The interaction between hedgehog and TGF-β signalling pathways in malignant mesothelioma

Keyuri Koriya
University of Notre Dame Australia

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The interaction between hedgehog and TGF-β signalling pathways in malignant mesothelioma.
Declaration

I declare that this thesis is my own work on account of my research during the Master of Philosophy degree unless indicated. The contents of this thesis have not been submitted for a degree at any tertiary institution.

________________________________
Keyuri. D. Koriya

________________________________
Date
Malignant mesothelioma (MM) is an aggressive cancer, the consequence of which is neoplastic transformation of mesothelial cells and is strongly associated with previous asbestos exposure. The disease is associated with poor patient survival due to difficulties in diagnosis and treatment options currently available. Therefore, it is essential to identify novel molecular targets that can be used to develop new treatment opportunities. Hedgehog (Hh) and transforming growth factor-beta (TGF-β) signalling pathways are involved in numerous overlapping developmental processes as well as tissue repair and tumorigenesis. Recently, TGF-β has been shown to induce the expression of Hh signalling effector molecules, the Gli transcriptional factors in various cancer cell types. Our laboratory has been interested in assessing the interaction between the TGF-β and Hh signalling pathways and how this interaction may contribute to tumour growth and metastasis in MM.

This study will test the hypothesis that TGF-β induces Gli1 and Gli2 expression in MM cells through activation of the SMAD signalling pathway. To test the hypothesis, this study addresses three aims. Aim 1, to examine the effect of TGF-β on Gli expression. Aim 2, to determine if TGF-β-induced Gli mRNA is independent of the Hh signalling pathway and to examine the effects of blocking TGF-β on Gli mRNA levels and aim 3, to determine the signalling pathway through which TGF-β regulates Gli expression.

MM and control mesothelial cells all expressed variable amounts of basal TGF-β1 and TGF-β2 mRNA with most MM cells expressing quantitatively higher TGF-β1 but lower TGF-β2 mRNA than controls. Basal levels of Gli1 and Gli2 mRNA were variable across the cell lines but Gli2 was upregulated in all cells upon TGF-β2 stimulation. TGF-β1 had no effect on Gli1 or Gli2 mRNA levels and TGF-β2 had no effect on Gli1 mRNA levels. These findings are different to previous studies in different cancers where TGF-β1 has been shown to upregulate early expression of Gli2 mRNA and subsequent upregulation of Gli1. This is the first time the effect of TGF-β2 has been examined in any cell line.

The mechanism of TGF-β2-mediated Gli2 mRNA expression was also examined using pharmacological inhibitors to the type I TGF-β receptor ALK5, and siRNA
against key mediators of the TGF-β SMAD signalling pathway, SMAD2 and SMAD3. Inhibition of ALK5 and SMAD2 and SMAD3 inhibited TGF-β2-induced Gli2 expression, confirming that TGF-β2-mediated Gli2 expression is SMAD2/3 dependent. Further studies are required to determine if TGF-β2-mediated Gli2 expression is independent of the classical Hh signalling Patched/Smoothened (Ptch/SMO) receptor complex.

In conclusion, TGF-β2 was identified as a potent transcriptional inducer of Gli2 transcription factor. These findings may be valuable in providing new therapeutic opportunities to treat MM using targeted TGF-β and Gli therapies. Further studies are now required to examine the functional importance of TGF-β2-mediated Gli2 expression in MM cell function and tumour growth.

In conclusion, TGF-β2 was identified as a potent transcriptional inducer of Gli2 transcriptional factor. These findings may be valuable in providing new therapeutic opportunities to treat MM using targeted TGF-β2 and Gli2 therapies. However, to make a definitive conclusion about this aspect, further studies need to be completed examining their functional importance in MM tumour growth.
Acknowledgements

I would like to thank my supervisors A/Prof. Steven E. Mutsaers, A/Prof. Cecilia M. Prêle, Dr. Bahareh Badrian and A/Prof. Gerard Hoyne for their guidance and support throughout the course of my Masters project. Thank you for your patience, kindness, encouragement and your academic experience which have been invaluable for me.

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I feel very privileged to have been provided with the financial support from the University Of Notre Dame. This aid has allowed me to focus on my project and greatly reduced the financial stress I would have otherwise felt for the duration of the course.

Finally, I would like to thank my family for their unconditional love and support both financially and emotionally throughout my degree, which have been my pillar, my joy and my guiding light to complete this project to the best of my ability.
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<td>ALK</td>
<td>Activin receptor Like Kinase</td>
</tr>
<tr>
<td>ALK5 inh</td>
<td>ALK5 inhibitor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium per Sulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAP1</td>
<td>BRCA-1 associated protein 1</td>
</tr>
<tr>
<td>BCC</td>
<td>Basal Cell Carcinoma</td>
</tr>
<tr>
<td>Bel-2</td>
<td>B-cell Lymphoma 2</td>
</tr>
<tr>
<td>BMI-1</td>
<td>B-lymphoma Insertion region-1 homolog</td>
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<tr>
<td>BMP</td>
<td>Bone Morphogenic Proteins</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>ºC</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>Cont</td>
<td>Control</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cm²</td>
<td>Centimeter²</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic Myeloid Leukaemia</td>
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<td>CSCs</td>
<td>Cancer Stem Cells</td>
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<tr>
<td>CSK</td>
<td>Casein Kinase</td>
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<tr>
<td>CTGF</td>
<td>Connective Tissue Growth Factor</td>
</tr>
<tr>
<td>Cyclo</td>
<td>Cyclopamine</td>
</tr>
<tr>
<td>dhh</td>
<td>Dessert Hedgehog</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double deionised water</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DNase</td>
<td>Dioxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide tri-phosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>ECM</td>
<td>Extra-cellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal Transition</td>
</tr>
<tr>
<td>EPP</td>
<td>Extrapleural Pneumonectomy</td>
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<td>Extracellular signa regulated Kinase</td>
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<td>Gli</td>
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<tr>
<td>GSK3</td>
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<td>H₂O₂</td>
<td>Hydrogen Peroxide</td>
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<tr>
<td>HCC</td>
<td>Hepatocellular Carcinoma Cells</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid</td>
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<td>Hh/hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>Hhip</td>
<td>Hedgehog interacting protein</td>
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<tr>
<td>HMGB1</td>
<td>High-mobility group box 1</td>
</tr>
<tr>
<td>Hr</td>
<td>Hour</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase Substrate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Ihh</td>
<td>Indian Hedgehog</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL8</td>
<td>Interleukin 8</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>Potassium Dihydrogen Phosphate</td>
</tr>
<tr>
<td>#L</td>
<td>#litres</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency Associated Peptide</td>
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<tr>
<td>L-Glut</td>
<td>L-glutamine</td>
</tr>
<tr>
<td>LN$_2$</td>
<td>Liquid Nitrogen</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of Heterozygosity</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
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<tr>
<td>mg</td>
<td>milli-grams</td>
</tr>
<tr>
<td>µg</td>
<td>Micro-grams</td>
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<tr>
<td>MgCl</td>
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</tr>
<tr>
<td>µm</td>
<td>micro-moles</td>
</tr>
<tr>
<td>MM</td>
<td>Malignant Mesothelioma</td>
</tr>
<tr>
<td>MPM</td>
<td>Malignant Pleural Mesothelioma</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>mL</td>
<td>milli-litres</td>
</tr>
<tr>
<td>µL</td>
<td>Micro-litres</td>
</tr>
<tr>
<td>mAmps</td>
<td>milli-amps</td>
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<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Na&lt;sub&gt;3&lt;/sub&gt;VO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Sodium Orthovnanadate</td>
</tr>
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<td>NaCl</td>
<td>Sodium Chloride</td>
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<tr>
<td>NaF</td>
<td>Sodium Fluoride</td>
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<td>Neurofibromatosis type 2</td>
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<td>Nuclear Factor κ B</td>
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<td>Nanogram</td>
</tr>
<tr>
<td>nmol</td>
<td>nano-moles</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitrogen Species</td>
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<tr>
<td>No si</td>
<td>No siRNA</td>
</tr>
<tr>
<td>NP40</td>
<td>Nonyl phenoxypolyethoxylethanol</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small Cell Lung Cancer</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phospho-buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PGK-1</td>
<td>Phosphoglycerate Kinase 1</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
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<tr>
<td>PMM</td>
<td>Peritoneal Mesothelioma</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethysulfonyl fluoride</td>
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<tr>
<td>P/S</td>
<td>Penicillin/Streptomycin</td>
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<tr>
<td>pSMAD2</td>
<td>Phosphorylated SMAD2</td>
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<td>pSMAD3</td>
<td>Phosphorylated SMAD3</td>
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<td>Ptch</td>
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PVDF       Polyvinylidene Fluoride
RNA        Ribonucleic acid
ROS        Reactive Oxygen Species
RNase       Ribonuclease
RT         Reverse Transcription
SB         SB-431542
SCLC       Small Cell Lung Cancer
sec        Second
SDS        Sodium dodecyl sulphate
SEM        Standard error of mean
shh        Sonic Hedgehog
shRNA      Small Hairpin RNA
siRNA      Small Interfering RNA
siSD2      siRNA to SMAD2
siSD3      siRNA to SMAD3
siSD2/3    combined siRNA to SMAD2 and SMAD3
SLB        Stress Lysis Buffer
sTGF-βR    small TGF-β type II receptor
sufu       Suppressor of Fused
Smo        Smoothened
SV40       Simian Virus 40
TB2        TGF-β2
TBS        Tris Buffer Saline
TBS-T      Tris buffer saline-Tween-20®
TEMED      Tetramethylenediamine
TGF-β      Transforming Growth Factor – β
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor α</td>
</tr>
<tr>
<td>TβRI</td>
<td>TGF-β receptor I</td>
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<tr>
<td>TβRII</td>
<td>TGF-β receptor II</td>
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<tr>
<td>YAP</td>
<td>Yes-associated Proteins</td>
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<td>V</td>
<td>Volts</td>
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<td>v/v</td>
<td>volume/volume</td>
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<td>w/v</td>
<td>Mass/Volume</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<td>ZPA</td>
<td>Zone of Polarizing Activity</td>
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Chapter 1
Introduction &
literature review
1.1 Introduction

The mesothelium comprises a monolayer of mesothelial cells extending over the surface of the serosal cavities and organs (Whitaker, Papadimitriou & Walters, 1982), with a main functional role of providing frictionless free movement between these tissues and organs, as well as protecting from infection and injury (Yung & Chan, 2007). Malignant transformation of mesothelial cells is closely linked to asbestos exposure (Spugnini, Bosari, Citro, Lorenzon, Cognetti, Baldi, 2006), and leads to the development of malignant mesothelioma (MM) (Mutsaers, 2004), MM an aggressive cancer with a poor prognosis. It has been well established that MM is largely unresponsive to traditional cancer treatments such as chemotherapy or radiation (Spugnini et al., 2006) and therefore novel therapeutic approaches are required.

In recent years the role of developmental signalling pathways has become an important focus of research in certain cancers and increasing evidence implicates the reactivation and aberrant expression of these pathways as critical to their pathogenesis. Two of these pathways are the hedgehog (Hh) and transforming growth factor β (TGF-β) signalling pathways. Both pathways have been individually studied in various cancers, however the molecular and cellular mechanisms behind their regulation of tumour growth are still unclear. Recent evidence has demonstrated a link between these pathways, and how they may work together to promote tumour growth and metastases in various cancers including gastric, breast and pancreatic adenocarcinomas. This current study will examine the interaction between the Hh and TGF-β signalling pathways in MM. The emerging picture of Glioma-associated oncogene homologue (Gli) transcription factors as an integrative platform of signalling pathways could have important implications in understanding tumour growth and development. Thus, examining how these pathways interact will expand the current understanding of the growth and development of this disease which may open new avenues for therapeutic approaches against MM progression.
1.2 The mesothelium

1.2.1 Structure

The mesothelium is derived from mesodermal tissue and extends over the entire surface of the pleura, pericardial and peritoneal surfaces (Mutsaers, 2004; Whitaker et al., 1982) and lines the sac that surrounds the testes (Mutsaers, 2004). The cells are 25µm in diameter and predominantly flattened, squamous like with surface microvilli resting on a thin basement membrane supported by connective tissue stroma and are present in greater numbers on the visceral (outer) surfaces compared to the parietal (inner) surfaces, Figure 1.1 & 1.2 (Whitaker et al., 1982).

Figure 1.1: A double layered mesothelium (arrows) on the visceral serosa of the lung of a 4 ½ week old human embryo (magnification x 500) (reproduced from Whitaker et al., 1982).

Despite the fact that the mesothelium is mainly composed of flattened cells, cuboidal cells can also be found in the milky spots of the omentum, within the septal folds of the mediastinal pleura and on the peritoneal side of the diaphragm overlying the lymphatic lacunae (Whitaker et al., 1982).
Figure 1.2: Electron micrograph of luminal aspect of mesothelium covered with a carpet of microvilli. Bar 3.2 µM (reproduced from Mutsaers, 2004).

1.2.2 Function

Although the structural organisation of the mesothelium indicates that it is a simple tissue, its functions are quite complex. The major functional roles of the mesothelium is to serve as a protective barrier against abrasion and infection, and to provide a frictionless interface to facilitate the free movement of opposing tissues and organs (Mutsaers, 2004). Tight junctions bind the mesothelial cells together. Secreted surface glycosaminoglycans, proteoglycans and surfactant lubricants secreted by the mesothelial cells, provide a non-adhesive glycocalyx and fluid lining that protects the serosal surface from abrasive damage and infection (Mutsaers, 2004; Whitaker et al., 1982).

Compelling evidence also highlights the importance of the mesothelium immune surveillance, where the mesothelium can initiate a specific immune response against an invading agent (Yung & Chan, 2007). The mesothelium can also transport fluid and solutes across the serosal membrane through pinocytic vesicles, intracellular junction and stromata (supporting framework of a cell), which allows rapid removal of fluid and cells from the serosal cavities to the underlying lymphatic system (Yung & Chan, 2007). In addition to these function, the mesothelial cells participate in
initiating and resolving serosal inflammation and repair, preventing adhesion formation through lysis of fibrin deposits and providing protection (a process of the immune system whereby cells capture antigen and enable their recognition by T-cells) against invading microorganisms (Mutsaers, 2004). The mesothelium also performs other functions that are essential for serosal homeostasis such as antigen presentation, synthesis of inflammatory growth factors and mediators (Mutsaers, 2004; Yung & Chan, 2007) and an active role in fibrin deposition and clearance (Yung & Chan, 2007).

1.3 Malignant Mesothelioma

MM is classified by its histological appearance, and according to the World Health Organization (WHO), this classification is in four main types; epithelioid, sarcomatoid, desmoplastic and biphasic (Izzi, Masuelli, Ttesoldi, Foti, Modesti & Bei, 2012). Patients with epithelioid MM often survive longer, while sarcomatoid MM is associated with poor prognosis (Sekido, 2010). The most important risk factor associated with MM is exposures to silicate asbestos fibres, but simian virus 40 (SV40) and genetic predisposition have also been linked to the disease (Husain et al., 2009). MM is resistant to chemotherapy and radiation therapy; in addition, current therapeutic approaches have so far proven to be ineffective to halt disease progression (Spugnini et al., 2006).

Malignant pleural mesothelioma (MPM) is the most common form of MM, followed by peritoneal malignant mesothelioma (PMM), then pericardial MM (Sekido, 2010). MPM patients commonly present with a pleural effusion associated with chest pain and dyspnoea (Boutin, Schlesser, Frenay, & Astoul, 1998). PMM is often found as a large tumour mass on the omentum, or as a diffuse tumour of the intestinal serosa (Moore, Parker & Wiggins, 2008). PMM presents with nonspecific symptoms including nausea, vomiting, constipation, diarrhoea and loss of appetite, with small bowel obstruction a late feature (Moore et al., 2008; Spugnini et al., 2006).
1.3.1 Epidemiology

MM before 1950 was so rare that pathologists questioned its existence (Garlepp & Leong, 1995). Wagner and colleagues established the link between asbestos and MM in 1960 based on the epidemiological data from South Africa (Wagner, Sleggs & Marchand, 1960). In Australia, the first reported case of MM was in a Wittenoom worker in 1962 and reports from other Australian states followed thereafter (McNulty, 1962). Although the use of asbestos was widely abandoned around 1980’s in the western world, the long latency period between the exposure to asbestos and the onset of MM, which can range from 15 to 60 years, meant that the mortality rates from MM have continued to rise (Moore et al., 2008). The occurrence of MM is higher in males (70-80%) than in females, and the diagnosis of the disease is usually made 20 to 40 years following exposures to asbestos (Moore et al., 2008). Based on this, the worldwide incidence of MM is predicted to peak in the next 20 years (Rudd, 2010). However, in non-western and developing countries where asbestos was used later then in western countries in some cases is still being used, there is a predicted corresponding delay in the anticipated peak incidences of MM (Leigh, Davidson, Hendrie, & Berry, 2002).

1.3.2 Aetiology

Asbestos refers to a group of naturally occurring hydrated mineral silicate fibres (Moore et al., 2008). The two main forms are serpentine asbestos; represented by chrysotile (white asbestos), and amphibole asbestos; which includes crocidolite (blue asbestos), amosite (brown asbestos), actinolite, tremolite and anthophyllite, (Rudd, 2010). Crocidolite, comprising of long thin fibres, are considered to be the most oncogenic type of asbestos fibre because they are able to penetrate the lungs resulting in repeated damage and tissue repair as well as local inflammation (Sekido, 2008).

The action of asbestos fibres in the pathogenesis of MM is still unclear, but the toxicity of most asbestos is dependent on the chemical and physical properties of the fibres and the time from the initial exposure (Kamp, 2009). Other factors such as
genetic predisposition are also likely to impact on the development of MM (Sekido, 2008). For example, studies examining Turkish families from three different villages, showed a cluster of MM cases with an autosomal dominant pattern, suggesting a possible genetic cause for the disease (Dogan, Baris, & Dogan, 2006).

SV40 is a DNA virus found in both monkeys and humans which can induce tumour development by blocking tumour suppressor genes (Rudd, 2010). SV40 virus has been proposed as an etiological agent for MM, but although its role in MM pathogenesis remains controversial (Murthy & Testa, 1999). It is thought to have a strong co-carcinogenic effect when associated with asbestos (Murthy & Testa, 1999).

### 1.3.3 Pathogenesis

MM induced by asbestos is thought to occur in four main processes. Firstly, asbestos has the ability to penetrate deeply into the serosal cavities and is thought to contribute to mechanical injury to the mesothelial cells which than initiates repeated cycles of damage, repair and inflammation (Rudd, 2010). Secondly, asbestos fibres may pierce the cells and disrupt the mitotic spindle of cells, affecting mitosis which may result in chromosomal damage such as aneuploidy (Sekido, 2010). Loss of chromosomes in MM, such as chromosome 22, causes aberrant or no expression of the Neurofibromatosis type 2 (NF2) tumour suppressor gene (Murthy & Testa, 1999). It has been noted that NF2 gene is inactivated by homozygous deletion, missense or nonsense mutations in 40-50% of MM cases (Sekido et al., 1995). A recent study has demonstrated that somatic mutations in the BAP1 (BRCA 1-associated protein 1) gene caused by the loss of an allele at 3p21 region, was found in 23% of MM patients (Rascoe, Jupiter, Cao, Littlejohn, & Smythe, 2012). Thirdly, asbestos can cause DNA strand breaks mediated by iron catalysed free radicals. Asbestos causes the release of reactive oxygen species (ROS) and nitrogen species (NOS), which can directly induce genotoxicity including base deletions, substitutions, insertion, sister chromatid exchange, rearrangements and chromosomal aberrations (Kamp & Weitzman, 2009). These may then lead to mutations in the mammalian cells, and subsequently the cell transforms and is able to resist apoptosis.
and undergo transformation (Kamp & Weitzman, 2009). Recently, Shukla et al. (2009) proposed that asbestos causes mesothelial cell death, primarily through programmed necrosis, involving H$_2$O$_2$ secretion, ATP depletion and high-mobility group box 1 protein (HMGB1) release, thereby promoting an inflammatory response. Fourthly, asbestos fibres induce the persistent activation of signalling pathways such as ERK1/2 involved in cell proliferation, protein metabolism and signal transduction, which are significantly altered in mesothelial cells after asbestos exposure (Kamp & Weitzman, 2009). Recent studies demonstrated critical roles for tumour necrosis factor-alpha (TNF-α) and nuclear factor-kB (NF-kB) in MM pathogenesis (Yang et al., 2006). Following asbestos exposure, macrophages accumulate in the pleura and lungs that secrete TNF-α, which binds to its receptor TNF-R1 and activates the NF-kB pathway. This activation allows mesothelial cells with asbestos induced DNA damage to divide rather than die, eventually developing MM (Yang et al., 2006). In addition to TNF-α, other cytokines and growth factors have been implicated in asbestos carcinogenesis, and their role in MM pathogenesis is being investigated (Sekido, 2010). Examples include: transforming growth factor-β (TGF-β), interleukin (IL) 6 and 8, which may have a role in stimulating tumour growth, and platelet derived growth factor (PDGF), which might act as regulatory factor in MM cell proliferation (Shukla et al., 2009).

1.3.4 Current Treatments

MM has a poor prognosis with an inevitable fatal course and very few long term survivors. MM cells have shown particular resistance to current available chemotherapy treatments with a median survival of 8 to 12 months and less than 20% response rate. Single line therapy (recommended initial treatment for MM) has not been successful. To date, the combination of pemetrexed and cisplatin has yielded the best results, with 12.1 months survival rate, and 41% response rate in a phase III clinical trial (Vandermeers et al., 2009). However, these agents interfere with the cell division process and in turn activates apoptosis (Spugnini et al., 2006), and the major concern with this treatment is rapid relapse, acute toxicity and shorter response duration. Radiation therapy has also been ineffective in prolonging patient
survival. However, some studies have shown gross disease regression after radiation therapy (Gregoire, 2010). In some studies, surgery has had beneficial responses with pleurectomy and extrapleural pneumonectomy (EPP). The most effective pleurectomy involves removal of the parietal (lines the inner pleural cavity and covers the diaphragm) and/or visceral (a thin serosal membrane around the inner walls of the lungs) pleura, leaving the underlying lung. EPP involves removal of the entire pleural envelop and its content as well as the affected lung, diaphragm and a portion of the pericardium. However, both pleurectomy and EPP have not consistently been shown to significantly prolong MM patient survival (Sugarbaker, Heher & Lee, 1991).

In general, therapeutic options including surgery, radiation, chemotherapy, immunotherapy and gene therapy, have had limited success to date. Therefore, current research is focussing on targeted molecular therapeutic approaches. Gene mutations and accumulation of these mutations, together with aberrant activation of growth regulatory pathways, has been linked to many cancers including MM. Hence, a better understanding of the underlying mechanisms regulating MM growth will help to identify molecular targets that can be used for diagnosis, prognosis and therapies. This is critical for many Australians with past asbestos exposure who have an increased future risk of developing MM.

1.4 Role of signalling pathways in MM
There has been a lot of interest in recent years in the role of cell signalling pathways in cancer. In particular the reactivation of classical developmental signalling pathways’, and their role in the early event of cell transformation. Reactivation of these pathways leads to various processes such as cell cycle regulation, apoptosis and angiogenesis. There is also increasing evidence of aberrant activation of these pathways. It is evident that distinct epigenetic and genetic events characterize the interplay between the molecules involved in these pathways. Involvement of the cytokine TNF-α and molecular pathway NF-kB in MM have been briefly described earlier. Similarly, studies have shown the involvement of other growth factors and
molecular pathways linked to MM, among these is TGF-β together with Ras, Hippo and Wnt signalling pathways (Zucali, Ceresoli, De Vincenzo, Simonelli, Lorenzi, & Gianoncelli, 2011). Another pathway, the Hedgehog pathway, has clearly been associated with tumorigenesis, with mutations in this pathway leading to hyperactivation of the pathway identified in a variety of cancers. However, its reactivation and aberrant activation in the growth and development in MM remains to be determined. In addition, recent evidence has shown crosstalk between the Hh and TGF-β signalling pathways. Several studies have shown that TGF-β can upregulate the important Hh pathway effector molecule Glioma-associated oncogene homologue (Gli) (Zucali et al., 2011). As we know that TGF-β is an important molecule for MM growth, studies into the interaction of these pathways in MM may identify novel therapeutic approaches to treat this disease.

1.5 The Hedgehog signalling pathway in development

Complex body plans requires the involvement of cell-cell signalling pathways. The discovery of the Hh gene was made through a genetic screen of mutations that disrupt the Drosophila larval body plan (Nusslein-Volhard & Wieschaus, 1980). The gene mutation resulted in disruption to the dorsal-ventral body plan of the larva leading to an abnormal distribution of hair-like projections on its ventral surface, called denticles. These hairs are distributed at regular intervals forming bands across the ventral surface and they appear along the entire length of the larva. The gene mutation identified by Nusslein, Volhard and Wieschaus lead to ectopic production of denticles on the ventral surface leading to the physical appearance of the larva that resembled that of a hedgehog. Thus, the gene and subsequent signalling pathway was given the name Hedgehog signalling pathway (Ingham & McMahon, 2001). Hh is a secreted protein that acts as an intercellular signalling protein. Hh proteins have been identified in a range of vertebrate species that function as key mediators of many fundamental processes. There are three mammalian Hh genes activating the same canonical signalling pathway, and these are sonic hedgehog (Shh), desert hedgehog (Dhh) and indian hedgehog (Ihh) (Ingham & McMahon, 2001). Shh is the most extensively studied ligand that has not only been associated with developmental
processes but also more recently has been implicated in some disease processes in man (Watkins, Berman, Burkholder, Wang, Beachy & Baylin, 2003).

1.5.1 Biological function
The Hh family of intercellular signalling proteins have been recognised as key mediators of several fundamental processes in embryonic development (Ingham & McMahon, 2001). The activities of these proteins are critical to the growth, morphogenesis and patterning of many different regions within the body plans of insects and vertebrates, and in most invertebrates (Ingham & McMahon, 2001). In embryonic development of a Drosophila, Hh is a central patterning signal in the wing, eye discs and leg (Herberlein, Singh, Luk, & Donohoe, 1995), together with regulating other processes such as germ cell migration, development of gonad, abdomen, tracheal system and optic lamina (Glazer & Shilo, 2001). Hh genes have also been identified in other species such as sea urchin and leech (Chang, Lopez, Von Kessler, Chiang, Simandl, Zhao, 1994). In vertebrate embryos, Shh expression has been observed in three key signalling centres, the floor plate, notochord and the zone of polarizing activity (ZPA), during limb bud development (Ingham & McMahon, 2001). The importance of notochord and floor plate is in the ventralization of the developing neural tube, whereas the ZPA plays a role in regulating anterior polarity (Ingham & McMahon, 2001). This suggests that the discovery of Shh provided an insight into neural tube and limbs formations thus, suggesting a molecular link in the development of distinct vertebrate body plans. In general, Shh signals can regulate cell proliferation, cell fate specification, and cell survival by inducing the expression of a range of target genes. For instance, Shh in blood cells plays a role in stem cell proliferation, thrombocyte differentiation and activation of haematopoiesis (Dyer, Farrington, Mohn, Munday & Baron, 2001). In the heart, Shh controls cardiac morphogenesis (Zhang, Santos & McMahon, 2001). In the limbs, Shh regulates anterior-posterior patterning of the skeleton (Dyer et al., 2001). In the lungs, Shh controls branching epithelium together with proliferation and survival of mesenchyme cells (Pepicelli, Lewis & McMahon, 1998). Shh also controls growth and morphogenesis in prostate and tooth (Ingham & McMahon,
Crucial developmental functions of Hh signalling in embryonic development is illustrated by defects in this pathway, which results in several foetal abnormalities in the brain, facial and midline (Belloni, Muenke, Roessler, Traverso, Siegel-Bartelt, & Frumkin, 1996). These defects are defined in holoprosencephaly, microencephaly and cyclopia (Gupta, Takebe & LoRusso, 2010). Holoprosencephaly is a disorder involving the development of midline and forebrain, in which the ventral cell types are lost, and in humans is caused by mutations that lead to Shh activity (Belloni et al., 1996).

In adults, the Hh pathway remains active, and is particularly involved in regulating tissue homeostasis and in continuous renewal and repair processes of adult tissues (Ingham & McMahon, 2001). The Hh pathway also plays a critical role in adult stem cell maintenance (Hooper & Scott, 2005). Most of the renewing tissues, such as skin and bone marrow, are maintained by stem cells that demonstrate the ability to both give rise to mature cell types of that specific tissue and to generate additional stem cells. The Hh signalling regulates stem cell activity through stimulating proliferation and self-renewal of stem cells in various tissues ((Reya, Morrison, Clarke & Weissman, 2001).

### 1.5.2 Signal Transduction

Hh signal transduction is induced after the initial modification of the Hh ligands. The Hh proteins are synthesised as 45kD precursor proteins that undergo intramolecular cleavage (Lee, Ekker, Von Kessler, Porter, Sun & Beachy, 1994). This modification is through the autocatalytic cleavage of the C-terminal domain, combined with binding of a cholesterol moiety at this cleavage site (Mahindroo, Punchihewa & Fuji, 2009). In addition to cholesterol coupling, the ligand is further lipid modified via palmitoylation of the N-terminal cysteine, giving rise to the active form of the Hh ligand (Ingham & McMahon, 2001). In embryonic development, the cells synthesizing Hh ligands are distinct from the Hh responsive cells that may be either situated adjacent to, or at some distance from the Hh secreting cell (Varjosalo & Taipale, 2007).
In certain conditions the Hh secreting cells release Hh ligands into the extracellular environment. They can then bind to the 12-span transmembrane protein receptor Patched (Ptc) located on the surface of the Hh responsive cell that function as a platform for intracellular signal transduction (Rietveld, Neutz, Simons & Eaton, 1999). In the absence of the Hh ligand, Ptc normally associates with the seven-transmembrane span receptor-like protein Smoothened (Smo) at the cell membrane and this interaction leads to inhibition of Smo dependent downstream intracellular signalling (Ingham & McMahon, 2001). This inhibition leads to phosphorylation of the glioma associated (Gli) zinc finger transcription factors that function downstream of the Smo receptor. In vertebrates there are three distinct Gli proteins, Gli1, Gli2 and Gli3 (Hui, Slusarski, Platt, Holmgren & Joyner, 1994), where Gli1 and Gli2 proteins activate Hh target genes functioning as transcriptional activators, and Gli3 is thought to act mainly as a transcriptional repressor (Choi, Omenetti, Syn & Diehl, 2010). Gli2 is the primary mediator of Hh signalling, as genetic approaches show the precedent role of Gli2 in Hh signalling for the induction of the Gli1 transcription factor (Choi et al., 2010).

The Gli proteins are phosphorylated in the cytosol by protein kinase A (PKA), glycogen synthetase kinase 3 (GSK3) and casein kinase (CSK) that targets them for degradation in the cytosol by proteasomes, preventing their nuclear translocation and transcription of the Hh target genes (Pan, Bai, Joyner & Wang, 2006; Pan, Wang & Wang, 2008).

On the other hand, when the extracellular environment of the responsive cell is enriched with Hh ligands, the Hh pathway is activated and the Hh ligand binds to the Ptc receptor and this releases Smo leading to its activation (Metcalfe & De Sauvage, 2011). Activation of Smo prevents the phosphorylation of Gli transcription factors, allowing their nuclear translocation and induction of transcription of Hh target genes such as sfrp2, neo1 and c-myc (Pan et al., 2006; Pan et al., 2008).

In vertebrates, in the presence of Hh ligand, the mechanism by which the Hh signal is transmitted from Smo to Gli transcriptional factors is not well understood. However
in flies, the Hh ligand binding leads to localisation of Smo, and involves complexes such as the serine threonine kinase fused (Fu), suppressor of fused (sufu) and kinesin-like molecule Costal-2 (cos2) (Ogden, Ascano, Stegman, Suber, Hooper & Robbins, 2003). The details of Gli activation remain obscure, however evidence suggests that in the absence of the Hh ligand, Gli interacts with multiprotein complexes such as sufu, but upon ligand binding the Gli proteins translocate to the nucleus initiating transcription (Figure 1.3, Ogden et al., 2003). This shows that the Hh signalling is regulated by positive and negative feedback mechanisms that are tightly conserved across species (Ingham & McMahon, 2001). Despite this, it seems to differentiate at some point amongst vertebrates and invertebrates (Varjosalo, & Taipale, 2006). Vertebrates have an additional transmembrane protein, the Hedgehog interacting protein (Hhip), which is also a Hh target gene. Hhip was discovered by screening mouse complementary DNA (cDNA) expression libraries for proteins that bind to Shh (Olsen et al., 2004). The Hhip competes with Ptch for binding to Hh ligands, that is, it has an affinity equal to Ptch (Olsen, Hsu, Glienke, Rubanyi & Brooks, 2004). Thus, when Ptch levels exceed that of Hh ligand, or when Hhip sequesters Hh ligands, this can inhibit the Hh pathway (Olsen et al., 2004).
Figure 1.3: the active and inactive states of the Hh signalling pathway. In the absence of the Hh ligand, inactive state (left), Ptch inhibits Smo, thus Gli is prevented from entering the nucleus through interactions with cytoplasmic protein complexes such as fused and sufu, and therefore transcription of the Hh target genes is repressed. In the presence of Hh ligand, active state (right), binding of the Ptch to Smo is released, leading to translocation of Gli to the nucleus, and Gli activates transcription of the Hh target genes (reproduced from Magliano & Hebrok, 2003)

Recent studies in vertebrates have shown that Hh signalling requires a non-motile cilium (Huangfu & Anderson, 2005). This was supported by genetic studies in mice which showed that disruption of several anterograde or retrograde intraflagellar transport components lead to limb and neural patterning defects caused by impaired Hh signalling between Smo and Gli (Huangfu & Anderson, 2005). This provides compelling evidence that activated mutations in these pathway components would increase cell proliferation, a hallmark of tumour formation.

Although the role of Hh signalling in embryonic development is well established, its functions in adult tissue repair and maintenance remains unclear. Additionally,
several reports indicate that Hh signalling is an important regulator of stem cell differentiation and maintenance.

1.5.3 Hh signalling and stem cells
A fundamental characteristic of stem cells is their ability to self-renew and generate differentiated cells within different organs (Liu, Dontu, & Mantle, 2006). A number of developmental pathways including Hh signalling have emerged as potentially regulating the self-renewal processes of normal stem cells in the skin, breast, nervous system and other haematopoietic systems (Taipale & Beachy 2001). Han et al. (2008) indicated that Hh signalling via the primary cilia was essential for the proliferation and maintenance of adult neural stem cells. Liu et al. (2006) studied the role of Hh signalling for stem cell self-renewal and multilineage differentiation in mammary glands. In normal breast development stem cells are capable of generating epithelial components of the mammary gland. They showed that the Hh signalling components Ptch, Gli1 and Gli2 were highly expressed in these human mammary stem or progenitor cells using both in vitro cell cultivation and in vivo mouse xenografts methods. Subsequently, they also showed that these genes were down regulated when the progenitor cells were induced to differentiate.

The function of Hh signalling in the haematopoietic system was well demonstrated by Zhao et al. (2009) whereby the Hh signalling receptor/modulator Smo was deleted in haematopoietic cells and injected into mice to examine the effect. The mice showed clear long term defects in the haematopoietic system. This loss of function clearly identified the requirement of Smo and Hh signalling in the haematopoietic cell self-renewal process in vivo. Forbes et al. (1996) identified that Hh protein is produced by the cap cells which controls the maintenance of somatic follicle stem cells. Subsequently, in another study conducted by Michel and colleagues (2012) they showed that Hh is an essential niche signal for maintenance and differentiation of somatic stem cells in the testis of a Drosophila.
1.5.4 Hh pathway and cancer

Hh genes can induce cell proliferation, and this function of the Hh pathway signalling is important in embryogenesis and tissue maintenance, but inappropriate Hh pathway activation can lead to tumorigenesis. In recent years, it has become clear that the aberrant activation of the Hh signalling pathway can lead to cancer, whereby abnormal upregulation of the Hh ligand or deregulated expression of downstream Hh pathway mediators is demonstrated. For example, loss of Ptc1 protein (Gailani, Stahle-Backdahl, Leffell, Glynn, Zaphiropoulos, Pressman & Unden, 1996; Raffel, 2004) can cause activating mutations of Smo, which subsequently results in gene disrupted Gli1 and Gli2 transcription (Xie, Murone, Luoh, Ryan, Gu, & Zhang, 1998).

The connections between the Hh pathway and cancer was first identified by the observation that mutations in the Ptc1 receptor were associated with the rare condition known as Gorlin’s syndrome (Johnson, Rothman, Xie, Goorich, Bare, & Bonifas, 1996). Patients with this condition develop sporadic basal cell carcinomas (BCCs) and are also at an increased risk of developing medullablastoma, a tumour of the progenitor cells of the cerebellum. This link was further developed when a majority of BCCs were observed with deletion and insertion mutations in the Ptc1 receptor protein; these mutations produce truncated proteins together with the loss of heterozygosity (LOH) which inhibits the ability of Ptc to suppress Smo resulting in dysregulated Hh signalling (Xie et al., 1998). In addition, Gli1 protein overexpression has also been noted in BCC like tumours in mice, establishing the importance of Gli transcription in this tumorigenic process (Nilsson, unden, Krause, Malmqwist, Raza & Zaphiropoulos, 2000).

Furthermore, many tumours exhibit autocrine, Hh ligand dependent, uncontrolled pathway activation; examples of these types of tumours include, stomach, lung, pancreas, prostate, brain, liver and breast (Kinzler, Bigner, Bigner, Trent, Law & O’Brien, 1987; Yauch, Gould, Scales, Tang, Tian, & Ahn, 2008). Most of these tumours demonstrate elevated Hh ligand or ectopic Ptc1 and Gli expression. A recent study conducted by Yauch et al. (2008) highlighted that tumorigenesis by Hh
signalling can occur through paracrine mechanisms. The epithelial tumours secret Hh ligands into the tumour stroma, and this directly stimulates proliferation of the mesenchymal cells. In another study, conducted by Fan and colleagues (2004) they showed that prostate cancer signals to the stroma; this was evident through Hh-induced elevated Ptc and Gli expressions in murine stroma.

Aberrant activation of Hh signalling has also been reported in human small cell lung cancer (SCLC) cell lines (Watkins et al., 2003). Activation of the Hh signalling molecule Smo promoted the clonogenicity of human SCLC in vitro and subsequently initiate and progress mouse SCLC in vivo (Park, Martelotto, Peifer, Sos, Karnezis, & Mahjoub, 2011). Reciprocally, when Smo was deleted in mutant lung epithelial cells, the initiation and progression of SCLC in mice was greatly suppressed. Additionally, following chemotherapy using pharmacological inhibitors of Hh signalling, these mice showed inhibited SCLC growth (Park et al., 2011). These roles of Hh signalling in SCLC demonstrate crucial functions in the development and maintenance of SCLC. Effects of Hh signalling blockage were also demonstrated by Berman et al. (2003), where cyclopamine (a compound that interferes with Smo activity preventing Hh induced Gli expression) treatment in murine medulloblastoma cells blocked cell proliferation and also induced changes in gene expression, thus establishing a specific role for Hh signalling in medulloblastoma growth.

The Gli transcription factors also regulate several cellular functions in malignant transformation such as cell cycle progression and apoptosis (Dennler, Andre, Alexaki, Li, Magnaldo, & Dijke, 2007). High levels of Gli1 expression have been noted in human Glioblastoma (Kinzler et al., 1987), and these high levels of Gli1 were also expressed in other varieties of glial tumours, with Gli1 overexpression leading to hyper-proliferation of the central nervous system (Dahmane, Sanchez, Gitton, Palma, Sun, & Beyna, 2001). Overexpression of Gli1 has also been identified in a variety of brain tumours ranging from low-grade to high-grade. This was evident in a study that identified overexpression of the Hh mediator Gli1 (Clement, Sanchez, Tribulet, Radovanovic & Altaba, 2007). Furthermore, inhibiting Gli1 expression
using small interfering RNA, affected cell proliferation in primary cultures of these tumour cells (Clement et al., 2007).

Recent evidence now indicates that deregulated tumour formation not only promotes tumour formation but is also required for tumour maintenance. This is evident in transformed cells that continue to require Hh activity for growth and survival. This was shown in a study conducted by Thayer et al. (2003) where all of the 26 pancreatic adenocarcinoma cell lines expressed Hh target genes. Furthermore, treatment of tumour cells with cyclopamine induced apoptosis and loss of proliferation in more than half of these cell lines. In support, a previous study conducted by Dahmane et al. (2001) a subset of Glioma cell lines responded to cyclopamine-mediated inhibition of Hh signalling. Similarly, other digestive tract derived tumours such as stomach and oesophagus showed increased levels of Hh pathway signalling, and as expected these cell lines were also susceptible to cyclopamine-mediated inhibition of tumour growth (Berman et al., 2003).

It is believed that tumour growth might depend on some cancer stem cells (CSCs) which are similar to normal stem cells, and regulate the same signalling molecules as normal stem cells. Increasing evidence supports the concept that abnormal formation and growth of cancer is due to deregulated signalling pathways in stem cells including the Hh pathway (Rubin & De Sauvage, 2006). Studies have shown that Hh signalling regulates self-renewal of CSC in breast, glioma and multiple myeloma (Clement et al., 2007). Self-renewal is required for the maintenance of malignant clones as reported in mouse models of chronic myeloid leukaemia (CML) which provides evidence that Hh signalling regulates this self-renewal property (Peacock et al., 2007). In this study, genetic disruption of Smo activity leading to loss of Hh signalling, lead to the inhibition of BCR-ABL (tyrosine-kinase inhibitor, first line therapy for patients with chronic leukaemia) expressing leukaemia stem cells and prolonged survival (Peacock et al., 2007). Active Hh signalling has also been identified in Glioblastoma CSCs, where the use of pathway inhibitors such as cyclopamine or siRNA (small interfering RNA) specifically targeting pathway components resulted in loss of tumorigenesis (Clement et al., 2007). Similarly, in
breast cancer, stimulating the activation of Hh pathways in CSCs using the Hh ligand and Gli1 or Gli2, or on the other hand inhibiting Gli1 or Gli2 expression using specific siRNA, altered the expression of BMI-1, which is a self–renewal regulator in normal stem cells (Liu et al., 2006).

CSCs have also been implicated in tumour progression and metastases development in solid tumours, and again Hh signalling has been reported to be playing a critical role in these processes (Mani, Guo, & Liao, 2008). For example, in colon carcinomas derived from primary clinical specimens, the Hh activity within the CSCs was evident by high expressions of Gli1, Gli2 and Ptc1 in their cellular compartments (Mani et al., 2008). Moreover, inhibiting Hh pathway activity in these cells with cyclopamine or siRNA to Gli1, Gli2, and Smo, reduced tumour cell proliferation and induced apoptosis (Mani et al., 2008). Furthermore, using short hairpin RNA (shRNA) against Smo eliminated metastatic development in these tumours. Tumours contain only small amount of CSC are typically resistant to chemotherapy and radiation because they are slow growing cells (Beachy, Karhadkar & Berman, 2004). The fact that Hh signalling has been identified in several types of CSCs would mean that inhibiting Hh signalling is a promising therapeutic target to deplete tumour forming CSCs.

It is certain that different components of the Hh pathway play pivotal roles in the biology of different cancers, and amongst these the Gli transcription factor especially Gli2, plays a predominant role. Kim et al. (2007) demonstrated that selective down-regulation of Gli2 inhibited proliferation of hepatocellular carcinoma cells (HCC), inhibited expression levels of cell survival markers such as Bcl-2 and e-Myc, and enhanced expression levels of cell cycle inhibitor marker p27. Subsequent studies following this showed that high expression of Gli2 in HCC tissues is associated with poor prognosis in patients (Zhang et al., 2013).
1.5.5 Hedgehog pathway and MM

Similar to other cancers, development of MM is considered to be a multi-step process that reveals mutations in different genes. These genes are commonly involved in growth and are regulated by pathways including the Hh signalling pathway (Greillier & Astoul, 2008). The aberrant expression and inappropriate activation of the Hh pathway has induced many cancers including non-small cell lung cancer and Medulloblastoma (Dennler et al., 2007). However, the significance of aberrant Hh pathway signalling in the development and growth of MM still remains to be determined.

A recent study conducted by Shi et al. (2012) was the first to document a role for Hh signalling in MM cell growth. They observed an increased expression of Ptch1, Gli1, Shh and Hhip in frozen tumour tissues from 39 MPM patients suggesting activation of the Hh pathway. They observed that high Gli1 expression levels were associated with poor patient survival. They then expanded their investigation to examine the activity of the Hh signalling pathway in MPM tumours, and the effects of inhibiting this pathway in primary mesothelioma cell cultures and in a mouse xenograft model. Activation of the pathway was also demonstrated in MM cells, as blocking Hh signalling with cyclopamine resulted in a downregulation of Gli1 expression in more than half the cell lines examined.

In vivo analysis using MPM xenografts (transplantation of cells from one species to another) determined that expression of Dhh correlated with human Gli1 and Ptch1 expressions, which suggests autocrine Hh activity in some MPM. Treatment of two primary MPM cultures obtained from xenografts in mice with Hh antagonist resulted in a dose dependent decrease of cell survival, indicating the role of Hh signalling in MPM growth. They also showed that inhibitors targeting Smo could decrease Gli expression and cell growth and subsequently, decreased cell proliferation in cell cultures. In conclusion, this study identified an upregulation of Hh signalling in MPM tumours. Additionally, observing that Smo inhibitors decreased cells growth both in vitro and in vivo.
Li et al. (2013) in a current study reported an essential role for Gli transcription factors in MPM tumour growth. They showed that a majority of MPM tumour tissues had significant higher levels of Gli1, Gli2 and their downstream target genes, and by inhibiting Gli1 and Gli2 using specific siRNAs, suppressed MPM cell proliferation in vitro and in vivo, thus highlighting Gli1 and Gli2 as important and potent therapeutic targets in MPM.

Hh signalling is unlikely to be a sole effector pathway in tumorigenesis and there is growing evidence to suggest that it works with several other oncogenic factors for tumour formation, including growth factors such as TGF-β. Similar to the Hh members, TGF-β has emerged as an important molecule to be involved in various physiological processes and under appropriate conditions contributes to the aggressiveness and neoplastic progression in a variety of tumour types.

1.6 TGF-β

TGF-β is a multifunctional peptide that was first identified for its ability to cause phenotypic transformation of rat fibroblasts (Sporn, Robers, Wakefield & Assoian, 1986). TGF-β has subsequently been shown to have various regulatory actions in a variety of normal and neoplastic cells, by regulating several functions including growth, differentiation, proliferation, adhesion and apoptosis (Sporn et al., 1986). TGF-β’s are important regulators of diverse developmental processes and homeostasis, and disruption of their activity has been implicated in a variety of human diseases including cancer (Sporn et al., 1986). More than 30 proteins have been identified as members of the TGF-β superfamily, including activins and bone morphogenic proteins (BMP) (Sporn et al., 1986). There are three mammalian TGF-β isoforms, TGF-β1, TGF-β2 and TGF-β3 and are synthesised by a wide variety of cell types including platelets, macrophages, fibroblasts and tumour cells (Savage, Das, Finelli, Townend, Sun, Baird & Padgett, 1996).

TGF-β was initially discovered in normal rat kidney fibroblasts, where TGF-β was measured for its ability to stimulate proliferation, and could also transform rat kidney
fibroblasts (Sporn et al., 1986). However, other contradicting studies also showed that TGF-β was essentially identical to a previously identified growth inhibitor, which was characterised in monkey kidney cells and which inhibited cell proliferation through autocrine mechanisms (Savage, Das, Finelli, Townend, Sun, Baird & Padgett, 1996). In support, Moses et al., 1987 showed that TGF-β is involved in the mitogenic process of fibroblasts, where it inhibits epithelial and endothelial cell proliferation in vitro. Other regulatory functions of TGF-β include initiating a cascade of events leading to neovascularization and matrix synthesis (Latterio, Geiser, Kulkarni, Roche, Sporn & Roberts, 1994), transport of glucose and amino acids, and glycolysis in fibroblasts, with a more specific action of enhancing fibronectin and collagen production in these cells (Smith et al., 1999). Thus the application of TGF-β to connective tissues, leads to fibrosis and angiogenesis (Sporn et al., 1986), suggesting that it may have application in tissue repair caused by trauma.

1.6.1 Regulation of TGF-β
TGF-β is synthesized as a large precursor protein which is then cleaved to generate mature the ligand (Mehra & Wrana, 2002). This pre-protein is about 100kDa in size and consist of an amino terminal and a mature carboxyl terminal (Gentry, Lioubin, Purchio & Marquardt, 1988). Cleavage is mediated by furins—which are pro-proteins that process latent TGF-β into biologically active forms (Matthews, Goodman, Gorman & Wells, 1994). The cleavage pro-region for TGF-β, also known as latency associated peptide (LAP), remains non-covalently bonded to the mature peptide to form a latent TGF-β complex. Thereafter, the latent TGF-β complex is secreted and undergoes further processes in the extracellular matrix (Thomsen & Melton, 1993). Various different cells synthesize TGF-β and essentially all have high affinity receptors for this peptide (Sporn et al., 1986).
1.6.2 TGF-β receptors

The TGF-β ligand binds to several specific cell membrane receptors. These membranes are of high affinity and are founds in essentially all normal and malignant, epithelial and mesenchymal cells (Clark & Coker, 1998). These transmembrane surface receptors carry TGF-β signal into the cell. Massague and colleagues 1998, characterised TGF-β receptors, whereby radioactively labelled TGF-β was cross linked with cell surface proteins which revealed that TGF-β binds to three types of receptors and were named as type I, II, and III receptors.

Type I and II receptors are glycoproteins of 55 kDa and 70 kDa respectively, with a core polypeptide of around 500-570 amino acids (Massague, 1998). The receptors have a relatively short extracellular region (approximately 150 amino acids), N-glycosylated, and around ten or more cystines which determine the general fold in that region, and three of these cystines form a cluster near the transmembrane sequence (Ebner, Chen, Shum, Lawler & Zoncheck, 1993). Lin and colleagues (1992), identified type II receptor with preferentially bound activin and TGF-β1 through expression cloning. The type I receptor has received many names. It is generally referred to as activin receptor like kinase (ALK) (Clark & Coker, 1998) or TβRI. Whereas type II receptor subfamily that specifically binds to TGF-β is known as TβRII (Wrighton, Lin & Feng, 2009). In contrast to type I and type II receptors, type III plays an ancillary role, whereby they modulate their activity by regulating ligand access to type I and type II.

The receptors comprise of an intracellular kinase domain which phosphorylates serine and threonine residues (Ebner et al., 1993). Signalling is initiated when the TGF-β ligand binds to the type II receptor, that interacts with a specific type I receptor (ALK5/TβRI) forming the mature signalling complex (Moustakas, Lin, Henis, Plamondon, O’Connor-McCourt & Lodish, 1993) Figure 1.4. This formation of ligand induced receptor complex leads to the phosphorylation of the type I receptor which activates the SMAD proteins that carry the signal to the nucleus and initiate transcription of the target genes (Ebner et al., 1993).
1.6.3 SMAD signalling pathway

After type I and II receptor activation, TGF-β signalling is mediated by the SMAD signalling pathway (Padgett, 1999). The SMAD proteins were named after the homologous proteins referred to as the Mad gene in *Drosophila* and the *sma* gene from *C. elegans* (Derynyck, Gelbart, Harland, Heldin, Kern & Massague, 1996). The first experimental evidence that SMADs functions downstream of receptors was performed through genetic mosaic analysis in *C. elegans* (Savage et al., 1996). Thereafter, eight different SMAD proteins were identified, divided into three functional categories: the receptor regulated SMADs (R-SMAD), common SMADs (co-SMAD) and inhibitory or antagonistic SMADs (I-SMAD). The intracellular proteins R-SMADs and co-SMADs are composed of three parts: the highly conserved Mad homology 1 and 2 (MH1 and MH2), N-terminal and a C-terminal. The I-SMADs are similar, except they have poorly conserved MH1 domain (Massague, 1998).

The R-SMADs (SMAD 1, 2, 3, 5 and 8) are phosphorylated by the type I receptors. The phosphorylation of the R-SMADS forms a complex with the co-SMAD, of
which SMAD4 is the only member (Massogue, Blain & Lo, 2000). The co-SMAD works as a co-activator or a co-suppressor, thereby possibly regulating positive and negative gene expression. In contrast, the I-SMADs (SMAD 6 and 7), act as antagonizing SMADs counteracting the effects of R-SMADs Figure 1.5 (Massague, 1998). These three SMAD groups represent the signalling pathway for TGF-β, which transmits signals from the receptors to the nucleus to regulate transcription activity of TGF-β target genes (Massogue et al. 2000).

Figure 1.5: The SMAD family members from vertebrates indicating highly conserved MH1 and MH2 collaborates with co-SMADs. The antagonistic SMADs counteract that activation (reproduced from Massague, 1998).

1.6.4 TGF-β Signal transduction through the SMAD pathway

TGF-β1 is the most abundant and most expressed isoform (Elliot & Blobe, 2005). TGF-β is secreted into the extracellular matrix as a latent protein complex, which is then modified into a biologically active form (Padgett, 1999). A cellular response is achieved when the activated TGF-β binds to receptor specific TBRII and ALK5/TβRI, which in turn initiates phosphorylation of the intracellular receptor regulated SMADs, SMAD2 and SMAD3 (Elliot & Blobe, 2005). These phosphorylated SMADs heterodimerise with the co-SMAD, SMAD4 forming a complex, which then translocate to the nucleus (Elliot & Blobe, 2005). Accumulation
of this SMAD complex in the nucleus initiates transcription of the TGF-β target genes (Figure 1.6, Elliot & Blobe, 2005). SMADs 1, 6 and 8 are activated in response to bone morphogenic proteins (BMP) signals.

![Figure 1.6: the TGF-β SMAD signalling pathway showing binding of the TGF-β to their receptors and downstream SMAD signalling (reproduced from Elliot & Blobe, 2005).](image)

Some studies suggest that TGF-β receptors can interact with a number of proteins, activating other signalling pathways. TGF-β induced signals are either transduced in SMAD-dependent (canonical) or SMAD-independent (non-canonical) pathways (Padgett, 1999; Massague, 1998).

### 1.6.5 Non-canonical TGF-β signalling pathways

Although TGFβRI & RII and SMADs comprise the TGF-β signalling pathway, other non SMAD pathways have been identified that signals through TGF-β, such as mitogen activated protein kinase (MAPK-JNK/ERK) (Engel, et al., 1999; Fink et al., 2001), Rho guanosine triphosphatase and PI3 kinase/Akt (Figure 1.7, Bhowmick et al., 2001), Evidence of TGF-β activating the MAPK pathway came from cells overexpressing dominant negative SMAD4 and SMAD4-deficient cells. In these
cells, activation with JNK/MAPK was enough to elicit TGF-β regulated response (Engel et al., 1999). The precise molecular mechanisms by which TGF-β activates MAPK remains unclear however, evidence suggests that in epithelial cells activation of ERK is involved in TGF-β mediated Ras signalling (Bhowmick et al., 2001; Zhang, 2009). Similarly, TGF-β induced epithelial to mesenchymal transition (EMT) involves non-SMAD pathways, signalling via the PI3K/Akt pathway (Bhowmick et al., 2001). These multistep SMAD and non-SMAD pathways activated by TGF-β involves a complex mechanism. A greater understanding of this TGF-β mediated pathways in different cell types and in the context of specific environments might provide insights into TGF-β signalling in various cancers including MM.

Figure 1.7: Representation of canonical and non-conical TGF-β signalling pathways (reproduced from Chaudhury & Rowe, 2009).

1.6.6 Role of TGF-β in cancer
TGF-β is an essential regulator of cellular and physiological processes, and alterations in the TGF-β signalling pathway and resistance to inhibition of proliferation is frequently observed in cancers. TGF-β function both as an anti-
proliferative factor and as a tumour promoter, and various components of the TGF-β signal transduction pathway that normally act as tumour suppressors, are mutated in different cancers (Massogue et al., 2000). Functional loss of TGF-β and its association with tumorigenesis has been demonstrated in gastric and colon carcinomas with somatic mutations in the type II TGF-β receptor (Suzuki et al., 2004). Pancreatic, lung and breast are other cancers identified carrying mutations in the TGF-β signalling pathway components (Suzuki et al., 2004). Therefore, these diverse activities have promoted investigation to broaden the understanding of how TGF-β family members signal to evaluate diagnostic, prognostic and predictive markers for cancer patients.

1.6.7 TGF-β signalling in MM

There is strong evidence to support the involvement of TGF-β in MM growth. Firstly, significant levels of TGF-β are produced by most human (Fitzpatrick et al., 1994; Shetty et al., 2009) and murine MM cell lines (Kuwahara et al., 2001). In addition MM patients have been documented with high levels of TGF-β, whereby immunoreactivity to TGF-β was observed in more than 90% MM patients (Jagirdar, Lee, Reibman, Gold, Aston & Begin, 1997). Maeda et al. (1994) measured TGF-β levels in pleural effusions from MM patients, which was approximately 280 pmol/L, nearly three times greater compared to pleural effusions from primary lung cancer patients and nearly six times higher compared to pleural effusions from non-small cell lung cancer (NSCLC) and breast cancer patients (Suzuki et al., 2004).

Secondly, involvement of TGF-β in MM pathogenesis has been observed in several studies. Fitzpatrick et al. (1994) transfected mouse MM cells with antisense RNA to TGF-β1 and TGF-β2 and observed a delay in tumour growth when these transfected MM cells were adoptively transferred to recipient animals. These findings were supported by Marzo et al. (1997) who demonstrated that administration of TGF-β2 antisense oligonucleotides in vivo reduced tumour growth in a MM animal model. Thirdly, there is evidence to suggest that TGF-β may potentially respond to immunological therapy. Suzuki et al. (2004) explored the effects of a soluble TGF-β
type II receptor (sTGF-βR) which specifically inhibits TGF-β₁ and TGF-β₃ isoforms (Smith et al., 1999) in AB12, AC29 and AB1 murine MM tumour models. AB12 and AC29 cells produce large amounts of endogenous TGF-β whereas AB1 did not (Christmas, Manning, Garlepp, Musk & Robinson, 1993). sTGF-βR treatment inhibited AB12 and AC29 tumour growth in mice. In contrast, AB1 growth was not affected by sTGF-βR treatment. Thus blocking of TGF-β is a promising anticancer therapy to treat MM patients.

In recent years there has been a strong focus on examination of signalling pathways in promoting tumour growth, which has lead to various anticancer treatments being developed. Most current therapies aim at blocking these signalling pathways. However, not all tumours are responsive to these inhibitors, and some tumours that may be responsive initially, become resistant during the course of the treatment. Therefore, there is need to develop a broader understanding of the regulation of signalling pathways in tumorigenesis process of various cancers to develop better therapeutic approaches.

Researchers are now exploring the interaction or cross talk between different signalling pathways in tumour growth. Initially, studies examined the effect of interacting signalling pathways on cellular functions, particularly in embryonic developmental and physiological processes. Recently, studies have looked at pathway interactions and how they are regulated in tumorigenesis.

1.7 TGF-β cross-talk with Wnt and Hippo signalling pathways
An example of these interacting pathways is between the TGF-β and Wnt signalling pathways. Attisano and Labbe. (2004) demonstrated that TGF-β and Wnt ligands can co-ordinate to regulate cell differentiation and control gene expression patterns, and described their co-ordination in promoting tumorogenesis in mice.

Wnt proteins are secreted glycoproteins that are important in various developmental processes including the formation of body axis during early embryogenesis (Moon,
Bowerman, Boutros & Parriman, 2002). In addition to its developmental role, inappropriate activation of the Wnt pathway has been implicated in various cancers (Cadigan & Nusse, 1997). β-catenin is the key component of the Wnt signalling pathway. In the absence of the Wnt ligand, the pathways intermediate molecules such as APC (Adenomatous Polyposis Coli), axin and GSK3 (Glycogen Synthase Kinase 3) forms a complex that results in phosphorylation and degradation of β-catenin, however, in the presence of Wnt ligand, this ligand binds to frizzled proteins which then stabilizes β-catenin, and accumulates in the nucleus associating itself with LEF1/LCF transcriptional factors to regulate target gene expression (Skromne & Stern, 2001). Mouse models have provided an insight into the cross-talk between TGF-β and Wnt signalling pathways, disrupted TGF-β signalling in the context of activated Wnt promoted tumorigenesis (Skromne & Stern, 2001). TGF-β signals through the SMAD pathway and Takaku and colleagues 1998, isolated SMAD4/APC heterozygotes and showed that these mice developed more severe form of malignant tumours from intestinal polps compared to mice with just the APC. In another study, SMAD4/APC heterozygote mice were challenged with carcinogens and these mice developed significant numbers of pancreatic acinar foci compared to the control APC mice (Cullingworth, Hooper, Harrison, Mason, Sirard, & Patek, 2002).

Similarly, interactions between TGF-β and Hippo signalling pathways have been noted in some cancers, including MM, whereby mutations in the Hippo cascade were observed in MM cases which then negatively regulated its pathway mediators. The Hippo signalling pathway controls organ size in animals by regulating cell proliferation and apoptosis (Hall, Wang, & Miao, 2010). This pathway induces expression of several organ growth genes such as cyclin E. Yes-associated proteins (YAP) human homolog is a key transcriptional target of the Hippo pathway (Hall et al., 2010). YAP plays a conserved role in the tumorigenic processes of various cancers. Fuji et al. (2012) observed a functional interaction between TGF-β and Hippo pathway in regulating connective tissue growth factor (CTGF). There was an induced CTGF expression in MM cells by the formation of YAP-TEAD4-SMAD3-p300 complex on the CTGF promoter (Fuji et al., 2012). In support, xenografted mice showed a prolonged survival rate when the CTGF expression was knocked
down in MM cell, suggesting CTGF as an important modulator of MM growth and pathology, providing a novel therapeutic approach for MM (Fuji et al., 2012).

Functional interaction between TGF-β and Hh signalling in cancers has also been identified by several investigators. TGF-β and Hh are involved in numerous overlapping processes during embryonic development and several studies have identified this interaction in cancers.

1.8 Interaction between Hh and TGF-β signalling pathways

As described earlier, the transcriptional response to Hh signalling is mediated by the Gli transcriptional factors. Gli regulates several cellular functions linked to malignant transformation, such as cell cycle progression, differentiation and apoptosis (Kasper, Frischauf & Abager, 2006). Also mentioned previously was the essential role of TGF-β essential in physiological processes including tissue repair, control of cell growth, differentiation and immune regulation. Subsequently, expression of TGF-β isoforms contributing to the severity and progression of neoplasms have also been identified in various tumour types (Derynck et al., 1996). Recent studies have shown that Gli is not solely regulated by Hh signalling but also by other pathways such as TGF-β. Studies have examined the capacity of TGF-β to affect the expression of Hh signalling molecule Gli1 and Gli2 in vitro and in vivo.

Dennler et al. (2007) identified Gli2 as the most transcriptionally active of the Gli proteins and a target of TGF-β/SMAD signalling, independent of the Hh signalling pathway. The capacity of TGF-β to rapidly induce Gli2 expression in the presence of Hh inhibitor cycloamine, revealed this pathway interaction. Interestingly, Gli1 is also induced by TGF-β but in a Gli2-dependent manner which was also independent of Hh signalling. In vitro studies confirmed Gli1 and Gli2 expressions induced by TGF-β in various cell types including breast carcinoma cells, human dermal fibroblasts, cutaneous melanoma, glioblastoma and keratinocytes. These findings were supported by in vivo studies, where transgenic mouse models overexpressing TGF-β1 activated Gli1 and Gli2 expression in a SMAD3 dependent manner. Finally
they showed TGF-β induced Gli1 and Gli2 expression was greatly inhibited inhibitors such as ALK5 inhibitors and transfected Gli2 siRNA oligonucleotides in cyclopamine resistant pancreatic adenocarcinoma cell lines (Dennler et al., 2007).

Yoo, Kang, Kim and Oh. (2008) investigated the role of Shh and TGF-β signalling in the metastatic process of human gastric cancers by examining ALK5 and Gli immunohistochemical staining on tumour biopsies from gastric cancer patients. ALK5 and Gli expression was classified as strong in 2% and 48% of biopsies respectively, Figure 1.8.

![Gli immunohistochemical stain x 200, Gli highly expressed in an area of gastric tissue (light brown staining) (reproduced from Yoo et al., 2008)](image)

Figure 1.8: Gli immunohistochemical stain x 200, Gli highly expressed in an area of gastric tissue (light brown staining) (reproduced from Yoo et al., 2008)

Several independent groups have confirmed induction of Gli, specifically Gli2 by TGF-β. Hu and colleagues, (2004) found that TGF-β signalling increased Gli2 expression, which then influenced the progression from ductal carcinoma in situ to invasive carcinoma. These findings were consistent with the findings of Alaxaki et al (2010), who found that TGF-β increased Gli2 expression which subsequently increased its downstream target Gli1 and this influenced cell differentiation and progress of myoepithelial cell to invasion. Edson and colleagues, (2010) found that in normal granulosa cells, Gli2 is a TGF-β-regulated gene and suggested that Gli may contribute to cancer progression in the ovary. Furthermore, a recent study by Johnson et al. (2010) observed that blocking Gli signalling with a Gli2 repressor gene in metastatic breast cancer cells reduced TGF-β-induced parathyroid hormone mRNA expression, thereby reducing tumour-induced bone destruction. TGF-β is a
molecule shown to be important in MM, however the aberrant activation of Hh signalling and the interaction of its mediators with TGF-β remains to be determined. While TGF-β contributes to the biological effects of the Hh pathway and its mediators, it is also highly likely that the Hh pathway could induce TGF-β activity. This opposite action was evident in non-small cell lung cancer (NSCLC), where pharmacological inhibitors of the Hh pathway were shown to inhibit TGF-β-induced epithelial to mesenchymal transition (EMT) (Maitah, Ali, Ahmed, Gadgeel & Sarkar, 2011). Hh and TGF-β signalling can form a vicious cycle where it can promote tumour growth and development in cancers. However the interaction between these pathways in MM is unknown.

1.9 Scope of thesis
Numerous studies have shown abnormal Hh signalling in various cancers. Similarly, TGF-β signalling aids to the pathogenesis in many cancers including MM. However, only a few studies have examined the interaction between the two pathways in cancer and there have been no published studies examining their interaction in MM. Current treatment regimens in MM are largely unsuccessful. Therefore, new innovative treatments such as molecular targeted approaches needs to be developed.

Previous studies by our laboratory and others have shown the contribution of TGF-β in MM. Our laboratory is now investigating the importance of Hh signalling in these cells. Importantly, we now have preliminary data suggesting that TGF-β affects the downstream effector molecules of the Hh pathway in MM cells. Its important then to understand how the TGF-β and Hh pathways interact and what regulates these pathways may identify novel targets for therapeutic intervention.

1.9.1 Research Direction
1.9.1.1 General Hypothesis
TGF-β induces Gli1 and Gli2 expression in MM cells through activation of the SMAD signalling pathway.
1.9.1.2 Aims

**Aim 1.** To examine the effect of TGF-β1 and TGF-β2 on the expression of Gli1 and Gli2 in MM cells.

  (a) Determine the levels of endogenous TGF-β1 and TGF-β2 mRNA in MM and control cell lines.
  (b) Validate the activity of recombinant TGF-β1/2 by measuring pSMAD2 proteins and human collagen type-1α1 mRNA expression in MM and control cells.
  (c) Examine the effect of TGF-β1 and TGF-β2 on Gli1 and Gli2 mRNA levels in MM and control cells.

**Aim 2.** To determine if TGF-β-induced Gli mRNA is independent of the Hh signalling pathway and examine the effects of blocking TGF-β on Gli1 and Gli2 mRNA levels.

  (a) Evaluate the effect of blocking ALK5 inhibitor (SB-431542) on TGF-β1 and TGF-β2 mediated Gli1 and Gli2 mRNA levels.
  (b) Evaluate the effect of ALK5 inhibitor on SMAD2/3 protein expression
  (c) Examine the effect of TGF-β1 and TGF-β2 on Gli1 and Gli2 mRNA levels in the presence of a Smo inhibitor

**Aim 3.** To determine the signalling pathway through which TGF-β regulates Gli1 and Gli2 expression.

  (a) Effect of TGF-β stimulation on SMAD2/3 protein expression
  Determine if TGF-β-induced Gli mRNA is mediated through the SMAD2/3 pathway.

To address aim 1 a) I will measure mRNA expression of TGF-â1/2 in MM, control cells (MeT-5A) and primary mesothelial cells by real-time PCR. In aim 1 b) I will isolate RNA and protein from MM and MeT-5A cells after TGF-â1/2 stimulation and measure phosphorylation of SMAD2 proteins and human collagen type-1α1 mRNA
levels. In aim 1 c) I will isolate RNA from MM and MeT-5A cells after TGF-â1/2 stimulation and measure Gli1/2 mRNA levels by real-time PCR.

For aim 2 and 3, based on aim 1(c) I will choose 1 MM and MeT-5A cell line for subsequent studies.

In aim 2 a) I will isolate RNA and protein from a 1 MM and MeT-5A cell lines after SB-431542 and TGF-β1/2 treatment. The expression of Gli1/2 mRNA levels will be measured by real-time PCR. In aim 2 b) the effectiveness of SB-431542 to inhibit TGF-β1/2 will be confirmed by SMAD2/3 protein expression by western blot analysis. For aim 2 c) cells will be pretreated with Hh pathway inhibitor cycloamine, then stimulate with TGF-β1/2 and isolate RNA to measure Gli1/2 mRNA levels by real-time PCR.

For aim 3 a) the effectiveness of the siRNA to delete SMAD pathway will be confirmed by measuring SMAD2/3 mRNA and pSMAD2 protein by real-time PCR and western blot analysis respectively. Thereafter, I will isolate RNA and protein from a 1 MM and MeT-5A cell line that have been transfected with SMAD2/3 and control siRNA and treated with TGF-β1/2. The expression of Gli1/2 mRNA will be measured using real-time PCR.
Chapter 2

Materials
2.1 Reagents

2.1.1 Tissue culture
Dulbecco’s modified eagle’s medium (DMEM) GIBCO® Life Technologies™
Foetal calf serum (FCS) SAFC Bioscience
Gentamycin {10mg/ml} GIBCO® Life Technologies™
L-glutamine (L-Glut) {200mM} GIBCO® Life Technologies™
Penicillin {10 000 units (U)/mL} GIBCO® Life Technologies™
/Streptomycin {10 000 µg/mL} (P/S) GIBCO® Life Technologies™
Trypsin-EDTA {0.25%} GIBCO® Life Technologies™

2.1.2 Reverse transcription (RT-PCR)
Omniscript reverse transcription kit Qiagen® Chadstone, Victoria, Australia
Deoxyribonucleoside triphosphates Qiagen®
(dNTPs) {5mM}
Omniscript reverse transcriptase {4U/µl} Qiagen®
Reverse transcriptase buffer {10x} Qiagen®
RNase inhibitor {4U/µl} Qiagen®
Random hexamers {3 µg/µL} Life Technologies™

2.1.3 Real-Time Polymerase Chain Reaction (PCR)
Quantifast™ Probe PCR Master Mix {2x} Qiagen®
Taqman® Universal PCR Master Mix Life Technologies™

2.1.4 RNA Isolation
Purelink™ RNA Mini Kit Life Technologies™
RNeasy Mini Kit Qiagen®
RNase Free DNase Set Qiagen®
2.1.5 Protein Isolations

Aprotinin
Sigma-Aldrich®

β-glycerophosphate
Sigma-Aldrich®

Dithiothreitol
Sigma-Aldrich®

EDTA
Life Technologies™

HEPES
Sigma-Aldrich®

Leupeptin
Sigma-Aldrich®

Magnesium Chloride (MgCl₂)
Life Technologies™

Nonyl phenoxypolyethoxylethanol (NP40)
Sigma-Aldrich®

Phenylmethanesulfonylfluoride (PMSF)
Sigma-Aldrich®

Sodium fluoride (NaF)
Sigma-Aldrich®

Sodium molybdate dehydrate (NaMolyb)
Sigma-Aldrich®

Sodium orthovanadate (Na₃VO₄)
Sigma-Aldrich®

Sodium pyrophosphate decahydrate (NaPyr)
Sigma-Aldrich®

Triton®X-100
Sigma-Aldrich®

2.1.6 Western blot

Acrylamide
BIO-RAD Gladsville, NSW, Australia

Ammonium per sulfate (APS)
BIO-RAD

Bromophenol blue
Sigma-Aldrich®

Coomassie Plus™ Protein Assay Kit
Thermo Scientific

Immobilon western HRP substrate
Millipore Klisiyth, Victoria, Australia

Instant Skim milk powder
Diploma Instant

Nitrocellulose membrane
Whatman® Dassel, Germany

Precision plus protein™
BIO-RAD
dual colour standard

Sodium dodecyl sulphate (SDS)
Sigma-Aldrich®

Tetramethylethylenediamine (TEMED)
Sigma-Aldrich®

Polyvinylidene fluoride (PVDF)
Millipore
2.1.7 Alphascreen® Surefire® Assay

GAPDH Surefire® Assay kit Perkin Elmer Life Sciences®
Massachusetts, U.S.A
Histone Surefire® Assay kit Perkin Elmer Life Sciences®
pSMAD2 Surefire® Assay kit Perkin Elmer Life Sciences®
Protein A acceptor beads Perkin Elmer Life Sciences®
Streptavidin coated donor beads Perkin Elmer Life Sciences®

2.1.8 Other reagents

Absolute ethanol Thermo Fisher Malaga, WA, Australia
β- Mercaptoethanol Sigma-Aldrich®
Bovine serum albumin Sigma-Aldrich®
Dimethyl sulfoxide (DMSO) Sigma-Aldrich®
Double deionised H₂O Sigma-Aldrich®
Glycerol Sigma-Aldrich®
Glycine Sigma-Aldrich®
Lipofectamine™ 2000 Life Technologies™
Methanol Chem Supply Gillman, SA, Australia
Sodium chloride (NaCl) Sigma-Aldrich®
Tris-base Sigma-Aldrich®

2.1.9 Antibodies

Anti-α-tubulin monoclonal primary antibody produced in mouse Sigma-Aldrich®
Secondary polyclonal anti-rabbit immunoglobulins/HRP Dakocytomation
Campbellfield, Vic, Australia
Secondary polyclonal anti-mouse immunoglobulins/HRP Dakocytomation
pSMAD2 primary antibody
produced in rabbit
Total SMAD2/3 primary antibody
produced in rabbit

2.1.10 Taqman® Primers/probes
Phosphoglycerate kinase 1 (PGK1) primer probe, VIC labelled
Gli1 primer probe,
FAM labelled. ID#: Hs01110766_m1
Gli2 primer probe,
FAM labelled. ID#: Hs01119974_m1
TGF-β1 primer probe,
FAM labelled. ID#: Hs00998129_m1
TGF-β2 primer probe,
FAM labelled. ID#: Hs00234244_m1
SMAD2 primer probe,
FAM labelled
SMAD3 primer probe,
FAM labelled
Ptch1 primer probe,
FAM labelled. ID#: Hs00970976_m1

2.1.11 Inhibitors/Small Interfering RNA (siRNA)
SB 431542, ALK5 inhibitor
SMAD2 siRNA
SMAD3 siRNA
Control siRNA
Control siRNA (FITC Conjugate)

2.1.12 Instruments and equipment
AGFA CP1000 X-ray processor
2.2 Buffers/Reagent Recipes

2.2.1 Glycine stripping buffer

Glycine 50 mM
SDS 35 mM
Tween-20® 1%

The components were dissolved in 1L ddH2O, the pH adjusted to 2.5 and stored at 4°C.

2.2.2 Phosphate Buffered Saline (PBS) {20x}

Na₂HPO₄ 0.2 M
NaCl 3 M
KH₂PO₄ 30 mM
KCl 55 mM

The components were dissolved in ddH₂O, adjusted to pH 7.4 and made up to a final volume of 1 L, sterilised and stored at room temperature. The solution was diluted 1:20 to 1x PBS in ddH₂O before use.

2.2.3 Sample Loading Buffer {4x}
Glycerol 40%
Tris/HCl pH 6.8 0.25 M
SDS 0.3 M
Bromophenol Blue 0.04%
β-mercaptoethanol 5%

The components were dissolved in 20 mL ddH₂O, aliquoted into eppendorf tubes and froze at -20°C until required.

2.2.4 SDS-PAGE Running Buffer {10x}
Tris-Base 0.25 M
Glycine 2 M
SDS 35 mM

Components were dissolved content in 1 L ddH₂O, the pH checked to be around 8.0, and then stored at room temperature before being diluted 1:10 in ddH₂O before use.

2.2.5 Stress Lysis Buffer (SLB)
HEPES 20 mM
MgCl₂ 55 mM
EDTA 0.02 mM
β-glycerophosphate 30 mM
NaCl 0.1 M

Dilution was made up to a final volume of 500 mL in ddH₂O, adjusted to pH 7.7, Aliquoted in 5mL sterile tubes and froze at -20°C until required for use.
2.2.6 SLB++

Triton®X-100 0.5%
NP40 1%
NaPyr 0.5 mM
Na₃VO₄ 0.2 mM
NaMolyb 1.0 mM
NaF 1.25 mM
Aprotinin 1 mM
Leupeptin 1 mM
PMSF 5 mM
DTT 5 mM

The components were added to 2 mL of SLB and used immediately.

2.2.7 Tris Buffered Saline (TBS) {10x}

Trizma Base 0.1 M
NaCl 1.5 M

The components were dissolved in 1 L ddH₂O, adjusted pH to 7.4 and stored at room temperature. The solution was diluted 1:10 with ddH₂O before use.

2.2.8 TBS/Tween-20® (TBST) {1x}

Tween-20® 1%
1x TBS 1 L

Tween-20® was dissolved in 1 L 1x TBS and thoroughly mixed then used immediately.

2.2.9 Transfer Buffer {10x}

Tris-Base 0.25 M
Glycine 2 M
SDS 3.5 M

Components were dissolved in 1 L ddH₂O, the pH checked to see it was in the range of 8.0-8.3, stored at 4°C and then diluted 1:10 in ddH₂O before use.
2.2.10 Tris/HCL at pH 6.8/8.8
Tris/HCL 1.5 M
The powder was dissolved in ddH₂O, pH adjusted to either to 6.8 or 8.8 and then made up to 50ml in ddH₂O before being stored at room temperature until required.

2.2.11 5% Milk + TBST
5% w/v Skim Milk Powder
1x TBS + 1% Tween 20®
Milk powder was dissolved in 1x TBST-T solution and used immediately.

2.2.12 10% w/v Ammonium Per sulphate (APS)
APS 1g
APS was dissolved in 10ml ddH₂O, aliquoted in 1.5ml eppendorf tubes and stored at -20°C until use.

2.2.13 Western Blot Gels

<table>
<thead>
<tr>
<th></th>
<th>Stacking Gel</th>
<th>Running Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/Biscarylammide</td>
<td>6%</td>
<td>10%</td>
</tr>
<tr>
<td>SDS {20%}</td>
<td>0.5%</td>
<td>0.5%</td>
</tr>
<tr>
<td>Tris/HCl {1M}</td>
<td>0.125 M at pH 6.8</td>
<td>0.375 M at pH 8.8</td>
</tr>
<tr>
<td>APS</td>
<td>75 µL</td>
<td>75 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µL</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

The components of 6% stacking gel and 10% running gel were mixed in separate falcon tubes and made up to 10 mL in ddH₂O.

2.3 Other Solutions
2.3.1 DNase 1
Qiagen® RNase free DNase set contains DNase 1 in a powder form, which was diluted according to manufacturer’s directions in 550 µL of RNase free water. Aliquoted in 0.6 mL eppendorf tubes and stored at -20°C until use. A 10 µL solution of DNase 1 was added to 70 µL of RDD buffer provided in the kit and used immediately.
Chapter 3

Methods
3.1 Tissue Culture

3.1.1 Cell lines
Human mesothelioma cell lines used in this study were isolated from pleural effusions (CRL2081, JU77, LO68, NO36 and VGE) (Manning, Whiteker, Murch, Garlepp, Davis & Musk, 1991) or obtained from Plural Diseases Unit at Lung Institute of Western Australia. An SV40 transformed non-tumorigenic normal human mesothelial cell line (MeT-5A) originating from the pleural effusion of a non-cancerous donor was also used (Ke at al., 1989). Primary mesothelial cells used in this study were isolated and cultured by Ms. Kimberly Birnie. These cells originated from pericardial fluids of patients undergoing heart surgery. Ethics approval for collection of these cells was provided by the Sir Charles Gardner Hospital Human Ethics Committee and RNA was isolated from these cells using the Qiagen® Reasy Mini Kit (section 3.6.1).

3.1.2 Cell Culture conditions
All cells were cultured in 75 cm² tissue culture flasks containing growth medium consisting of Dulbecco’s modified eagle’s medium (DMEM) with 10% foetal calf serum (FCS), 4 mM L-glutamine, and either 100 U/mL penicillin and 100 µg/mL Streptomycin or 5 mg/mL gentamycin. Cell cultures were switched to gentamycin in the middle of the study due to issues with bacterial contamination. Cells were incubated in 5% (v/v) CO₂ at 37°C and passaged when confluent.

3.1.3 Cell Thawing and Freezing
Cell stocks were stored in liquid nitrogen (LN₂) and when required for culture, an aliquot was removed and thawed at 37°C in a water bath. Cells were then added to a 25 cm² flask containing 4 mL of growth medium. The next day the medium was changed to 5 mL growth medium. Once the cells were confluent, they were passaged and transferred to a 75 cm² flask.

For cell freezing, 1 x 10⁶ cells were resuspended in 1 mL of freezing medium (DMEM with 50% FCS and 10% DMSO). The cell suspension was added to a
cryogenic vial and stored at -80°C overnight in a Mr Frosty container. The following day, the vial was transferred to LN₂.

3.1.4 Cell passage
Once the cells were confluent, the medium was aspirated from the flask and the cells were washed twice with sterile 1x PBS to remove any remaining medium. Thereafter, 1 mL of 0.25% trypsin/EDTA was added. Once the cells detached from the surface of the flask, 1 mL of growth medium was added to neutralise the trypsin. A single cell suspension was formed by adding 9 mL of growth medium to the flask and pipetted up and down. Cells were then transferred to a sterile 15 mL falcon tube and centrifuged at 1200 rpm for 5 min at room temperature. The supernatant was aspirated and cells resuspended in fresh growth medium at appropriate concentrations. Cell lines CRL2081, JU77 and LO68 were split at a ratio of 1:10, NO36, VGE and MeT-5A were split at a ratio of 1:5. These cells were then made up to 10 mL growth medium in a new 75 cm² flask.

3.1.5 Mycoplasma Testing
Cell cultures were assessed for Mycoplasma spp. infection every time a cell line was removed from liquid Nitrogen (LN₂). Mycoplasma are a group of micro-organisms that may cause changes in cells by altering their cell metabolism, morphology, as well as protein and RNA/DNA synthesis (Drexler & Uphoff, 2003). Following trypsinisation and cell resuspension in growth medium during cell passaging, 1mL of the cell suspension was added to a 1.5 mL microcentrifuge tube and centrifuged at 400 x g for 5 min. The supernatant was aspirated and 1mL of 0.9% saline (NaCl) was added to the pellet and centrifuged at 400 x g for 5 min. The saline wash was repeated twice. The pellet was left in the tube and sent to PathWest Laboratory Medicine WA for mycoplasma testing. All the cell cultures used in this study were negative for Mycoplasma spp.

3.1.6 Cell counting and seeding
Cells were counted in a haemocytometer to determine the number of cells available for seeding. Following trypsinization and centrifugation, the cell pellet was re-
suspended in 5 mL of fresh growth medium and a 10 µL aliquot of the cell suspension was added to 10 µL of trypan blue and 10 µL loaded into a haemocytometer. The cell concentration was calculated using the following equation:

\[
\text{Number of cells per mL} = \frac{\text{Total number of cells} \times 10^4}{4}
\]

Based on this cell calculation, the correct concentration of cells were seeded into assay plates. Cells were plated in growth medium to a total volume of 3 mL in 6 well plates and 1 mL each in 12 well plates. Cells were then incubated in 5% (v/v) CO₂ at 37°C. The number of cells plated in each type of plate type for different experiments is shown in table 3.1.

**Table 3.1: Cell densities seeded for each experiment and the type of culture plate used.**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Experiment</th>
<th>Cell density</th>
<th>Type of plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>LO68, CRL2081, JU77</td>
<td>Basal gene expression</td>
<td>0.75 x 10⁵</td>
<td>12 well</td>
</tr>
<tr>
<td>NO36</td>
<td>Basal gene expression</td>
<td>1.0 x 10⁵</td>
<td>12 well</td>
</tr>
<tr>
<td>MeT-5A, VGE</td>
<td>Basal gene expression</td>
<td>1.5 x 10⁵</td>
<td>12 well</td>
</tr>
<tr>
<td>LO68, CRL2081, JU77, MeT-5A</td>
<td>Gene expression with stimulation</td>
<td>2.5 x 10⁵</td>
<td>12 well</td>
</tr>
<tr>
<td>LO68, MeT-5A</td>
<td>Protein expression with stimulation</td>
<td>5.0 x 10⁵</td>
<td>6 well</td>
</tr>
<tr>
<td>Met-5A, CRL 2081</td>
<td>Gene expression with transfection</td>
<td>1.0 x 10⁵</td>
<td>12 well</td>
</tr>
<tr>
<td>MeT-5A, CRL 2081</td>
<td>Protein expression with transfection</td>
<td>5.0 x 10⁵</td>
<td>6 well</td>
</tr>
</tbody>
</table>
3.2 Basal gene expression
Following cell passage, cells were seeded in 12 well plates in triplicates at the optimal seeding density for different cell lines as outlined in table 3.1. The correct number of cells made up to 1mL per well in full growth medium were seeded in 12 well plates and incubated for 48 hr at 37ºC in 5% v/v CO₂. Following 48 hr incubation, cells were harvested and mRNA levels for TGF-β₁ and 2 and Gli1 and 2 determined according to section 3.6.

3.3 TGF-β stimulation
Following cell passage, cells were seeded in appropriate plates for mRNA expression and protein experiments as outlined in table 3.1 and incubated overnight at 37ºC in 5% v/v CO₂. The next day the medium was aspirated and replaced with 1mL of starvation medium (DMEM, 0.4% FCS, L-glutamine, Penicillin/streptomycin or Gentamycin) in 12 well plates or 2mL in 6 well plates and incubated overnight. The following day the starvation medium was aspirated and cells incubated with 5 ng/mL TGF-β₁ or TGF-β₂ was made up to 1 mL with starvation medium in each well for 12 well plates and up to 2 mL/well for 6 well plates. The control wells contained 2mg/mL BSA vehicle control in starvation medium. Cells were then incubated for 4 to 48 hr at 37ºC in 5% v/v CO₂ for mRNA expression and from 0-2 h for protein measurement. At the appropriate time point cells were harvested for Gli1 and 2 mRNA and pSMAD2, total SMAD2/3 and α-tubulin protein expression analyses according to section 3.6 and 3.7 respectively.

3.4 TGF-β receptor inhibitor studies
CRL 2081 and MeT-5A cells were used for all TGF-β receptor inhibition and Gli studies. As per table 3.1, Met-5A and CRL 2081 cells were seeded in 12 well plates at 2.5 x 10⁵ per well and in 6 well plates at 5.0 x 10⁵ for protein expression. The CRL2081 cell line represents MM cell line and MeT-5A represents a non-malignant mesothelial cell line. Following overnight incubation, the medium was replaced with 1mL and 2mL starvation medium in 12 well and 6 well plates respectively. The next day cells were pre-treated with 20 µM of the TGF-β type 1 receptor (ALK5) inhibitor, SB-431542. Cells were also treated with 5-10 µM of the Hh pathway
inhibitor cyclopamine to determine if TGF-β-induced Gli expression occurs independently of the classic Ptch/SMO-mediated hedgehog pathway. Control wells were incubated with the same volume of 100% DMSO or ethanol as vehicle controls for the ALK5 inhibitor or cyclopamine respectively. Cells were pre-treated for 1h with negative inhibitors followed by stimulation with 5 ng/mL TGF-β₂ for 6 hr for mRNA expression and up to 2 h for protein measurement and then lysed according to section 3.6.1. The protocol for mRNA expression and protein detection was followed as outlined in section 3.6 and 3.7 respectively.

3.5 Transfection of cells

Cells were seeded for mRNA or protein expression studies as shown in table 3.1. After overnight culture, the medium was changed to DMEM with 10% FCS but no antibiotics or L-glutamine for at least 45 minute prior to transfection, as outlined in table 3.2.

<table>
<thead>
<tr>
<th>Type of plate</th>
<th>Volume of DMEM with 10% FCS, no antibiotics or L-glutamine added</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 well</td>
<td>2800 µL</td>
</tr>
<tr>
<td>12 well</td>
<td>900 µL</td>
</tr>
</tbody>
</table>

Cell transfection was performed using two mixtures. The first transfection mixture contained appropriate concentrations of SMAD2, SMAD3 or control siRNA made up in DMEM (containing no FCS, L-glutamine or antibiotic). The volume of transfection mixture per well depended on the type of plate used (outlined in table 3.4). The second transfection mixture contained Lipofectamine™ 2000 reagent also made up in DMEM, and amounts of this mixture used determined by plate size are shown in table 3.3. The first and second mixture were combined and incubated for 20 min at room temperature to allow complexing of the reagents. The total volume of mixture as indicated in table 3.4 was then added drop wise to the appropriate well.
The plates were gently rocked to combine the mixtures and returned to the incubator. The siRNA to SMAD2, SMAD3 and control were initially transfected into cells at a final concentration of 1 nM, 10 nM and 20 nM for optimisation experiments. 10 nM concentration was subsequently chosen as the optimal concentration for SMAD2 and SMAD3 inhibition studies.

Table 3.3: Volume of Lipofectamine™ 2000 made up in DMEM, and total volume of the mixture per well according to plate type.

<table>
<thead>
<tr>
<th>Plate</th>
<th>Lipofectamine™ 2000/well (µL)</th>
<th>DMEM/well (µL)</th>
<th>Total volume of Lipofectamine™ 2000 + DMEM/well (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 well</td>
<td>2</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>12 well</td>
<td>1</td>
<td>49</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 3.4: Volumes per well used for transfection of cells according to plate type.

<table>
<thead>
<tr>
<th>Plate</th>
<th>Volume of transfection mixture (µL)</th>
<th>Volume of Lipofectamine™ 2000 + DMEM/well (µL)</th>
<th>Total volume of complexes added/well (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 well</td>
<td>100</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>12 well</td>
<td>50</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

At 24 hours following transfection, the medium was aspirated and 0.4% FCS starvation medium was added to the cells. The following day, appropriate wells were stimulated with TGF-β₂ and the cells lysed for Gli mRNA expression at 6 hr post stimulation and phosphorylated and total SMAD2/3 protein measurement at 2 hr as per section 3.6.1 and 3.7.1 respectively.
3.6 mRNA expression analysis
Following treatments, cells were lysed and RNA isolated using the Qiagen® RNeasy mini kit. The RNA was then converted to cDNA and analysed by Real-Time PCR.

3.6.1 Cell lysis, RNA isolation and quantification
The medium in 12 well plates was aspirated and 200 µL of the Lysis buffer from the RNeasy mini kit was added to each well. A 1% v/v β-mercaptaethanol solution was added to the kit lysis buffer immediately before use. All experiments were set up in triplicate wells for each treatment and the lysates were combined into one microcentrifuge tube. RNA was extracted according to the Qiagen® RNeasy mini kit protocol with a minor modification to include DNase 1 treatment. To degrade any DNA contamination in the sample, 350 µL of wash buffer was added to spin columns and centrifuged at 12 000 x g for 15 sec. The waste was discarded and 80 µL of RNase free DNAse 1 solution (section 2.3.1) was added to the spin columns and left for 15 min at room temperature. A 350 µL aliquot of wash buffer was added to the spin columns and centrifuged at 12 000 x g for 15 sec. The rest of the protocol was performed according to the manufacturer’s instructions. RNA was eluted in 50 µL of RNase free water. The extracted RNA was quantified using a NanoDrop 2000 spectrophotometer and samples stored at -20ºC until use.

3.6.2 cDNA synthesis
cDNA was synthesised from RNA using the Qiagen® Omniscript Reverse Transcriptase kit. The reaction mixture contained 1 µg of RNA, 1x RT buffer, dNTP (500 µM), random hexamer primers (10 µM), RNase inhibitor (10 U), reverse transcriptase (4 U) and RNase free water giving a total reaction volume of 20 µL. A no template control was also included that contained everything except RNA, which was replaced with RNase free water. The cDNA synthesis reaction was carried out at 37ºC for 60 min in the PCR thermocycler. Samples were stored at -20ºC until use.
3.6.3 Real-Time PCR
Following cDNA synthesis, Real-Time PCR was carried out to determine the levels of various mRNAs of interest in the human MM and control cell lines. The reactions were performed in duplicate with a total volume of 10 µL per well in a 96 well reaction plate. Each reaction contained 1uL of template cDNA, 1x TaqMan® gene expression Master Mix, 1x TaqMan® probe and RNase free water. Controls included the no template control from the cDNA synthesis, an RNA control (containing 2µL of each RNA template used in the cDNA synthesis reaction) to detect any genomic DNA contaminations and a water control to detect any real time PCR master mix contamination. An adhesive optical film was used to cover the plate and the plate centrifuged to collect the samples at the bottom of the wells. The samples were then run on the StepOne Plus™ Real-time PCR system. The program consisted of 1 cycle each of 50ºC for 2 min followed by 95ºC for 10 min followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min. The data was collected and stored during the run by the StepOne software v2.1.

3.7 Protein analysis (Western blot)
3.7.1 Protein extraction
Cells seeded in 6 well plates as previously outlined in table 3.1 were used for protein analysis. The medium was aspirated from wells and cells washed twice with ice cold 1x PBS. SLB++ buffer (section 2.2.6), 220 µL for 100% confluent cells and 110 µL for 70-90 confluent cells was then added to the wells. The cells were scraped from the plate and the suspension transferred into a microcentrifuge tube. The tube was then vortexed and incubated on ice for 10 min followed by 10 min centrifugation at 13 000 x g at 4ºC. The supernatant was transferred to a new microcentrifuge tube and stored at -20ºC until use.

3.7.2 Protein quantification (Coomassie plus Bradford Assay)
The Coomassie plus Bradford assay was used to quantify protein samples. Different concentrations of BSA were prepared from 2 mg/ml stock BSA and deionised water to use as a standard curve. Concentrations of the BSA ranged from 0.05 mg/ml to 0.75 mg/ml. Protein samples were diluted 1:10 and 1:20 in deionised water and 10
µL of that was plated in duplicates in a 96 well plate followed by 200 µL of Coomassie plus reagent. Solutions were mixed by tapping the plate then incubated at room temperature for 5 min before reading at 595 nm on the Wallac Victor Multilable Counter.

3.7.3 Sample preparation
The concentration of protein in each sample was calculated from the Coomassie plus Bradford assay. Samples were thawed at room temperature and the appropriate volume of sample required for 10-20 µg of protein was made up to 16 µL with deionised water and added to 4 µL of loading buffer, giving a total of 20 µL. The tubes were then heated to 95ºC for 10 min and centrifuged briefly. Samples were then loaded onto the polyacrylamide gel for electrophoresis.

3.7.4 Gel electrophoresis
The polyacrylamide gel consisted of a 10% running gel and 6% stacking gel. The protein samples were loaded into the wells. A 5 µL Precision Plus protein™ dual coloured standard was also loaded. The running tank was then filled with 1x SDS-PAGE running buffer and run at 180V for approximately 45 min.

3.7.5 Electrophoretic protein transfer
Once the gel electrophoresis was complete, the PVDF (soaked in methanol for 10 min) or nitrocellulose transfer membrane, sponges and filter paper, were all soaked in 1x transfer buffer for approximately 10 min. A cassette was prepared and the gel and membrane were sandwiched between sponge and filter paper with sponge first then filter papers, gel, membrane, filter papers, and another sponge. These were all sandwiched together after ensuring no air bubbles formed between the gel and the membrane. The cassette and an ice block were placed in the transfer tank and the tank filled with cold 1x transfer buffer (4ºC). The proteins were transferred at 100v for 1.5 hr at room temperature.
3.7.6 Protein detection

After protein transfer was complete, the cassette was dissembled and the membrane blocked for 1 hr at room temperature in TBST with 5% skim milk powder (chapter 2, Section 2.2.1) to block non-specific binding. The membrane was then washed 4 times with 1 x TBST (section 2.2.8) for 5 min each before being incubated with the primary antibody at 4ºC overnight. Primary antibodies used in this study were diluted in 5% skim milk TBST as shown in table 3.5. Following overnight incubation, the membrane was washed 4 times in TBST for 5 min each at room temperature and then incubated for 1.5 hr in secondary antibody diluted in 1% skim milk TBST as described in table 3.5. Membranes were washed 4 times with 1 x TBST for 5 min each prior to the addition of Immobilon Western HRP Substrate. Protein bands were detected at different exposures with CL-Xposure™ Film using the AGFA developer in a CP1000 X-ray processor.

Table 3.5: Dilutions of primary and secondary antibody used for western blot analysis

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Dilution used</th>
<th>HRP conjugated secondary antibody</th>
<th>Dilution used</th>
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3.7.7 Membrane Stripping

Membranes were stripped with two 15 min washes in glycine stripping buffer. The membranes were then washed 4 times in 1 x TBST for 5 min each before being blocked for 1 hr in 5% skim milk TBST. The membrane was then incubated in primary antibody overnight at 4ºC and the western blot process continued as described in section 3.7.6.
3.7.8 Western blot analysis optimisation

Following analysis of baseline TGF-β1/2 and Gli1/2 in MM and control cells, 3 MM and control MeT-5A cell lines were selected for TGF-β1/2 stimulation studies to examine Gli expression. Activity of TGF-β can be measured by examining the protein expression of its downstream signalling molecules such as SMAD2/3 and specifically the phosphorylation of SMAD2 and SMAD3. For optimisation of pSMAD2, pSMAD3 and total SMAD2/3, antibodies for western blot analysis were used at various concentrations: primary (1:1,000 to 1:4,000) and secondary (1:1,000 to 1:5000).

During the optimisation process, it was discovered that the PVDF transfer membrane produced excessive background and band fuzziness (figure 3.1 A), therefore I decided to use nitrocellulose membranes for all further experiments. Total SMAD2/3 had to be optimised so that there was a clear separation of the two protein bands. The SDS-page gel was initially run at 100V for 2 hr to produce band separation for accurate quantitation (figure 3.1 B), but in the later experiments the gel was run at 180V for 45 min which produced better band separation (figure 3.1 C).

After several optimisation runs it was determined that the best western blot conditions were to run the SDS-page gel at 180V for 45 min and the protein transfer onto a nitrocellulose membrane and probed with the optimised concentrations of primary and secondary antibodies.

Figure 3.1 Optimisation of total SMAD2/3 antibody with different western blot conditions. (A) SDS page gel run at 100V for 1.5 hr and proteins transferred onto a
PVDF membrane showing fuzziness of the bands and high background. (B) SDS page gel run at 100V for 1.5 hr and proteins transferred onto a nitrocellulose membrane produced cleaner bands. (C) SDS page gel run at 180V for 45 min and proteins transferred onto a nitrocellulose membrane produced clean and crisp bands.

3.8 Surefire protein assay

3.8.1 Protein quantitation and sample preparation

Cells were seeded into 6 well plates at optimal seeding densities as outlined in table 3.1. After overnight incubation at 37°C, the medium was aspirated from each well and 2 mL of starvation medium added. After 16-24 hr incubation the wells were stimulated with 5 ng/µL TGF-β₂ and protein lysed using 1 x Lysis buffer supplied by the Alphascreen® Surefire® Assay kit. The samples were then incubated on ice for 10 min before being centrifuged at 12 000 x g for 3 min to pellet insoluble material. The supernatants were transferred to a new microcentrifuge tube and stored at -20°C until use. To ensure equal loading of protein, the concentration was quantified using a Bradford assay as described in section 3.7.2. A 1µg aliquot of protein for each sample was made up to 4 µL in ddH₂O water and added to a Proxiplate 384 well plus plate.

3.8.2 Alphascreen® Surefire protein assay

After the addition of 4 µL of the prepared protein lysates (section 3.7.1) to the Proxiplate 384 Plus plate, 5 µL of acceptor mix was added to the wells. The acceptor mix contained acceptor beads diluted 1:50 in activation buffer and reaction buffer mix (the activation buffer diluted 1:5 in reaction buffer). The plate was then sealed with a top seal black adhesive cover, wrapped in aluminium foil and incubated for 2 hr at room temperature in a dark environment. The plate was then unsealed and 2 µL of donor mix containing the donor beads diluted 1:20 in dilution buffer was added to the wells. The plate was again resealed with the black adhesive seal and wrapped in aluminium foil and incubated at room temperature in a dark environment for 2 hr or overnight for better protein detection. The plate was unsealed and fluorescence in each sample quantified using the EnSpire plate reader.
3.9 Flow Cytometry

3.9.1 Cell setup and harvest
Cells were transfected according to section 3.5 with fluorescent labelled siRNA. From this point the cells were kept in the dark where possible. After 24 hr of incubation, the medium was aspirated and the cells washed twice with 1xPBS. A 50 µL aliquot of trypsin/EDTA was then added to each well followed by 200 µL of 1 x PBS. Once the cells detached from the surface of the well, 50 µL of growth medium was added to each well to neutralise the trypsin/EDTA. The suspension was then added to 3 mL sterile glass tubes and centrifuged at 1200 for 5 min at room temperature. The supernatant was then discarded, the pellet resuspended in 1 mL of 1 x PBS and centrifuged again at 1200 for 5 min. The supernatant was then removed, the pellet resuspended in 500 µL 1 x PBS and the cells immediately examined using the Olympus® IX-81® inverted fluorescence microscope.

3.10 Statistical Analysis
Microsoft Excel and GraphPad Prism 5.0 were used to analyse data. Data is presented as mean ± standard error of the mean (SEM) with three or more replicates. Statistical analysis was performed by a student’s unpaired t-test or one way analysis of variance for multiple comparisons using one way ANOVA. A p value of equal to or less than 0.05 was considered to be significant. Data with only two replicates are presented as mean.
Chapter 4

Results
4.1 Cell Morphology

MM cell lines used in this study were morphologically consistent with those previously published (Manning et al., 1991) (figure 4.1). At confluence the CRL2081 and LO68 cells expressed a spindled shaped morphology (figure 4.1 A and C), whereas JU77, NO36 and VGE cells had a cobblestone, epithelial-like morphology at confluence (figure 4.1 B, E and F). The control human transformed mesothelial cell line MeT-5A, was also epithelial-like when confluent but had a more fibroblastic morphology when sub-confluent, which is consistent with their original morphology at isolation (Ke et al., 1989) (figure 4.1 D).

![Figure 4.1: Morphology of confluent cell lines. (A) CRL2081 (B) JU77 (C) LO68 (D) MeT-5A (E) NO36 and (F) VGE cells. Bar = 0.2 mm.](image-url)
4.2 Baseline levels of TGF-β1/2 and Gli1/2 in MM and control cells.
Baseline levels of TGF-β1/2 and Gli1/2 mRNA were measured in 5 MM cell lines and control MeT-5A and primary mesothelial cells by real-time PCR. These cells were the once available at the laboratory and thus screened for the baseline levels. Determining the basal gene expression levels of TGF-β and Gli in these cells will allow comparisons to be made between normal and malignant cells and provide a reference point for differences in gene expression for subsequent experiments.

4.2.1 Baseline levels of TGF-β1 and TGF-β2 mRNA in MM and control cells.
TGF-β plays an important role in modulating the growth and immune function of many types of cancer and is also produced by several different cancer cell types including MM (Fitzpatrick et al., 1994). In this study the baseline levels of TGF-β₁ and TGF-β₂ mRNA in 5 MM and MeT-5A cell lines were compared to control normal mesothelial cells. RNA isolated from primary mesothelial cells was kindly provided by Ms. Kimberly Birnie in our laboratory. Human PGK-1 was used as an endogenous control. The level of TGF-β₁ mRNA varied between different MM cell lines. MeT-5A, LO68 and JU77 cells had high levels of TGF-β₁ mRNA (p-value of 0.0209, 0.0004 and 0.0018 respectively), while VGE (p-value of 0.0485) and NO36 had lower TGF-β₁ mRNA levels than control mesothelial cells but the difference wasn’t significant (figure 4.2).

The level of TGF-β₂ mRNA was also measured in all cell lines and control cells. The levels were lower in MeT-5A and MM cells compared with control mesothelial cells (figure 4.3), but the differences were not significant in other cell lines except CRL2081 (p-value of 0.0100).
Figure 4.2: Baseline TGF-β₁ mRNA levels in MM and control cells. TGF-β₁ mRNA levels were quantified by real time PCR and the results normalised to PGK-1. MM and MeT-5A cell lines (dark grey bars) were compared to primary mesothelial cells (light grey bar). Data represent the mean of three experiments. Error bars = SEM (standard error of mean). *= p ≤ 0.05. **= p ≤ 0.01. ***= p ≤ 0.001.
Figure 4.3: Baseline TGF-β2 mRNA levels in MM and control cells. TGF-β2 mRNA levels were quantified by real time PCR and the results normalised to PGK-1. MM and MeT-5A cell lines (dark grey bars). Primary mesothelial cells (light grey bar). Data represent the mean of three experiments. Error bars = SEM. * = p ≤ 0.05.

4.2.2 Baseline Gli1 and Gli2 mRNA levels in MM and control cells.
The effector molecules of the Hh signalling pathway are the Gli transcription factors (Forbes et al., 1996). This study measured Gli1 and Gli2 mRNA levels in MM, MeT-5A and primary mesothelial cells. Gli1 expression was higher in MeT-5A and 4 out of 5 MM cell lines compared with primary mesothelial cells (figure 4.4), although the difference was only significant in three MM cell lines, LO68, CRL2081 and NO36 (p-value of 0.0022, 0.0085 and 0.0002 respectively). JU77 cells had significantly lower Gli1 mRNA levels compared to primary cells (p-value of 0.0101). Similarly, Gli2 expression was higher in MeT-5A and 3 out of 5 MM cell lines compared to primary mesothelial cells, however only LO68 was significantly different (p-value of 0.0128) (figure 4.5).
Figure 4.4: Baseline Gli1 mRNA levels in MM and control cells. Gli1 mRNA levels were quantified by real time PCR and the results normalised to PGK-1. MM and MeT-5A cell lines (green bars) were compared to primary mesothelial cells (white bar). Data represent the mean of three experiments. Error bars = SEM. *= p ≤ 0.05. **= p ≤ 0.01. ***= p ≤ 0.001.
Figure 4.5: Baseline Gli2 mRNA levels in MM and control cells. Gli2 mRNA levels were quantified by real time PCR and the results normalised to PGK-1. The MM and MeT-5A cell lines (maroon bars) were compared to primary mesothelial cells (white bar). Data represent the mean of three experiments. Error bars = SEM. *= p ≤ 0.05.

4.3 Confirmation of TGF-β1 and TGF-β2 activity.
TGF-β is known to induce phosphorylation of SMAD2 and SMAD3 and TGF-β1 is known to stimulate the production of many extracellular matrix (ECM) proteins including collagen type-1 (Ignatz & Massague, 1986). Therefore, I wanted to see if my preparation of recombinant TGF-β1/2 induced phosphorylation of SMAD2 and stimulated collagen type-1α1 mRNA production in one of my MM cell lines JU77. Based on previous studies in my laboratory and from published data, I expected pSMAD2 increase over time with TGF-β stimulation and maximal collagen mRNA expression at 24 hr, therefore this confirmation experiments were done on the cell line previously shown to be inducing this.

4.3.1 TGF-β signalling is mediated by the SMAD2/3 pathway in MM cells.
The SMAD proteins transmit signals from the TGF-β receptors at the cell membrane to the nucleus and initiating transcription of TGF-β target genes (Massague et al.,
2000). Based on previous findings and antibody database, the cells were incubated with recombinant TGF-β2 and protein isolated at 0, 1/2, 1 and 2hrs. JU77 cells were treated with 5 ng/mL TGF-β1 for up to 2 hr and results compared with untreated control. TGF-β1 induced an increase in pSMAD2 over time as expected confirming activity of the TGF-β1 preparation I was using (figure 4.6 A). MM and MeT-5A cells were incubated with recombinant TGF-β2 for 30, 60 and 120 min and pSMAD2 and total SMAD2/3 protein examined by western blot. All three cell lines showed significant induction of pSMAD2 in response to TGF-β2 stimulation within 2 hr of stimulation (figure 4.8 A, B, C and D).

4.3.2 TGF-β induces human collagen type-1α1 mRNA expression in MM and control cells.

For collagen mRNA expression, cells were stimulated with TGF-β1 or TGF-β2 for 6, 24 and 48 hr. Using JU77 cells, TGF-β1 significantly upregulated collagen type-1α1 mRNA at 24 hr compared with unstimulated control (p-value of 0.0168) (figure 4.6 B), similar observations were made with JU77 cells stimulated with TGF-β2 (figure 4.7). These results were consistent with previous findings in my laboratory.
Figure 4.6: TGF-β1 induces the expression of human collagen type-1α1 mRNA and pSMAD2 in JU77 cells. (A) pSMAD2 and total SMAD2/3 protein expression were determined by western blot following 30 min 1h and 2hr TGF-β1 stimulation. Data represents one experiment. (B) Collagen type-1α1 mRNA levels were quantified by real time PCR and the results normalised to PGK-1. Data is presented as fold change above control at three different time points. Red bars show expression at 6 hr, green bars show expression at 24 hr and blue bars show expression at 48 hr. Data represents the mean of three experiments. Error bars = SEM. Significance compared to respective control: *= p ≤ 0.05.
Figure 4.7: TGF-β2 induces human collagen type-1α1 mRNA in MM and control cells. The data is presented as fold change for each cell line at three different time points (6, 24 and 48 hr) compared with their unstimulated controls. mRNA levels were quantified by real time PCR and the results normalised to PGK-1. Red bars show expression at 6hr, green bars show expression at 24hr and blue bars show expression at 48hr. Each bar represents one experiment.
Control

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**Ratio of pSMAD2 normalised to total SMAD2/3**

Time points

---

**B**

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**C**
### Figure 4.8: TGF-β2 induces phosphorylation of SMAD2 in MM and MeT-5A cells.

Total SMAD2/3 and pSMAD2 was examined by western blot 2 h after TGF-β2 stimulation. White bars show unstimulated control. Pink bars show TGF-β2 stimulated cells. (A) Representative western blot for pSMAD2 and total SMAD2/3. pSMAD2 expression was normalised to total SMAD2/3 by densitometry for (B) CRL2081 (C) LO68 and (D) MeT-5A. Data represent the mean of three experiments. Error bars = SEM. Significance compared to control: *= p ≤ 0.05. **= p ≤ 0.01. ***= p ≤ 0.001.

### 4.4 The effect of TGF-β1 and TGF-β2 on Gli1 and Gli2 mRNA levels in MM and control cells.

Recent studies have shown an interaction between Hh and TGF-β signalling pathways in a variety of cell types (Dennler et al., 2007). This interaction occurs downstream of SMO and targets Gli2 in some normal and malignant cell types (Alexaki et al., 2010). Gli1 expression is subsequently activated in a Gli2 dependent manner (Dennler et al., 2007). Therefore in this study the effect of TGF-β1 and TGF-β2 on Gli1 and Gli2 mRNA levels were examined in MM and control MeT-5A cells at various times. LO68 cells were selected as they showed high expression of Gli1 and Gli2 mRNA expression, whereas JU77 cells showed low levels and CRI2081 cells displayed variable levels. The earlier time point (4-6hr) was set to examine...
early Gli2 expression while the later time points (24 and 48hr) for the subsequent Gli1 expression.

4.4.1 Effect of TGF-β₁ on Gli1 and Gli2 MM and control cells.

TGF-β₁ and TGF-β₂ are both important for MM growth (Kuwahara et al., 2001), therefore in this study the effect of both TGF-β isoforms on Gli1 and Gli2 mRNA levels in MM and control cells were examined.

Figure 4.9 and 4.10 show relative Gli1 and Gli2 mRNA levels after TGF-β₁ stimulation in MM and MeT-5A cells at 6, 24 and 48 hr. TGF-β₁ did not increase Gli1 or Gli2 mRNA levels in any of the cell lines when compared with unstimulated controls. In fact there was a significant decrease in Gli1 mRNA levels at 24 h compared to control in LO68 cells (p value of 0.0134) but this was due to high levels of Gli1 mRNA in the 24 hr control.
Figure 4.9: TGF-β1 does not stimulate an increase in Gli1 mRNA levels in MM and control cells. Gli1 mRNA levels were quantified by real time PCR and the results normalised to PGK-1. White bars show unstimulated cells. Green bars show TGF-β1 stimulated cells. Data is presented as mRNA expression at 6, 24 and 48 h after TGF-β1 stimulations in (A) CRL2081 (B) JU77 (C) LO68 and (D) MeT-5A cells. Data represent the mean of three experiments. Error bars = SEM. Significance compared to untreated control: *= p ≤ 0.05. Abbreviations: control (C).

Figure 4.10: TGF-β1 does not increase Gli2 mRNA levels in MM and control cells. Gli2 mRNA levels were quantified by real time PCR and the results normalised to PGK-1. White bars show unstimulated cells. Red bars show TGF-β1 stimulated cells. Data is presented as mRNA expression at 6, 24 and 48 h after TGF-β1 treatment in (A) CRL2081 (B) JU77 (C) LO68 and (D) MeT-5A cells. Data represent the mean of three experiments. Error bars = SEM.
4.4.2 Effect of TGF-β₂ on Gli1 and Gli2 expression in MM and control cells.

The effect of TGF-β₂ on Gli1 and Gli2 mRNA was examined in three MM and control MeT-5A cell lines at 4, 6, 24 and 48 hr. TGF-β₂ had no effect on Gli1 mRNA expression compared to unstimulated controls in all cell lines tested (figure 4.11). Interestingly, Gli1 mRNA levels were high in the control samples at 24 hr consistent with the observations made with TGF-β₁ stimulations. TGF-β₂ caused a significant down regulation of Gli1 mRNA levels in MeT-5A cells at 24 hr compared to its unstimulated control (p-value of 0.0242).

TGF-β₂ stimulated a significant increase in Gli2 mRNA levels at 4-6 hr in all of the cell lines, including control MeT-5A cells (figure 4.12). A 2.5 and 3 fold increase in Gli2 mRNA expression was observed in CRL2081 and MeT-5A cell lines (p-value of 0.02 and 0.0039 respectively) at 6 h and approximately a 2 fold change in LO68 and JU77 cells at 4h (p-value of 0.0179 and 0.0367 respectively). CRL2081 and control MeT-5A cell lines were subsequently selected for further studies.
Figure 4.11: TGF-β2 does not increase Gli1 mRNA levels in MM and control cells. Gli1 mRNA levels were quantified by real time PCR and the results normalised to PGK-1. White bars show unstimulated cells. Green bars show TGF-β2 stimulated cells. Data is presented as mRNA expression at 4, 6, 24 and 48h in (A) CRL2081 (B) JU77 (C) LO68 and (D) MeT-5A cells. Data represent the mean of three experiments. Error bars = SEM. Significance compared to respective controls: *= p ≤ 0.05.
Figure 4.12: TGF-β2 stimulated upregulation of Gli2 mRNA in all cell types. Gli2 mRNA levels were quantified by real time PCR and the results normalised to PGK-1. White bars show unstimulated cells. Red bars show TGF-β2 stimulated cells. Data is expressed as mRNA expression in (A) CRL2081 (B) JU77 (C) LO68 and (D) MeT-5A cells. Data represent the mean of three experiments. Error bars = SEM. Significance compared to respective controls: *= p ≤ 0.05.

4.4.3 Effect of TGF-β2 on Ptch1 mRNA expression in CRL2081 and control cells.

Several studies have shown Ptch1 to be a target gene for Gli1 (Cigna et al., 2012), and Gli2 (Kim et al., 2007; Regl et al., 2004; Lipinski et al., 2008). In this study I examined the effect of TGF-β2 on Ptch1 mRNA levels in CRL2081 and MeT-5A cells 24 hr and 48 hr post treatment by real-time PCR. CRL2082 cells were selected as they showed greater response with TGF-β2 for Gli2 mRNA expression, and the MeT-5A cells served as control. Ptch1 mRNA levels did not change at 24 h but
increased at 48h compared to control in both the cell lines (figure 4.13), but was only significant in MeT-5A cells (p-value of 0.0127).

Figure 4.13: Ptch1 expression after TGF-β2 treatment in CRL2081 and MeT-5A cells. Ptch1 mRNA levels were measured by real time PCR and the results
normalised to PGK-1. White bars show unstimulated cells. Blue bars show TGF-β2 stimulated cells. Data is expressed as mRNA expression for (A) CRL2081 and (B) MeT-5A cells. Bars represent the mean of three experiments. Error bars = SEM. *= p ≤ 0.05.

CRL2081 and MeT-5A cell lines were selected for further inhibition studies. As CRL2081 had highest Gli2 activation after TGF-β2 stimulation and MeT-5A served as a non-malignant control cell line.

4.5 Effect of blocking TGF-β receptor on Gli2 expression.
MM cells produce TGF-β (Marzo et al., 1997). Therefore to further explore the potential interaction between TGF-β and Hh pathways in MM cells, I examined the effect of blocking TGF-β2 signalling on Gli2 mRNA levels. ALK5 receptor inhibitor SB-431542 compound was used to block the TGFβRI/ALK5 receptor which mediates TGF-β signalling in a wide range of cell types (Ebner et al., 1993). Blocking TGFβRI/ALK5 should inhibit TGF-β2 mediated phosphorylation of SMAD2/3, preventing transcription of TGF-β2 target genes including Gli2 (Inman et al., 2002).

4.5.1 SB-431542 reduced phospho-SMAD2 protein in CRL2081 and MeT-5A cells.
To confirm that inhibiting the TGF-β2 receptor complex abrogated downstream signalling, I examined pSMAD2 by western blot analysis. Incubation of CRL2081 and MeT-5A cells with TGF-β2 lead to robust induction of pSMAD2 which was inhibited when cells were pretreated with SB-431542 prior to TGF-β2 exposure. Figure 4.14 A shows a western blot of CRL2081 and MeT-5A cells treated with SB-431542 and DMSO control in the presence or absence of TGF-β2 and assessed for pSMAD2, total SMAD2/3 and α-tubulin. As shown is figure 4.14 B and C there was a 4.2 fold inhibition of pSMAD2 in CRL2081 cells pretreated with SB-431542 then treated with TGF-β2 and a 15.3 fold decrease in MeT-5A cells.
A

![Western Blot Images]

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**CRL2081**

**MeT-5A**

60 kDa

52 kDa

50 kDa
Figure 4.14: SB-431542 inhibits pSMAD2 protein expression in CRL2081 and MeT-5A cells. pSMAD2 and total SMAD2/3 levels were assessed by western blot. α-tubulin was used as a lane loading control. White bars show treatment in the absence of TGF-β2. Orange bars show TGF-β2 stimulated cells. (A) Western blots showing pSMAD2, total SMAD2/3 and α-tubulin expression for CRL2081 and MeT-5A cells. Densitometric analysis showing pSMAD2 normalised against total SMAD2/3 for (B) CRL2081 and (C) MeT-5A. Each bar represents the mean of two
4.5.2 SB-431542 blocks TGF-β₂-mediated Gli2 mRNA expression in CRL2081 and MeT-5A cell lines.

Initial studies optimised SB-431542 inhibition of TGF-β signalling by treating cells with different concentrations of the inhibitor. The optimal concentration was 20 μM and therefore this concentration was used in all subsequent studies (figure 4.15).

**Figure 4.15: Optimisation of SB-431542 treatment in CRL2081 cells.** Gli2 mRNA was quantified by real-time PCR and the results normalised to PGK-1. White bars show controls and red bars show different concentration of SB-431542 treated cells. Data is presented as mRNA expression. Each bar represents one experiment. Abbreviations: ALK5 inhibitor – SB431542 (SB).
CRL2081 and MeT-5A cells were pretreated with SB-431542 or its vehicle control for 1h then stimulated with TGF-β2. After 6hr the cells were harvested and Gli2 mRNA expression was analysed. Consistent with previous findings both CRL2081 and MeT-5A cells showed significant Gli2 induction by TGF-β2 compared to its DMSO or untreated controls (p-value of 0.0254, 0.0328 and 0.0269 respectively). Gli2 mRNA expression levels in cells treated with SB-431542 in presence or absence of TGF-β2 was compared to cells treated with vehicle control stimulated with TGF-β2. SB-431542 significantly suppressed TGF-β2 induced Gli2 mRNA expression in both cell lines (figure 4.16) (p-value of 0.0458 and 0.0248 in CRL2081 cells and 0.0010 and 0.0107 in MeT-5A cells).
Figure 4.16: SB-431542 abrogates TGF-β2-induced Gli2 mRNA expression. Gli2 mRNA was quantified by real time PCR and the results normalised to PGK-1. White bars show different treatments and red bars show cells with different treatments and stimulated with TGF-β2. Data is presented as relative expression for (A) CRL2081 and (B) MeT-5A cells. Data represents the mean of three experiments. Error bars = SEM. Significance compared to TGF-β2 unstimulated controls: *= p ≤ 0.05. Significance compared to cells treated with DMSO + TGF-β2: ^= p ≤ 0.05. ^ ^ ^= p ≤ 0.001.

4.6 Effect of blocking the SMAD2/3 pathway on TGF-β2 induced Gli2 expression.

To determine if the SMAD2/3 pathway is involved in TGF-β2 mediated Gli2 expression in MeT-5A and CRL2081 cells, SMAD signalling was blocked using siRNA to SMAD2/3 genes. The concentration of siRNA was optimised to successfully knock down SMAD2 and SMAD3 mRNA and protein in MeT-5A cells. Cells were then transfected with siRNA against SMAD2 or SMAD3 and treated with TGF-β2. Gli2 mRNA expression was evaluated by real-time PCR and pSMAD2 and total SMAD2/3 protein expression was assessed by western blot.
4.6.1 Optimisation of siRNA concentrations to block SMAD2 and SMAD3

siRNA concentrations were optimised in MeT-5A cells. Cells were transfected with 1, 10 or 20 nM siRNA against SMAD2 or SMAD3. The SMAD2 and SMAD3 mRNA and pSMAD2 and total SMAD2/3 protein were measured after 48 hr. As shown in figure 4.17. Transfection of 10nM SMAD2 or SMAD3 siRNA resulted in a 3.2 fold reduction in SMAD2 mRNA and a 4.3 fold reduction in SMAD3 respectively relative to controls. At 20nM siRNA there was non-specific suppression of SMAD targets. Therefore 10nM siRNA was used for all subsequent experiments.

The specificity of the siRNA knockdown was further investigated by analysing SMAD2/3 protein expression by western blot. Figure 4.18 A shows western blot of MeT-5A cells treated with 10 and 20nM siRNA to SMAD2 and SMAD3 and assessed for pSMAD2, total SMAD2/3 and α-tubulin protein expression. Treatment of cells with 10nM SMAD2 siRNA knocked down pSMAD2 and total SMAD2 proteins by 50% compared with control (figure 4.18 B and C), and SMAD3 siRNA knocked down approximately 40% total SMAD3 protein (figure 4.18 D). Consistent with mRNA data, treatment of MeT-5A cells with 20nM siRNA led to a non-specific decrease in pSMAD2 protein.
Figure 4.17: SMAD2 and SMAD3 siRNA at 10nM effectively suppressed SMAD2/3 mRNA in MeT-5A cells. SMAD2/3 mRNA levels were quantified by real time PCR and the results normalised to PGK-1. White bars show untransfected or control siRNA transfected cells. Purple and green bars show siRNA transfected cells. Data is presented as mRNA expression of (A) SMAD2 and (B) SMAD3. Data represents the mean of two repeat experiments. Abbreviations: control siRNA (cont), SMAD2 siRNA (si SD2) and SMAD3 siRNA (si SD3).
Figure 4.18: SMAD2 and SMAD3 siRNA suppressed pSMAD2 and total SMAD2/3 protein expression in MeT-5A cells. pSMAD2 and total SMAD2/3
levels were examined by western blot analysis. α-tubulin was used as an endogenous and lane loading control. White bars show untransfected or control siRNA transfected cells. Purple and green bars show siRNA transfections. (A) Western blot showing pSMAD2, total SMAD2/3 and α-tubulin expression in MeT-5A cells. Densitometric analysis showing pSMAD2 (B), total SMAD2 (C) and total SMAD3 (D) expression was normalised against α-tubulin. Each bar represents the mean of two experiments.

4.6.2 Validation of SMAD2/3 mRNA expression knockdown.

The effect of SMAD2 and SMAD3 siRNA on SMAD2 and SMAD3 mRNA levels was examined in CRL2081 and MeT-5A cells. Transfection with SMAD2 and SMAD3 siRNA reduced the basal levels of SMAD2 and SMAD3 respectively compared to control siRNA (figure 4.19).

SMAD2 siRNA reduced SMAD2 mRNA levels by 60% in CRL2081 cells (figure 4.19 A), and by 50% in MeT-5A cells (figure 4.19 B) (p-value of 0.0409 and 0.0060 respectively). SMAD3 siRNA reduced SMAD3 mRNA levels by 45% in CRL2081 cells (figure 4.19 C) and approximately 40% in MeT-5A cells (figure 4.19 D) (p-value of 0.0384 and 0.0321 respectively). Similar effects were observed when combining siRNAs to SMAD2/3. In CRL2081 cells SMAD2/3 siRNA reduced SMAD2 mRNA levels by 50% and by 60% in MeT-5A cells (figure 4.18 A and B) (p-value of 0.0500 and 0.0076 respectively). There was approximately 30% inhibition in SMAD3 mRNA levels in CRL2081 cells and 60% in MeT-5A cells with combined SMAD2/3 siRNA (figure 4.19 C and D).
Figure 4.19: SMAD2 and SMAD3 siRNA supresses SMAD2/3 mRNA in CRL2081 and MeT-5A cells. Cells were transfected with siRNA against SMAD2 or SMAD3 (coloured bars) or controls (white bars). SMAD2 and SMAD3 mRNA levels were quantified by real time PCR and the results normalised to PGK-1. Data is presented as mRNA expression of SMAD2 in (A) CRL2081 and (B) MeT-5A and SMAD3 in (C) CRL2081 and (D) MeT-5A. Data represent the mean of four experiments. Error bars = SEM. Significance compared control siRNA transfected cells: * = p ≤ 0.05. Abbreviations: Control siRNA (Cont siRNA), SMAD2 siRNA (si SD2), SMAD3 siRNA (si SD3), SMAD2 and SMAD3 siRNA (si SD 2/3).

4.6.3: Validation of SMAD2/3 protein expression knockdown.

To confirm that siRNA to SMAD2 and SMAD3 inhibits SMAD proteins, CRL2081 and MeT-5A cells transfected with SMAD2 and SMAD3 siRNA and total and pSMAD2 and pSMAD3 proteins analysed by western blot. These cells were also stimulated with TGF-β2 after SMAD2 and SMAD3 siRNA transfections to examine reduction in pSMAD2 and pSMAD3 proteins compared to TGF-β2 induced levels.
Staining for α-tubulin was used as an endogenous control and lane loading control. Phosphorylated SMAD2 and SMAD3 were reduced when cells were transfected with siRNA to SMAD2 and SMAD3 respectively (figure 4.20 A and 4.21 A). SMAD siRNAs also knocked down TGF-β2 mediated pSMAD2 and total SMAD2/3 respectively. However the knock down was only around 40-50% for phosphorylated SMAD2 (figure 4.20 B and 4.21 B) and 25% for SMAD3 (figure 4.20 C and 4.21 C) compared with controls for both cell lines. In both cell lines, partial knockdown of total SMAD2 and SMAD3 protein was also observed with respective siRNAs (figure 4.20 and 4.21 C and D).
Figure 4.20: SMAD2 and SMAD3 siRNA transfections shows a trend towards reduced pSMAD2/3 and total SMAD2/3 protein in CRL2081 cells. Coloured bars show transfections followed by TGF-β2 stimulation. White bars show treatment in the absence of TGF-β2. (A) Western blots for pSMAD2, total SMAD2/3 and α-tubulin loading control. Densitometric analysis showing pSMAD2 (B), pSMAD3 (C), total SMAD2 (D) and total SMAD3 (E) normalised to α-tubulin. Data represent mean of two experiments.
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**Experiment 1**

**Experiment 2**

**Notes:**
- pSMAD2 and pSMAD3 are phosphorylated forms of SMAD2 and SMAD3.
- Total SMAD2/3 represents the total level of SMAD2 and SMAD3.
- α-tubulin is used as a loading control.
No si
No si +TB2
Cont si
Cont si +TB2
si SD2
si SD2 +TB2
si SD3
si SD3 +TB2
si SD2/3
si SD2/3 +TB2

B

Ratio of total SMAD2 normalised to α-tubulin

Treatment

C

Ratio of pSMAD3 normalised to α-tubulin

Treatment

D

Ratio of total SMAD2 normalised to α-tubulin

Treatment
Figure 4.21: SMAD2 and SMAD3 siRNA transfections shows a trend towards reduced pSMAD2/3 and total SMAD2/3 protein in MeT-5A cells. Coloured bars show transfections followed by TGF-β2 stimulation. White bars show controls in the absence of TGF-β2. (A) Western blots for pSMAD2, total SMAD2/3 and α-tubulin loading control. Densitometric analysis showing pSMAD2 (B), pSMAD3 (C), total SMAD2 (D) and total SMAD3 (E) normalised to α-tubulin. Data represent mean of two experiments. Abbreviations: No siRNA (No si), Control siRNA (Cont si).

4.6.4 Gli2 induction by TGF-β2 is SMAD2/3 dependent.
To determine if TGF-β2-induced Gli2 expression is SMAD2/3 dependent, CRL2081 and MeT-5A cells were transfected with 10nM siRNA to SMAD2, SMAD3 or control siRNA treated with TGF-β2 and Gli2 mRNA levels measured. Following TGF-β2 treatment, there was strong induction of Gli2 mRNA in both cell lines. This induction was reduced when the cells were transfected with SMAD2 or SMAD3 siRNA (figure TGF4.22). SMAD2 and SMAD3 siRNA showed greatest inhibition of Gli2 mRNA expression in CRL2081 cells (p-value of 0.0093 and 0.0044 respectively) (figure 4.22 A). Although there was a trend for a decrease in Gli2 levels in MeT5A cells transfected with SMAD2 and SMAD3 siRNA (figure 4.22 B).
Figure 4.22: SMAD2 and SMAD3 siRNA suppress TGF-β2-mediated Gli2 mRNA expression in CRL2081 and shows a trend towards a decrease in MeT-5A cells. Gli2 mRNA levels were quantified by real-time PCR and the results normalised to PGK-1. Cells transfected with siRNA targeting SMAD2, SMAD3 or control siRNA were treated with TGF-β2 (red bars) or left untreated (white bars) and Gli2 mRNA levels measured. Data is presented as mRNA expression for (A)
CRL2081 and (B) MeT-5A cells. Data represents the mean of four experiments. Error bars = SEM. Significance compared to TGF-β$_2$ unstimulated controls: *= p ≤ 0.05. Significance compared control siRNA transfected and TGF-β$_2$ treated cells: ^= p ≤ 0.05. ^^= p ≤ 0.01.

4.6.5 Transfection efficiency of siRNA in CRL2081 and MeT-5A cells.
As the knockdown effects of siRNA to SMAD2 and SMAD3 were only moderate, I wanted to measure the transfection efficiency in the cell lines. The SMAD2/3 siRNA used in the study did not have a label that could be used to assess transfection efficiency. Therefore I transfected cells with control siRNA containing a fluorescein conjugate (FITC) designed to measure transfection efficiency. FITC is a light reactive dye that can be quantified by FACS analysis. Similar to other siRNA transfection experiments, 10nM FITC labelled control siRNA was transfected into cells and efficiency assessed 24 hr after transfection. The cell population was gated based on the dimensions and complexity of the cells analysed (figure 4.23 A for CRL2081 and figure 4.24 A for MeT-5A cells). The cell population was separated into FITC negative and FITC positive cells using untransfected cells as negative controls (figure 4.23 B and C for CRL2081 and figure 4.24 B and C for MeT-5A cells). Transfection was only about 6% in CRL2081 and 27% in MeT-5A cells (figure 4.23 D and 4.24 D).
Figure 4.23: Transfection efficiency of FITC labelled control siRNA in CRL2081 cells. The percentage of cells expressing FITC label was determined. (A) representative scatter plot showing side scatter height (SCC-H) versus forward scatter height (FSC-H) and gating the cell population and creating a (B) histogram of FITC fluorescence intensity showing an untransfected cell population and the gating for FITC positive cells and (C) histogram of FITC fluorescence intensity showing transfected cell population and gating for FITC positive cells. (D) Percentage of FITC positive cells. Data is the mean of three wells in one experiment. Error bars = SEM.
Figure 4.24: Transfection efficiency of FITC labelled control siRNA in MeT-5A cells. The percentage of cells expressing FITC fluorescence intensity was determined. (A) Representative dot points plotting side scatter height (SCC-H) verses forward scatter height (FSC-H) and gating the cell population and creating a (B) histogram of FITC fluorescence intensity showing an untransfected cell population and the gating for FITC positive cells and (C) histogram of FITC fluorescence intensity showing transfected cell population and gating for FITC positive cells. (D) Percentage of FITC positive cells. Data is the mean of three wells in one experiment. Error bars = SEM.
4.7 Effect of cyclopamine on TGF-β2 mediated Gli2 expression in cell lines.

Studies in different tours have shown that induction of Gli by TGF-β is independent of Hh signalling (through Ptch/Smo axis) (Dennler et al., 2007). To determine if TGF-β2-induced Gli2 is also independent of Hh signalling in MM, cells were treated with TGF-β2 in presence or absence of cyclopamine (a SMO inhibitor) and Gli2 mRNA levels measured. If TGF-β2 induced Gli2 upregulation is independent of Hh signalling, cyclopamine will have no effect on induction of Gli2.

4.7.1 Effect of cyclopamine on TGF-β2 mediated Gli2 mRNA expression

The effect of blocking Hh signalling with cyclopamine on TGF-β2-mediated Gli2 mRNA expression was examined in MM and control cells. As shown in figure 4.25, samples pretreated with vehicle control then stimulated with TGF-β2 had an unexpected reduction in Gli2 mRNA levels in both MeT-5A and CRL2081 cells. Although cyclopamine treated cells had reduced Gli2 mRNA, no conclusion could be drawn from this experiment. Further optimisation studies are required to eliminate the effect of the control before the effect of blocking the Hh pathway on TGF-β2-induced Gli2 mRNA can be adequately assessed.
Figure 4.25: Cyclopamine and vehicle control reduced TGF-β2 induced Gli2 expression in CRL2081 and MeT-5A cells. CRL2081 and MeT-5A cells were treated with cyclopamine and controls and then stimulated with TGF-β2. Gli2 mRNA was quantified by real time PCR and the results normalised to PGK-1. White bars show treated cells in the absence of TGF-β2. Red bars show treated stimulated with...
TGF-β₂. Data is presented as mRNA expression (A) MeT-5A and (B) CRL2081 cells. Data represents mean of two experiments in MeT-5A and one experiment in CRL2081 cells. Abbreviations: Cyclopamine (Cyclo).

4.7.2 Effect of cyclopamine on Ptch1 expression in MeT-5A cells.
As cyclopamine and the vehicle control affected Gli2 levels, I examined their effect on another target gene Ptch1. MeT-5A cells were selected as these served as the control cells, and I wanted to investigate the effect of cyclopamine in these cells before progressing to MM cells. MeT-5A cells were pretreated with cyclopamine or vehicle control for 1h before incubation with TGF-β₂. Ptch1 mRNA expression levels were then measured after 6 hr. Ptch1 levels were reduced by 5 µM but not the 10 µM cyclopamine compared with the vehicle treated controls but this effect was restored in the presence of TGF-β₂ (figure 4.26). Further experiments need to be performed to confirm this observation.
Figure 4.26: Treatment with cyclopamine down regulated Ptc1h1 expression in MeT-5A cells. MeT-5A cells were treated with cyclopamine or vehicle control, stimulated with TGF-β2 and Ptc1h1 mRNA levels quantified by real time PCR. The results were normalised to PGK-1. White bars show treated cells without TGF-β2 stimulation. Blue bars show treated cells with TGF-β2 stimulation. Data represents the mean of two repeat experiments.

4.8 Quantitative measurement of pSMAD2 protein using alphascreen® Surefire® technology.

The Alphascreen® Surefire® technology by Perkin and Elmer is a recently developed method used to quantify signalling proteins within cell lysates. This technique requires less protein and is fully quantitative rather than the semi-quantitative western blots technique. This method was used to generate a quantitative measure of pSMAD2 protein levels expressed in cell lysates and to support the western blot data. The pSMAD2 units obtained from the assay were divided by those of GAPDH or Histone endogenous control to obtain a ratio.
4.8.1 Quantitative analysis of pSMAD2 using Alphascreen analysis.

To optimise the assay for measuring pSMAD2 levels using Alphascreen technology, the MeT-5A cells were stimulated with TGF-β2 and lysed at different time points. I wanted to firstly investigate the effect in MeT-5A cells for the optimization experiments. GAPDH protein expression was used as an endogenous control to normalize levels of pSMAD2 protein. Results show a trend towards an increase in the expression of pSMAD2 in TGF-β2 stimulated cells over time compared with unstimulated control (figure 4.27), but the increase was only observed at 2 hr (1.8 fold increase p-value 0.0230). This increase was less than the results obtained by western blot analysis (4.5 fold change in pSMAD2 at 2 hr compared to unstimulated control) (figure 4.8).

![Graph](image)

**Figure 4.27:** pSMAD2 protein levels normalised to GAPDH in MeT-5A cells using Alphascreen technology. pSMAD2 protein expression following TGF-β2 stimulation normalised to GAPDH detected using the Surefire® assay. White bar shows unstimulated control. Green bars show TGF-β2 stimulated cells. Data represent the mean of three experiments. Error bars = SEM. Significance compared to untreated control: *= p ≤ 0.05.
4.8.2 pSMAD2 levels normalised to Histone in MeT-5A cells.

For MM cells GAPDH may not be the most suitable endogenous control as previous unpublished data in our laboratory showed that GAPDH levels can change upon different treatments. Therefore, data was normalised to Histone levels and expressed as a ratio. There was no significant change in the level of pSMAD2 in MeT-5A cells at any of the time points examined compared with unstimulated control (figure 4.28). In contrast, western blot analysis revealed an increase in pSMAD2 over time (figure 4.8). Further examination of the result showed that there was a high variation in Histone expression, which skewed the data.

![Graph showing ratio of pSMAD2 normalised to histone over time points](image)

**Figure 4.28: pSMAD2 protein levels normalised to Histone in MeT-5A cells using Alphascreen technology.** pSMAD2 protein expression following TGF-β2 stimulation normalised to Histone. White bar shows unstimulated control. Green bars show TGF-β2 stimulated cells. Data represent the mean of three experiments. Error bars = SEM.
Chapter 5
Discussion
Malignant mesothelioma MM is a fatal cancer of the mesothelium (Spugnini et al., 2006) associated with poor prognosis due to difficulties in diagnosis and the lack of treatment options currently available (Chen & Pace., 2012). Therefore, novel targeted therapies are required (Kao et al., 2010). Recent studies have focused on the role of aberrantly expressed developmental signalling pathways in the pathogenesis of several cancers (Dennler et al., 2007; Cigna et al., 2012). Two of these pathways are the Hh and TGF-β signalling pathways. There is increasing evidence that these pathways work together to promote tumour growth. Some studies have identified TGF-β as a potent inducer of the primary Hh effector molecule and transcription factor, Gli (Alexaki et al., 2004; Dennler et al., 2007; Javelaud et al., 2012; Mohseny et al., 2012). The current study aimed to investigate the interaction of these two pathways at the level of Gli, in MM. Identifying new pathways that regulate tumour cell growth may lead to the development of novel therapies.

5.1 TGF-β expression in MM cells

TGF-β is a multifunctional cytokine and has a defined, yet complex role in mediating or regulating a series of stochastic events that occur in cancers. (Sporn et al., 1986). There are three mammalian isoforms of TGF-β (Massague, 1998). This study examined basal mRNA expression levels of the most abundant forms of TGF-β, TGF-β₁ and TGF-β₂ in MM cells. Previous studies have demonstrated substantial production of TGF-β isoforms in human and mouse MM cell lines (Gerwin et al., 1987; Lauber et al., 1992; Fitzpatrick et al., 1994). The effect of blocking endogenous TGF-β on MM cell and tumour growth using antisense oligonucleotides to TGF-β₁ and TGF-β₂ showed significant biological effects on MM cell function and blocking TGF-β₂ inhibited tumour growth in vivo (Fitzpatrick et al., 1994; Marzo et al., 1997). Therefore it is clear that TGF-β is an important molecule in MM growth but how it mediates it’s effects are not clear. To investigate the interaction between TGF-β and Hh signalling I first needed to determine how much of each TGF-β isoform was produced in the cells I used in my study.
Basal levels of TGF-β1 mRNA was generally expressed higher in control MeT-5A cells but had variable expression across the MM cell lines studied compared to primary mesothelial cells. Basal levels of TGF-β2 mRNA was generally expressed lower in MM and MeT-5A cells compared to primary mesothelial cells. However, this study only measured mRNA levels and future studies should also measure protein. A previous study examined TGF-β protein in MM tumour tissue by immunohistochemistry, and showed differential localisations of TGF-β isoforms, where TGF-β1 was shown to be localised more in the stroma than in cells and TGF-β2 more in MM tumour cells than in the stroma (Jigardar et al., 1997). Although I saw more TGF-β1 than TGF-β2 mRNA in the cells this may not reflect protein levels. Also, as cells secrete TGF-β in the latent form, immunolocalisation studies would only measure total TGF-β and not the amount of active TGF-β present. Therefore a more specific type of assay is required which would measure active TGF-β isoforms such as enzyme linked immunosorbent assay (ELISA). The assay detects latent TGF-β, which can then be activated with acids to measure the amount of active TGF-β isoform protein present in cell supernatants. In this study, I performed initial optimisation experiments using ELISA to measure active and latent TGF-β1 and TGF-β2 in MM cells (results not included). However, the latent level was below the level of detection. Therefore, this technique needs further optimisation, such as concentrating the cell culture supernatants using ultrafiltration approaches. Despite only measuring mRNA levels of TGF-β1 and TGF-β2 in this study, the findings show that both isoforms are produced by MM cells with possibly more TGF-β1 related to malignancy that TGF-β2. However, the importance of TGF-β2 cannot be overlooked given that TGF-β2 antisense oligonucleotides blocked MM tumour growth \textit{in vivo} (Marzo et al., 1997). Knowing the basal mRNA levels of TGF-β for each cell line also provided a reference point for subsequent experiments.

5.2 Gli expression in MM cells

I also examined the mRNA expression of the Hh signalling pathway effector molecules Gli1 and Gli2. Baseline mRNA levels of both Gli1 and Gli2 was variable across the MM cell lines and MeT-5A cells had higher Gli1 and Gli2 levels
compared to primary mesothelial cells. Although there was variable expression of Gli across cell lines, most cells produced Gli mRNA, supporting reports from other studies and suggesting a role for Gli and the Hh pathway in MM growth (Shi et al., 2012; Li et al., 2013). Expression of Gli3 transcription factor mRNA was not measured in this study as it is known to be a transcriptional repressor (Matice & Joyne, 1999), and other studies have shown a minimal effect of TGF-β on Gli3 expression (Dennler et al., 2007; Cigna et al., 2012).

5.3 TGF-β signals through the SMAD pathway in MM

Among the immediate transduction pathways activated by TGF-β, the canonical signalling pathway is the SMAD cascade (Massague et al., 2005). SMADs are carriers of TGF-β receptor signals into the nucleus and phosphorylation of SMAD2 and SMAD3 by the TGF-β receptor complex is a critical step in TGF-β ligand signalling and transcriptional regulation (Ebner et al., 1993). Binding of TGF-β ligand to cell specific receptors activates and phosphorylates TβRI, and this then phosphorylates SMAD2 and SMAD3 which form a complex with SMAD4. The resulting complex moves to the nucleus and transcription of the target genes is achieved (Padgett, 1999).

I firstly wanted to confirm activity of the human recombinant TGF-β1 and TGF-β2 preparations used in this study. This was confirmed by phosphorylation of SMAD2 proteins and its known downstream target gene human collagen type-1α1. TGF-β1 increased pSMAD2 over time in JU77 cells and also stimulated collagen type-1α1 mRNA maximally at 24 hr, consistent with previous findings in our laboratory. However due to the nature of the total SMAD2/3 antibody used in this project, it was difficult to obtain a band each of SMAD2 and SMAD3 proteins by western blotting and therefore the quantitation of SMAD2 proteins with imageJ was difficult to perform. Similar to the TGF-β1 isoform, TGF-β2 also induced an increase in pSMAD2 levels as well as collagen production in MM cells, confirming the activity of both TGF-β preparations. To my knowledge, this is also the first time the effect of TGF-β2 on pSMAD2 has been examined in MM cells. Interestingly, the maximal
fold increase in collagen mRNA induced by TGF-β₂ was higher than for TGF-β₁. For JU77 cells, TGF-β₁ stimulated a 2.8 fold increase in collagen mRNA at 24 hr compared with a 14 fold increase after TGF-β₂ stimulation. This suggests differential effects of the two TGF-β isoforms in these cells.

Although western blot analysis showed that TGF-β increased pSMAD2, this technique is semi-quantitative, and estimates of protein are prone to factors that may alter the final outcomes (Gassman et al., 2009). As a result, better and more quantitative measurements of protein changes are required. Recently, a newer approach called the Alphacreen® Surefire® assay has been developed to detect these proteins in a much more efficient, accurate and timely manner (Hurt & Titus, 2010). This assay is a proximity based sandwich immunoassay which uses a combination of antibodies, donor beads and acceptor beads, which bind with the target protein creating protein complexes that release quantifiable light directly correlating to the amount of target protein in that sample (Hurt & Titus, 2010). Several studies have successfully optimised and utilised this assay to quantify protein expression in vitro (Crouch et al., 2010). Results obtained to date in this study did not reflect the western blot data and so most likely needs to be further optimised. However, due to time constraints I was unable to fully optimise this technique.

5.4 Effect of TGF-β₁/₂ on Gli1/2 mRNA levels in MM cells.

The effect of TGF-β₁ and TGF-β₂ on Gli mRNA expression was then examined in MM and control cell lines. TGF-β₁ has previously been shown to stimulate an increase in Gli2 which in turn upregulates Gli1 in a Gli2-dependent manner (Dennler et al., 2007; Cigna et al., 2012). In glioblastoma, pancreatic adenocarcinoma and lung fibroblast cell lines, a rapid induction of Gli2 in response to TGF-β₁ was observed, peaking between 2-8 hr and levels remaining above basal expression up to 24 hr, whereas delayed induction of Gli1 was observed between 16-24 hr (Dennler et al., 2007; Cigna et al., 2012). Interestingly, TGF-β₁ had no effect on either Gli2 or Gli1 levels in the 4 MM and control MeT-5A cell lines examined. As MM cells also produce TGF-β₂ and blocking TGF-β₂ inhibits MM tumour growth (Marzo et al,
1997), I decided to examine the effect of TGF-β2 on Gli expression. TGF-β2 induced Gli2 mRNA between 4-6hr in all MM cell lines and control MeT-5A cells in a similar fashion to previous reports for TGF-β1, but interestingly had no effect on Gli1 mRNA expression up to 48 hr after treatment.

Previous studies have shown that TGF-β isoforms have a similar structure but serve different biological functions in different cell types in vitro and in vivo (Millan et al., 1991). For example, TGF-β1 gene knockdown mice experience defective haematopoiesis and endothelial differentiation, while TGF-β2 null mice exhibit cardiac, lung, ear and urogenital defects and TGF-β3 gene ablation mice results in delayed pulmonary development (Dunker & Kreiglstein, 2001; Sanford et al., 1997). This illustrates diverse biological roles of these isoforms which can influence cell growth and differentiation in different cell types in a positive or negative manner (Sporn et al., 1986). Thus, despite most studies having shown that TGF-β1 induces Gli expression in various cancers, this study showed that TGF-β1 was ineffective in inducing Gli in MM cells but TGF-β2 could, highlighting the differential role of the two isoforms in respect to Gli activation.

This differential response may be because it has been reported that TGF-β2 has a different mode of receptor activation (Rotzer et al., 2001). As previously described, signalling via TGF-β1 is initiated when TGF-β1 binds to TGFβRII, and this recruits the binding and phosphorylation of TGFβR1 (Wrana et al., 1994). Activated TGFβRI phosphorylates its cytoplasmic effectors SMAD2/3, which phosphorylates and forms a complex with SMAD4, translocates to the nucleus and initiates transcription of target genes (Massague et al., 1999). In contrast, TGFβRII has a low intrinsic affinity for the TGF-β2 isoform (Lin et al., 1995). TGFβRIII or betaglycan acts as a co-receptor and is required for the responsiveness of TGF-β2, TGFβIII binds to TGF-β2 and presents it to TGFβRII, and this has been described in different cell types (Sankar et al., 1995; Brown et al., 1999). However it is incompletely understood why direct binding of TGF-β1 to TGFβRII does not have the same effect (Rotzer et al., 2001). Therefore, Brown et al. (1999) proposed that TGF-β2 alters the activity and composition of TGFβRII-TGFβRI complex in order to activate downstream signalling molecules, resulting in specific TGF-β2 effects, and that TGFβRIII has a
preferential affinity for the TGF-β2 isoform. Supporting this, an earlier study by Mitchell et al. (1992) demonstrated that compared to mesenchymal enriched primary placental cells, trophoblast enriched primary placental cell cultures were more responsive to TGF-β2 than TGF-β1 due to the presence of an active form of betaglycan receptor at the cell surface. Eickelberg and colleagues, (2001) examined the function of betaglycan in renal epithelial LLC cells and L6 myoblast cells and observed that expression of betaglycan in LLC cells inhibited TGF-β1 signalling, measured by collagen expression and phosphorylation of downstream SMAD2/3 proteins. In comparison, the expression in L6 cells enhanced TGF-β1 signalling. The expression of betaglycan in LLC cells prevented association between TGFβRI and TGFβRII. Therefore it is possible that in MM cells, betaglycan inhibits TGF-β1 signalling but supports TGF-β2 signalling by acting as a co-receptor. However, this needs to be confirmed by further studies such as measuring the expression of TGFβRIII in these cells.

It was also interesting to note that TGF-β2 induced maximum Gli2 expression in all cells lines studied between 4 and 6 hr but the levels then reduced back to basal levels at 24 and post TGF-β2 stimulation. These observations are not consistent with the findings by Dennler et al. (2007). As previously described, they observed rapid and persistent Gli2 induction by TGF-β1, peaking between 2-8 hr and remaining at levels higher than their basal expression up to 24 hr. This could possibly be an effect of a negative regulator of TGF-β signalling, SMAD7 forming stable complexes with active TGF-β receptor complexes and interrupting the phosphorylation of SMAD2/3, blocking transcription of target genes (Zhu, Chen & Chen, 2011). Therefore, this observation in the current study could mean that although TGF-β2 induces an effect on Gli2 expression, this effect could be short lived and may only be functionally important in that time frame. Examination of Gli2 target genes at later times might suggest functional importance.

Another interesting observation following TGF-β1 and TGF-β2 treatment in MM cells is that Gli1 levels were upregulated in the control 24 hr sample in all cell lines examined. A possible reason for this response could be initiation of cell survival mechanisms through the Hh signalling pathway. Cells are cultured in 0.4% FCS
supplemented medium for 24 hr before and after TGF-β stimulation. Therefore, cells are starved of serum and hence growth nutrients for an extended period of time. This stress response may in itself stimulate Gli1 expression. The Hh pathway is required for growth and survival of tumour cells (Bar et al., 2007). Previous studies showed that Hh pathway activation, as evidenced by elevated expression of its target gene Gli1, leads to increased transcription of anti-apoptotic molecules such as Bcl-2 in medullablastoma (Bar et al., 2007). Other studies have shown that expression of Bcl-2 is predominantly regulated by Gli2 instead of Gli1, such as in plasmacytoma tumour cells (Regl et al., 2004). In this study, induction of Gli expression at 24 hr in controls may be a defence mechanism but ultimately masks any increased expression of Gli1 at 24 hr in response to TGF-β. This mechanism is worth exploring.

The functional significance of TGF-β2-induced Gli2 induction can be assessed by measuring downstream targets for Gli2. Previous studies have shown Ptch1 to be a target gene for Gli1 (Cigna et al., 2012), while others have shown that it is a Gli2 target (Kim et al., 2007; Regl et al., 2004; Lipinski et al., 2008). Therefore, to confirm functional significance of Gli2 activation, the regulation of Ptch1 mRNA expression levels were examined in TGF-β2 stimulated CRL2081 and MeT-5A cell lines. Ptch1 was upregulated at 48 hr post TGF-β2 stimulation compared to its unstimulated control in both cell lines, however only MeT-5A showed significance. Although this may be due to the direct effect of Gli2 upregulation, the timing of expression of Ptch1 is not consistent with previous reports (Cigna et al., 2012). As Gli2 is induced at earlier time points in response to TGF-β2, I expected upregulation of Ptch1 at around 24 hr rather than 48 hr if it was a Gli2 target gene in these cells. Therefore it is possible that Gli1 was upregulated in response to TGF-β2 at 24 hr and hence the delay in upregulation of Ptch1. Clearly this is impossible to confirm when control levels of Gli1 were also upregulated after 24 hr with TGF-β2 treatment, but this requires further investigation.

In this study I measured the effect of TGF-β isoforms on Gli mRNA levels rather than protein. This was because of difficulty measuring Gli isoforms using currently available antibodies for western blot analysis. An alternative approach would have been to use a Gli1 or Gli2 reporter assay following TGF-β1/2 treatment. A reporter
construct (such as Luciferase) is attached to the gene of interest (Gli1 or Gli2) to create gene fusion. This construct is integrated into cellular genomic DNA. Following appropriate stimulation there is activation of transcription of the reporter gene which releases the reporter protein. In the case of luciferase, this is measured by a luminometer, and the intensity of the signal is a direct reflection of the level of activity of the gene of interest (Smale, 20010).

I then wanted to examine the mechanisms regulating TGF-β2-mediated Gli2 upregulation. To do this I chose two cell lines; MM cell line CRL2081 and control MeT-5A (Reddel et al, 1989). Both cell lines were induced by TGF-β2 to up-regulate Gli2 mRNA expression. Ideally normal mesothelial cells would have been used as controls for all experiments but cultures of normal mesothelial cells were not available during the study. Although TGF-β2 induced Gli2 activation, it was unclear if TGF-β2 acted directly on Gli2 or was dependent on the classical Hh Ptch/SMO axis. Dennler et al. (2007) showed that cyclopamine abrogated shh induced Gli2 expression but has no effect on TGF-β1 mediated Gli2 expression.

However, Cigna et al. (2012) showed that in human lung fibroblasts (HLFs), TGF-β induced mRNA and protein expression of α-SMA (Smooth muscle actin), collagen and fibronectin. Blocking Hh signalling using cyclopamine inhibited the expression of these proteins showing the requirement of Hh signalling in TGF-β-mediated expression of certain proteins in these cells. Similarly, Yoo et al. (2008) showed that Shh promotes motility and invasiveness of gastric cancer cells through TGF-β-mediated activation of the ALK5-SMAD3 pathway.

To examine the role of the classic Hh signalling pathway in MM, cells were treated with TGF-β2 in the presence or absence of the SMO inhibitor cyclopamine. Cyclopamine is a steroidal alkaloid isolated from corn lily, and blocks Hh pathway signalling by blocking the Ptch/SMO axis and influencing the function of SMO (Yao et al., 2011). (Ingham & McMahon, 2001). If TGF-β2 was acting through Ptch/SMO, then cyclopamine would block the actions of TGF-β2. If not, then cyclopamine would have no effect. Studies have analysed the optimal concentration of cyclopamine for its ability to block cellular response and thus, 5 and 10µM
concentrations were used in this study (Chen et al., 2002; Yao et al., 2011). Unfortunately, although I saw a reduction in Gli2 mRNA levels following TGF-β2 treatment in the presence of cyclopamine, there was an equal reduction in vehicle (ethanol) controls. Therefore, no conclusions could be drawn from this study. Due to the unexpected effect of the vehicle control, further optimisation of this experiment needs to be performed, possibly using a different vehicle control such as DMSO or water, depending on the solubility properties of the cyclopamine preparation. Unfortunately there was not enough time available for me to perform these optimisation experiments in this study.

An alternative to measuring Gli2 levels in cyclopamine and TGF-β2 treated mesothelial cells is to measure an alternative downstream target gene for Gli such as Ptch. As expected, cyclopamine blocked Ptch1 mRNA expression in Met-5A cells, but the levels were restored in the presence of TGF-β2. This suggests that TGF-β2 induces Ptch1 independent of Ptch/SMO-mediated signalling in MeT-5A cells. However, this experiment was only performed once and only in MeT-5A cells. Therefore these findings need to be confirmed and the effect on MM cells examined.

5.5 SB-431542 inhibits TGF-β2-induced Gli2 expression in MM cells.

I then wanted to determine if TGF-β2 was stimulating Gli2 mRNA upregulation through the classic TGF-β signalling receptor TβRI/ALK5 via the SMAD signalling pathway. The TGF-β ligand brings together type 1 and type II receptor complexes. The type II receptor phosphorylates and activates the type I receptor (ALK5) (Moustakas et al., 1993) and the signals are transduced to the nucleus primarily through activation of the SMAD2/3 complex (Ebner et al., 1993). To assess the role of the ALK5 receptor in TGF-β2-mediated Gli2 expression, I measured TGF-β2-induced Gli2 mRNA expression after blocking TGF-β2 signalling using the ALK5 inhibitor SB-431542. This is a potent inhibitor of the ALK5 receptor that acts as a competitive ATP binding site kinase inhibitor, and has been shown to inhibit the in vitro phosphorylation of SMAD2 and SMAD3 (Inham et al., 2002). To examine the effectiveness of this inhibitor, cells were pretreated with SB-431542 followed by
TGF-β2 stimulation. TGF-β2 alone increased phosphorylation of SMAD2 in both CRI2081 and MeT-5A cells. However, in the presence of SB431542, pSMAD2 induction by TGF-β2 was prevented. Consequently, TGF-β2-induced Gli2 was also inhibited by SB-431542. Therefore it was concluded that TGF-β2 acts through the ALK5 receptor to induce Gli2 mRNA expression.

5.6 TGF-β2-induced Gli2 is mediated through the SMAD2/3 signalling cascade.

I then wanted to determine if TGF-β2-induced Gli2 was mediated through SMAD2/3 signalling. Western blot data clearly showed that TGF-β2 induced pSMAD2/3 in MM and MeT-5A cells. However, TGF-β can also signal through other pathways such as MAPK and PI3K. The approach I used was to block SMAD2 and SMAD3 using specific siRNA and measure its effect on TGF-β2-mediated Gli2 expression.

I first tested the transfection efficiency of this approach. Unfortunately, the siRNA available to SMAD2 and SMAD3 were not tagged with a reporter molecule and so I could not work out the efficiency directly from my SMAD transfection studies. Therefore, I used an FITC labelled scrambled siRNA. Both cell lines demonstrated a low percentage of cells carrying the label, with only 6% efficiency in CRL2081 cells and 25% in MeT-5A. The experiment was performed using live cell harvest for FACS analysis which would compromise the mortality of cells and the transfection efficiency. Therefore, this low transfection efficiency could be due to technical issues when performing the experiment such as extended spinning time, or delay during cell harvest. If I had more time I would optimize the technique with other harvesting and cell fixing techniques before FACS analysis to avoid technical issues and get a better readout.

Despite the relatively low transfection efficiency, I tried to obtain effective mRNA and protein knockdown of pSMAD2 and total SMAD2/3 using SMAD2 and SMAD3 siRNA at a range of concentrations. The 1 nM concentration had limited effect, whereas I got significant knockdown at 10 and 20 nM siRNA although at 20 nM some of the effects may have been non-specific. Previous studies have shown that
high concentrations of siRNA can have off-target effects and relatively low concentrations (e.g. 1 nM) are sufficient to silence the intended target (Caffrey et al., 2011). The 10 nM siRNAs led to efficient knockdown at the mRNA and protein level, and therefore this concentration was used for subsequent experiments. siRNA to SMAD2 and SMAD3, individually and combined, effectively knocked down SMAD2 and SMAD3 mRNA and protein phosphorylation in both CRL2081 and MeT-5A cells in the presence or absence of TGF-β2, with the greatest effect when the SMAD2 and SMAD3 siRNA were combined. However, as the transfection efficiency in CRL2081 cells was so low, it is unclear if this result was real or non-specific. Further optimisation studies are needed to confirm siRNA results in CRL2081 cells. Analysis of Gli2 mRNA levels following TGF-β2 stimulation in cells transfected with SMAD2 and SMAD3 siRNA, clearly showed suppression of TGF-β2-mediated Gli2 mRNA, confirming the role of the SMAD pathway. These results are consistent with the findings by Dennler et al. (2007) where they showed that SMAD3 siRNA markedly impaired TGF-β-induced Gli2 mRNA expression in fibroblasts and keratinocytes.

5.7 Summary and Conclusions

MM remains a disease with a dismal outcome. Hh and TGF-β signalling pathways are involved in numerous overlapping processes during development and recent studies have discovered this interaction may be important in the pathogenesis of some cancers (Perrot et al., 2013; Javelaud et al., 2012; Dennler et al., 2007). Therefore the role of this interaction in diagnosis, prognosis and treatment options in MM have to be investigated. This study aimed to determine whether this pathway interaction occurs in MM, particularly if TGF-β induces the Gli transcription factors.

TGF-β1/2 isoforms and Gli1/2 are variably expressed in MM cell lines. TGF-β1 had no effect on Gli1 or Gli2 expression. However, TGF-β2 induced Gli2 but not Gli1 mRNA expression. Both TGF-β1 and TGF-β2 signals through the canonical SMAD signalling pathway and blocking TGF-β2 signalling at the TβRI receptor level inhibited TGF-β2 mediated Gli2 mRNA expression and pSMAD2 protein expression.
Knocking down SMAD2/3 using siRNA also inhibited increased TGF-β2-mediated Gli2 mRNA levels, confirming the role of the SMAD2/3 signalling pathway in TGF-β2-mediated Gli2 upregulation. However, it is yet to be determined if TGF-β2-mediated Gli2 activation is independent of Hh signalling, and if this interaction plays a functional role in MM tumour growth.

From the results of this study, it can be concluded that TGF-β2 is a potent inducer of Gli2 and mediates its effects through the SMAD2/3 signalling pathway. Although further studies are required to determine that targeting TGF-β/Gli interaction may provide therapeutic benefits for MM patients.

**5.8 Future directions**

This study has confirmed the interaction of TGF-β with Hh signalling pathway effector molecules Gli, but further studies are required to delineate the signalling mechanisms and elucidate the biological significance of this interaction. The current study was limited by only examining this interaction at the level of Gli mRNA. Therefore to further address the interaction of Hh and TGF-β signalling pathways, the study needs to be expanded to investigate the role of TGF-β1/2 on Gli1/2 protein levels and on other Hh mediators such as Ptc1, Ptc2, SMO and Hhip and their downstream target genes, and how this may contribute to TGF-β2 mediated tumour growth.

It would also be valuable to examine the role of TGF-β2-induced Gli2 and the expression of its downstream target genes in MM. Also, the functional effect of TGF-β2-induced Gli2 on cell proliferation, differentiation, viability, apoptosis, migration and invasion. This study also needs to be expanded to investigate the failure of TGF-β1/2 to induce Gli1 using other methods and assays, and subsequently the functional significance of TGF-β2-mediated effects compared with TGF-β1-mediated effects reported by others. To determine if TGF-β2 mediated Gli2 induction is dependent or independent of Hh signalling, the cyclopamine experiment should be
further optimised, as well as examining the effect of other Hh signalling inhibitors on TGF-β2-induced Gli2 for comparison.

Further studies should also investigate the effect of exogenous TGF-β2 on Gli2 expression and the effects of blocking this activation in vivo. A murine model would provide the opportunity to assess the role of this pathway interaction in vivo tumour growth. To achieve this, MM cell lines could be transfected with adenoviral vectors expressing single or combinations of active TGF-β2, SMAD2 or SMAD3 siRNA or control constructs and transplanted into mice or treated with a Gli inhibitor such as GANT61 (Mazumdar et al., 2011), and tumour formation compared. The animals would be killed at various times or once the tumour reached 1 cm in diameter, and Hh pathway mediators such as Gli1, Gli2, Ptc1, Ptc2, Smo and Hhip levels measured as well as features such as histopathology, cell proliferation and apoptosis.

Interfering with TGF-β2 signalling has shown promising results in preventing tumour growth in a variety of tumour types including MM. Similarly, the effect of combined TGF-β and Gli inhibitors need to be investigated in vitro and in vivo in MM to define effective therapeutic strategies.
Chapter 6
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6. References


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