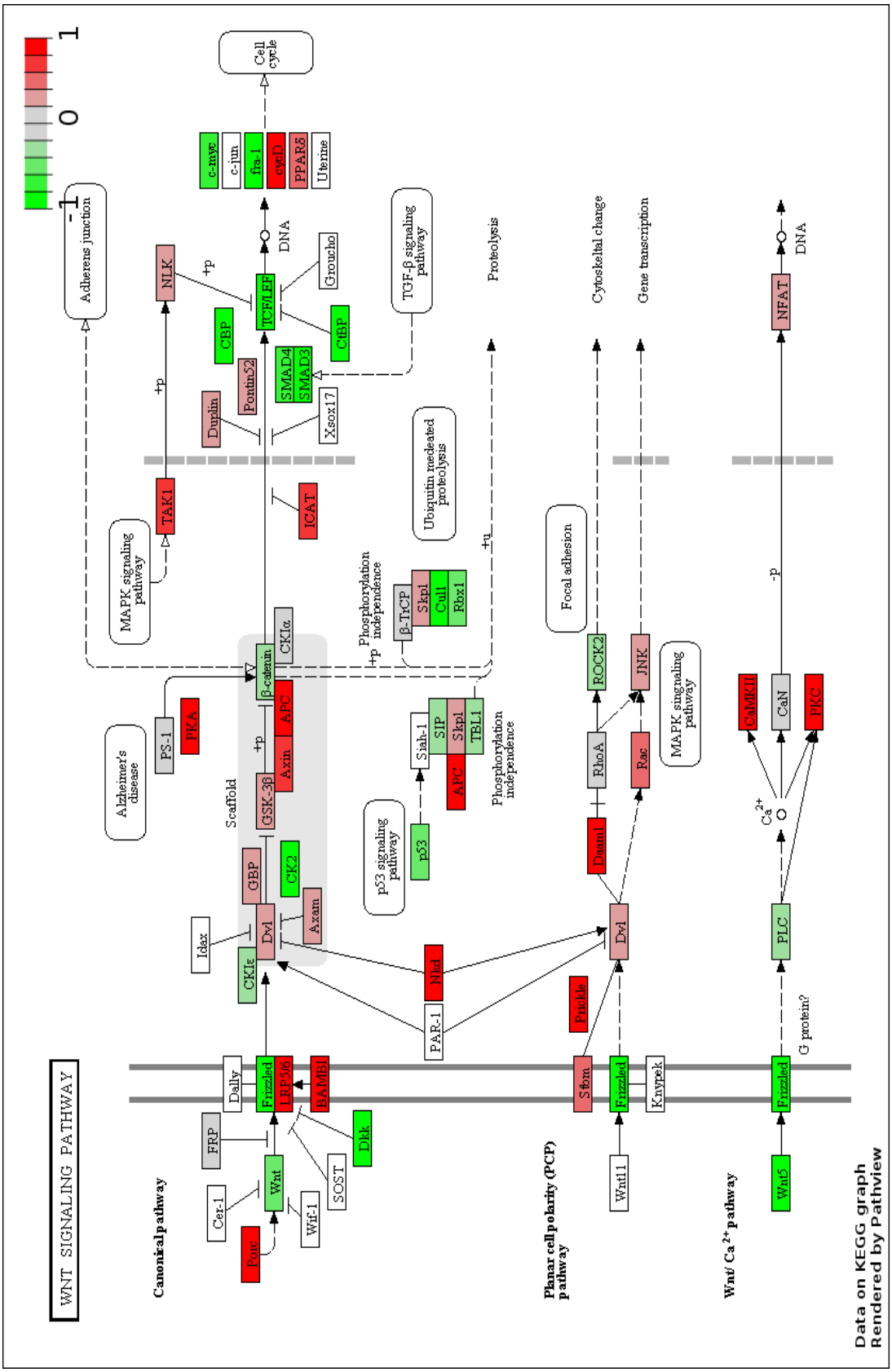

Figure 6.6. Differentially expressed gene (DEG) distribution in KEGG Wnt pathway for JU77 cells in response to SAHA. Gene expression is shown as colour map at individual nodes as indicated in the legend. Multi gene nodes represent sum values.

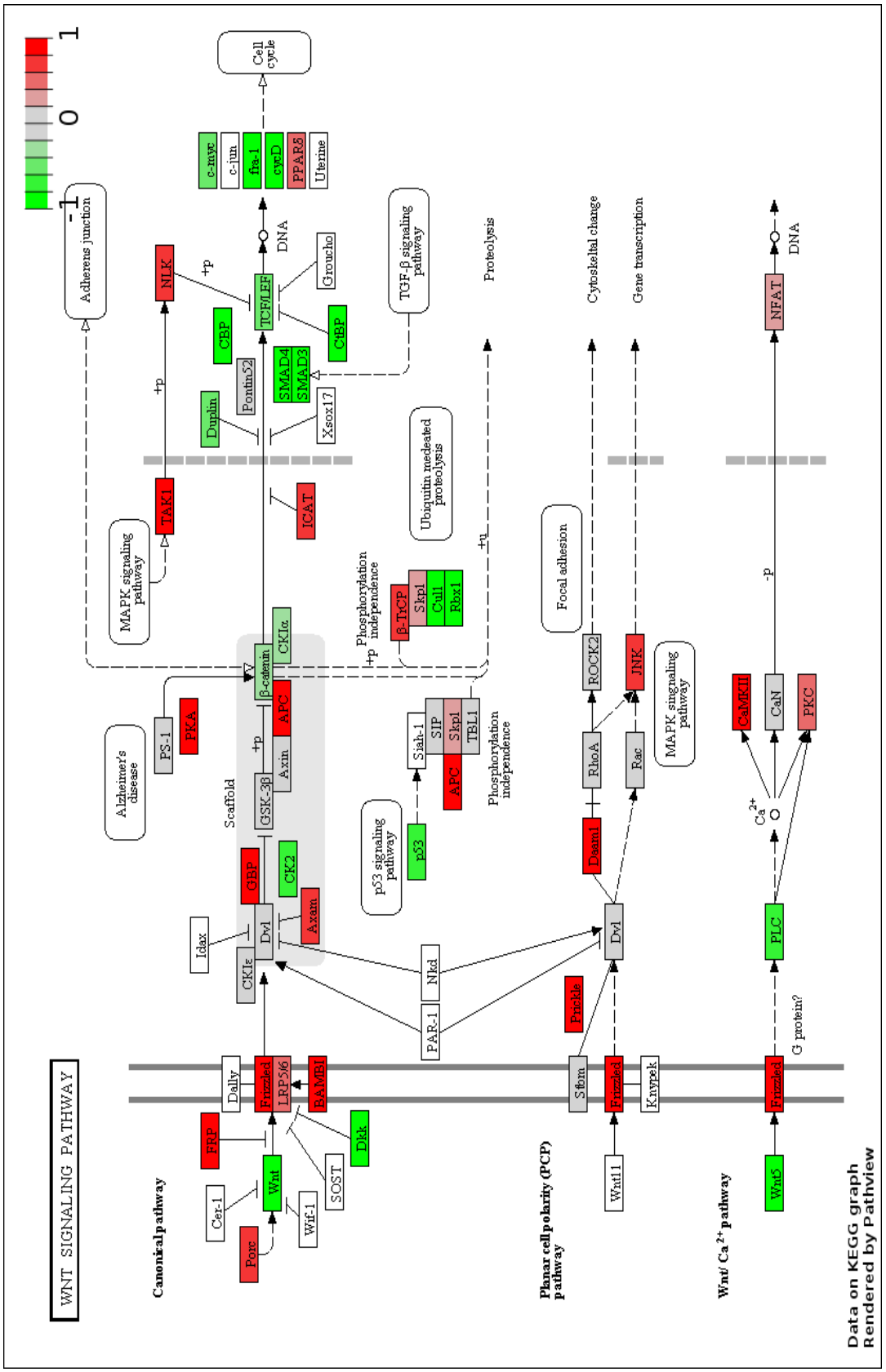

Figure 6.7. Differentially expressed gene (DEG) distribution in KEGG Wnt pathway for ONE58 cells in response to SAHA. Gene expression is shown as colour map at individual gene nodes.

6.7. Gene ontogeny analysis of differentially expressed genes following SAHA treatment in JU77 and ONE58 cells.

Having examined the Wnt pathway, we then undertook a broader analysis of pathways regulated by SAHA in mesothelioma cells by using gene ontology analysis. In general, an overall profile of pathway downregulation was seen and the greatest degree of change being in the expression of cell-cycle control pathways in both cell lines. In JU77 cells, a total of 2865 genes were differentially expressed (> 1.5 fold change, $p < 0.05$) with 1203 genes downregulated and 1662 genes upregulated. For ONE58 cells, 3627 genes were differentially expressed (>1.5 fold change, $p < 0.05$) with 1674 genes downregulated and 1953 genes upregulated.

The gene ontology analysis for JU77 cells treated with SAHA is shown in Figure 6.8 and demonstrates a widespread downregulation of pathways with the greatest degree of relative transcriptional change being in genes associated with cell cycle control. Modulation of gene expression in cancer pathways is also prominent across several different categories of cancers and the upregulation of genes controlling axon guidance was unique compared to the general profile of pathway downregulation. The roles of β catenin-independent Wnt signalling in axon guidance and nerve tract formation are shared between the Wnt/PCP and Wnt/Ca^{2+} pathways (see Section 1.10 for pathway descriptions) and their interplay has been extensively reviewed by Clark and colleagues (2012). The observed upregulation is consistent with the increased expression of β catenin-independent Wnt signalling pathway components seen in Figure 6.6. A more diverse response was seen in ONE58 cells treated with SAHA (Figure 6.9) but the greatest association between transcriptional modulation and gene ontology still being that of cell cycle control. Interestingly, modest effects are seen not only in cancer pathways but also in homeostatic processes including ubiquitination, nucleic acid metabolism and lysine degradation. Other aspects of cellular metabolism such as phosphoinositide metabolism and mineral absorption are also implicated in the effects of SAHA although the upregulation of axon guidance genes is less prominent in ONE58 cells.

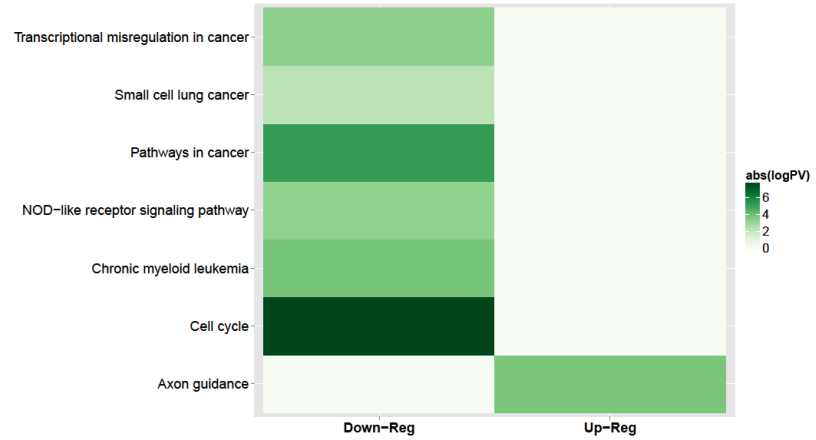

Figure 6.8. Gene ontology analysis of differentially expressed genes in KEGG pathways in JU77 cells. Functional association of magnitude of differential expression is expressed in terms of colour map.

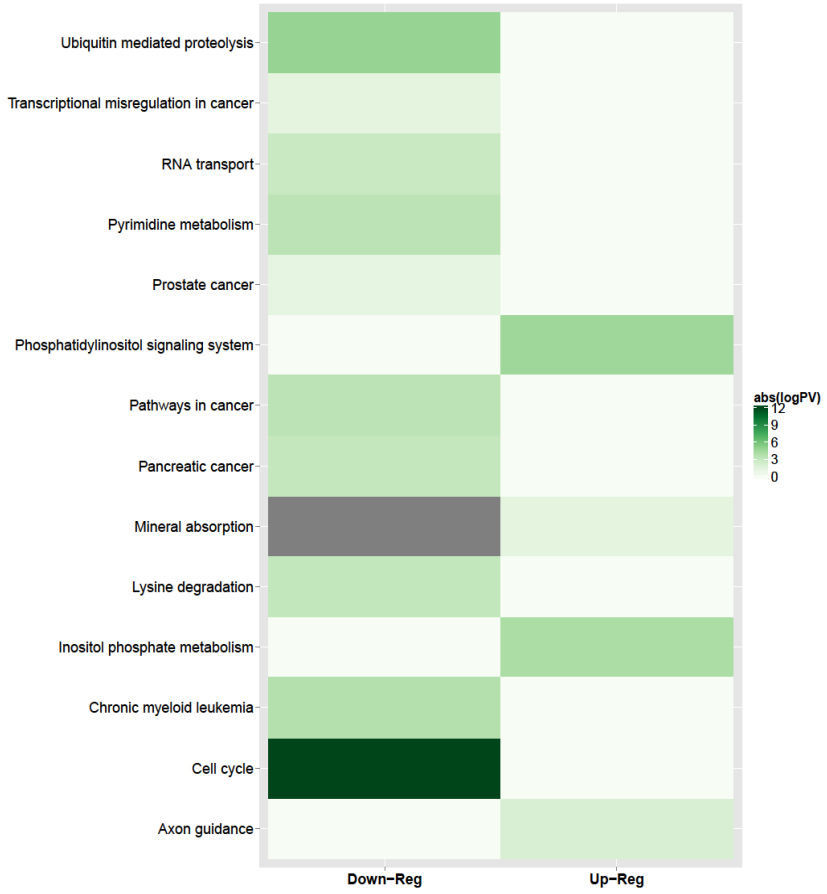

Figure 6.9. Gene ontology analysis of differentially expressed genes in KEGG pathways in ONE58 cells. Functional association of magnitude of differential expression is expressed in terms of colour map.

6.8. Discussion.

At the time of these experiments, investigations into the epigenetic effects of SAHA (Vorinostat) upon Wnt signalling in mesothelioma are still lacking although its pharmacological effects have been characterised in other malignancies. This study however, aimed to provide some *in vitro* insight into the biological effects of histone deacetylase inhibition upon the Wnt signalling system in order to explore potential therapeutic targets. Unlike the modest effects of XAV939 after 48h, treatment with SAHA produced a greater degree of cell death over the same treatment time and interestingly, a loss of viability with SAHA treatment was observed in both 2D and 3D culture methods unlike treatment with XAV939.

The greater loss of viability was observed in 3D with SAHA treatment compared to treatment with XAV939 is consistent with the overall effectiveness of SAHA in these cells and is indicative of effectiveness in the generally more resistant 3D model. The multicellular resistance of spheroids *in vitro* may be due to the tissue specific nature of the extracellular matrix which consists of a mixture of collagen, elastin, fibronectin and heparan sulphate (Santini et al., 2000). Other considerations include the nature of integrin/matrix interactions upon cell survival, cell adhesion molecule (CAM) expression profiles and concentrations of growth factors within a cell aggregate – all of which may contribute to multicellular resistance of spheroids *in vitro* (Santini et al., 2000). It would therefore be of interest to investigate the expression of these genes in this 3D model, however, for practical reasons, this was not feasible in this study.

Investigation into the common differentially expressed Wnt and Wnt-related genes in JU77 and ONE58 cells as a result of SAHA treatment raises several interesting observations and questions which further exemplify the context-dependent nature of Wnt pathway component expression. The expression of Wnt ligands in these cell lines has been described in Section 3.1 and the expression of relatively few Wnt ligands is apparent. The common upregulation of Wnt9a and the common downregulation of Wnt3 and Wnt5b will also require careful, context dependent analysis in order to determine their physiological roles in mesothelioma. Previous analysis by RT-PCR of JU77 and ONE58 cells amongst others (Section 3.1) showed the presence of Wnt3 in both cell lines and therefore its downregulation by histone deacetylase inhibition is of significance in these studies.

The significance of Fzd receptor expression patterns in mesothelioma cells observed in Section 3.2 also requires clarification, as differential expression of the individual Fzds may indicate either an induction or repression of Wnt signalling via a β catenin mediated or alternate pathway. The associations of individual Fzds and their respective malignancies has been expertly reviewed by Ueno and colleagues (2013) and provides some information on the associations of the Fzds as a group in human cancers. The expression of Fzd2 in JU77 and ONE58 cells was also confirmed by RT-PCR analysis and its downregulation in these experiments following SAHA treatment is consistent with its upregulation in other cancers such as lung squamous cell carcinoma and melanoma with its role in mesothelioma being confounded due to its ability to activate either of β catenin-mediated or β catenin independent Wnt signalling (Ueno et al., 2013). Similarly for Fzd3 which was unique to ONE58 cells and also has a ligand-specific role of promoting proliferation or inhibiting of cancer cell motility (Ueno et al., 2013), thereby making it either an oncogene or a therapeutic target albeit in a context-specific manner. The upregulated expression of Fzd4 in both cell lines is contrast to previous reports of the association between Fzd4 downregulation and increased motility in bladder cancer cells (Ueno et al., 2012; Ueno et al., 2013). No significant changes were observed in the expression of Fzd7 in these studies having been detected only in ONE58 cells and poorly expressed in the other cell lines tested (Section 3.2). The role of Fzd7 has been characterised in colon cancer cells (Ueno et al., 2009) and its association with several other human cancers reviewed (King et al., 2012) but little is known about its actions in mesothelioma.

Expression of c-Myc and cyclin D1 has been considered the hallmark response to a β catenin mediated Wnt signal (He et al., 1998; Shtutman et al., 1999; Logan and Nusse, 2004) and the downregulated expression of the common Wnt target gene cyclin D1 in particular is consistent with attenuation of β catenin mediated Wnt signalling and the anti-proliferative effects of SAHA. The downregulation of LEF1 in JU77 cells may also contribute to attenuation of Wnt signalling in these cells, although non-Wnt influences cannot be discounted in this case. Conversely, the upregulation of cyclin D2 in JU77 cells and cyclin D3 in ONE58 cells is somewhat counterintuitive to the effects expected from a cytotoxic drug and represents another opportunity for further examination in studies of mesothelioma.

Downregulation of the critical regulatory component APC was also observed with the upregulation of APC2 being common to both cell lines but not APC itself. The APC2 homologue has been characterised in colorectal cancer as a truncated mutant, thereby rendering it less effective in β catenin regulation compared to wild-type APC (Schneikert et al., 2013) and its upregulation subsequent to SAHA treatment in both cell lines deserves further attention. The differing expression patterns of the Axin genes was observed with the upregulation of Axin2 in JU77 cells but a common downregulation of Axin1 in both cell lines. The significance of this difference is unknown as Axin2 is the predominant, active isoform in the control of β catenin dependent Wnt signalling (Mazzoni and Fearon, 2014). The binding of protein phosphatase 2A (PP2A) to Axin as part of the β catenin degradation complex has been described (for review, see Stamos and Weis, 2013) and that the role of PP2A in particular is one of β catenin *dephosphorylation*, thereby liberating it from the degradation complex (MacDonald et al., 2009) although the precise functions and effects may depend on the composition of the particular PP2A heterotrimer complex (Kimelman and Xu, 2006) thereby explaining the biological relevance of the α and γ regulatory 'B'-subunit upregulation observed in these experiments in response to SAHA treatment.

Several previous studies have described a series of genetic 'signatures' representing the transcriptional profile of various cancer cell type following histone deacetylation by either SAHA or agents belonging to other, distinct chemical categories. Considerable knowledge of these transcriptional signatures has been gained from microarray studies in order to determine critical differentially expressed genes. However, the use of Next Generation RNA sequence analysis has not been previously applied to investigations of histone deacetylation in mesothelioma. An early study by Glaser et al., (2003) described a set of 13 commonly differentially expressed genes in breast and bladder carcinoma cells which showed a strong association between SAHA treatment the expression of genes controlling apoptosis and cell cycle progression. A subsequent study in multiple myeloma cells also produced a set of pathway-specific transcriptional profiles pertaining to apoptosis, cell cycle control, proteosomal regulation and DNA synthesis together with the finding that SAHA can also act as a chemosensitising agent to other apoptotic agents (Mitsiades et al., 2004). Further investigation into the effects of SAHA on apoptotic pathways in T-cell leukaemia have also been carried out (Peart et al., 2005) and studies in renal cell carcinoma lines have produced distinct transcriptional profiles consisting of a set of 20 differentially expressed genes following SAHA treatment and a smaller set of 10 genes after combination treatment

with a retinoid (Tavares et al., 2008). A similarly small set of 11 differentially expressed genes has also been described in colorectal cancer with the suggestion that transcriptional signatures arise in a cell-specific manner and affect only a small number of genes once appropriate analysis has been carried out (LaBonte et al., 2009). The use of autophagy inhibitors (chloroquine and bafilomycin A1) to potentiate the effects of SAHA have also been explored in gastric cancer cell lines with a set of 16 genes differentially expressed as a result of SAHA treatment alone (Claerhout et al., 2011). The gene ontogeny analysis in this thesis study is consistent with the pro-apoptotic / deregulated cell cycle transcriptional profiles described in other studies (Mitsiades et al., 2004; Peart et al., 2005) and these experiments represent the first investigations into the effects of SAHA-mediated histone deacetylase inhibition on Wnt signalling in mesothelioma cell lines. An emphasis was placed on the Wnt signalling system in this thesis study, however, further analysis across the entire transcriptome is currenly under way in the supervisor's laboratory as only the Wnt-related portion of the data pool was used.

Clinically, SAHA (Vorinostat) is indicated for the treatment of cutaneous T-cell lymphoma (CTCL) and its pharmacokinetic properties have led to the assessment of SAHA as an adjunct to other chemotherapy regimens such as proteasome inhibitors, platinum alkylating agents, microtubule inhibitors, topoisomerase inhibitors and anthracyclines (Iwamoto et al., 2013). Other hydroxamic acid derivatives such as belinostat, panabinostat, givinostat, resminostat, PCI24781 and JNJ26481585 are also under evaluation in solid tumours and haematological cancers (Khan and LaThangue, 2012; Lakshmaiah et al., 2014).

Taken together, SAHA has been of considerable scientific value not only in a clinical setting but also *in vitro* as a pharmacological tool with which to investigate the effects of histone deacetylation in mesothelioma and in particular, the Wnt signalling system. This thesis study is to our knowledge, the first effort at utilising RNA sequence analysis in order to elucidate further knowledge in this topic.

Chapter 7.
Summary, general discussion, and future perspectives.

Table of contents.

7.0. Introduction.

The aims of this thesis were to identify and characterise the Wnt signalling system in malignant mesothelioma cells in order to determine potential targets for therapeutic intervention and to describe the molecular mechanisms affected by its modulation. A selective approach of Wnt signal inhibition by tankyrase enzyme inhibition was employed by the use of XAV939 and the effects of histone deacetylase inhibition by SAHA on Wnt signalling in mesothelioma further explored at a molecular level. The presence of β catenin mediated Wnt signalling was established as well as the downregulation of the expression of Wnt4 and SFRP4 in the JU77, LO68 and ONE58 mesothelioma cell lines (Fox et al., 2013). A 3D culture model was developed and utilised to evaluate the effects of the compounds XAV939 and SAHA on cellular proliferation. In addition to proliferation assays in 2D and 3D culture, a series of functional assays were developed to assess the phenotypic effects of Wnt signalling in the form of colony formation, adhesion and migration assays.

Evaluation of the effects upon the expression of Wnt signal components following tankyrase and histone deacetylase inhibition was also undertaken by RNA sequence analysis with application of this analysis to both β catenin dependent and independent pathways. Targeted inhibition of β catenin mediated Wnt signalling by XAV939 produced a moderate effect on Wnt component expression but also yielded a distinct set of genes of interest. The expression of some of these genes has already been shown to be controlled by Wnt signalling with the basis of expression control of other genes remaining unexplored. Deregulation of Wnt and Wnt-associated gene expression by SAHA produced a transcriptional profile consistent with Wnt pathway downregulation along with the transcriptional downregulation of gene groups associated with growth, proliferation and cell cycle control as observed through gene ontology analysis. The antiproliferative effects of XAV939 were more pronounced in 2D culture compared to the marked effects of SAHA in both 2D and 3D culture, but phenotypic effects were still observed with XAV939 in functional assays performed in 2D culture. Taken together, these experiments have allowed a degree of insight into the role of Wnt signalling in mesothelioma.

7.1. Exploration of Wnt signalling in mesothelioma.

The biological consequences of a Wnt signal will require careful interpretation due to the several layers of complexity that characterise the context of a Wnt signal. The particular combination of Wnt ligand and Fzd receptor has been shown to produce differing effects with activation of either the β catenin dependent or independent pathway (Mikels and Nusse, 2006; Rao and Kuhl, 2010; Ring et al., 2014) with the additional consideration of the temporal setting of a cell in either of development (Logan and Nusse, 2004), tissue homeostasis (Yang et al., 2016), disease (Kahn, 2014) and cancer (Yu and Virshup, 2014). The interpretation of a Wnt signal must also include considerations beyond the relatively schematic view of Wnt/Fzd/LRP binding followed by β catenin accumulation or the activation of a β catenin independent pathway to include the influence of secreted mediators such as the SFRPs and WIF which may regulate Wnt signalling through modulating changes in ligand concentration (van Amerongen and Nusse, 2009). This has been demonstrated by Grumolato and co-workers (2010) through studies in the structural basis of differential Wnt signal responses using Wnt3a and Wnt5a which are conventionally considered as activators of β catenin dependent and independent pathways respectively. These authors also propose a role for Dvl in the assignment of a response to a given Wnt/Fzd binding event (Grumolato et al., 2010). The experiments in this thesis have revealed the expression of a selection of Wnt ligands in the JU77, LO68 and ONE58 mesothelioma cell lines in which the expression of Wnts 2b and 4 were downregulated. Also observed, was the varied nature of Fzd receptor and SFRP expression as well as the detection of β catenin expression and the downregulation of the β catenin destruction complex components APC and Axin2.

Considering the large number of potential combinations, the need to elucidate the results of these combinations is an issue deserving of continued attention in order to determine the nature of a Wnt signal and how to modulate it (for review, see van Amerongen and Nusse, 2009). The context and pathway activation(s) of Wnt signalling in cancer is of importance in view of the interplay between the β catenin mediated and β catenin independent divisions of Wnt signalling and their effects upon cellular metabolism (for review, see Sherwood, 2015) and Wnt-induced expression of drug resistance genes such as P glycoprotein (Pinzon-Daza et al., 2014). Furthermore, Wnt signalling as a whole may also be influenced through crosstalk with other signalling pathways such as the Sonic Hedgehog

(SHH) and Transforming Growth Factor β (TGF-β) pathways via common components such as GSK3β in addition to potentially exerting an influence on other non-Wnt like these (for review, see Zhang et al., 2016).

One of the aims of these studies was to describe β catenin-mediated aspect of Wnt signalling in the JU77, LO68 and ONE58 cell lines derived from Western Australian cases of mesothelioma in order to provide some insight into Wnt signalling in this malignancy. Further research into the essential biology of Wnt signalling in all its forms and careful context-dependent interpretation of results may provide a more detailed background with which to understand the pathobiology and refractory nature of mesothelioma.

7.2. Applications of 3D culture in mesothelioma research.

The resistance of cells to a treatment in the 3D setting was first described in a study of irradiated spheroids and termed "the contact effect" (Durand and Sutherland, 1972). Three potential mechanisms were proposed through subsequent investigations in which (i) intercellular communication aids post-exposure recovery, (ii) a potentially enhanced DNA repair efficiency due to DNA compaction and (iii) changes in DNA repair associated genes (Olive and Durand, 1994). Characterisation of resistance in 3D culture to chemotherapeutic agents led to an extension of the 'contact effect' to what is currently referred to as Multicellular Resistance (MCR), a reversible phenomenon that is distinct to multidrug resistance (MDR) mediated by the P-glycoprotein / MDR1 pump. Key differences between MCR and the 'contact effect' concept include (i) the inhibition of apoptosis and/or a decrease in cellular permeability to external reagents, (ii) cell quiescence, (iii) hypoxia and necrosis towards the core region in addition to the changes in DNA repair mechanism efficiency proposed in the contact effect model (Desoize and Jardillier, 2000).

An association between 3D attachment and growth arrest was made between ε-cadherin and the cyclin dependent kinase inhibitor $p27^{KIP1}$ whereby ε-cadherin complexation with receptor tyrosine kinases such as those associated with growth factor signalling produced and upregulation of $P27^{KIP1}$ to produce cell cycle arrest (St. Croix and Kerbel, 1997). The discovery of three novel candidate genes in colon carcinoma cells by Timmins and co-workers (2004) also lends support to proposed MCR mechanisms. Their findings include

the development of an apoptosis-resistant phenotype through downregulation of SKIP3, and hypoxia-stress related gene and negative regulator of the NFκB pathway (Timmins et al., 2004). Other potential mechanisms identified included the upregulation of p48 (a DNA repair associated transcription factor) and S100A4 (a calcium binding protein associated with metastasis) (Timmins et al., 2004). Tissue hypoxia such as that in a spheroid is also associated with the increased expression of the Hypoxia Inducible Factors (HIFs) which regulate the expression of genes associated with energy production, proliferation and metastasis through possible effects on $p27^{KIP1}$, c-Myc and cyclin D1 (Brocato et al., 2014; Kumar and Choi, 2014) and the effects of HIF isoforms on cell migration have been investigated in a low-oxygen 3D culture setting (Ma et al., 2016). Interestingly, a reduction in MDR1 (P-glycoprotein pump) expression in colon cancer spheroids by HIF1α gene silencing has been demonstrated and illustrates a potential crossover point or therapeutic target between multicellular and multi-drug resistance (Chen et al., 2014).

Studies of mesothelioma in 3D culture have been limited to a small number of investigations into the sensitisation to apoptosis of mesothelioma cells grown either as tumour fragments or spheroids. To date, Wnt signalling in mesothelioma has only been examined in 2D culture and the experiments performed in this thesis are the first to explore the effects of Wnt signal inhibition by XAV939 in the 2D and 3D culture of mesothelioma cells. Histone deacetylase inhibition however, has been more widely studied in 2D and 3D culture as well as being used clinically, but its effect upon Wnt signal pathway component expression has been examined for the first time in these experiments.

The first 3D study of mesothelioma used resected tumour fragments and showed inhibition of the phosphoinositide-3 kinase/Akt (PI3K/Akt) pathway with LY294002 and inhibition of mTOR by rapamycin, producing sensitivity to cycloheximide and induction of apoptosis by TRAIL (TNF-related apoptosis inducing ligand) (Kim et al. 2005). The same approach of mTOR blockade was also used to overcome chemotherapy resistance in M28 and REN mesothelioma spheroids where resistance in 3D culture to protein synthesis (anisomycin), histone deacetylase inhibitors (trichostatin A, sodium butyrate) and proteosomal inhibition (MG132) was overcome to various (Barbone et al., 2008). Another similar investigation reinforced the role of the mTOR/PI3K complex in chemotherapy resistance in mesothelioma by demonstrating apoptosis in tumour masses resistant to cycloheximide

and gemcitabine (Wilson et al., 2008). A novel mTOR inhibitor, GDC0980 has also been evaluated in 3D culture and tumour fragments along with the standard therapy of cisplatin and pemetrexed together with bortezomib (a proteasome inhibitor) (Barbone et al., 2015).

The regulation of mesothelioma cell apoptosis by members of the Bcl2 family has also demonstrated in 3D culture using tumour fragments and cultured spheroids by inhibition of Bcl2 function with ABT-737 (Barbone et al., 2011). Resistance to bortezomib, an accumulation of the pro-apoptotic protein Bim and sensitisation to apoptosis was demonstrated (Barbone et al., 2011). Further studies by these authors demonstrated sensitisation to apoptosis and abolition of bortezomib resistance by histone deacetylase inhibition with SAHA together with the potentiation of the cisplatin/pemetrexed response in tumour fragments and cultured spheroids (Barbone et al., 2012).

The use of 3D culture in mesothelioma research is a field that has yet to reach its full potential as a model for research into not only Wnt signalling, but also for investigations into multicellular resistance against clinically utilised agents. Opportunities exist for the continued study of Wnt signalling and the development of Wnt-selective pharmacological agents in a culture model that resembles the physiological microenvironment *in vivo*.

7.3. Application of tankyrase inhibition by XAV939 in cancer.

The development and characterisation of tankyrase inhibition by XAV939 was conducted in colorectal cancer (Huang et al., 2009; Wu et al., 2016) and has recently expanded to include other gene silencing / knockdown methods in other cancers such as neuroblastoma (Tian et al., 2013), lung carcinoma (Lu et al., 2013) and mantle cell lymphoma (He et al., 2015). The experiments conducted in this thesis represent the first investigations of XAV939 in mesothelioma which have shown reductions in viability, colony formation, motility and migration in four mesothelioma cell lines. These observations, together with the studies mentioned above suggest implications for the regulation of metastasis in highly aggressive cancers such as mesothelioma.

A paucity of information exists on the mechanism(s) that resulted in the reduced motility produced by XAV939 treatment with only a single study of XAV939-mediated cytoskeletal deregulation performed in monkey epithelial cells in which, the dissociation of an actin/vinculin/ε cadherin cell-to-cell adhesion complex was described (Lafon-Hughes et al., 2014). Although species and cell context specific differences may exist, some inference may be made in studies of mesothelioma and provide a basis for refined investigations on this topic in mesothelioma. The induction of apoptosis subsequent to XAV939 treatment is the most widely reported form of cell death (Tian et al., 2013; Lu et al., 2013; He et al., 2015; Wu et al., 2016) and an alternate mechanism of cell death via 26S proteasome inhibition has also been proposed (Cho-Park and Steller, 2013). The stabilisation of APC2 by XAV939 treatment has recently been reported in *Drosophila* by Croy and co-workers (2016) and provides further information on the pharmacological effects of this agent.

Selective inhibition of β catenin-mediated Wnt signalling by tankyrase inhibitors in order to produce the stabilisation of the β catenin degradation complex represents an opportunity with which to explore changes in target gene expression and functional phenotype. These studies have yielded a set of target genes differentially expressed as a result of tankyrase inhibition, some of which are not previously associated with Wnt signalling. These may represent cell-specific or novel Wnt target genes and may be influenced by potential crosstalk between the β catenin dependent and independent arms of Wnt signalling.

7.4. Histone deacetylase inhibition and its effects on Wnt signalling and mesothelioma.

Comparatively little information is known about the effects of histone deacetylase blockade in cancer and even less so with regard to its effects in mesothelioma with only a small number of studies on this topic carrying an emphasis on the apoptotic process in particular. The reduction of the expression of the anti-apoptotic protein Bcl-X_L using sodium butyrate has been demonstrated in two mesothelioma cell lines (Cao et al., 2001) and later work by Hurwitz et al., (2011) showed the reversal of the anti-apoptotic properties of FLIP (FLICE Inhibitory Protein) using SAHA in the 2D and 3D culture of seven mesothelioma cell lines. To date, no prior studies have been conducted to investigate the transcriptional modulation produced by histone deacetylase inhibition in mesothelioma and the specific effects upon Wnt signalling have been investigated in this thesis.

An interesting paradox has been described in which Wnt signal activation occurs as a result of histone deacetylase inhibition in a developmental model of mouse oligodendrocytes (Ye et al., 2009), breast cancer cell lines (Debeb et al., 2012), colon cancer cell lines (Godman et al., 2008) and acute T-lymphoblastic leukaemia (Shao et al., 2012), which appears to contradict the generalised assumption of Wnt signal attenuation from HDACi treatment or loss of histone deacetylase function (Kim et al., 2012; Gotze et al., 2014; Chen et al., 2015). Therefore, the possibility of cell type-specific Wnt responses to agents such as SAHA in order to produce an antiproliferative effect is one that requires consideration. The association between the use of valproic acid (a non-hydroxamic acid HDACi) and developmental defects both *in vitro* and *in vivo* has been reviewed by Wiltse (2005) with the proposal that β catenin accumulation as a result of Wnt pathway activation together with deregulated cell adhesion produced developmental defects *in vivo*. Other non-Wnt mechanisms suggested included disruption of the expression of genes related to apoptosis or cell cycle control and interference with folate metabolism (Wiltse, 2005). A comparison of different HDACis in colorectal cancer has also showed an increase in Wnt signalling resulting from treatment with SAHA, Trichostatin A (both hydroxamic acids), sodium butyrate (a short-chain fatty acid) and MS275 (a benzamide derivative) leading to the suggestion that cancers with high Wnt activity may respond by apoptosis but only growth arrest in cancers with lower Wnt dependence (Bordonaro et al., 2007).

The phenomenon of contact resistance in 3D culture of four mesothelioma cell lines and primary tumour tissue fragment spheroids was also partly overcome with the use of SAHA in conjunction with bortezomib resulting in the augmented effects of cisplatin/pemetrexed combination therapy through the restoration of Noxa expression (Barbone et al., 2012). A similar potentiation of cisplatin/pemetrexed by valproic acid has also been demonstrated *in vitro* and *in vivo* by production of reactive oxygen species as a direct effect of valproic acid treatment (Vandermeers et al., 2009). These authors also considered the possibility of using valproate clinically due to its longer half life and more favourable toxicity profile compared to that of SAHA (Vandermeers et al., 2009).

The unique and significant (28.6 fold) upregulation of CXCR4 observed in ONE58 mesothelioma cells as a result of SAHA treatment was an unexpected result, despite it not being associated with classical elements of the Wnt signal pathway. Under physiological conditions, CXCR4 acts as the receptor for the chemokine CXCL12α but its overexpression has been associated with numerous human cancers via mechanisms such as HIF-1 mediated induction of VEGF signalling and effects on cell motility (Sun et al., 2010). A similar finding in primary tissue and mesothelioma cell lines was also made by Li et al., (2011) who also characterised the involvement of Akt (protein kinase B) in the CXCR4/CXCL12α pathway in mesothelioma.

The use of SAHA as a single-agent alternative to the alkylating agent/antifolate regimens was first explored in a Phase I trial of 13 patients with advanced mesothelioma (Krug et al., 2005). However, a larger subsequent study of 661 patients in the VANTAGE-014 trial produced no significant differences in survival time between treated patients (30.7 weeks) compared to control patients (27.1 weeks) leading to the conclusion that SAHA by itself as either a second or third line option did not improve survival time in mesothelioma (Krug et al. 2015). Although unsuccessful in the treatment of mesothelioma, RNA sequence analysis has demonstrated the widespread transcriptional modulation produced by SAHA in this study of mesothelioma. A total of 2865 genes were differentially regulated in JU77 cells and 3627 genes in ONE58 cells as a result of SAHA treatment including a distinct set of 31 Wnt genes as part of the response. Therefore, the outcomes of histone deacetylase inhibition in mesothelioma may be complex and include potentially useful observations in relation to Wnt signal modulation as an avenue for therapy development in the future.

7.5. Limitations of this study.

The concepts explored in these experiments have involved the use of four cell lines in order to investigate the profile of Wnt signalling in mesothelioma. However, the increasing availability of datasets such as RNA sequence analysis or microarray studies derived from clinical tumours will enable the validation of results oberved in cell lines and in turn, allow a greater correlation between *in vitro* and the clinical situation. The application of 3D culture to the mesothelioma cell lines used has allowed the development of 3D models that will be useful for further studies in these and other cell lines.

Analysis of gene expression by RNA sequencing has proved useful in these studies and confirmation of the results observed may be possible by applying this method to alternate strategies of Wnt pathway modulation. The use of nonselective HDAC inhibitors such as SAHA may provide the basis for refined inhibition strategies through the use of agents selective for particular HDAC isoforms. An isoform-selective method would allow further insight into the differential effects of HDAC inhibitors seen between the *in vivo* and *in vitro* settings. The optimisation of pharmacological and pharmacokinetic parameters associated with administration, distribution, metabolism and elimination through structure-activity relationship studies for both tankyrase and histone deacetylase inhibitors may also lead to the development clinically effective treatment regimens. The pharmacological improvement of these agents is required in order to overcome therapeutic considerations such as inhibition selectivity to minimise off-target effects or biological barriers including the composition/penetration of the tumour stroma.

Transcriptome analysis through the use of 'deep sequencing' technologies such as RNA Seq analysis have provided a great degree of insight into the genetic basis of cellular behaviour at the transcriptional level in response to experimental manipulation. Transcriptome analysis serves to corroborate the results observed in functional assays developed using conventional laboratory methods by not only quantifying transcript abundance (as normalised counts) but also variations in transcript sequence and structure as a result of post-transcriptional modifications (for review, see Wang et al., 2009). Information from broad, pathway studies like these may be gained from samples taken from conventional culture methods or adapted to a cell based method of single cell transcriptome analysis with equal reliability (Wu et al., 2014).

The RNA Seq analysis performed in this thesis is an initial effort in understanding the responses of the four mesothelioma cell lines to the inhibition of tankyrase and histone deacetylase enzymes using the basic parameter of normalised transcript counts to evaluate relative changes in gene expression. However, further analysis of transcriptional variants is possible using software suites such as ALEXA Seq which has been validated in a colorectal cancer model using both reverse transcription PCR and quantitative Q-PCR analysis (Griffith et al., 2010). Data obtained by RNA sequence analysis has been successfully demonstrated with results that correlate closely with with these validation methods in mammalian and

non-mammalian models (Asmann et al., 2009; Shi and He, 2014) as well as in a single-cell format (Wu et al., 2014). It is widely believed that studies of systems biology and large scale genome-wide methods such as RNA Seq analysis do not require validation through other methods due to their greater sensitivity and reproducibility. An alternate form of validation has been suggested by Hughes (2009) who recommends analysis of the data to be conducted at the level of the raw data readout, through peer review using identical software in order to detect errors in the raw data. This then obviates the need for additional time and expense associated with the need for other validation modalities which may, in fact be processing faulty data in what may be described as a point-analysis of a large body of bioinformatic results (Hughes, 2009).

7.6. Future research and functional significance.

The experiments performed in this thesis have facilitated the exploration of three key areas of interest within the field of mesothelioma research; namely the expression of Wnt pathway components, the effects of tankyrase inhibition at a genetic and phenotypic level by XAV939 and similarly for histone deacetylase inhibition by SAHA. The differential expression of genes described in chapter 3 presents two interesting questions concerning the expression profile of two Wnt pathway genes; LEF1and Wnt4. The expression of LEF1 was observed in the JU77, LO68 and ONE58 cell lines and conversely, a profound downregulation of Wnt4 expression seen in the same cell lines. Additional verification of LEF1 expression in clinical RNA sequence data sets and subsequent investigation of the functional consequences in a relevant cell model would will allow further insight into the LEF1 dependent targets of β catenin-mediated Wnt signalling. The role of Wnt4 has been described in a developmental context and its downregulation in the three mesothelioma cell lines tested is of significance *in vitro* together with its lack of upregulation subsequent to tankyrase and histone deacetylase inhibition. A comparative analysis with clinical tumour samples may provide further information regarding its role in malignancy. The addition of recombinant Wnt4 did not produce any experimental effects and an alternative approach using endogenous overexpression may be investigated in future studies if supported by clinical tumour expression data.

A novel set of differentially expressed genes as a result of tankyrase inhibition by XAV939 also provides a wide-ranging basis for deeper examination of their role(s) and/or possible modulation by β catenin mediated Wnt signalling. Validation of these genes as Wnt targets in mesothelioma will require an alternative approach to the abolition of β catenin-mediated Wnt signalling through methods such as gene knockdown or RNAi silencing as part of ongoing studies. . The relatively greater degree of transcriptional modulation produced through SAHA-mediated histone deacetylase inhibition has provided insight into the differential expression of Wnt signal pathway components in mesothelioma and will also assist in the establishment of a transcriptional profile in this disease.

Taken together, the description and modulation of Wnt signalling in mesothelioma through the inhibition of two distinct enzyme types will assist in the extension of this topic to other cancers. The introduction of 3D culture to studies of Wnt signalling may also provide a platform for investigating tumour and matrix phenomena such as vascularisation and metastasis in order to understand the aggressive nature of mesothelioma and other, refractory cancers.

Appendix:

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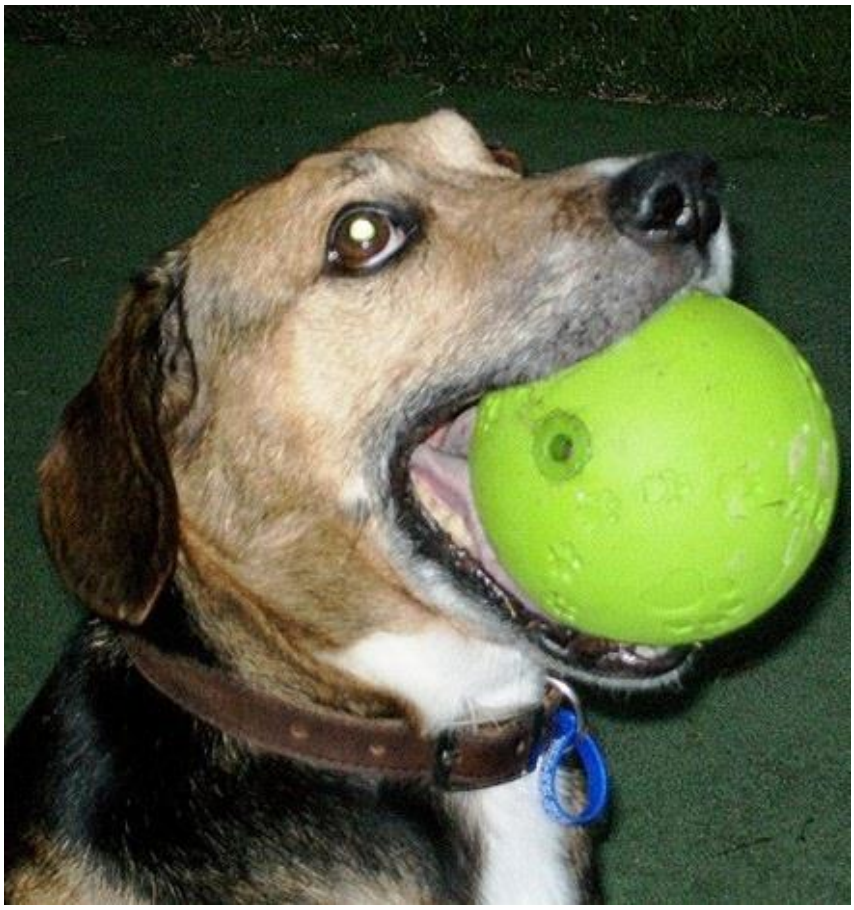

To Dodge.

In death, as you were in life – you will always be The Best.

Thanks mate.