Targeting Hedgehog Signalling as a Drug Therapy in Aggressive Fibromatosis

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Laboratory Medicine and Pathobiology
University of Toronto

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2012

Abstract

Aggressive fibromatosis is a benign fibroproliferative tumour that can occur as a sporadic lesion or a manifestation in patients with familial syndromes, such as familial adenomatous polyposis. Tumours are characterized by the stabilization of β-catenin and the activation of β-catenin-mediated transcription. Current treatment results are far from ideal, and recurrence rates are high. As a result, there remains a need for more effective therapeutic strategies. In this work, we demonstrate the effect of hedgehog signalling inhibition on aggressive fibromatosis tumour development and β-catenin modulation. We found that hedgehog inhibition decreased cell viability and proliferation as well as total β-catenin levels in human aggressive fibromatosis tumour cells in vitro. Furthermore, following hedgehog inhibition in Apc+/Apc1638N aggressive fibromatosis mouse model, the number and volume of the tumours formed was reduced. Together, this work suggests that hedgehog signalling inhibitor agents are potential candidates to effectively manage aggressive fibromatosis.
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<tbody>
<tr>
<td>FAP</td>
<td>familial adenomatous polyposis</td>
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<td>AF</td>
<td>aggressive fibromatosis</td>
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<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drugs</td>
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<tr>
<td>IFNs</td>
<td>interferons</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>PCP</td>
<td>planar cell polarity</td>
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<tr>
<td>FZ</td>
<td>frizzled</td>
</tr>
<tr>
<td>Dsh</td>
<td>disheveled</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>glycogen synthase kinase 3-beta</td>
</tr>
<tr>
<td>CK1γ</td>
<td>casein kinase 1-gamma</td>
</tr>
<tr>
<td>β-TrCP</td>
<td>beta-transducin repeats-containing protein</td>
</tr>
<tr>
<td>SCF</td>
<td>Skp1/Cullin/F-box protein</td>
</tr>
<tr>
<td>TCF</td>
<td>T cell factor</td>
</tr>
<tr>
<td>LEF</td>
<td>lymphoid enhancer factor</td>
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<tr>
<td>LRP</td>
<td>LDL receptor-related protein</td>
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<tr>
<td>Arm</td>
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<tr>
<td>Pyg</td>
<td>pygopus</td>
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<tr>
<td>MMP-7</td>
<td>matrix metalloproteinase-7</td>
</tr>
<tr>
<td>SAMP</td>
<td>Ser-Ala-Met-Pro</td>
</tr>
<tr>
<td>EB1</td>
<td>end-binding protein 1</td>
</tr>
<tr>
<td>DLG</td>
<td>discs large</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
</tr>
<tr>
<td>Min</td>
<td>multiple intestinal neoplasia</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
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<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>MPC</td>
<td>mesenchymal progenitor cell</td>
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<tr>
<td>CFU-f</td>
<td>colony forming unit-fibroblasts</td>
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<tr>
<td>Hh</td>
<td>hedgehog</td>
</tr>
<tr>
<td>Smo</td>
<td>smoothened</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>BCC</td>
<td>basal cell carcinoma</td>
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<tr>
<td>HSPG</td>
<td>heparan sulfate proteoglycans</td>
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<td>hedgehog-interacting protein 1</td>
</tr>
<tr>
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<td>glioma-associated</td>
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<td>protein kinase A</td>
</tr>
<tr>
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<td>processing determining domain</td>
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<td>costal2</td>
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</tr>
<tr>
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<td>β-arrestin 2</td>
</tr>
<tr>
<td>FIF</td>
<td>familial infiltrative fibromatosis</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
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1.1 Abstract

Aggressive fibromatosis (also known as desmoids), is a locally invasive soft tissue tumour, generally arising in connective tissues. They are benign and unable to metastasize systemically. Tumours can occur either as sporadic lesions or as part of pre-neoplastic conditions, such as familial adenomatous polyposis (FAP). The stabilization of β-catenin and the up-regulation of β-catenin-dependent transcriptional activity are observed in tumours, indicating the association between dysregulated Wnt/β-catenin signalling pathway and aggressive fibromatosis. The standardization of therapeutic strategies for aggressive fibromatosis has been challenging due to the rarity of these tumours. This chapter will discuss the development of aggressive fibromatosis, current therapeutic strategies, cell of origin, and possible signalling pathways that could be targeted to modulate the phenotype of aggressive fibromatosis as well as introduce the research covered in the rest of this thesis.
1.2 Clinical Perspectives of Aggressive Fibromatosis

1.2.1 Overview

Aggressive fibromatosis (AF) tumour, also known as desmoid tumour from the Greek word desmos for ‘band of tendons’, is a benign locally invasive soft tissue lesion composed of spindle-shaped fibroblastic cells surrounded by collagen fibres (Figure 1.) (1). Tumours appear to originate from the fascia membrane or musculoaponeurotic structures, and lack a capsule. Desmoid tumours grow slowly and do not have metastatic potential; however, they possess infiltrative capacity to invade into local tissues and vital organs. The lesions show little or no inflammatory components (2, 3). They constitute about 3.5% of all fibrous tissue tumours and 0.03% to 0.1% of all neoplasms, with a frequency of occurrence of 2 to 4 cases per 1 million annually (3-5). Histopathologically, bipolar fibroblast cells expressing vimentin, a marker of mesenchymal cells and lacking the expression of epithelial markers such as E-cadherin are observed (6). The anatomical location, cellular morphology, and histological profile of these lesions suggest that they are of mesenchymal origins (7, 8). The most common site of occurrence is the abdominal wall and, the muscles of the shoulder girdle, especially the deltoid (most common site of extra-abdominal desmoids occurrence) (3).

Studies of clonality of aggressive fibromatosis tumours have demonstrated that it is a neoplasia derived from mesenchymal precursors (9). Cytogenetic studies of aggressive fibromatoses reveal the presence of trisomy 8 and trisomy 20 (non-random clonal aberrations acquired during neoplastic progression), and the absence of 5q in some cases (10-12). Strikingly, telomeric fusion, which is regarded as a rare phenomenon and a feature of several mesenchymal neoplasms, was also observed in some cases (10). Also, examining the non-random inactivation of X chromosome in tumours showed that desmoids are a monoclonal disorder, indicating that tumours derive from a single progenitor cell with a growth advantage and are not composed of normal fibroblasts stimulated by proliferative growth factors (9). Analysis of recurrent fibromatosis tumours by another study showed that the inactivation pattern of recurrent tumours was comparable to that of primary tumours (13). This data suggests that recurrent tumours are derived from the same clone as the primary tumour.
1.2.2 Morbidity and Mortality

Mortality associated with extremity aggressive fibromatosis is uncommon. Four deaths were reported out of total 51 pediatric patients who were analyzed in two studies (14, 15). Two deaths were reported due to local tumour progression in the head and neck region, while the other two cases were associated with chemotherapy toxicity. However, these lesions can result in severe morbidity by impeding movement and disfigurement following surgical removal of the lesion (16, 17).

Intra-abdominal AF tumours, however, are often of serious significance to the patients due to the impingement of critical organs and obstruction of normal functions (2, 3, 17, 18). For example, renal function may be impaired by pressure on the ureters, while surgical removal of the lesion, which is the principal method of treatment of desmoids, may be technically impossible. Mortality in intra-abdominal AF patients with FAP ranges from 10% to 50% with a mean of 20% (2, 17, 19).

1.2.3 Therapeutic Management of Aggressive Fibromatosis

Aggressive fibromatosis tumours are often treated using a multimodality approach including surgery, radiotherapy, and chemotherapy. However, treatment results are far from ideal, and recurrence rates are high.

1.2.3.1 Surgical Excision

The primary therapy of choice is wide local surgical excision to a normal tissue margins, but this approach is limited to mainly extremity and small mesenteric AF (3-5, 14, 16, 17). Complete excision of large intra-abdominal AF is more difficult as it may involve sacrificing critical structures. Local recurrence rates are high (5-50%), despite microscopically clear surgical margins (3, 20, 21). Recurrence after surgical excision is generally regarded as evidence of incomplete surgery. As a result, it is certainly necessary to remove the tumour with surrounding tissues. When feasible, repeated surgery is worthwhile for recurrences and it should be followed by close observation and further intervention (16, 22).
1.2.3.2 Radiotherapy

Early reports of radiotherapy in controlling AF were controversial with positive response in a small number of cases (5, 19, 20, 23, 24). However, it is indicated that radiotherapy can be beneficial especially if used in combination with surgery and in the treatment of unresectable or recurrent lesions or in the patients when the risk of surgery is high (3, 4, 16, 21-23). The commonly used dose ranges from 50 to 60 Gy in adults (25-27) and if used in conjunction with surgery, offers an average local control rate of 70-95% compared to only 40-75% with surgery alone (28, 29). Still, the addition of radiotherapy for patients has demonstrated a relapse rate of 31% for unresectable tumours (25). Furthermore, studies have shown that high dose treatment modality may have unacceptable radiotherapy-associated morbidity in patients, such as growth abnormality and increased risk of secondary malignancy (30-33).

1.2.3.3 Chemotherapy

Chemotherapy as another form of treatment for AF has been studied because of the existence of high recurrence rates following surgery and the radiotherapy-associated morbidity, in patients received high dose of radiation. There is limited information on single-agent activity of chemotherapy against AF. However, combination chemotherapy regimens were used more frequently in AF patients, and most information regarding the effectiveness of cytotoxic chemotherapy is mainly derived from them (34-37). An overall response rate of 50% is typically accompanied by severe side effects including nausea, vomiting, acute and chronic toxicity such as cardiotoxicity and myelotoxicity (36, 38). Most commonly used regimens include doxorubicin in combination with others such as dacarbazine, cyclophosphamide and vincristine (35, 37, 39); actinomycin-D-based chemotherapy (35, 40); or a combination of methotrexate with a vinca alkaloid (vinblastine) (36, 41).

1.2.3.4 Hormonal Therapy

As it is suggested by a number of observations, regarding the natural history of AF tumours, they may be hormonally responsive. About 80% of all AF tumours occur in women (3). It was observed by Reitamo et al. that the speed of growth of AF tumours is higher during pregnancy, in premenopausal compared with postmenopausal women, and in females relative to males (5). However, studies on the incidence of aggressive fibromatosis in FAP patients have contradicted
these previous findings, while some demonstrate an increased incidence in females (42-44); others show no significant differences between two genders (3, 18).

Estrogen receptor expression has been demonstrated in AF tumours of FAP patients (1), although the detected receptor levels were low (45, 46). The theory of hormone dependency of AF tumours has been supported also by preclinical and in vitro studies. In guinea-pigs following prolonged estrogen administration, fibrous tumours histologically similar to human AF were formed in the abdominal organs, anterior abdominal wall, and thorax. Also, the formation of these tumours was prevented by the administration of testosterone, progesterone and desoxycorticosterone (47). Due to the rarity of AF tumours, there are no randomized studies conclusively showing a causative role of estrogen or supporting the use of anti-estrogen agents as a standardized method of treatment. A number of hormonal agents have been tested and found to be effective in AF. Such agents include tamoxifen, progesterone, and testolactone (48-54).

1.2.3.5 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

The efficacy of a variety of NSAIDs such as indomethacin, sulindac, and colchicines were explored in AF patients. They were associated with partial and complete responses in several non-randomized retrospective studies (54-56). Also, in vivo studies on the efficacy of NSAIDs against AF have indicated its potential (57). However, total regression of tumours has yet to be achieved. As such, NSAIDs alone may not suffice as a stand-alone treatment but may prove to be an effective adjuvant therapy.

1.2.3.6 Other Therapeutic Strategies

There are studies to support the use of interferons (IFNs) in AF. It has been shown that the proliferation and collagen synthesis by fibroblasts can be inhibited by use of IFN-alpha and IFN-gamma, respectively (58, 59). Clinical studies demonstrate moderate efficacy of IFN treatment on aggressive fibromatosis. However, no conclusive statements can be made due to the small number of patients analyzed thus far, and whether IFN can be used as a non-cytotoxic alternative in patients whose endocrine therapy has failed needs further studies (30, 60-62).
1.3 Molecular Etiology of Aggressive Fibromatosis

Most aggressive fibromatosis in children occurs sporadically with the highest frequency in the extremities; however, it can also be found in intra-abdominal regions (16). In adults, most sporadic AF tumours occur in extremities, and only a small percentage is found in intra-abdominal regions. However, adult FAP-linked AF tumours occur mainly in abdominal sites (2, 4, 5, 17-20, 22). The association between AF development and trauma or surgical intervention at the site of the tumour (63, 64), family history, endogenous hormonal environment and exogenous sex hormones has been demonstrated. Female sex is also a risk factor in adult sporadic AF (4, 22) but not in the FAP-associated tumours (3, 17-19, 43). There are some reports indicating that an antecedent of trauma is present in approximately 15% of cases of aggressive fibromatosis tumours (64, 65). Genetic predisposition is also a risk factor.

Aggressive fibromatosis tumours can occur either as sporadic lesions or as a manifestation of familial syndromes, such as FAP. FAP is an autosomal dominant hereditary condition associated with a substantial increase in risk of colorectal and other cancers. FAP patients develop more than 100 colorectal adenomas at a young age, and malignant transformation is observed at the age of about 50 years in untreated patients. Although aggressive fibromatosis tumours are rare in the general population, they are a common extracolonic manifestation in FAP patients (66). Patients with FAP have a 1000-fold increased risk of developing AF tumours, compared with the general population (18, 67). Aggressive fibromatosis tumours are reported to be the second most important cause of death in patients with FAP, after colorectal carcinoma (19, 42). The germline mutations in the adenomatous polyposis coli (APC) tumour suppressor gene leading to a truncation and loss of function of the APC protein are present in FAP, which predispose patients to develop colonic polyps as well as AF tumours. The frequency of occurrence of aggressive fibromatosis in FAP patients is reported between 3.6 to 34% by different studies (3, 18, 68). In contrast to FAP-linked tumours, in sporadic cases most tumours are associated with somatic mutations in the CTNNB1 gene (gene that codes for β-catenin) or in the APC gene (6, 69-71). In a study by Tejpar et al., mutational analysis was performed on 42 cases of sporadic AF (69). β-catenin point mutations were found in 22 of the 42 tumours studied, while inactivating somatic APC mutations were detected in nine tumours. Ten β-catenin mutations resulted in a substitution of alanine to threonine at codon 41 and twelve resulted in a substitution of phenylalanine to
serine at codon 45. Taken together, 60% of sporadic AF tumours contain β-catenin mutations, and 15% contain APC mutations. This gives AF tumours one of the highest reported incidences of β-catenin mutations. About 25% of tumours still remain as unexplained. Beside APC gene mutations, no mutations were detected in other tumour suppressor genes such as TP53 (gene that codes for tumour protein 53, p53) or RB1 (gene that codes for retinoblastoma protein, pRb) in AF tumours (72, 73). However, loss of mitotic spindle cell cycle checkpoint and a lack of Rb immunohistochemical staining and decreased Rb mRNA expression were observed in AF (72, 73).

Regardless of the causative mutation, all AF tumours demonstrate increased β-catenin protein expression, its nuclear localization, and activation of tcf-mediated transcription (Figure 2.) (74). As both protein products of the APC gene and CTNNB1 gene are implicated in the canonical WNT/β-catenin pathway, this suggests that the deregulation of WNT/β-catenin pathway plays a key role in the pathogenesis of AF tumours.

1.4 Overview of WNT/β-catenin Pathway

1.4.1 Non-canonical and Canonical Wnt Pathways

The Wnt signalling pathway plays a critical role in animal development. Wnt ligands are a large family of secreted glycoproteins (19 different genes in the mouse and human genomes) that regulate cell fate specification, tissue patterning and cell growth in receptive cells (75). Abnormal Wnt signalling has been associated with many human diseases such as cancer. Wnt ligands produce their effects through activation of at least three major signalling pathways. The best-studied one is the canonical Wnt pathway which acts through β-catenin (76). By contrast, non-canonical Wnt pathways seem to act independently of β-catenin and regulate cell movement and organization (77). There are many non-canonical pathways, but the best characterized ones are the planar cell polarity (PCP) and Wnt/calcium pathways. The Wnt/PCP pathway is initiated by binding of Wnt ligand to Frizzled (Fz) and ROR2 receptors. Upon this binding is the recruitment of Dishevelled (Dsh), which forms a complex with Daam1. Daam1 subsequently activates the small G-protein RhoA, Rac, ROCK, and JNK to modulate cell polarity and motility (78). The Wnt/calcium pathway initiates by binding of Wnt ligands to Fz and activating heterotrimeric G-proteins (79), which results in the release of intracellular calcium. This
pathway involves activation of phospholipase C, protein kinase C, and calmodulin-dependent protein kinase II (80).

In the canonical Wnt pathway and in the absence of Wnt ligands, the pool of free cytoplasmic β-catenin is regulated by the cytoplasmic multiprotein complex (also called the destruction complex) through the ubiquitin-dependent pathway (81). The destruction complex is composed of glycogen synthase kinase 3-beta (GSK-3β), Axin (a scaffolding protein) (82), APC (a scaffolding protein) (83), and casein kinase 1-gamma (CK1γ) (84). β-catenin is sequentially phosphorylated. First, it is phosphorylated by CK1γ at serine 45, which in turn enables GSK-3β to phosphorylate threonine 41, serine 33, and serine 37 (85). GSK-3β does not directly bind to β-catenin; however, it interacts with β-catenin through Axin (contains binding sites for APC, GSK-3β, and β-catenin) and APC (contains interaction sites for Axin and β-catenin) (83). Upon β-catenin binding to the destruction complex, GSK-3β phosphorylates APC, which in turn increases APC’s affinity for β-catenin and strengthens their interaction. Phosphorylation of serine 33 and serine 37 residues of β-catenin triggers ubiquitylation of β-catenin by beta-transducin repeats-containing protein (β-TrCP) and β-catenin degradation in proteasomes (86). β-TrCP is a component of the Skp1/Cullin/F-box protein (SCF) E3 ubiquitin ligase complex that recruits an E2 ubiquitin conjugating enzyme and promotes β-catenin ubiquitination and degradation by the 26S proteasome system (81). In the absence of β-catenin in the nucleus, high-mobility group DNA-binding T cell factors (TCF) or lymphoid enhancer factors (LEF), which have dual functions as both transcriptional repressors and activators, act as a transcriptional repressor through interaction with members of repressor families such as Groucho, Gro-related gene and transducer-like enhancer proteins. TCFs/LEFs are architectural transcriptional factors that introduce bends into DNA; however they are weak transcriptional factors by themselves and require additional co-repressors or co-activators. Upon binding to Wnt-responsive elements, the nucleotide sequence of CCTTTGWW (W indicated either T or A) is recognized by TCF/LEF. Then, along with transcriptional repressors, TCF/LEF transcriptional factors cause significant DNA bending that alter local chromatin structure and ultimately inhibit expression of downstream Wnt target genes (87-91).

In the presence of Wnt ligands, the pathway is activated upon binding of ligands to a seven-pass transmembrane receptor of the Fz family (92) and single-pass transmembrane co-receptor LDL receptor-related protein (LRP) 5/6 (93). This binding recruits Dsh to the plasma membrane
where it becomes phosphorylated by CK1. This leads to the recruitment of Axin-GSK-3β complex (94). Membrane-bound GSK-3β phosphorylates LRP5/6, which in turn recruits additional Axin-GSK-3β to phosphorylate other residues on LRP5/6. Following recruitment of Axin and GSK-3β to the plasma membrane, the destruction complex becomes destabilized, and β-catenin remains unphosphorylated (95). The unphosphorylated β-catenin accumulates in the cytoplasm, as it cannot be recognized by β-TrCP and is not degraded by ubiquitin-dependent pathway. The non-degraded β-catenin translocates into the nucleus via a nuclear localization signal and importin-independent manner, possibly by binding directly to the nuclear pore machinery proteins through its central armadillo (Arm) repeats (96, 97). In the nucleus, β-catenin engages TCF/LEF to activate expression of downstream target genes by displacing histone deacetylase-associated transcriptional co-repressors from TCF/LEF (98). Therefore, TCF/LEF is converted into a transcriptional activator upon interaction with β-catenin. Transcriptional activation is mediated through the interaction of β-catenin with the histone acetyltransferase p300/CBP, the chromatin remodeling SWI/SNF complex, and BCL9 bound to pygopus (Pyg) (Figure 3.) (99, 100).

Although β-catenin and TCFs are universal Wnt pathway activators, their target genes appear to be cell-type specific depending on the presence of certain co-activators or co-repressors. Some of the identified Wnt pathway target genes are c-jun (101), c-myc (102), cyclin-D1 (103), matrix metalloproteinase-7 (MMP-7) or matrilysin (104), axin2 (105), and multidrug resistance-1 (106). Overall outcomes of the activation of different transcriptional programs by canonical Wnt signalling include promoting cell proliferation and tissue expansion, cell fate determination or terminal differentiation of postmitotic cells, maintenance or activation of stem cells, and development and progression of cancer pathology (75).

1.4.2 APC

The APC gene was first identified as the gene mutated in FAP. It is located on human chromosome 5q21-22 and consists of 15 exons, with 8535 base pairs of coding sequences, coding for an approximately 310KDa protein composed of 2843 amino acids (107, 108). The largest exon, exon 15, is the target of most germline mutations in FAP patients and somatic mutations in tumours. There are several structural motifs in the APC protein. The first third of APC, near its N-terminal, contains the oligomerization domain and the seven Arm repeats, which
bind to APC-stimulated guanine nucleotide exchange factor and kinesin superfamliy associated protein 3. Three 15-amino acid, seven 20-amino acid repeats, and three SAMP (Ser-Ala-Met-Pro) repeats are present within the central third of APC. The 15-amino acid repeats bind β-catenin but do not affect β-catenin level; however the 20-amino acid repeats bind and down-regulate β-catenin. The three SAMP repeats bind axin. The carboxy-terminal end of APC is enriched with basic amino acids that bind to tubulin. Additionally, it consists of the end-binding protein 1(EB1) domain which binds EB1, and discs large (DLG) domain which binds DLG (Figure 4.) (109, 110). Being able to bind EB1 and DLG, and having nuclear export and import signal domains, APC was suggested to play roles in cell migration and adhesion, cell cycle regulation, and chromosomal stability (109, 111). However, one of the main functions of APC is to promote the degradation of β-catenin and is considered to be an important tumour suppressor protein (112). Therefore, mutations in APC are associated with several human cancers such as aggressive fibromatosis. APC derives this tumour suppressing capacity from its ability to modulate the oncogenic Wnt signal transduction cascade through the effect on the cellular levels of β-catenin.

As a tumour suppressor, APC does not entirely follow the Knudson’s ‘two-hit’ hypothesis (113) for tumourigenesis. The position and type of the second hit in the tumour of FAP patients depends on the localization of the germline mutation, and different germline-somatic APC mutations provide a cell with different selective growth advantages such as AF tumour formation or colorectal adenomas growth. In FAP patients with a germline APC mutation distal to codon 1449, the somatic allelic loss was found in their AF tumours; however, the region of germline APC mutations associated with somatic allelic loss in colorectal adenomas is close to codon 1300 (114). Mutations in the 3’ end of the APC gene predispose patients with FAP to develop primarily AF tumours but few, if any, colorectal polyps. Mutations in this region result in an early stop codon, and eventually a truncated APC protein lacking β-catenin regulatory domains (115, 116). This suggests that tumours may be initiated by mutations in the APC gene with the loss of β-catenin regulation capacities. An APC mutation is found in about 15% of cases of sporadic aggressive fibromatoses. Alman et al. demonstrated biallelic truncation mutations of the APC gene in 3 of 6 sporadic aggressive fibromatosis tumours analyzed. Two cases showed frame-shift mutations in both alleles of the APC gene at either codon 1324 or 1371 resulting in downstream stop codons at codon 1331 or 1414, respectively. The third case showed a
substitution of T to G in one allele causing a stop codon at codon 1493 and loss of heterozygosity in the wild-type allele (6). Additionally, in a mutational analysis on 42 cases of sporadic AF, somatic APC mutations were detected in 9 tumours studied. These mutations were clustered between codons 1324 and 1567 resulting in an early stop codon. One case had mutation in both alleles, while the other 8 cases showed a loss of heterozygosity (69). When full-length APC gene was transfected into primary cell cultures derived from sporadic AF tumours with APC mutations, both cell proliferation and β-catenin protein level were decreased. However, after transfection of both full-length APC gene and ΔN89β-catenin (a stable form of protein that is not degraded by APC) into the same primary cell cultures, the cell proliferation was not decreased. Thus, the APC truncating mutations provided AF tumour cells with a proliferation advantage through β-catenin (70). Furthermore, the involvement of the APC gene in the development of AF tumours was shown by the generation of Apc1638N mouse model (117). This mouse model carries a targeted missense mutation at codon 1638 of the APC gene. In addition to the tumours of upper gastro-intestinal tract, heterozygous animals are characterized by developing fully penetrant and multifocal AF tumours and cutaneous cysts, representative of the clinical manifestations observed in FAP patients (115).

1.4.3 β-catenin

1.4.3.1 Structure of β-catenin

The CTNNB1 gene which codes for β-catenin is located on human chromosome 3p22-p21.3 and consists of 13 exons. This gene codes for a 92-94KDa protein composed of 781 amino acids. β-catenin protein contains a central region of 12 imperfect copies of the 42 amino acids Arm repeat flanked by a 130 amino acids N-terminal domain and a 100 amino acids C-terminal domain. The amino terminus of the protein contains phosphorylation sites for GSK-3β and tyrosine kinases and is involved in β-catenin degradation by ubiquitination. The central Arm repeats interact with cadherins, APC, axin, and TCF/LEF. The carboxy terminus contains transcriptional transactivation elements for target genes activation (118-120).
1.4.3.2 Functions of β-catenin

β-catenin protein has two cellular key functions. It participates as a structural protein in the
cadherin-mediated cell-cell adhesion in the cytoplasm and is involved in the nucleus as a
transcriptional co-activator during Wnt signalling.

In cell adhesion, β-catenin is a central component of the cadherin/catenin adhesion complex
located at the cell membrane. Cadherins are type-1 single-transmembrane glycoproteins which
mediate calcium-dependent intercellular adhesion. These proteins contain an extracellular
domain which interacts with adjacent E-cadherin molecules on adjacent cells and a cytoplasmic
domain which binds to either β-catenin or γ-catenin (Plakoglobin). β-catenin links the
cytoplasmic domain of cadherin via alpha-catenin indirectly to the actin cytoskeleton (121-123).
The absence of functional beta-catenin protein results in a loss or reduction of cadherin-mediated
cell-cell adhesion and a disintegration of embryonic and adult tissues (124). β-catenin mediated
cell adhesion depends primarily on its interactions with the cytoplasmic domain of cadherin and
the N-terminal domain of alpha-catenin molecules.

The E-cadherin/catenins complex can prevent the invasion of tumour cells. Therefore, the more
invasive phenotype in certain cancers is associated with an inactivation of one component of the
complex which results in the destabilized complex (125, 126). The stability of this complex is
also dependent on the phosphorylation status of both β-catenin and cadherins. It has been shown
that in vitro phosphorylation of specific serine residues in cadherin enhances the binding affinity
of β-catenin to cadherins. For example, phosphorylation of serine residues 834, 836, and 842 but
not 846 (by CK1), increases the affinity of cadherins for β-catenin and strengthen their
interaction (118, 127). Additionally, the ligand-binding region of β-catenin is a substrate of Src
kinase. This kinase specifically phosphorylates tyrosine 654 of β-catenin, which is located on the
last Arm repeat. This phosphorylation status results in the reduction of cadherin binding and
adhesive functions (128, 129).

During cell-to-cell signalling, β-catenin is a key mediator in the Wnt signalling cascade which
modulates transcription in target cells. Somatic β-catenin mutations at its phosphorylation sites
were reported in about 60% of sporadic AF cases by different groups (69, 130, 131). These
mutations were point mutations at either codon 41 or 45, which are two of the four
phosphorylation and ubiquitination sites of β-catenin, and result in producing a stabilized form of
β-catenin protein. The point mutations cause threonine (ACC) to alanine (GCC) or serine (TCT) to phenylalanine (TTT) substitution at codons 41 and 45, respectively. Saito et al. also found nine point mutations of β-catenin from codon 21 to codon 67 in 7 out of 18 cases of sporadic AF tumours. In addition, they showed that β-catenin nuclear expression correlates with cyclin-D1 overexpression, suggesting that the accumulation of β-catenin within the nuclei could affect the regulation of the cyclin-D1 gene. Furthermore, all cases of sporadic AF, regardless of their mutation status, showed diffuse β-catenin nuclear staining (132). Finally, using a transgenic mouse that overexpresses a stabilized form of β-catenin, Cheon et al. showed that β-catenin stabilization is the fundamental cause and sufficient to form AF tumours (133).

1.4.4 β-catenin Dysregulation and Tumourigenesis

Mutations that promote constitutive activation of the Wnt signalling pathway are observed in many human cancers. These mutations result in an inappropriate target gene activation linked to β-catenin dysregulation. There are three mechanisms involved in this neoplastic process including improper activation of the Wnt signalling pathway, direct mutations in β-catenin gene that prevent its downregulation, and mutations resulting in an inactivation of the destruction complex, such as mutations in APC gene (134).

Regarding the improper activation of Wnt pathway, there is no report of Wnt genes being mutated and directly involved in human cancers. However, the ectopic expression of Wnt-1 in murine mammary gland promotes tumour formation (135). Although the overexpression, and sometimes underexpression of Wnt genes was reported by numerous studies in human cancers, still strong genetic alteration evidence, such as amplification, rearrangement, or mutations of genes encoding Wnt ligands or receptors has not been documented (136, 137).

Most mutations in the β-catenin gene occur in or around exon 3 at specific amino terminus serine and threonine residues which contain phosphorylation sites for GSK-3β. As a result, mutated β-catenin protein becomes refractory to phosphorylation by GSK-3β and subsequent recognition and degradation by ubiquitin-proteasome pathway (Figure 5.). These mutations occur in a wide variety of human cancers (138). In addition to AF, hepatoblastoma also occur with an increased incidence in FAP individuals. In both AF (69, 130, 131) and hepatoblastoma (139), activating β-catenin mutations localized to the exon 3 of the CTNNB1 gene were identified at high frequency.
Other types of tumours such as hepatocellular carcinoma, nephroblastoma, and medulloblastoma also contain \textit{CTNNB1} mutations in their sporadic forms (140, 141).

Among mutations which result in an inactivation of the destruction complex, \textit{APC} mutations are the most common type. \textit{APC} is a tumour suppressor in human cancers and its mutation relates strongly to the regulation of \(\beta\)-catenin (109, 112). The \textit{APC} mutations, which typically truncate the protein, are selected against \(\beta\)-catenin regulatory domains, but not necessarily against \(\beta\)-catenin binding domains (138). The \textit{APC} gene was first identified as the gene mutated in FAP. In FAP-associated tumours in addition to the original germline mutations, there are additional somatic \textit{APC} mutations or loss of heterozygosity (LOH) in the wild-type \textit{APC} allele (107, 108). As a result of these mutations, truncated proteins with different length and stability as well as variable \(\beta\)-catenin regulatory capacity are produced. Therefore, a great deal of variability is seen in FAP patients. The most severe and common human APC mutations are located between codons 450 and 1578 resulting in stable truncated proteins (142). In addition to the high colorectal polyp counts, patients with truncations between codons 1395-1578 develop desmoids, osteomas, epidermoid cysts, and polyps of the upper gastrointestinal tract. On the other hand, mutations in the extreme 5' end of the \textit{APC} gene result in a less severe phenotype of FAP, and mutations in the 3' end, which often result in an undetectable level of truncated protein, also confer an attenuated form of FAP but with an increased risk for extraintestinal manifestations (142-144).

One possible explanation for this interdependence of mutations is the “just-right” signalling hypothesis. This model suggests that APC function must be impaired sufficiently to allow the accumulation of \(\beta\)-catenin, but not above the certain limit (145). Albuquerque et al. analyzed somatic \textit{APC} mutations and LOH in 133 colorectal adenomas from FAP patients. They found that in cases with germline mutations resulting in truncated proteins without any of the seven \(\beta\)-catenin downregulating repeats, most of the corresponding somatic point mutations retain one or, less frequently, two of the same \(\beta\)-catenin regulatory repeats. In contrast, most second hits remove all of the seven \(\beta\)-catenin downregulating repeats in cases where the germline mutation results in a truncated protein with one \(\beta\)-catenin regulatory repeat. The latter frequently occurs due to the allelic loss. Their results indicate that \textit{APC} genotypes that are likely to retain some activity in downregulating \(\beta\)-catenin signalling are selected, and this selection process is based at
a specific level of β-catenin signalling optimal for tumour formation, rather than at its constitutive activation by deletion of all of the β-catenin downregulating domains in APC.

Mouse models have been the best tools to further understand the role of Wnt signalling in cancer and the genotype-phenotype association observed in FAP patients. The Apc\textsuperscript{Min} (multiple intestinal neoplasia) mice carry an ethyl-nitroso-urea-induced nonsense mutation at codon 850, and express stable truncated Apc protein, approximately 95KDa, lacking all β-catenin regulatory domains (146). Heterozygous Apc\textsuperscript{Min} mice are characterized by the development of multiple intestinal polyps, more than 100 lesions mainly located in the upper gastrointestinal tract, and no aggressive fibromatosis tumours. Due to the intestinal bleeding, these mice seldom live longer than 140 days. However, a targeted chain-terminating missense mutation at codon 1638 of the Apc gene results in nearly undetectable amounts of the protein with minimal β-catenin regulatory capacity (117). Heterozygous Apc\textsuperscript{1638N} mice are characterized by the development of many AF tumours, but only a few intestinal tumours, 5-6 lesions mainly located in the upper gastrointestinal tract. The position of the two responsible mutations along the Apc gene, which results in a different protein level of β-catenin, seems to be the reason for the phenotypic differences observed in these two mouse models.

Inactivating mutations are also found in other components of the destruction complex other than those observed in the APC gene. For example, inactivating mutations in AXIN have been reported in a few colorectal cancer cell lines (147). Furthermore, Satoh et al. reported the biallelic inactivation in AXIN1 in human hepatocellular carcinomas and cell lines. Interestingly, these mutations were identified in cases without activating mutations in CTNNB1 gene, and result in truncated axin proteins lacking β-catenin binding sites (148).

Inappropriate gene activation following stabilization of β-catenin, which is caused by mutations in Wnt signalling pathway components, results in the development of cancer (75). Tumours mediated by aberrant Wnt/β-catenin signalling show distinct gene expression patterns. This distinct cell-type specific gene expression is regulated by β-catenin recruiting various co-activators and co-repressors (149). Finally, nuclear β-catenin interacts with TCF/LEF transcription factors to activate different Wnt target genes. Tejpar et al. showed that nuclear β-catenin specifically binds to TCF3 in aggressive fibromatosis (74). This is in contrast to colonic neoplasia, in which β-catenin acts primarily through binding to TCF4 (150). Overall
consequence of aberrant Wnt/β-catenin signalling includes dysregulated proliferation, apoptosis, migration, and differentiation (151, 152). Some of the identified Wnt target genes involved in the aforementioned processes are \textit{C-MYC}, \textit{CYCLIN-D1}, \textit{MMP}, and cyclooxygenase \textit{(COX)} (102-104).

C-myc is a very strong oncogene that promotes G1/S cell cycle transition. Also, it is very often found to be upregulated in many types of cancers such as colorectal tumours (153). Conversely, it was shown that c-myc’s overproduction may induce apoptosis (154). Additionally, c-myc protein was shown to be overexpressed in the nucleus in approximately 45% of AF tumours analyzed (151). Similarly, immunohistochemical staining of cyclin-D1, which is also involved in cell cycle regulation, revealed its overexpression in 40-70% of AF tumours by two different studies (132, 151).

MMPs are a large family of zinc-dependent endopeptidases, and capable of degrading extracellular matrix (ECM) proteins. Aside from their main function, which is to degrade the ECM during normal biological processes, including embryonic development and wound healing, they are also involved in multiple stages of cancer progression including invasion and metastasis of tumour cells (155, 156). Expression of various MMPs has been found to be upregulated in many types of human cancers. For example, overexpression of MMP-7 has been demonstrated in human colorectal cancer (104). Furthermore, Denys et al. found a striking overexpression of mRNA levels of MMP-1, MMP-3, MMP-7, MMP-11, MMP-12, and MMP-13 in human AF tumours, compared to unaffected fibroblasts from the same patients. This overexpression was accompanied by a downregulation of MMP inhibitors (tissue inhibitors of metalloproteinases, TIMPs) including TIMP-1 and TIMP-3 (157). Moreover, studies of Mmps and their inhibitors in murine aggressive fibromatosis revealed that when mice predisposed to developing AF tumours treated with an inhibitor of Mmps or crossed with a transgenic mouse that overexpresses \textit{Timp-1}, resulted in a significant reduction in tumour volume (158). These data suggest that MMPs play a crucial role in modulating tumour progression and invasiveness.

COX, also called prostaglandin H synthase, is an enzyme responsible for formation of prostaglandins from arachidonic acid. A role for COX in carcinogenesis was suggested by studies in which elevated levels of arachidonic acid metabolites such as prostaglandin E2 were found in some neoplastic processes, of which colonic neoplasia was found to overexpresses
Furthermore, COX-2 levels were elevated in both human and murine AF tumours. Using a COX-2 blocking agent, the proliferation in human tumour cell cultures was reduced, and crossing $Apc^{1638N}$ mice with Cox-2$^{-/-}$ mice resulted in a smaller tumour size (57).

### 1.5 Mouse Models of Aggressive Fibromatosis

#### 1.5.1 Beta-catenin Stabilized Mouse Model

Previously, a transgenic mouse model was generated by our lab, which overexpresses an inducible, stabilized form of $\beta$-catenin in mesenchymal cells under the control of a tetracycline-regulated promoter (133). Phosphorylation of amino terminus serine and threonine sites of the $\beta$-catenin protein results in its ubiquitin-mediated degradation. Using an *in vitro* mutagenesis, these phosphorylation sites at codons 33, 37, 41, and 45 were changed to alanine, resulting in stabilized form of $\beta$-catenin. After three months of transgene induction, AF tumours were found in 18 of 24 mice analyzed and gastrointestinal polyps were found in all of the mice. These observations suggested that $\beta$-catenin stabilization is the fundamental cause and sufficient to form AF tumours. Aggressive fibromatosis tumour formation by inducing stabilized $\beta$-catenin is similar to adult-onset tumour formation caused by sporadic mutations that occur later in life, in contrast to a genetic neoplastic syndrome, in which a germline mutation is causative. Therefore, this provides an animal model that is analogous to adult-onset, sporadic aggressive fibromatosis.

#### 1.5.2 $Apc^{1638N}$ Mouse Model

Fodde et al. generated the $Apc^{1638N}$ mouse model by introducing the neomycin gene at codon 1638 in the transcriptional orientation opposite that of $Apc$ (117). Western blot analysis of embryonic stem cells and several tissues from heterozygous $Apc^{1638N}$ mice revealed the presence of the wild-type Apc protein, but failed to detect the expected 182KDa truncated polypeptide. However, immunoprecipitation analysis of homozygous $Apc^{1638N}$ cell lines revealed very low amounts (1-2%) of the expected Apc protein. Therefore, the $Apc^{1638N}$ mutant allele should be regarded as ‘leaky’ and not as a complete inactivation (null) of the $Apc$ gene (Figure 6.). Homozygosity for this mutation is embryonic lethal. The heterozygous mice on the C57BL/6J background develop an average of 5-6 intestinal tumours per mouse. In addition to these tumours, the $Apc^{1638N}$ model develops a broad spectrum of extra-colonic manifestations such as
multifocal aggressive fibromatosis tumours and cutaneous cysts. The AF tumours are significantly higher in the male mice, with an average of 45.7 lesions compared with 16.2 in females (160). The study of age of onset in AF tumours revealed that the majority of tumours arise between 1.5 and 2 months of age, when the average number of tumours increases from 6.7 to 27 per mouse. However, mice older than 2 months of age still continue to develop new tumours but at a significantly slower rate (160). The progression pattern of the intestinal tumours and their combination with extra-colonic manifestations such as AF lesions observed in $Apc^{1638N}$ mice, which resemble that observed in FAP patients, provide a useful tool for studying relevant extra-abdominal manifestations of FAP. The $Apc^+ / Apc^{1638N}$ is a mouse model that is used in this study.

1.6 Cell of Origin of AF

The identification of the primary cell of origin of tumours is a key step toward an understanding of the pathology of this disease and having this knowledge can lead to the development of strategies to suppress tumour in pre-neoplastic conditions. However, the cellular origins of most tumours are poorly defined, especially in mesenchymal neoplasms (161).

Aggressive fibromatoses generally arise in connective tissues. The anatomical location, cellular morphology, and histological profile of these lesions suggest that they derive from mesenchymal sources (7, 8); however, these observations are still not enough to identify the cells responsible for AF oncogenesis. A recent study by our lab provided evidence regarding the involvement of cells with mesenchymal stem cell (MSC) like characteristic in both the initiation and maintenance of aggressive fibromatosis tumours (162). By using a mouse model genetically predisposed to AF ($Apc^{+/1638N}$), a positive correlation between mesenchymal progenitor cell (MPC) numbers and AF tumour formation was observed. Furthermore, when MPC deficient mice ($Sca1^{-/-}$) were crossed with $Apc^{+/1638N}$ mice, a reduction in the number of AF tumours was found in mature mice. However, Sca-1 deficiency did not impact the formation of intestinal neoplasms, which are epithelial derived tumours. Finally, MPCs isolated from $Apc^{+/1638N}$ mice and mice expressing activated $\beta$-catenin allele were subcutaneously injected into immunocompromised mice, where they initiated aberrant cellular growth. Taken together, these findings suggest that the formation and development of AF is influenced by MPCs. As a result, it is possible that protecting this progenitor cell population in patients with FAP can prevent
aggressive fibromatosis. Also, understanding MSC biology and its molecular mediators which regulate its proliferation and differentiation can be used to develop new treatments.

1.7 Mesenchymal Stem/Progenitor Cells

The identification of bone marrow-derived cells with the potential to differentiate into the various lineages of mesenchymal tissue was first described by Friedenstein and his coworkers in 1968 (163-165). Adult bone marrow is a site of origin of several types of hematopoietic and non-hematopoietic stem cells with distinct functions. For instance, MSCs, also known as bone marrow stromal cells or as suggested by the International Society for Cytotherapy: multipotent mesenchymal stromal cells (166), are a group of clonogenic adherent cells with multilineage differentiation capacity into mesoderm-type cells such as osteoblasts, adipocytes, and chondrocytes (167, 168). MSCs have been isolated from multiple species, including human (169-171), and multiple organs, including bone marrow, adipose tissue (172), and umbilical cord blood (173). In addition to their differentiation capacity, MSCs provide supportive stroma for growth and differentiation of hematopoietic stem cells and hematopoiesis (174). Moreover, having the immunomodulatory capacities, MSCs are a good candidate for use as a cell therapeutic agent in immunological diseases (175, 176). MSCs are fibroblast-like cells which form colonies (colony forming unit-fibroblasts [CFU-f]) during their initial growth in vitro (164, 177). Isolating a true MSC population is challenging due to the lack of specific MSC markers. The common accepted tests for the identification of MSCs include the capacity of these cells to form CFU-f in culture, analysis of their surface markers such as CD73, CD90, CD105, and STRO-1, and their multilineage differentiation potential (166, 178).

There is growing evidences suggesting that mesenchymal progenitors can give rise to mesenchymal neoplasms following acquiring oncogenic changes. For example, Riggi et al. demonstrated that overexpression of EWS-FLI-1 fusion protein, the transforming event in Ewing’s sarcoma, can transform primary bone marrow-derived mesenchymal progenitor cells and generate tumours that display hallmarks of Ewing’s sarcoma (179). In another study, it was illustrated that human MSCs can be transformed via inhibition of the Wnt/β-catenin signalling pathway to form malignant fibrous histiocytoma (a commonly diagnosed mesenchymal tumour)-like tumours in nude mice (180). Finally, another group showed the development of sarcomas in mice injected with ex vivo-expanded MSCs. Furthermore, primary bone marrow-derived MSCs
from two strains of mice showed cytogenetic changes after several passages \textit{in vitro} in the same study (181). Taken together, these studies suggest that mesenchymal neoplasms may indeed originate from MPCs.

Developmentally important signalling pathways such as Wnt and hedgehog (Hh) signalling regulate proliferation and differentiation of MSCs. There are several lines of evidence to support the role of Wnt/β-catenin signalling in regulating MSC self-renewal, expansion, and commitment/differentiation. Briefly, Wnt activation has been shown to be necessary for MSC differentiation to osteocytes, chondrocytes, myoblasts, and adipocytes; conversely, Wnt inhibition increases MSC proliferation and promotes their entry into the cell cycle (182-185). There is also growing evidence suggesting the role of Hh signalling in modulating the proliferation and differentiation of MSCs. Using human MSCs, it has been shown that Hh signalling decreases during osteoblast differentiation, which is associated with a decrease in Smoothened (Smo) expression, a key partner that transmits the Hh signal upon stimulation. Consistently, activation of Hh signalling by the treatment of cells with sonic Hh or two molecules able to activate Hh signalling inhibits osteoblast differentiation. Furthermore, the role of Hh signalling was studied on the human MSC proliferation which revealed that inhibition of Hh signalling, using cyclopamine or siRNA against Gli2, results in a decrease in human MSC proliferation and their ability to form clones. This proliferation inhibition is through blocking cells in the G0/G1 phases of the cell cycle (186, 187). These observations suggest the critical function of Hh morphogen in human MSC biology. As a result, targeting this pathway can be used to develop new therapies for cancers originating from MSCs with deregulated proliferation/differentiation pattern.

\section*{1.8 Hedgehog Signalling Pathway}

\subsection*{1.8.1 \textit{Hh} Gene Discovery}

The Hh signalling pathway is another signalling cascade that directs patterning and is crucial for proper development. Hh signalling is most active during embryogenesis controlling cell proliferation, differentiation and tissue patterning in a dose-dependent manner and it is mainly quiescent in adults (188-190). However, it plays a role in stem cell maintenance, tissue repair and regeneration during adult life (191-193). Not surprisingly, aberrant activation of Hh signalling is
associated with numerous human disorders including birth defects, such as Gorlin syndrome, and cancers including basal cell carcinoma (BCC) and medulloblastoma (194-196).

The $hh$ gene was first identified in 1980 as a gene encoding for a secreted signalling protein which was required for embryonic segment polarity in *Drosophila melanogaster* (197). In 1993, vertebrate $hh$ genes were first reported following a cross-species (fish, chick, and mouse) collaborative work by three groups (198-200). However, there were several differences in these initial findings compared to what was observed in fly. For example, unlike the fly, which has a single $hh$ gene, there are several $hh$ homologs in vertebrate species. In mammals, there are three Hh ligands, including Sonic Hh (SHh), Desert Hh (DHh), and Indian Hh (IHh). SHh is the most studied of the three proteins due to its role in patterning of the neural tube in mice. IHh was found to be involved in skeletal bone formation and DHh’s function is restricted to the testis (201-203). Importantly, Hh signalling can act over a long range and is concentration-dependent; causing distinguished molecular responses at distinct concentration thresholds, which is a classic morphogen activity (188).

### 1.8.2 Production, Secretion, and Reception of Hh Ligand

In cells that produce Hh, newly made protein undergoes post-translational modifications, including autoprocessing and lipid modifications (204, 205). The 45KDa precursor protein, which consists of a C-terminal protease domain and an N-terminal signalling unit, undergoes an intramolecular cleavage that is catalyzed by its C-terminal portion. This reaction releases a 19KDa active signalling domain of Hh called HhN (206). During this cleavage, the C-terminal of HhN becomes covalently modified by coupling a cholesterol molecule which results in the processed form of the signalling moiety, called HhNp (207, 208). In addition to cholesterol coupling, another lipid modification occurs within the Hh protein. The most N-terminal cysteine of Hh, which is highly conserved in all Hh proteins, becomes modified by the fatty acid palmitate (209). These lipid modifications are necessary for the proper movement and reception of the ligands (210, 211). Subsequent release of Hh depends on Dispatched (Disp), a multi-pass transmembrane protein (212). In the absence of Disp, Hh-expressing cells fail to secrete the accumulated Hh; as a result, the signalling pathway is not activated in the responding cells (213). Also studies in *Drosophila* have revealed a role for heparan sulfate proteoglycans (HSPG), cell-surface and extracellular matrix proteins, in the regulation of Hh protein distribution (214).
In addition to Hh ligand dispersal, its reception is also regulated by different modulators. Glypican-3, a member of the glypican subfamily of HSPGs, was reported as a negative regulator of the pathway by competing with Patched (Ptch) for the binding of Hh at the cell surface and promoting Hh endocytosis and its subsequent lysosomal degradation in mammals (215). Hedgehog-interacting protein 1 (Hhip 1), a membrane-bound glycoprotein, is another negative regulator in the Hh-receiving cells which also competes with Ptch for Hh binding and sequesters Hh ligands (216). Hhip 1 binds all three mammalian Hh proteins, and its expression is induced by Hh signalling that forms a negative regulatory feedback loop, thereby attenuating the Hh signalling rather than activating it (217). In contrast to the fly, where a single *ptch* gene is present, vertebrates have two *PTCH* genes giving rise to Ptch 1 and Ptch 2, a 12-pass transmembrane protein related to Disp, that act as Hh receptors (218, 219). Ptch belongs to a family of integral-membrane proteins that contain a sterol-sensing domain, a motif identified in proteins implicated in cholesterol intracellular trafficking (220). Unlike other receptors that function as signal transducers to activate signalling pathways upon the ligand binding, Ptch acts as an unusual receptor by blocking pathway activation in the absence of Hh and so, acting as a pathway inhibitor. Like Hhip 1 where its expression is induced by Hh, Ptch expression on the cell surface is also elevated by Hh signalling which results in sequestration of the Hh ligand, a negative feedback mechanism (221). Therefore, Hh controls its own distribution by manipulating the expression of its receptors.

There are also positive regulators on the cell surface of the Hh receiving cells, including Cdo and Boc which contain the immunoglobulin/ fibronectin-repeat, and the glycosylphosphatidylinositol-anchored membrane-bound Gas1. These positive regulators enhance Hh binding to Ptch (222, 223). Another positive regulator of the pathway is Smo; a seven-pass transmembrane protein belongs to the superfamily of G-protein-coupled receptor whose activity is essential for all aspects of Hh signalling both in *Drosophila* and vertebrates (224, 225). Smo activity is inhibited by Ptch in the absence of Hh ligand. As a consequence of Hh ligand binding to Ptch, the negative interaction between Ptch and Smo is relieved, which leads to the intracellular transduction of the Hh signal downstream of Smo (218, 226).
1.8.3 Localization of Hh Pathway Components in Cilia

The mechanism by which Ptch regulates Smo activity has been questionable (227). Before the discovery of primary cilia as a cellular organelle associated with mammalian Hh signalling, there were a few models proposed. One possibility involves a direct interaction between Ptch and Smo resulting in suppression of Smo activity by Ptch, and this suppression is relieved upon Hh binding to Ptch without causing dissociation of the Smo-Ptch complex (218). The second and simpler stoichiometric model implies to a dissociation of the receptor complex upon Hh binding, and therefore releasing Ptch suppression of Smo (226). The alternative to these stoichiometric models is that Ptch catalytically regulating Smo activity, either directly or indirectly (227). However, with the discovery that Hh signalling in vertebrates is dependent on the primary cilium, a microtubule-based antenna-like organelles projecting from the surface of most, if not all, mammalian cells, a new explanation for how this regulatory mechanism works was established (228, 229). Recent discoveries revealed that all of the major Hh pathway components, including Ptch1, Smo, and Gli proteins at least partially localize to the primary cilium. The cilium is essential for ligand-induced activity of the Hh pathway (230, 231). This new model implicates trafficking of the two proteins in and out of the cilium as a key event in regulating Smo activity (232). In this model and in the absence of Hh ligand, Ptch localizes on cilia and inhibits accumulation of Smo in cilia; however, binding of Hh to Ptch triggers the internalization of the receptor-ligand complex in endosomal vesicles and accumulating of Smo in cilia. This ciliary localization of Smo correlates with the activation of Hh pathway and its downstream target genes. This ciliary localization of Smo is also observed when cells are treated with Smo agonists, such as SAG or oxysterols, or in cells with an oncogenic Smo mutation (232, 233).

1.8.4 Hh Signal Transduction Downstream of Smo

Ciliary localization of Smo is thought to initiate a signalling cascade, leading to the activation of the glioma-associated (Gli) family of zinc finger transcription factors which are the ultimate effectors in the mammalian Hh signalling pathway downstream of Smo (234, 235). There are three Gli proteins, Gli1, 2 and 3, in vertebrates; however, Drosophila has a single Gli protein that is encoded by the cubitus interruptus (ci) gene (236-238). In Drosophila, Ci is the key regulator of Hh target genes which functions as both a transcription repressor and activator. In the absence
of Hh ligand, Ci is proteolytically processed into a repressor form. This cleavage results in a truncated form of the protein which lacks the carboxy-terminal region but contain the amino-terminal portion and the zinc-finger DNA-binding domains (239). By contrast and in the presence of Hh ligand, this cleavage is inhibited and a full-length protein that functions as a transcriptional activator is generated (237, 240, 241). In contrast to Ci, Gli1 is not proteolytically processed and only occurs as a full-length transcriptional activator; while Gli2 has both activator and repressor functions, and Gli3 is mostly a repressor, although it can also have positive effects (242-245). In the absence of Hh signalling, Gli1 is transcriptionally silent, Gli2 is expressed but is not an activator and Gli3 is present as a cleaved repressor to inhibit Hh targets. In the presence of Hh and activation of Smo, Gli repressors are lost and Gli activators are made (245).

In mice, Gli2 and Gli3 are essential genes, whereas Gli1 is dispensable for embryonic development and encodes a secondary mediator of Hh signalling (246-248). For example, Gli1^{zfd/zfd} (zinc finger deletion) mutants are viable and appear normal; however Gli2^{zfd/zfd} mutants are embryonic lethal and show many developmental defects including shortened limbs (249). In Gli2^{zfd} mutants a 2.4 kb region of Gli2 that contains the exons coding for zinc fingers 3-5 as well as 71 amino acid residues carboxyl to the zinc fingers are deleted. Since zinc fingers 4 and 5 are known to be important for DNA binding, this truncated protein should not be able to bind DNA. In this study, we used Gli2^{zfd} mutants which do not show any detectable abnormalities. These mice were crossed with our Apc^{+/Apc1638N} mice to generate Apc^{+/Apc1638N; Gli2^{zfd}} and Apc^{+/Apc1638N; Gli2^{+/+}} littermates.

Regulation of Gli activity involves multiple mechanisms including protein phosphorylation, proteasome-mediated proteolysis and cytoplasmic-nuclear shuttling. Protein kinase A (PKA), GSK-3β, and CK1 sequentially phosphorylate multiple phosphorylation sites that are present in Gli3 C-terminus. This results in the recruitment of the F-box subunit of an SCF E3 ubiquitin ligase (β-TrCP) which targets the C-terminal half of Gli3 for proteasome-mediated degradation (250, 251). How the proteasome selectively degrades the C-terminal half of Gli3 is not fully understood; however, a processing determining domain (PDD) of Gli3 seems to be crucial for this limited degradation (252). The PDD in Gli2 appears to be less effective than the one in Gli3 which explains why Gli3 is processed more efficiently than Gli2 and is the predominant Gli repressor form. Gli1 lacks the PDD domain and as a result only exists in a full-length activator form.
The suppressor of fused (Sufu) is found downstream of Smo in the regulation of mammalian Gli activity (253-256). A remarkable difference between Drosophila and mammalian Hh signal transduction lies in the role of Sufu, since the genetic deletion of Sufu in Drosophila does not lead to constitutive activation of the Hh pathway (257), in contrast to the situation in mice where it causes ectopic Hh pathway activation, similar to loss of Ptch1 (258, 259). Sufu binds to all three Glis (253, 260, 261) and co-localizes together with Smo and Gli to the primary cilium (230), and may regulate Gli activity by controlling their processing and/or degradation and as a result the ratio between Gli activators and repressors. It may also inhibit Gli nuclear localization (254) or suppress Gli activity by recruiting a transcriptional co-repressor complex (262, 263).

In Drosophila, Ci is associated with a cytoplasmic protein complex composed of a serine-threonine kinase, Fused (Fu), and a kinesin-related protein, Costal2 (Cos2), and interacts with Sufu. While Fu is thought to play an essential role in the fly Hh signalling (264-267), it is dispensable for mammalian Hh signalling (268, 269). Instead, mammalian Fu was shown to play an essential role in motile ciliogenesis through an interaction with Kif27, a mammalian Cos2 homologue(270). Kif7, another Cos2 homologue in mammals, is another evolutionary conserved regulator of Gli transcription factors. Recent studies in mice have revealed that Kif7 is required for the proper regulation of Hh signalling and its activity is dependent on the presence of the primary cilium. Kif7 acts both negatively and positively in mammalian Hh signalling (Figure 7.) (271-273).

The three ubiquitously expressed direct target genes of Hh pathway, and hence of the Gli transcriptional responses, are GLI1, PTCH1, and HHIP (217, 221). While GLI1 amplifies the initial Hh signal at the transcriptional level and its mRNA level is a reliable indicator of the pathway activity, the other two target genes mediate negative feedback on the pathway and, thus, it is more difficult to predict pathway activity from their levels. Other verified target genes include CYCLIN-D2 (274, 275), BCL2 (276, 277), SNAIL (278), NKX2.2 (279), and NMYC (280, 281). Upregulation of these genes result in different developmental fate responses, such as cell proliferation, cell survival, and epithelial-mesenchymal transition in metastasis. Therefore, it is not surprising that dysregulated Hh signalling can lead to a variety of cancers.
1.8.5 Hh Signalling in Tumourigenesis

Three basic proposed models for the involvement of aberrant Hh pathway in cancer development are as follows:

1.8.5.1 Type I: ligand-independent, mutation driven

The identification of *PTCH1* inactivating mutation leading to constitutively activated Hh signalling in the absence of ligand, as the cause of Gorlin’s syndrome (or basal cell nevus syndrome), was established as the initial link between Hh pathway and cancer (282, 283). These patients develop BCC with a high incidence, in addition to medulloblastomas and rhabdomyosarcomas (284). Mutations in other Hh pathway components include activating mutations in *SMO* which is seen in BCC and medulloblastoma, or inactivating mutations in *SUFU* in medulloblastoma and rhabdomyosarcoma (285-287). Additionally, *GLI1* gene amplification in glioma would also belong to this category (288). One hallmark of all these tumours is constitutive pathway activation in the absence of Hh ligand. The use of Hh pathway inhibitors could be effective in patients harboring such mutations. Tumours with activating mutations in pathway components downstream of Smo are definitely insensitive to the majority of the Hh pathway inhibitors, as most of them act at the level of Smo, and need inhibition further downstream in the pathway. Therefore, since the inhibition at the level of GLI activator function should cover all possible situations of aberrant Hh pathway activation, this would be the ideal strategy.

1.8.5.2 Type II: ligand-dependent, autocrine

In this model the tumour cells both secrete Hh and respond to it in an autocrine manner without having a genetic aberration in Hh pathway components. This autocrine response of tumour cells to Hh stimulates proliferation and survival of tumour cells leading to tumour growth. This is based on a fact that tumour cells were shown to express Hh in addition to downstream Hh signalling components, and their growth was inhibited by the Hh ligand-neutralizing antibody 5E1, RNAi-mediated knockdown of *SMO* or *GLI1*, and cyclopamine treatment when cultured *in vitro* or when grown as *in vivo* xenografts. This has been identified in a wide variety of cancers,
including upper gastro-intestinal tract (289, 290), colorectal (291), lung (292), pancreas (293), prostate (294, 295), breast (296), and melanoma cancers (297).

### 1.8.5.3 Type III: ligand-dependent, paracrine

In contrast to the autocrine model, tumour cells in paracrine manner secrete Hh but do not respond to it directly; however the adjacent stromal cells respond to secreted Hh and feed other signals back to the tumour to promote its growth and survival (298). This was first reported by Bushman and colleagues in a model of prostate cancer (299), and later was shown in several naturally Hh-overexpressing pancreatic and colorectal xenografts of human primary tumours and cell lines, where the secreted tumour-derived Hh stimulate expression of Hh target genes in the infiltrating stroma but not in the tumour cells (298). Additionally, all the murine target genes were downregulated and the tumour growth was slowed down upon treatment with either the Hh-blocking antibody 5E1 or a Smo inhibitor.

A variation of the paracrine signalling model that works in the opposite direction occurs when tumours receive Hh secreted from stromal cells such as the bone marrow or lymph nodes. This ‘reverse paracrine’ signal (Type IIIb) leads to the pathway activation in the tumour, upregulating survival genes in addition to GLI1 and PTCH1. Thus far, this has only been observed in experimental models of glioma (300, 301), and diverse B cell malignancies (302, 303) (Figure 8.).

### 1.9 Thesis Summary and Rationale

The goal of this research is to investigate the role of Hh signalling pathway in pathogenesis of AF tumours. AF is a fibroproliferative tumour that occurs as a sporadic lesion or a manifestation in FAP patients. Mutations frequently occur in either the APC gene or CTNNB1 gene. In both cases, genetic alterations cause stabilization of β-catenin and activation of β-catenin-mediated-TCF-dependant transcriptional activity, which appears to be a general occurrence in AF tumours. There are a variety of therapeutic strategies available for AF; however, current therapies still do not demonstrate total success for primary and recurrent tumours. As such, AF remains a clinical challenge and there remains a need for more effective therapies. AF derives from MSCs, and it is possible that signalling pathways which regulate MSC differentiation also alter the phenotype in
AF. Since hedgehog signalling maintains MSCs in a less differentiated state, this pathway could also play a similar function in AF.

1.9.1 Hypothesis

Hedgehog signalling plays a role in modulating tumor phenotype in aggressive fibromatosis.

1.9.2 Research Aims

The role of Hh signalling pathway in AF pathogenesis is examined and detailed in chapter 2. Specifically, I investigate the effect of Hh pathway inhibition on cell behavior using AF tumour cells in vitro. Additionally, using $Apc^{+/1638N}$ mouse model for AF and $Gli2^{+/+}$ mouse model for modulated-Hh pathway, I described the phenotypic effect of Hh inhibition on the development of AF tumours, in addition to the possible positive feedback loop between Hh and Wnt/β-catenin pathways. I demonstrate that Hh inhibition may serve as a potential novel adjuvant therapeutic strategy for FAP-associated AF.

Conclusion from this work will set the stage for further exploration of the role of Hh pathway in AF. Also, the mechanism by which Hh may act in AF pathogenesis needs to be further elucidated. Such future experimental approaches are outlined in chapter 3. It will be essential to determine how exactly the Hh inhibition modulates β-catenin levels.
Figure 1. Histology analysis of aggressive fibromatosis.

(A) H&E staining of human aggressive fibromatosis tumour. Spindle-shaped cells in a collagenous matrix are illustrated. (B) H&E staining of mouse aggressive fibromatosis tumour. Spindle-shaped cells surrounded by collagen fibres infiltrating into muscles are present.
Figure 2. β-catenin immunohistochemistry in aggressive fibromatosis.

(A) Human aggressive fibromatosis tissue showing intense staining of the cytoplasm in most cells, and potential nuclear staining in others. (B) Normal marginal fascial tissue from the same patient, showing significantly decreased β-catenin staining to the fibrocytes (69). Reprinted by permission from Macmillan Publishers Ltd: Oncogene (69), copyright (1999).
Figure 3. The canonical Wnt/β-catenin signalling pathway.

(A) In the absence of an activating Wnt ligand, cytoplasmic protein Dsh remains unphosphorylated. This in turn allows a multiprotein complex, which includes the scaffolding proteins APC and Axin, CK1γ and GSK3β to degrade β-catenin. Axin binds and phosphorylates β-catenin through the kinases CK1γ and GSK3β. Phosphorylated β-catenin is then targeted for ubiquitin-mediated degradation. As a result, Wnt target gene expression is repressed by transcriptional inhibitors. (B) In the presence of Wnt ligands, Frizzled and LRP5/6 inhibit the multiprotein complex through phosphorylated Dsh. As a result, β-catenin is stabilized in the cytoplasm and is able to translocate into the nucleus. β-catenin can then bind to TCF/LEF transcription factors, thereby regulating expression of Wnt target genes (304). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Rheumatology (304), copyright (2008).
Figure 4. Structural features of the APC protein.

The molecule is a 310KDa protein composed of 2843 amino acids. There are oligomerization domain and seven Arm repeats in the first third of the protein near its N-terminal. Three 15-amino acid, seven 20-amino acid, and three SAMP repeats are located in the central third of the APC. The carboxy-terminal end is enriched with basic amino acids binding to tubulin, in addition to, other domains binding to EB1 and DLG. Most of the mutations in APC occur in the mutator cluster region (MCR) and create truncated proteins contain ASEF and β-catenin binding sites in the Arm repeat domain but loses the β-catenin regulatory activity which is located in the 20-amino acid repeat domain. Somatic mutations are selected more frequently in FAP patients with germline mutations outside of the MCR (305). Reprinted by permission from BioMed Central.
The amino acid sequence of residues 32-45 is presented in the single letter code. β-catenin expression levels are post-translationally regulated through sequential phosphorylation at codon 33, 37, 41, and 45 and subsequent ubiquitin-mediated degradation. Aggressive fibromatosis patients have shown a high frequency of mutations at codon 41 and 45, resulting in the inability of β-catenin to become phosphorylated by GSK-3 β and CK1 gamma and the stabilization of β-catenin in the cytoplasm (138). Reprinted from Current Opinion in Genetics & Development, 9, Polakis P, The oncogenic activation of β-catenin, 15-21, Copyright (1999), with permission from Elsevier.
Figure 6. *Apc*\(^{1638N}\) mouse model.

The *Apc*\(^{1638N}\) mouse model was generated by introducing the neomycin gene in codon 1638, in the transcriptional orientation opposite to that of *Apc*. For unknown reasons, homozygous *Apc*\(^{+/1638N}\) cell lines contain very low amounts (1–2%) of the expected 182 kDa truncated protein. *Apc*\(^{+/1638N}\) mice develop, on average, 5–6 intestinal tumours per mouse, and a broad spectrum of extra-intestinal manifestations, including multifocal desmoids and cutaneous cysts (116).

In the absence of Hh ligands (left panel), the Ptch receptor at the base of the primary cilium suppresses the function of Smo by preventing its entry into the cilium. Full-length Gli proteins, mainly Gli3, are converted to a c-terminally truncated repressor form (Gli-R). Formation of the Gli-R is promoted by sequential phosphorylation of full-length Gli by GSK3β, PKA, and CK1, which creates binding sites for the adaptor protein β-TrCP. Then, the Gli/TrCP complex becomes subject to ubiquitination mediated by the Cull1-based E3 ligase, which results in partial Gli degradation by the 26S proteasome and formation of the Gli-R. The Gli-R mediates transcriptional repression of Hh target genes. Whether Gli, Sufu, and Kif7 exist in a complex that traffics the cilium in the absence of Hh signalling remains to be resolved. In the presence of Hh ligands (right panel), binding inhibits Ptch’s function, which results in the movement of Smo from intracellular vesicles to the primary cilium. Upon binding, the Hh/Ptch complex becomes internalized in endosomes and later degraded. Smo trafficking is promoted by GRK2 phosphorylation, thereby recruiting β-arrestin2 (Arrb2), which interacts with the anterograde IFT motor kinesin-II and this may facilitate the movement of Smo along the cilia microtubule.
becomes activated and promotes the activation of full-length Gli proteins, mainly Gli2, which enters the nucleus and promotes transcription of target genes. The cell surface protein Hhip competes with binding of the Hh ligands and limits their range of action while the Hh-binding Gas1, Cdo and Boc cell surface proteins positively affect the Hh signalling outcome (306). Reprinted from Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 1805, Stephan Teglund and Rune Toftgård, Hedgehog beyond medulloblastoma and basal cell carcinoma, 181-208, Copyright (2010), with permission from Elsevier.
Figure 8. Different models of Hh pathway activation contributing to cancer development.

In the type I of ligand-independent signalling (top left panel), the Hh pathway is activated in a cell-intrinsic manner through loss-of-function mutations in negative-acting components, such as PTCH1 and SUFU, or through gain-of-function mutations in positive-acting components, such as SMO (indicated by asterisks). In the type II of ligand-dependent, autocrine signalling (top right panel), Hh ligands are produced by the tumour cells and causes cell-autonomous Hh pathway activation. In the type IIIa model of ligand-dependent, paracrine signalling (bottom left panel), the tumour cells produce Hh ligands that activate the Hh pathway in the neighboring stromal cells. This, in turn, elicits production of factor(s), X, that feed back upon the tumour cells to indirectly promote tumour progression. In the reverse paracrine model, type IIIb (bottom right panel), the stromal cells secrete Hh ligands to affect Hh pathway activation in the tumour cells.

In analogy with the paracrine feedback mechanisms in the IIIa model, the tumour cells may induce factor(s), Y, that sustain the Hh secretion or induce other tumour-promoting factors (306). Reprinted from Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 1805, Stephan...
Teglund and Rune Toftgård, Hedgehog beyond medulloblastoma and basal cell carcinoma, 181-208, Copyright (2010), with permission from Elsevier.
1.11 References


2.1 Abstract

Aggressive fibromatosis is a benign fibroproliferative tumour that can occur as a sporadic lesion or a manifestation in patients with familial syndromes, such as familial adenomatous polyposis. Tumours are characterized by the stabilization of β-catenin and the activation of β-catenin-mediated transcription. Current treatment results are far from ideal, and recurrence rates are high. As a result, there remains a need for more effective therapeutic strategies. In this work, we demonstrate the effect of Hedgehog signalling inhibition on aggressive fibromatosis tumour development and β-catenin modulation. We found that Hedgehog inhibition decreased cell viability and proliferation as well as total β-catenin levels in human aggressive fibromatosis tumour cells \textit{in vitro}. Furthermore, following Hedgehog inhibition in \textit{Apc}^{+/Apc^{1638N}} aggressive fibromatosis mouse model, the number and volume of the tumours formed was reduced. Together, this work suggests that Hedgehog signalling inhibitor agents are potential candidates to effectively manage aggressive fibromatosis.
2.2 Introduction

Aggressive fibromatosis (AF) tumour, also known as desmoid tumour, is a benign locally invasive soft tissue lesion composed of monoclonal proliferation of mesenchymal cells arising in connective tissues (1). Histologically, bipolar fibroblast cells expressing vimentin, a marker of mesenchymal cells and lacking the expression of epithelial markers such as E-cadherin are observed (2). Despite their tendency to infiltrate into surrounding normal tissues, these lesions do not metastasize to distant sites. Morbidity and mortality result from the pressure effects and obstruction of vital organs (3, 4). Therapy available to patients with AF includes surgery, radiotherapy and chemotherapy. However, these treatment methods have demonstrated limited success to manage aggressive fibromatosis. Surgical excision is associated with high recurrence rates, and effective systemic therapy remains elusive despite several reports of successful regression of tumours with drug treatments and radiation therapy (5, 6). Although rare in the general population with an incidence of 2-4 cases per 1 million annually (7), these tumours are a common extracolonic manifestation of familial adenomatous polyposis (FAP). Patients with FAP have a 1000-fold increased risk of developing AF, compared with the general population (8). Aggressive fibromatosis tumours are reported to be the second most common cause of death in patients with FAP, after colorectal carcinoma (9). The molecular etiology of aggressive fibromatosis is well characterized (10). These tumours can occur either as a sporadic lesion or as a manifestation of familial syndromes, such as FAP or familial infiltrative fibromatosis (FIF). In sporadic cases, most tumours are associated with somatic mutations in either a gene that codes for β-catenin (CTNNB1) or adenomatous polyposis coli gene (APC), whereas in both FAP and FIF, tumours contain germline mutations in the APC gene (2, 11). In both sporadic and familial cases, the genetic alterations cause stabilization of β-catenin and activation of β-catenin-mediated T-cell factor (TCF)-dependant transcriptional activity (12). Consistent with this, Cheon et al. showed that overexpressing a stabilized form of β-catenin is sufficient to cause aggressive fibromatosis in mice (13). However, the Apc^{1638N} mouse that carries a targeted mutation in the 3’ end of the Apc gene develops aggressive fibromatoses at a higher frequency similar to what is observed in FAP (14). By 6 months of age, male mice develop an average of 45 aggressive fibromatosis tumours, whereas female mice develop significantly fewer tumours. These mice also develop gastrointestinal polyps, although the frequency of polyp formation is less than that of the Min mouse, which harbors an Apc mutation at a different location (15).
β-catenin is a key signalling molecule in the canonical Wnt/β-catenin signalling pathway involved in several developmental and regenerative processes as well as neoplasia. In the absence of activating Wnt ligands, the cytoplasmic multiprotein complex or destruction complex composed of scaffolding proteins APC and Axin, casein kinase 1-gamma (CK1γ) and glycogen synthase kinase 3-beta (GSK3β) regulates the pool of free cytoplasmic β-catenin by phosphorylating its specific serine-threonine sites and targeting the phosphorylated form of the protein for ubiquitin-dependent degradation. This in turn enables the architectural TCF/Lymphoid enhancer factor (LEF) transcriptional factors to interact with members of transcriptional repressor families to inhibit the expression of downstream Wnt target genes. The pathway is initiated when activating Wnt ligands bind to a receptor complex comprised of a seven-pass transmembrane receptor of the Frizzled (Fz) family and single-pass transmembrane co-receptors, LDL receptor-related proteins (LRP) 5/6. This binding recruits another cytoplasmic protein Dishevelled (Dsh) to the plasma membrane followed by the recruitment of Axin-GSK3β complex, which results in the formation of destabilized destruction complex and unphosphorylated β-catenin. The unphosphorylated β-catenin accumulates in the cytoplasm and translocates into the nucleus where it binds to transcription factors in the TCF/LEF family to activate transcription and expression of cell-type specific target genes (16, 17).

The location, cellular morphology, and histologic profile of AF tumours suggest that they derive from mesenchymal sources (3, 4). In addition, a recent study in mice revealed more evidence on mesenchymal stem cell (MSC) origin of AF tumours (18). In this study by using a gene profiling technique, cells from aggressive fibromatosis tumours were compared to MSCs. Tumour cells expressed cell surface markers and genes that are expressed in MSCs. Furthermore, a positive correlation between the number of MSCs and the number of AF tumours formed was observed, when a mouse model of AF (Apc1638N) was used. Also, when Apc1638N mice were crossed with Sca-1−/− mice, which develop fewer MSCs, the number of aggressive fibromatosis tumours formed was less than that of the wild-type controls; however the formation of intestinal neoplasms with the epithelial origin was not affected. Last but not least, MSCs isolated from Apc1638N mice and mice expressing activated β-catenin allele had the capacity to initiate tumour formation when subcutaneously injected into immunodeficient NOD/SCID mice. However, the MSCs isolated from the wild-type animals did not form any tumours. Taken together, these observations suggest that the formation and development of aggressive fibromatosis is
influenced by MSCs; therefore, protecting this progenitor cell population in FAP patients may prevent AF development. Additionally, targeting the molecular mediators that regulate MSC proliferation and differentiation can be used to develop new treatments for AF. One such mediator is hedgehog (Hh) signalling pathway that acts to maintain MSCs in a less differentiated state with more proliferative capacity (19, 20). As a result, inhibition of Hh signalling pathway could be developed to a novel therapeutic approach to treat aggressive fibromatosis tumours.

The hedgehog pathway plays central roles at both embryonic and post-embryonic stages. During embryogenesis, it regulates body patterning and organ development, and during adult life, it functions in stem cell renewal, tissue repair and regeneration. More importantly, its aberrant activation is associated with numerous human disorders including birth defects and cancers (21, 22). In mammals, there are three Hh ligands, including Sonic Hh, Desert Hh, and Indian Hh. Hedgehog pathway in cells receiving its signal is regulated at multiple levels. Initiation of the pathway response involves Hh protein binding to Patched-1 (Ptc1), a 12-pass transmembrane Hh receptor, which in turn relieves its inhibition on the seven-pass transmembrane protein Smoothened (Smo). The ultimate target of Smo action in mammals is the activation of glioma-associated (Gli) family of zinc-finger transcription factors that mediate signal transduction to the nucleus and activate the expression of the pathway target genes such as GLI1, PTCH1, and HHIP (23). Three Gli proteins have been identified in vertebrates, Gli1-3. Different form of Gli proteins function as transcriptional activators and repressors. Regulation of Gli activity involves multiple mechanisms including protein phosphorylation, proteasome-mediated proteolysis and cytoplasmic-nuclear shuttling. The Gli proteins are sequentially phosphorylated by protein kinase A (PKA), GSK3β, and CK1. Gli1 is not proteolytically processed and only occurs as a full-length transcriptional activator. However, Gli2 shows both activator and repressor functions and Gli3 is mostly a repressor despite its possible positive effects (24, 25). In mice, Gli2 and Gli3 are essential genes, but not Gli1 (26-29). Studying various transgenic mice have shown that the zinc-finger DNA binding domain of Gli1 protein is not required for proper sonic Hh signalling, whereas lack of Gli2 zinc-finger DNA binding domain results in a severe phenotype in mutant animals and is required for proper signalling. For example, Gli1<sup>zfd/zfd</sup> (zinc finger deletion) mutants are viable and appear normal; however Gli2<sup>zfd/zfd</sup> mutants are embryonic lethal and show many developmental defects including shortened limbs (26-29). In the absence of Hh ligand, Ptc1 suppresses Smo activity by preventing its localization to the cell surface.
Moreover, phosphorylation of Gli transcription factors by protein kinases results in proteasome-mediated cleavage of them into an NH2-terminal truncated form, which acts as a repressor of Hh target genes expression (30, 31).

The identification of Ptch1 heterozygous mutations, as the cause of Gorlin’s syndrome, was established as the initial link between Hh pathway and cancer (32). Cancers caused by aberrant activation of Hh pathway are categorized into three groups: ligand-independent, ligand-dependent/autocrine, and ligand-dependent/paracrine. In the ligand-independent model, tumours harbor a mutation in Hh pathway components, such as Ptch1 or Smo, leading to pathway activation in the absence of Hh ligand which has been mainly studied in basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma. However, in tumours having ligand-dependent constitutive pathway activation, there is no known mutational basis in the pathway components. In the autocrine model, the tumour cells both secrete Hh and respond to it; however in paracrine model, the adjacent normal cells respond to the Hh that is secreted by the tumour cells. Such cancers include glioma, gastrointestinal, prostate, breast, colon, and small-cell lung carcinoma (21, 22, 33).

These observations suggest that Hh signalling pathway may play a similar role in aggressive fibromatosis as in MSCs, and as such, its inhibition could be used as a novel therapeutic approach for this tumour type. Also, studies of other β-catenin mediated tumours, such as intestinal tumour, have demonstrated the interaction and communication between β-catenin and Hh pathways (34-38). Moreover, accumulating evidence indicate that efficient inhibition of Hh pathway can prevent formation or progression of various types of cancer. Therefore, in this study, we examined the role of Hh signalling pathway in aggressive fibromatosis tumourigenesis as well as the possible interaction between Hh pathway and β-catenin, using the Apc^{+/Apc^{1638N}} mouse model for aggressive fibromatosis as well as the primary cultures derived from human tumours.
2.3 Materials and Methods

2.3.1 Human Tumor Samples

Primary human aggressive fibromatosis tumors were obtained at the time of surgery. Tumor and surrounding normal fibrous tissues were harvested and processed immediately following the surgical excision. Tissues were cryopreserved and stored in the -80 freezer for future use. As previously reported, cells were also collected from tumors immediately after surgery to establish primary cell cultures (39, 40).

2.3.2 Mouse Models

The generation and phenotype of the \(Apc^{1638N}\) and \(Gli2^{zfd/+}\) mice have been reported previously (14, 26). As a model of aggressive fibromatosis, we used \(Apc^{1638N}\) mice which harbor a targeted chain-termination mutation at the codon 1638 of exon 15 of the \(Apc\) gene. These mice develop 5-6 gastrointestinal lesions, and male mice develop an average of 45 fibromatoses by 6 months of age. The females develop a lower number of tumors than the male mice (15). These mice were used to study the effect of Hh pathway inhibition on AF tumour formation following a pharmacologic treatment. As a model for modulated Hh signalling, we used \(Gli2^{zfd}\) mice. In \(Gli2^{zfd}\) mutants, a 2.4 kb region of \(Gli2\) that contains the exons coding for zinc fingers 3-5 as well as 71 amino acid residues carboxyl to the zinc fingers are deleted. As a result they are unable to transactivate transcription (26, 41). Homozygous mutants are embryonic lethal, while heterozygous mutants are viable but demonstrate a decrease in Hh-mediated transcriptional activity. The \(Gli2^{zfd/+}\) mice were used to study the effect of Hh pathway modulation at the genetic level on AF formation. To study fibroblasts with stabilized \(\beta\)-catenin allele, we used the \(Catnb^{lox(ex3)}\) mice which contain \(loxP\) sites flanking exon 3 (42). When subjected to Cre recombinase, this results in the expression of a functional \(\beta\)-catenin protein missing the N-terminal phosphorylation sites and as such is a constitutively stabilized and transcriptionally active protein. Isolated fibroblast cells from these mice were used to examine the effect of \(\beta\)-catenin stabilization on Hh signalling.
2.3.3 Inhibition of Hedgehog Signalling *In Vivo*

Fourteen six-week old *Apc<sup>1638N</sup>* male mice were treated by oral gavage with the Hh inhibitor, triparanol (43, 44), (C27H32ClNO2, Hoechst Marion Roussel, Cincinnati, OH, USA), three times a week at a dose of 400mg/kg. Fifteen control mice received olive oil as a carrier. Mice were treated until they were 6 months of age, after which they were sacrificed and examined for tumour formation, as previously reported, by an observer blinded to the treatments (15, 45). At autopsy, tumours and intestinal polyps were scored macroscopically. Tumours and normal tissues were harvested for further RNA and protein extraction. Because triparanol inhibits cholesterol biosynthesis and thus reduces its serum level (43, 46), this was measured to determine if the mice were able to absorb the drug using the Amplex red cholesterol assay according to the manufacturer’s instruction (Invitrogen) (47). To determine whether triparanol treatment inhibited Hh signalling *in vivo*, the level of expression of Hh pathway target gene, *Ptch1*, was measured in tissues from treated and control mice by real-time PCR.

*Gli2* has not been shown to be processed to an inhibitor form *in vivo*. Mice lacking *Gli2* show a similar phenotype to that seen in mice lacking Hh ligands (48). Therefore, to inhibit the Hh signalling pathway at the genetic level and study its effect on AF tumours, we used the *Gli2<sup>zfd/+</sup>* mice and crossed them with *Apc<sup>1638N</sup>* mice, using a previously described strategy (45) to generate *Apc<sup>+</sup>/Apc<sup>1638N</sup>; *Gli2<sup>zfd/+</sup>* and *Apc<sup>+</sup>/Apc<sup>1638N</sup>; *Gli2<sup>+/+</sup>* littermates. In this way, mice heterozygous for *Gli2* were compared with their littermate controls. Ten *Apc<sup>+</sup>/Apc<sup>1638N</sup>; *Gli2<sup>zfd/+</sup>* and fourteen *Apc<sup>+</sup>/Apc<sup>1638N</sup>; *Gli2<sup>+/+</sup>* male mice were investigated. Mice were sacrificed at 6 months of age, and the number and volume of tumours formed were scored as previously reported by an observer blinded to the genotypes (15, 45). To determine if Hh signalling was inhibited following the cross, the level of expression of Hh pathway target gene, *Ptch1*, was measured in tissues from both genotypes by real-time PCR.

2.3.4 Cell Culture Studies and Treatments

Primary cell cultures from the human aggressive fibromatosis tumours were obtained as previously described (39, 40). Briefly, the cultures were initially established in DMEM supplemented with 10% fetal bovine serum (FBS) and maintained at 37° C in 5% CO<sub>2</sub>. Cells were divided when confluent and experiments were performed between the second and fourth
passages. To treat the aggressive fibromatosis cells in vitro, further experiments were performed in low serum (1%) media containing 5µM of triparanol diluted in DMSO to inhibit Hh signalling and the same concentration of DMSO was used as a control. Cells were studied for the effect of Hh inhibition after 48h of incubation. To determine whether the treatment inhibited Hh signalling in cultured cells, the level of expression of GLI1 was measured in treated and control cells. All the experiments were performed in triplicate. To examine the level of β-catenin protein after the treatment, the cell lysates were extracted from treated and control cells in triplicate. Proliferation was measured using an antibody against proliferating cell nuclear antigen (PCNA) by western blot analysis. The viable cell mass was assayed using the CellTiter96 reagent (Promega) (49) as described. Apoptosis was measured using the Caspase-Glo 3/7 Assay (Promega) (50).

Primary dermal fibroblast cultures from mice expressing the stabilized allele of β-catenin, Catnb\textsuperscript{lox (ex3)}, were established by enzymatic digestion of dorsal dermal skin using (39, 51) collagenase I. The cultures were initially established in DMEM supplemented with 10% FBS and maintained at 37\(^\circ\) C in 5% CO\(_2\). Cells were divided when confluent and experiments were performed on the passage one. At the day of transfection, the media were removed and cells were washed twice with PBS. Then, half of the cells were treated with an adenovirus expressing Cre recombinase and green fluorescent protein (GFP) as a marker, and the other half with an adenovirus expressing only GFP with a MOI of 230 virus particles per cell (Vector Biolabs). After 48h of incubation, cells were checked under the fluorescent microscope and the GFP positive cells representative of a successful recombination were observed. Also, to verify the effectiveness of Cre to cause recombination in cells, both Cre and GFP treated cells were examined for β-catenin protein expression using western blot analysis (42). RNA from all cells was also isolated for further gene expression study.

2.3.5 Gene Expression Studies

Total RNA was isolated from human tissues, murine tissues, and human and murine cell cultures using TriZol reagent, Qiagen RNeasy fibrous tissue mini kit, or Qiagen RNeasy mini kit, respectively, according to the manufacturers’ instructions. A total of 1µg (human samples), 500ng (murine samples), or 300ng (cell cultures) RNA was used to generate single-strand cDNA by using random hexamer primer and the superscript II first strand synthesis system (Invitrogen)
for reverse transcription. Quantitative real-time PCR was performed using TaqMan primers for human GLI1, PTCH1, GAPDH and murine Gli1, Gli2, Ptc1, Hhip, Axin2, Gapdh, and β-Actin (Applied Biosystem). The reactions were performed in duplicate in 20µl using TaqMan universal master mix (Applied Biosystem) on a 96-well plate format. The threshold cycle, C_t, was determined using the analysis software SDS2.1. The ΔΔC_t method was used for the analysis of the data (52). The expression levels were presented as the fold change or relative expression from normal control tissues.

### 2.3.6 Western Blot Analysis

To extract the protein, total lysate was extracted using 1x reporter gene lysis buffer containing complete mini protease inhibitor and phosphatase inhibitor tablets (Roche). Lysates were centrifuged for 5 minutes to remove cell debris and quantified using the Bicinchoninic Acid (BCA) protein assay (Pierce). Equal amounts of total protein were separated by electrophoresis through an SDS-polyacrylamide gel. Nitrocellulose membranes (Amersham) were probed with an antibody against protein of interest. Polyclonal goat anti-HGli1 (R&D, AF3324), monoclonal mouse anti-β-catenin (BD Biosciences, 610154), monoclonal mouse anti-PCNA (Cell Signalling, 2586), monoclonal mouse anti-β-Actin (Sigma, A5441), and monoclonal mouse anti-Actin (Calbiochem, CP01) were used as the primary labeling antibodies. Target protein bands were detected using appropriate horseradish peroxidase-conjugated secondary antibodies (BD Bioscience) and the enhanced chemiluminescence detection system (Millipore, WBLUR0100 and WBLUF0100) according to the manufacturer’s instruction. Densitometry was performed using the Image J software (National Institutes of Health).

### 2.3.7 Fibroblastic Colony-Forming Unit (CFU-F) Assay

The femurs and tibias of 4-month old male Apc+/Apc1638N; Gli2zfd/+ and Apc+/Apc1638N; Gli2+/+ (littermates) mice were harvested for bone marrow isolation to measure colony-forming units as previously reported (53). Cells (1.9 x10⁶) were seeded in triplicate in 12-well cell culture plates and grown for 8 days in αMEM (alpha modification of Eagle’s medium) with high glucose supplement, Glutamine, 10% FBS, and 1X antibiotic/antimycotic. After 96 hours, the medium was changed to remove non-adherent cells. After 8 days, cells were stained with 0.25% crystal
violet solution (Sigma, C3886) and the number of colonies > 1mm was counted by an observer blinded to the genotypes (54).

2.3.8 Statistical Analysis

Means, standard deviations, and 95% confidence intervals were calculated for each experiment. Student’s t-test was used to compare means between different experimental conditions.

2.4 Results

2.4.1 Hedgehog signalling pathway is activated in human and murine aggressive fibromatosis

The level of expression of hedgehog direct target genes, \( GLI1 \) and \( PTCH1 \) was compared between human aggressive fibromatosis tumours and adjacent normal tissues from the same patients. Both \( GLI1 \) and \( PTCH1 \) were up-regulated up to 10-fold and 4-fold, respectively, in aggressive fibromatosis tissues \( (p<0.05, \text{ Figure 9.A}) \). Murine aggressive fibromatosis and adjacent normal tissues were also examined using real-time PCR for the expression of \( Gli1 \), \( Gli2 \), and \( Ptch1 \). While \( Gli1 \) and \( Gli2 \) were significantly up-regulated up to 12-fold and 2-fold, respectively, in the tumours \( (p<0.05, \text{ Figure 9.B}) \), the level of \( Ptch1 \) up-regulation did not reach to statistically significant level \( (p>0.05, \text{ Figure 9.B}) \). To investigate the level of expression of \( Gli1 \) protein, western analysis was conducted on human aggressive fibromatosis tumours and adjacent normal tissues from the same patients. \( Gli1 \) protein level was 3-fold higher in the tumours than the normal tissues (Figure 9.C). \( Gli1 \) immunostaining revealed the presence of positive cytoplasmic and nuclear staining in some of the tumour cells (Figure 9.D). Thus, Hh signalling pathway is activated in aggressive fibromatosis.

2.4.2 Hedgehog pathway inhibition results in a reduction in the number and volume of aggressive fibromatosis \( in vivo \)

Since hedgehog target genes were upregulated in both human and murine aggressive fibromatoses, we investigated the role of Hh signalling in this tumour type \( in vivo \). First, to examine whether the pharmacologic modulation of Hh signalling might alter the tumour
phenotype, we treated fourteen $Apc^+/Apc^{1638N}$ male mice with the Hh-inhibitor, triparanol, and fifteen male mice with olive oil as a carrier. At 6 months of age, these mice were sacrificed and the number and volume of tumours that formed were scored as previously reported (15, 45). Tumours in triparanol-treated mice decreased in volume by 30% when compared to the control mice ($p<0.01$, Figure 10.A). However, there was no significant difference between the numbers of tumours in the two study groups ($p>0.05$, data not shown). The Amplex red cholesterol assay showed that the serum cholesterol of mice treated with triparanol was almost half of that of mice treated with the carrier, indicating that the mice were able to absorb triparanol ($p<0.01$, Figure 10.B). To confirm that treatment with triparanol inhibited Hh signalling in mice, the level of expression of $Ptch1$ was measured in the tissues from each study groups by real-time PCR. $Ptch1$ expression was decreased significantly in the tissues of triparanol treated group compared to the vehicle treated group ($p<0.05$, Figure 10.C).

To genetically modulate Hh signalling in aggressive fibromatosis tumours, we crossed $Gli2^{zfd/+}$ mice with $Apc^+/Apc^{1638N}$ mice. At 6 months of age, ten $Apc^+/Apc^{1638N}; Gli2^{zfd/+}$ and fourteen $Apc^+/Apc^{1638N}; Gli2^{+/+}$ male mice were sacrificed and the number and volume of tumours that formed were scored as previously reported (15, 45). The number of tumours in $Apc^+/Apc^{1638N}; Gli2^{zfd/+}$ mice was decreased by 38% when compared to their littermates control ($p<0.01$, Figure 10.D). However, there was no significant differences in tumour volumes between the two study groups ($p>0.05$, data not shown). To confirm that the genetic alteration of $Gli2$, down-regulated Hh signalling in the crosses, the level of expression of $Ptch1$ was measured in the tumours that formed in the two groups by real-time PCR, which was significantly decreased in $Apc^+/Apc^{1638N}; Gli2^{zfd/+}$ mice compared to their littermates control ($p<0.05$, Figure 10.E). Thus, the Hh inhibition in vivo regulates both the number and size of aggressive fibromatoses. $Apc^{1638N}$ mice also develop intestinal polyps (15). Interestingly, we did not observe any significant differences in the numbers of intestinal polyps formed at 6 months of age ($p>0.05$, data not shown). Therefore, this suggests that in $Apc^{1638N}$ mice, epithelial neoplasms were not affected by inhibition of Hh signalling pathway.
2.4.3 Hedgehog signalling regulates the number of mesenchymal progenitors

Hh signalling inhibition leads to a decrease in proliferation and clonogenicity of mesenchymal progenitor cells (19). Because aggressive fibromatosis tumours have mesenchymal cell origin, we then examined the possibility that Hh signalling could regulate the population of mesenchymal progenitor cells present, and this could affect the tumourigenesis of aggressive fibromatosis. A decrease in the number of mesenchymal progenitors in \( Apc^+/Apc^{1638N} ; \text{Gli}^{2-}/+ \) mice could result in a decrease in the number of cells that could become tumours. To explore this possibility, we compared the numbers of CFU-F between \( Apc^+/Apc^{1638N} ; \text{Gli}^{2-}/+ \) mice and \( Apc^+/Apc^{1638N} ; \text{Gli}^{2+}/+ \) littermates. CFU-F which has the potential to differentiate into various mesenchymal lineages is a surrogate measure of MSCs present in the bone marrow, and as such, CFU-F can be used as an estimation of MSCs present. There was a significant reduction in the numbers of CFU-F in four month old \( Apc^+/Apc^{1638N} ; \text{Gli}^{2-}/+ \) compared with \( Apc^+/Apc^{1638N} ; \text{Gli}^{2+}/+ \) littermates (\( p<0.05 \), Figure 11.A and B). This shows that Hh signalling plays a role in regulating the number of mesenchymal progenitors, and this could be a mechanism by which \( Apc^+/Apc^{1638N} ; \text{Gli}^{2-}/+ \) mice develop fewer aggressive fibromatosis tumours.

2.4.4 Hedgehog signalling alters cell behaviours of aggressive fibromatosis \textit{in vitro}

To explore how Hh may regulate cell behaviors such as viability, proliferation, and apoptosis, we studied primary cell cultures derived from human tumours. Cultures were prepared as described earlier (39, 40), and cells were examined for viability, proliferation, and apoptosis after 48h of incubation with 5\( \mu \)M of triparanol. The expression of \textit{GLI1} was measured as a control for our treatment. \textit{GLI1} expression was significantly down-regulated following triparanol treatment of cells (\( p<0.001 \), Figure 12.A). Triparanol treatment significantly inhibited growth of AF cells as cell viability was measured by CellTiter96 reagent (\( p<0.05 \), Figure 12.B). Using an antibody against PCNA, we measured the PCNA protein level to investigate the proliferation rate of cells. Triparanol-treated cells showed a decrease in PCNA protein expression compared to DMSO-treated cells (Figure 12.C and D). However, there was no significant difference in apoptosis rate.
as measured by caspase 3/7 activity ($p>0.05$, data not shown). This suggests that triparanol may inhibit the tumour cell viability by reducing the rate of proliferation.

### 2.4.5 Hedgehog regulates β-catenin level and β-catenin regulates hedgehog activity in aggressive fibromatosis and in fibroblasts

To investigate if β-catenin protein level was modulated by Hh in tumour cells in vitro, we tested the level of β-catenin in the primary cell cultures from human tumours. Western blot analysis using an antibody against total β-catenin protein revealed a reduction in the amount of protein in the triparanol-treated cells compared to the vehicle treated cells ($p<0.001$, Figure 13.A). Then, we examined tumours from $Apc^{1638N}$ and $Gli2^{zi/zi}$ crosses, and found a reduction in β-catenin protein level after Hh inhibition (Figure 13.B). This shows that Hh signalling positively regulates β-catenin in aggressive fibromatosis.

To determine if β-catenin-mediated signalling could regulate the expression of hedgehog signalling target genes, the expression of Hh pathway target genes, $Gli1$, $Ptch1$, and $Hhip$ was examined in the primary dermal fibroblast cultures derived from mice expressing the conditional stabilized β-catenin allele (42). GFP positive cells, as a marker of a successful recombination, were observed under fluorescent microscope (Figure 14.A). Also, western blot analysis using an antibody against total β-catenin showed successful recombination resulting in the deletion of exon 3 of the protein and formation of stabilized form of β-catenin in Cre treated cells but not in GFP treated cells (Figure 14.B). To further explore whether the stabilized β-catenin was functional, we looked at $Axin2$ gene expression level as a universal canonical Wnt/β-catenin pathway target gene (55). Real-time PCR analysis showed a significant increase in the level of $Axin2$ expression in Cre treated cells compared to GFP treated cells ($p<0.01$, Figure 14.C). Real-time PCR analysis revealed an up-regulation in $Gli1$, $Ptch1$, and $Hhip$ expression level in cells expressing stabilized β-catenin but not in the control cells ($p<0.01$, $p<0.05$, and $p<0.01$ respectively, Figure 14.C). Thus, the Hh and β-catenin signalling pathways regulate each other in aggressive fibromatosis and fibroblasts.
2.5 Discussion

Here, we showed that both human and mouse aggressive fibromatosis exhibit an elevated level of Hh signalling activation compared with adjacent normal tissues. Mice predisposed to aggressive fibromatosis formation developed fewer tumours when the Hh pathway was inhibited genetically or smaller tumours when the pathway was inhibited pharmacologically. These mice also developed fewer CFU-F compared with their wild-type littermates, potentially explaining why these animals developed fewer tumours as well. Moreover, we found that the Hh and β-catenin signalling pathways regulate each other in this tumour type. β-catenin protein level was decreased following Hh inhibition in tumours both in vitro and in vivo, and Hh pathway was up-regulated after stabilizing β-catenin in fibroblasts in vitro. Finally, we showed that the viability and proliferation rates of aggressive fibromatosis cells were affected by Hh inhibition in vitro but not the apoptosis rate. Thus, our results revealed a novel role for Hh signalling pathway in pathogenesis of aggressive fibromatosis.

During embryonic development, the Hh signalling pathway regulates cell proliferation and differentiation in a time-and position-dependent manner (21). Therefore, it is not surprising that Hh pathway mutation or deregulation contributes to the onset of tumourigenesis or accelerates the rate of tumour growth, like other important signalling pathways, Notch and Wnt signalling that have crucial roles during both embryogenesis and tumourigenesis (56, 57). Hh signalling can be activated as a result of a mutation in one of the pathway components (ligand-independent) or by expression of a Hh ligand (ligand-dependent) (58). There is growing evidence that inhibiting Hh signalling in tumours without a mutation in the pathway components could be a useful therapeutic approach (59-62). Whether the pathway is activated as a result of a paracrine or autocrine signalling, yet inhibiting its activity by an antibody against the ligand (5E1) or by a small molecule against Smo (cyclopamine) can suppress the tumour growth. For example, the involvement of Hh pathway signalling in pancreatic cancer and the effectiveness of its blockade in reducing the tumour cell proliferation by cyclopamine both in vitro and in vivo have been shown by several studies (59, 60). Moreover, in pancreatic xenograft mouse models, both the growth of the primary tumour and the metastatic spread was inhibited by Hh pathway blockade. Another ligand-dependent, Hh activated cancer is prostate cancer. Xenografted prostate cancer cell lines were found to be responsive to the administration of Hh pathway inhibitors
cyclopamine and GANT61 which blocked subcutaneous tumour growth. Moreover, the pathway manipulation was shown to modulate the invasiveness and metastasis of this tumour type (61, 62). Hh inhibition by cyclopamine also found to induce apoptosis in colorectal tumour cells (63). This evidence supports the notion that Hh signalling regulates different cell behaviours and contributes to tumourigenesis. Aggressive fibromatosis is a fibroproliferative process that is marked by dysregulated Wnt/β-catenin signalling; where mutations are found in either APC or β-catenin coding genes, the two major mediators of the canonical Wnt/β-catenin signalling pathway. The involvement of Hh signalling pathway has not been studied in aggressive fibromatosis. Here, we show that Hh signalling is also activated in aggressive fibromatosis; however, likely through a ligand-dependent mechanism, as mutations have not been identified in Hh signalling components in this tumour type (64). The exact mechanism of this activation needs to be elucidated further, as it can help to design more effective therapies that involve Hh inhibitors. Hh has also been reported to regulate cell behaviour such as proliferation in several cell types through various downstream mechanisms. For example, it controls proliferation through an increase in G1 cyclins and modulation of pRb phosphorylation via cyclin D and E in cerebellar granule neuron precursors (65) and vascular smooth muscle cells (66), or an increase in E2F1, CDC2 and CDC45L in human keratinocytes overexpressing Gli2 (67). We also found that Hh inhibition in aggressive fibromatosis cells suppresses the proliferation rate. Like other tumours in which ligand-dependent Hh signalling is activated, Hh inhibition might be an effective therapeutic approach in aggressive fibromatosis.

In addition to its well-established roles in directing the patterning of embryonic tissues and structures, the Hh signalling has also been implicated in the maintenance of adult stem or progenitor cells such as stem cells of the hair follicle epithelium (68), of the gastrointestinal tract (69), or in the particular case of the cancer stem cells (70). In these adult stem cells, Hh is known to stimulate their proliferation. Recently, human MSCs were found as a new adult stem cell type regulated by Hh signalling which need a basal level of Hh activity for the optimal proliferation and clonogenicity (19). This is in contrast to what is observed in other non-cancer stem cells. Plaisant et al. showed that inhibition of Hh signalling decreases MSCs proliferation and clonogenicity which is a result of blocking cells in the G0/G1 phase of the cell cycle. In two other studies, differentiation of human MSCs was shown to be affected by Hh signalling (20, 71). It was found that Hh signalling decreases during both adipocytes and osteoblast
differentiation, and Hh activation impairs their differentiation which is associated with a decrease in the expression of adipocytic and osteoblastic genes. Aggressive fibromatosis most likely arise from mesenchymal precursor cells (18). As such, signalling pathways that regulate mesenchymal precursor cell behaviour, such as Hh pathway, may also regulate cell behaviour in this tumour type. Therefore, inhibiting the Hh pathway in aggressive fibromatosis mouse model could cause the MSC population to attain a more differentiated cellular phenotype with a reduced capacity to proliferate, and as a result, could lower the number of cells that have the potential to become a tumour. Consistent with these ideas, in our study, we found that both numbers of CFU-F and tumours in mice with reduced Hh activity were less than that of their wild-type littermates.

Multiple levels of cross-talk exist between Wnt and Hh signalling during embryonic development and postnatal cell differentiation. For example, in Drosophila, the F-box protein and β-TrCP homolog, Slimb, regulates the degradation of Armadillo (β-catenin homolog) and Ci (Gli homolog) (72). There is also evidence that Gli proteins regulate the expression of Wnt genes (73). Another study showed that IHh is a negative regulator of Wnt signalling and is required for differentiation of colonic epithelial cells (74). The association between these two signalling pathways has been also reported in different cancers. Positive regulation of the Wnt pathway by Hh signalling has been suggested in tumour models of pancreas, skin, and medulloblastoma (34-36). However, there are conflicting results from studies on intestinal tumourigenesis. One study reported that Smo overexpression contributes to intestinal tumourigenesis by increasing β-catenin-dependent Wnt signalling (37). On the other hand, other studies report that the Wnt pathway is negatively regulated by the Hh signalling in colon cancer cell lines (38, 74). While one study shows that overexpression of IHh inhibits the Wnt signalling in DLD1 (an APC mutant colon cancer cell line) cells (74); the other study shows that overexpression of GLI1 suppresses the Wnt signalling in SW480 and HCT116 colon cancer cell lines (38). Last but not least, Sufu, which is a negative regulator of Hh signalling, has been found to regulate the activities of both β-catenin and Gli1 through a CRM1-mediated nuclear export mechanism in human colon cancer cell lines (75). In the present study, we indicated that Hh and β-catenin-mediated Wnt signalling pathways regulate each other in a positive feedback loop in aggressive fibromatosis and in fibroblasts.

In this study, we used triparanol to inhibit Hh signalling. This resulted in the development of smaller tumours in Apc1638N mice, but no difference in the number of tumours formed was
observed. Triparanol is a distal inhibitor of cholesterol biosynthesis, and is not a specific Hh inhibitor. It inhibits Hh signalling pathway upstream of Gli transcription factors (46). The reason we did not observe a difference in the tumour number might be due to the age of mice when we started the treatment. $Apc^{1638N}$ mice develop a low number of aggressive fibromatosis at one month of age (15). However, the majority of tumours arise between 1.5 to 2 months of age, and mice older than 2 months develop new tumours at a considerably slower rate. We started the treatment at 6 weeks of age. Possibly by that time, there were number of tumours formed and our treatment did not target the peak of tumour formation. Another explanation would be due to the presence of small tumours formed in the treated group but not in the control group. It is possible that the very small tumours were missed by our tumour counting; thus we did not observe any changes in the tumour number between the two study groups. The triparanol was administered by oral gavage, and it could have systemic effects on mice which resulted in the development of smaller tumours in the treatment group compared to the controls. In the other hand, when Hh signalling was inhibited at the genetic level by crossing $Apc^{1638N}$ mice with $Gli2^{zfd/+}$ mice, it resulted in the development of a smaller number of tumours in $Apc^+/Apc^{1638N}; Gli2^{zfd/+}$ group compared to $Apc^+/Apc^{1638N}; Gli2^{+/+}$ group. Aggressive fibromatosis is derived from mesenchymal progenitor cells (18), and Hh signalling is known to regulate MSCs proliferation and differentiation (19, 20). Thus, to explain our observation, we looked at the CFU-F number, a gross measure of mesenchymal progenitors, from the same mice. The number of CFU-F was lower in $Apc^+/Apc^{1638N}; Gli2^{zfd/+}$ mice, which had fewer tumours, compared to the control group, $Apc^+/Apc^{1638N}; Gli2^{+/+}$. Therefore, our genetic model of Hh modulation provides a stronger evidence for the role of Hh signalling in regulating the formation of aggressive fibromatosis than using a pharmacological agent to inhibit the Hh signalling pathway.

Our data identified a role for Hh signalling in a mesenchymal tumour, aggressive fibromatosis. It also suggests that Hh inhibitors could serve as a novel therapeutic approach to this tumour type.
2.6 Figures

Figure 9. Expression comparison of downstream transcriptional targets of Hedgehog pathway in aggressive fibromatosis and normal fibrous tissues.

(A) Expression of Hedgehog target genes including GLI1 and PTCH1 in human aggressive fibromatosis samples and their matched normal controls analyzed by real-time PCR (n=4). There is a 10 fold increase in expression of GLI1 and a 4 fold increase in expression of PTCH1 in tumour tissues when compared to normal tissues from the same patients. Expression of genes of interest was determined by taking the ratio of gene expression over the expression of a housekeeping gene. Fold increases were then determined by taking the ratio of expression of genes of interest in tumour tissue over their expression in normal tissue. Data shown is the mean.
of 3 independent trials. (B) Expression of Gli1, Gli2, and Ptch1 in murine aggressive fibromatosis samples and normal tissues from the same mice using real-time PCR. There is a 12 fold, 2 fold, and 8 fold increase in expression of Gli1 (n=5), Gli2 (n=4), and Ptch1 (n=5), respectively. Fold changes were determined in the same way as human study. Data shown is the mean of 3 independent trials. (C) Expression of GLI1 and Actin (Control) by western blotting in the human aggressive fibromatosis tissues and their matched normal controls (n=2). (D) Immunohistochemistry for Gli1 in a human aggressive fibromatosis sample showing positive nuclear staining in some of the tumour cells. All the error bars represent 95% confidence intervals. *p<0.05.
Figure 10. Inhibition of Hedgehog pathway impacts aggressive fibromatosis development.

(A) Male Apc\(^+\)/Apc\(^{1638N}\) mice were divided into two study groups: 1) Olive oil (n=15) and 2) Triparanol at 400mg/kg body weight (n=14). Mice were sacrificed at 6 months of age and tumour number and volume were scored. Data represent mean volume (mm\(^3\)) per tumour. Mice treated with triparanol developed significantly smaller tumours compared with their littermate controls (10.20 ± 1.39 mm\(^3\) in olive oil treated versus 7.49 ± 1.04 mm\(^3\) in triparanol treated). (B) Triparanol treatment decreased the serum cholesterol levels of mice (n=4). Data represent mean of serum cholesterol level per mouse. (C) Real-time PCR from tissues of triparanol and olive oil treated mice shows that Hh inhibition by triparanol significantly decreased the expression of \(Ptch1\). (D) Number of tumours formed by the male Apc\(^+\)/Apc\(^{1638N}\); Gli2\(^{+/+}\) (n=14) and male Apc\(^+\)/Apc\(^{1638N}\); Gli2\(^{+-}\) (n=10) was compared at 6 months of age. Data represent mean number of tumours per genotype. The mice carrying Gli2\(^{+-}\) developed significantly fewer tumours than their wild-type controls (54 ± 10.87 in Apc\(^+\)/Apc\(^{1638N}\); Gli2\(^{+/+}\) versus 33 ± 7.28 in Apc\(^+\)/Apc\(^{1638N}\); Gli2\(^{++}\)). (E) Real-time PCR from tumour samples of Apc\(^+\)/Apc\(^{1638N}\); Gli2\(^{+/+}\) and Apc\(^+\)/Apc\(^{1638N}\);
Gli2<sup>−/−</sup> mice shows that Gli2 knock down significantly decreased the expression of Ptch1. All the error bars represent 95% confidence intervals. *p<0.05, **p<0.01.
Figure 11. Inhibition of Hedgehog pathway decreased the CFU-F numbers.

(A) *Apc\(^+/+\)/Apc\(^{1638N}\) mice with a reduced Hh activity have decreased number of CFU-F when compared with their wild-type littermates at 4 months of age. Data represent mean number of CFU-F per mouse in each genotype. 3 mice per given genotype were used. Error bars represent 95% confidence intervals. *\(p<0.05\). (B) A representative pictures demonstrating the difference in the numbers of CFU-C between *Apc\(^+/+\)/Apc\(^{1638N}\); *Gli2\(^++\) and *Apc\(^+/+\)/Apc\(^{1638N}\); *Gli2\(^+/-\) mice.
Figure 12. Triparanol modulates viability and proliferation of human aggressive fibromatosis tumour cells \textit{in vitro}.

Primary cells derived from human aggressive fibromatosis tumours treated with DMSO or Triparanol in triplicate for 48h and the following assays were performed. (A) Real-time PCR from cells treated with DMSO and triparanol shows that triparanol significantly decreased $GLI1$ expression in cells ($n=4$). (B) Significant growth inhibition in cells treated with triparanol. Data represent average relative viable mass in DMSO and triparanol treated cells ($n=3$). Cell viability was measured using the CellTiter96 reagent. (C&D) A representative western blot and a bar graph of treated and control cells show a decrease in PCNA expression, as a proliferation marker, in triparanol treated cells ($n=1$). All the error bars represent 95% confidence intervals. $^* p<0.05$, $^{** *} p<0.001$. 
Figure 13. Hedgehog regulates β-catenin activity in aggressive fibromatosis.

(A) Expression of β-catenin and Actin (Control) analyzed by western blotting in the aggressive fibromatosis tumour cells treated with DMSO and triparanol. Following triparanol treatment, β-catenin protein level significantly decreased. Data represent relative protein level in treated and control cells (n=3). (B) Expression of β-catenin and Actin (Control) analyzed by western blotting in tumours of Apc<sup>+/Apc<sup>1638N</sup>; Gli2<sup>+/+</sup> and Apc<sup>+/Apc<sup>1638N</sup>; Gli2<sup>+/-</sup> mice shows the decreased protein level of β-catenin in mice with reduced Hh activity (n=2). All the error bars represent 95% confidence intervals. ***p<0.001.
Figure 14. β-catenin regulates Hedgehog activity in fibroblasts.

Primary dermal fibroblasts derived from mice carrying the conditional stabilized β-catenin allele subjected to Cre recombinase to express the constitutively stabilized β-catenin protein. (A) A fluorescent microscopy image shows the presence of GFP positive cells as a result of a successful recombination. (B) Expression of the stabilized β-catenin protein in the Ad-Cre-treated cells confirmed by western blot analysis. Position for the wild-type and stabilized β-
catenin are shown on the left. (C) Real-time PCR shows a 8 fold increase in expression of *Axin2*, as a universal Wnt/β-catenin target gene, in the Cre treated cells confirming the activation of β-catenin-mediated transcription. Also, following stabilizing β-catenin in fibroblasts, there was a slight increase in the expression of Hedgehog target genes including *Gli1, Ptc1*, and *Hhip*. All the error bars represent 95% confidence intervals. *p*<0.05, **p**<0.01.
2.7 References


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Chapter 3
Summary and Future Directions

3.1 Summary

Aggressive fibromatosis is a benign fibroproliferative tumour that is marked by dysregulated Wnt/β-catenin signalling; where mutations are found in either APC or β-catenin coding genes, the two major mediators of the canonical Wnt/β-catenin signalling pathway. These mutations result in the stabilization of β-catenin and activation of β-catenin-mediated transcription. Growing evidence suggests that aggressive fibromatoses arise from mesenchymal precursor cells. We were interested in the hedgehog (Hh) signalling pathway, as a pathway that regulates proliferation and differentiation of mesenchymal stem cells (MSCs) and possibly has the capacity to inhibit the development of aggressive fibromatosis tumours.

In this study, we compared the level of activity of Hh signalling pathway in aggressive fibromatosis tumours and normal tissues, and found that Hh signalling is up-regulated in tumours compared with the normal tissues. Upon this finding, we used two strategies to inhibit Hh pathway \textit{in vivo}. We used the Hh inhibitor triparanol or genetically suppressed the Hh pathway to demonstrate that the suppression of this pathway effectively inhibit the development of aggressive fibromatosis tumours. Tumour counts from male $Apc^{+}/Apc^{1638N}$ mice treated up to 6 months of age indicated that triparanol has the capacity to significantly reduce the volume of tumours formed \textit{in vivo}. We also observed a reduction in the number of tumours formed in 6 month old male $Apc^{+}/Apc^{1638N}$ mice having their $Gli2$ knocked down. Moreover, we found that the number of mesenchymal progenitors was decreased upon Hh inhibition in mice. This further supports the possible MSC origin of aggressive fibromatosis tumours.

Furthermore, primary cells derived from a number of aggressive fibromatosis tumours were treated with triparanol. We demonstrated that triparanol inhibits the viability and proliferation of tumour cells. Finally, we found that \textit{in vivo} and \textit{in vitro} Hh inhibition reduces the total β-catenin protein levels in aggressive fibromatosis tumours, and stabilization of β-catenin in fibroblasts increases the activity of Hh pathway. These findings show that Hh and β-catenin signalling
regulate the activities of each other in aggressive fibromatosis and fibroblasts. Our data supports the notion that aggressive fibromatosis tumours derive from MSCs and points to the Hh signalling pathway as a potential target for developing new therapies for this tumour type.

3.2 Future Directions

There are several research questions that arise from this study:

3.2.1 Effects of Hedgehog Inhibition on Aggressive Fibromatosis

At 6 months of age, a decrease in the numbers of aggressive fibromatosis tumours in \( Apc^{+}/Apc^{1638N}; Gli2^{zf+/+} \) mice correlated to diminished numbers of mesenchymal progenitors (as marked by a decrease in the number of CFU-F formed); however, it is unclear if this result was due to impaired de novo tumourigenesis or altered fate determination of pre-existing tumour cells. Hedgehog signalling is known to regulate the proliferation and differentiation of MSCs by inducing their proliferation and inhibiting their differentiation. In our study, we did not observe any differences in either the size of the tumours or the cellularity of the tumours formed in two study groups (\( Apc^{+}/Apc^{1638N}; Gli2^{zf+/+} \) and \( Apc^{+}/Apc^{1638N}; Gli2^{+/-} \)). However, the numbers of tumours was fewer in \( Apc^{+}/Apc^{1638N}; Gli2^{zf+/+} \) mice than the wild-type group. This suggests that Hh inhibition at the genetic level altered the tumour formation more effectively than the tumour progression. Therefore, it would be of interest to know how the \( Gli2 \) deficiency impacts the mesenchymal progenitor cells in vivo to determine whether de novo tumour formation was impaired or not. If \( Gli2^{zf+/+} \) mice have normal numbers of mesenchymal progenitors before and at 2 months of age (when aggressive fibromatosis tumours begin to develop in \( Apc^{+}/Apc^{1638N} \) mice), then de novo tumour formation cannot be impacted. As such, it would be interesting to observe the \( Apc^{+}/Apc^{1638N}; Gli2^{zf+/+} \) mice at different time points, and compare the numbers of mesenchymal progenitor cells at each time points to determine at which stage the depletion in MSCs initiates. Also, to determine if tumour numbers would be further diminished as mice age, the \( Apc^{+}/Apc^{1638N}; Gli2^{zf+} \) mice can be observed for longer than 6 months. In addition, examination of differentiation potential of progenitors within aggressive fibromatosis in \( Apc^{+}/Apc^{1638N}; Gli2^{zf+/+} \) mice would reveal any differences in lineage commitment as increased differentiation may lead to diminished number of progenitor cells remaining to maintain the neoplastic lesions.
Moreover, at 6 months of age, a decrease in the volume of the tumours was observed in $Apc^+/Apc^{1638N}$ mice treated with the Hh inhibitor triparanol; however, the cellularity of the tumours in two groups (triparanol treated and vehicle treated) was the same. Aggressive fibromatosis tumours are composed of spindle-shaped fibroblastic cells surrounded by a network of collagen fibres. In addition, hedgehog signalling has been shown to stimulate the release of collagen from fibroblasts (1). It would be interesting to examine the collagen content of tumours in the triparanol treated and vehicle treated tumours to determine whether the Hh inhibition suppresses the collagen production in the tumours and this results in the formation of smaller tumours. Collagen synthesis can be quantified by real-time PCR (collagen type I mRNA level), biochemical assays (such as Sircol assay), or staining (such as Picrosirius Red Staining).

### 3.2.2 Mechanism of β-catenin Regulation

Aggressive fibromatosis is characterized by stabilization and elevated levels of β-catenin (2). Our study demonstrated that Hh inhibition decreases β-catenin levels. In the canonical Wnt pathway, β-catenin is mainly regulated through phosphorylation at specific serine and threonine residues by GSK-3β, leading to its ubiquitination and degradation (3). As a result, the investigation of the mechanism by which β-catenin is regulated could begin by performing western blot analysis for the phosphorylation status of GSK-3β, as the activity of GSK-3β is determined by its phosphorylation at particular sites including serine 9 and 21. Like GSK-3β, β-catenin stability and activity could also be determined through western blot analysis for phosphorylation at specific serine or threonine sites, which targets β-catenin for ubiquitin-mediated degradation. Alternatively, regulation of β-catenin levels could also be mediated through its subcellular localization. In epithelial tissues, β-catenin links the cytoplasmic domain of E-cadherin via alpha-catenin to the actin cytoskeleton (4, 5). The cadherin-bound pool of β-catenin could be released to participate in downstream signalling. As such, the localization may account for changes observed in the levels of β-catenin and could be detected by performing immunohistochemical analysis. Although previous studies have demonstrated positive staining for N-cadherin in aggressive fibromatosis tumours, it is still unclear as to whether cadherin-catenin complexes play a role in this tumour type. β-catenin interactions with cadherin could be mediated by phosphorylation at specific tyrosine sites by receptor tyrosine kinases which could be detected by western blot analysis. Alternatively, β-catenin levels could be regulated through ubiquitination and degradation (6), which could be detected by performing ubiquitination assays,
as well as through its production at the transcriptional levels, which could be detected by measuring \textit{CTNNB1} mRNA levels using quantitative PCR.

3.2.3 Regulation and Expression of Wnt Target Genes

It has been previously shown that β-catenin is localized in the nucleus in aggressive fibromatosis tumours (7). Stabilized β-catenin is able to translocate into the nucleus and together with TCF/LEF transcription factors activate the expression of downstream cell-type specific Wnt target genes. In our study, we found that Hh inhibition decreased total β-catenin levels in aggressive fibromatosis tumours. To determine whether this inhibition has an effect on the subsequent transcriptional function of β-catenin, a TCF-reporter assay could be performed on primary cells transiently transfected with TCF-LEF luciferase reporter construct pTOPFLASH or the control reporter p fopflash, which contains mutant TCF-LEF consensus binding sequence. Transfected cells treated with the pharmacological agent of interest to inhibit Hh pathway would be measured for luciferase enzyme activity detected by a luminometer. Relative luminescence activity would indicate the potential effects that the treatment may have on transcriptional activity as a result of modulating total β-catenin protein levels.

In addition, we observed that Hh inhibition decreased viability and proliferation of primary cells derived from human aggressive fibromatosis tumours. Such biological changes would be better understood upon establishing differential gene expression profiles of treated tumours. To determine the gene expression profile modified by the treatment, real-time PCR reaction could be performed on extracted RNA. Differential gene expression profiles would be established and indicate downstream genetic consequences of Hh inhibition to elicit such phenotypic observations both \textit{in vivo} and \textit{in vitro}. One example is to look at the cell-cycle regulators expression upon Hh inhibition in tumour cells, as Hh inhibition is known to have anti-proliferative effects on tumour cells through modulating the cell proliferation regulators, such as c-Myc and cyclins (8).
3.3 References


