Identifying Pharmacological Therapeutics for Aggressive Fibromatosis

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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University of Toronto

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Abstract

Aggressive fibromatosis is a fibroproliferative tumour that can occur as a sporadic lesion or a manifestation in FAP patients. Tumours are characterized by the stabilization of beta-catenin. Current therapies have yet to offer complete success for primary and recurrent tumours, and there remains a need for more effective therapeutic strategies. In this work, we demonstrate the anti-neoplastic and beta-catenin modulating capacities of Nefopam, a currently approved analgesic agent. We found that Nefopam was able to decrease cell viability and proliferation as well as total beta-catenin levels in human aggressive fibromatosis tumour cells in vitro. Furthermore, Nefopam reduced the number of tumours formed in the Apc+/Apc1638N aggressive fibromatosis mouse model. We also demonstrated that androgens contribute to the development of tumours and could also modulate beta-catenin levels as indicated in Testosterone-treated orchidectomized Apc+/Apc1638N mice. Together, this work suggests that Nefopam and androgen signaling-blocking agents are potential candidates to effectively manage aggressive fibromatosis.
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<tbody>
<tr>
<td>AF</td>
<td>aggressive fibromatosis</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>CK</td>
<td>casein kinase</td>
</tr>
<tr>
<td>COX</td>
<td>cycloxygenase</td>
</tr>
<tr>
<td>CTNNb1</td>
<td>catenin beta 1</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>FAP</td>
<td>familial adenomatous polyposis</td>
</tr>
<tr>
<td>Fz</td>
<td>frizzled</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GSK</td>
<td>glycogen synthase kinase</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HDD</td>
<td>hereditary desmoids disease</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>LEF</td>
<td>lymphoid enhancer-binding factor</td>
</tr>
<tr>
<td>LRP</td>
<td>LDL receptor-related protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metallopeptidase</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PGK</td>
<td>phosphoglycerate kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SRB</td>
<td>sulforhodamine B</td>
</tr>
<tr>
<td>TCF</td>
<td>T-cell factor</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
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Chapter 1

1 Current Ideas of Aggressive Fibromatosis

1.1 Abstract

Aggressive fibromatosis, also known as desmoids, is a rare fibroproliferative tumour. They are benign and unable to metastasize systemically but have the capacity to invade locally into nearby tissues and organs. Tumours occur either as sporadic lesions or as a manifestation in familial syndromes, indicating an association with dysregulated molecular pathways and genetic programming. The stabilization of beta-catenin is observed in aggressive fibromatosis tumours, implicating a tumourigenic role of the canonical Wnt/beta-catenin signaling pathway. The rarity of these tumours has so far made the standardization of therapeutic strategies challenging. This chapter discusses the development of aggressive fibromatosis and current therapeutic strategies as well as introduces the research covered in the rest of this thesis.
1.2 Clinical Perspectives of Aggressive Fibromatosis

1.2.1 Histology

Aggressive fibromatosis (also known as desmoids) is a rare and benign tumour composed of elongated spindle (fibroblast-like) cells surrounded by collagen fibers. Although tumours are considered benign, lacking the common malignant characteristic of metastases, they possess infiltrative capacities to invade into local tissue and vital organs nearby. Morbidity and possibly mortality result from the impingement of critical organs and obstruction of normal function. Immunohistochemical analysis demonstrated the expression of vimentin, a marker of mesenchymal cells, and lack the expression of epithelial markers such as E-cadherin, suggesting that aggressive fibromatosis tumours are derived from mesenchymal precursors (Shields et al., 2001).

1.2.2 Epidemiology

Aggressive fibromatosis can occur as sporadic lesions or as a manifestation of familial syndromes, including familial adenomatous polyposis (FAP) and hereditary desmoids disease (HDD). It has been observed that aggressive fibromatoses tumours are the first presentation of FAP (Bandipalliam et al., 2004; Benoit et al., 2007). While tumours are rare with a reported incidence of 2 to 5 cases per million in the general population, there is a reported higher incidence of nearly 850 times in patients with familial syndromes (Pikaar et al., 2002). Aggressive fibromatosis occurrence represents 0.03% to 0.1% of all tumours, 3.5% of fibrous tissue tumours and between 10 to 25% of FAP patients (Jarvinen, 1987; Jones et al., 1986; Naylor et al., 1979; Richards et al., 1981), strongly suggesting that tumours result from inappropriate molecular signaling and dysregulated genetic programming.

1.2.3 Neoplastic Nature

Studies of clonality of aggressive fibromatoses tumours have demonstrated that it is a neoplasia derived from mesenchymal precursors (Alman et al., 1997b). Investigation of the clonal nature initially involved the analysis of trisomy 8 and trisomy 20, which are non-random clonal aberrations acquired during neoplastic progression. Cytogenic abnormalities, including trisomy 8, trisomy 20 or absence of 5q, have been observed in some cases (Bridge et al., 1992; Fletcher et al., 1995). By examining the non-random inactivation of X-chromosome in tumours, it was
found that aggressive fibromatosis is 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 (Alman et al., 1997b). Another study reported that the inactivation pattern of recurrent fibromatosis tumours was comparable to that of primary tumours, suggesting that recurrent tumours are derived from the same clone as the primary tumour (Li et al., 1996). Understanding the clonality of aggressive fibromatosis has guided research towards the examination of the underlying aberrant mechanisms that confer a growth advantage to cells resulting in neoplasia.
1.3 Molecular Insights

Aggressive fibromatosis tumours occur in 10 to 25% of FAP patients (Jarvinen, 1987; Jones et al., 1986; Naylor et al., 1979; Richards et al., 1981) with a reported higher incidence of nearly 850 times in patients with familial syndromes as compared to the general population (Pikaar et al., 2002). Increased occurrence of tumours in familial syndromes suggests a genetic disposition. Germline mutations at particular loci in the adenomatous polyposis coli (APC) gene predispose patients with FAP to develop colonic polyps as well as aggressive fibromatosis tumours. In contrast to FAP-associated tumours, which are associated with germline APC mutations, sporadic tumours are usually characterized by somatic mutations in the CTNNb1 gene (Gebert et al., 2007; Tejpar et al., 2005). Protein products of the APC gene and CTNNb1 gene are both implicated in the canonical Wnt signaling pathway.

1.3.1 Non-canonical and Canonical Wnt Pathway

Upon binding of particular Wnt ligands to Frizzled (Fz) receptor, three major Wnt signaling pathways are potentially turned on: 1) the canonical Wnt/beta-catenin signaling pathway (Figure 1), 2) the planar cell polarity (PCP) pathway, and 3) the Wnt/Ca\(^{2+}\) pathway. Both the Wnt/PCP and Wnt/Ca\(^{2+}\) pathway are thought to be the non-canonical, beta-catenin-independent Wnt activated pathways, which are involved primarily in cell movement and polarity (Veeman et al., 2003). The Wnt/PCP pathway is initiated with Wnt ligand interacting with Fz and ROR2 receptors and signals through the recruitment of Dishevelled (Dsh) to activate Rac, MAP3K, Jun, Daam, RhoA and Cdc42 to modulate cell polarity and motility (Camilli et al., 2010). The Wnt/Ca\(^{2+}\) pathway is initiated through binding particular Wnt ligands to Fz and activating heterotrimeric G proteins (Malbon, et al., 2001). This results in the release of intracellular calcium, which activates calmodulin-dependent protein kinase II (CAMKII) and protein kinase C (PKC) (Kuhl, et al., 2001). Of these three major signaling pathways, the canonical Wnt/beta-catenin signaling pathway is best understood and will be the focus of the remainder of this thesis.

The canonical Wnt signaling pathway is involved in a number of developmental and regenerative processes. In the absence of an activating Wnt ligand, an intracellular multiprotein complex promotes the destruction of cytoplasmic beta-catenin. This complex is comprised of APC, Axin (a scaffolding protein), casein kinase 1-gamma (CK1gamma) and glycogen synthase kinase 3-beta (GSK3beta) (Price, 2006). Beta-catenin is sequentially phosphorylated first by CK1gamma
at serine 45 and then by GSK3beta at threonine 41, serine 37 and serine 33. Phosphorylation at serine 33 and 37 creates a binding site for the F box/WD repeat protein beta-TrCP, a component of the E3 ubiquitin ligase, which catalyzes the covalent modification of lysine residues of beta-catenin with ATP-activated ubiquitin (Aberle et al., 1997). GSK3beta does not directly bind to beta-catenin but interacts with beta-catenin through Axin, which contains binding sites for APC, GSK3beta and beta-catenin (Rubinfeld et al., 1996). In the nucleus, high-mobility group DNA-binding T cell factors (TCF) or lymphoid enhancer factors (LEF) act as a transcriptional repressor through interaction with members of repressor families, including Groucho, Gro-related gene and transducer-like enhancer proteins. Upon binding to Wnt-responsive elements characterized as CCTTGTWW (W represents either T or A), TCF/LEF along with transcriptional repressors induce significant DNA bending that alter local chromatin structure and ultimately inhibit expression of downstream Wnt target genes (Cavallo et al., 1998; Jennings and Ish-Horowicz, 2008; Roose et al., 1998).

Wnt signaling is initiated when an activating ligand binds to Fz, seven-pass transmembrane receptor (Bhanot et al., 1996), and single-pass transmembrane co-receptor LDL receptor-related proteins (LRP) 5/6 (Pinson et al., 2000). Binding of an activating Wnt ligand induces phosphorylation of LRP6 at its PPPSPxS motifs (P, proline; S, serine or threonine; x, a variable residue) by CK1 and GSK3. These dually phosphorylated motifs are docking sites for the Axin complex (Tamai et al., 2004). Cytoplasmic scaffolding protein, Dsh, interacts with Fz and is required for Axin recruitment to the plasma membrane (Cliffe et al., 2003). Both Dsh and Axin each harbor a homologous DIX domain that exhibits dynamic polymerization, which helps to further tether Axin to the cell membrane (Schwarz-Romond et al., 2007). Subsequently, the multiprotein complex becomes destabilized, allowing for beta-catenin to escape ubiquitin-dependent degradation and accumulate in the cytoplasm. Beta-catenin translocates into the nucleus in a nuclear localization signal- and importin-independent fashion by interacting directly with multiple FXFG motifs (F, phenylalanine; G, glycine; x, a variable residue) on nuclear pore proteins via the central armadillo repeats of beta-catenin (Fagotto et al., 1998; Henderson and Fagotto, 2002). Beta-catenin displaces HDAC-associated transcriptional co-repressors from TCF/LEF (Daniels and Weis, 2005), and TCF is converted into a transcriptional activator upon interaction with beta-catenin (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997). While bound to Wnt responsive elements, TCF/LEF-beta-catenin recruits a variety of
coactivator complexes including BCL9 and Pygo, Mediator (for transcriptional initiation), p300/CBP and histone acetyltransferases, MILL1/2 histone methyltransferases, SWI/SNF family of ATPases for chromatin remodeling, and the PAF1 complex, for transcription elongation and histone modification (Mosimann et al., 2009).

Downstream gene expression appears to be cell-type specific, resulting in the activation of different transcriptional programs involved in cell proliferation and tissue expansion, cell fate determination or terminal differentiation of post-mitotic cells, maintenance or activation of stem cells, and development and progression of cancer pathology. Some of the identified Wnt target genes include c-jun (Mann et al., 1999), c-myc (He et al., 1998), cyclin D1 (Saito et al., 2001; Tetsu and McCormick, 1999), matrix metallopeptidase (MMP) 7 or matrilysin (Brabletz et al., 1999; Crawford et al., 1999), axin 2 (Jho et al., 2002; Yan et al., 2001), and survivin (Zhang et al., 2001).

1.3.2 APC

APC is one of the main regulators of beta-catenin signaling and is considered to be an important tumour suppressor protein. Mutations in the APC gene are associated with several human cancers and tumour formation such as aggressive fibromatosis (Figure 2). This tumour suppressing capacity derives from its ability to regulate beta-catenin in the canonical Wnt signaling pathway and the role it plays as a key regulator in the multiprotein complex. However, its tumour suppressing activities may also encompass additional functions including the regulation of migration, cell adhesion and chromosomal segregation (Fodde et al., 2001; Polakis, 1997).

The occurrence of aggressive fibromatosis tumours seems to increase with incidence of FAP and other familial syndromes. Genetic analysis has found that FAP patients carry an autosomal dominant, germline mutation in the tumour suppressor gene APC, leading to premature termination and truncation of the protein product (Tejpar et al., 1999). FAP patients with reported aggressive fibromatosis tumours consistently carry mutations confined to a 3’ mutation cluster region located between codons 1250 and 1500 (Fodde and Smits, 2001). Mutations in this region result in an early stop codon, which selects for a truncated APC protein lacking beta-catenin regulatory domains, suggesting that tumours may be initiated by mutations in the APC gene with the loss of beta-catenin regulation capacities (Li et al., 1998). When primary cells
derived from human aggressive fibromatosis tumours containing APC mutations were cultured and transfected transiently or stably with the full-length APC gene, both beta-catenin levels and cell proliferation were decreased (Li et al., 1998). The involvement of APC gene in aggressive fibromatosis was further demonstrated using the Apc\(^{+}/\text{Apc}^{1638N}\) mouse model, which carries a targeted inactivating missense mutation at codon 1638 of the mouse Apc gene (Fodde and Smits, 2001). Heterozygous Apc\(^{+}\)/Apc\(^{1638N}\) mice display full penetrance of polyps in the upper gastrointestinal tract as well as aggressive fibromatosis tumours (Fodde and Smits, 2001), representative of the clinical manifestations of FAP patients.

### 1.3.3 Beta-catenin

Beta-catenin is a crucial regulator of E-cadherin-mediated cell adhesion at the plasma membrane and an effector protein of the canonical Wnt signaling pathway. Depending on its location in the cell, it has the capacity to interact with a variety of proteins and elicit different biological functions at the plasma membrane, cytoplasm and nucleus. These protein-protein interactions occur through 12 armadillo repeats in the armadillo repeat domain, the binding site for alpha-catenin as well as phosphorylation sites for GSK3\(\beta\) and CK1\(\gamma\) in the N-terminal domain, and transcriptional transactivation domain in the C-terminal domain (Jiang and Struhl, 1998; Rimm et al., 1995; Wu et al., 2003; Xing et al., 2003). In this study, we focus on the crucial role of beta-catenin in the Wnt signaling pathway in the context of tumour initiation and progression.

Given that the main function of tumour suppressor APC is to downregulate beta-catenin levels, mutational analysis of the CTNNb1 gene and APC gene were performed using 42 sporadic AF tumours (Tejpar et al., 1999). In their study, there was a predominance of tumours with beta-catenin stabilizing mutations and a few tumours containing inactivating APC mutations. Tumours containing mutations in APC gene were exclusive to those with mutations in CTNNb1 gene (Tejpar et al., 1999). Mutations found in the CTNNb1 gene were consistently and specifically confined to codons 41 and 45 at the N-terminus, where alanine was substituted for threonine at codon 41 and phenylalanine for serine at codon 45 (Figure 3) (Tejpar et al., 1999). These serine and threonine residues as well those at codons 33 and 37 are important for the functional regulation of beta-catenin as they are specific phosphorylation sites for GSK3\(\beta\) and
CK1gamma (Rubinfeld et al., 1996). Mutations at codons 41 and 45 confer a sufficient level of resistance to phosphorylation, resulting in the stabilization of beta-catenin in the cytoplasm.

1.3.4 Dysregulation of beta-Catenin Signaling in Tumourigenesis

The stabilization of beta-catenin can occur as a result of inactivating mutations found in the APC gene, direct mutations in the CTNNb1 gene, or mutations in Wnt receptor genes (Figure 4) (Polakis, 1999).

Many APC mutations identified in FAP produce truncated proteins of different length and stability as well as variable beta-catenin regulatory capacity. The multiple intestinal neoplasia Apc$^{\text{Min}}$ mice carry an ethyl-nitroso-urea-induced nonsense mutation at codon 850, resulting in a truncated Apc protein lacking all beta-catenin regulatory domains. These mice display a severe intestinal phenotype characterized by the development of more than 100 intestinal polyps and no aggressive fibromatosis tumours (Fodde et al., 1994; Silverman et al., 2002; Su et al., 1993). On the other hand, Apc$^{1638N}$ mice, which carry a targeted missense mutation at codon 1638, produce nearly undetectable amounts of the Apc protein with minimal beta-catenin regulatory capacity. These mice develop only a few intestinal tumours and extra-abdominal manifestations including aggressive fibromatosis tumours (Fodde et al., 1999).

While it has been reported that carriers of identical APC mutations have variable tumour phenotypes, suggesting the presence of environmental and or genetic modifying factors, it is also believed that there exist general genotype-phenotype correlation in both humans and mice. APC mutations up to codon 1400 produce stable truncated proteins and are generally associated with high numbers of intestinal neoplasia and relatively low incidence of extra-abdominal manifestations. On the other hand, APC mutations beyond codon 1600 do not produce a stable truncated protein and are associated with reduced intestinal neoplasia and more severe extra-abdominal lesions such as aggressive fibromatosis tumours (Fodde and Khan, 1995).

One explanation for these observations is the “just-right” signaling hypothesis. According to this model, APC function must be impaired sufficiently to allow for functional nuclear accumulation of beta-catenin. APC mutations are selected for based on a specific level of beta-catenin signaling optimal for tumour formation or growth advantage, rather than its constitutive activation by deletion of beta-catenin regulatory domains of APC. This model is supported by an
APC dose-dependent reduction in TCF/beta-catenin reporter activity in several transfection experiments (Dihlmann et al., 1997; Dihlmann et al., 1999; Korinek et al., 1997). Different dosages of nuclear beta-catenin initiate different tissue-specific programs (Hecht and Kemler, 2000; Mosimann et al., 2009), suggesting that beta-catenin can become oncogenic unless it is precisely regulated.

Inappropriate regulation of beta-catenin signaling results in the aberrant downstream activation of Wnt target genes leading to tumour formation (Fearon and Vogelstein, 1990). Differential gene expression among tumours mediated by aberrant beta-catenin signaling is observed. Downstream target genes display distinct temporal and spatial expression patterns. Beta-catenin is able to regulate differential gene expression by recruiting various coactivators (Hecht and Kemler, 2000). Precise beta-catenin signaling functions in parallel with the tight regulation of nuclear beta-catenin levels available to interact with TCF transcription factors and to activate Wnt target genes. A threshold of nuclear beta-catenin required for binding to TCF is achieved through binding of nuclear beta-catenin buffers, which compete with beta-catenin-TCF interactions, as do Groucho and other transcriptional repressors that bind to TCF. These cell and tissue-specific factors dictate beta-catenin-TCF binding interactions and determine the activation of certain Wnt target genes (Cadigan, 2008). It is thought that while colorectal cancers result from beta-catenin acting primarily through binding TCF4 (Morin et al., 1997), nuclear beta-catenin in aggressive fibromatosis specifically binds to TCF3 (Tejpar et al., 2001).

Consequences of dysregulated beta-catenin signaling include aberrant proliferation, apoptosis, migration and differentiation (Jilong et al., 2007; Tejpar et al., 2005), which are mediated by several gene targets of the Wnt/beta-catenin pathway such as c-myc, cyclin-D1, MMP, and cyclooxygenase (COX) among others (Kikuchi, 2000; Sparks et al., 1998).

C-myc is an oncogene that promotes G1/S cell cycle transition and is upregulated in colorectal tumours (Sikora et al., 1987); conversely, overproduction of c-myc may induce programmed cell death (Thompson, 1998). Staining for c-myc demonstrated a nuclear overexpression in nearly 45% of aggressive fibromatosis tumours (Jilong et al., 2007). Similarly, cyclin D1, which is also involved in cell cycle regulation, was overexpressed in 50% of tumour samples (Jilong et al., 2007; Saito et al., 2001).
MMPs are a large family of zinc-dependent endopeptidases, which degrade most components of the extracellular matrix (Curran and Murray, 1999; Kahari and Saarialho-Kere, 1999). While its main function is to degrade or disrupt the extracellular matrix during normal biological processes including embryonic development, bone remodeling, and wound healing, it is also implicated in the progression of neoplastic processes. It is believed that MMPs mainly contributes to the invasion and metastasis of tumour cells; however, mounting evidence is also placing a prominent role for MMPs in cancer initiation and progression (Matono et al., 2008; Schwartz et al., 2003). Expression profiles demonstrated that MMP-7 is overexpressed in human colorectal cancer (Brabletz et al., 1999). A striking overexpression of mRNA levels of MMP-1, MMP-2, MMP-3, MMP-7, MMP-11, MMP-12 and MMP-13 was demonstrated in aggressive fibromatosis tumours, accompanied by a downregulation of MMP inhibitors (tissue inhibitors of metalloproteinases, TIMPs) including TIMP-1, TIMP-2 and TIMP-3 (Denys et al., 2004a). Mice predisposed to developing aggressive fibromatosis tumours treated with an inhibitor of MMPs or crossed with a transgenic mouse that overexpresses Timp-1 resulted in a significant reduction in tumour volume (Kong et al., 2004), suggesting that MMP activity may modulate tumour size and invasiveness.

COX, also called prostaglandin H synthase, catalyzes the production of prostaglandins from arachidonic acid. Its potential role in neoplasia was suggested by the presence of elevated levels of arachidonic acid metabolites including prostaglandins. For instance, COX-2 was found to be overexpressed in neoplastic lesions including colonic neoplasia (Williams et al., 1999). COX-2 levels were elevated also in murine aggressive fibromatosis tumours and the use of a COX-2 blocking agent reduced the proliferation of cells derived from human aggressive fibromatosis tumours (Poon et al., 2001b).

Other studies have further demonstrated that the expression of Wnt/beta-catenin target genes is dysregulated in aggressive fibromatosis tumours. Insulin-like growth factor binding protein-6 was consistently downregulated in two APC-mutated and two beta-catenin-mutated tumours (Denys et al., 2004b). In addition, genes involved in cell-cell or cell-matrix interactions such as ADAM12 (a disintegrin and metalloprotease protein) and FAP (fibroblast activation protein-alpha), and in determining cell fates or embryonic development such as WISP-1 (Wnt inducible signaling pathway protein) and SOX-11 (SRY-related HMG-box), were shown to be highly expressed in 12 cases of aggressive fibromatosis tumours (Skubitz and Skubitz, 2004).
1.4 Aggressive Fibromatosis Mouse Models

1.4.1 Beta-catenin Stabilized Mouse Model

Our lab previously generated a transgenic mouse model, which expresses an inducible, stabilized form of beta-catenin under the control of a tetracycline-regulated promoter (Cheon et al., 2002). Phosphorylation of serine and threonine sites of beta-catenin results in its ubiquitin-mediated degradation. In vitro mutagenesis modified phosphorylation sites at codons 33, 37, 41 and 45 from serine or threonine to alanine, rendering beta-catenin stabilized. Stabilized beta-catenin protein is detectable by 6 weeks of age, and 3-month-old mice fully develop aggressive fibromatosis tumours as well as gastrointestinal tumours, suggesting that beta-catenin stabilization alone is sufficient for tumour development (Cheon et al., 2002). Aggressive fibromatosis tumour formation under an induced stabilization of beta-catenin is similar to adult-onset tumour formation caused by sporadic mutations that occur later in life. As such, this provides an animal model of adult-onset, sporadic aggressive fibromatosis (Cheon et al., 2002).

1.4.2 Apc+/Apc1638N Mouse Model

The Apc+/Apc1638N mouse model was generated by introducing the PGK-neomycin gene at codon 1638 in the transcriptional orientation opposite that of Apc (Fodde et al., 1994). Western blot analysis of embryonic stem cells and several tissues from Apc+/Apc1638N mice did not demonstrate the expected 182 kDa truncated Apc protein. However, immunoprecipitation analysis of Apc1638N/Apc1638N cell lines revealed very low amounts (1-2%) of the expected Apc protein (Fodde et al., 1994). These mice on the C57BL/6J background develop with 100% penetrance an average 5-6 upper gastrointestinal polyps per mouse as well as extra-colonic manifestations, including an average of 46 aggressive fibromatosis tumours in males and 16 in females by 6 months of age (Poon et al., 2001a; Smits et al., 1998). The majority of tumours arise between 1.5 and 2 months of age during which the average number of tumours increased from 6.7 to 27 per mouse. Mice older than 2 months of age continue to develop tumours but at a significantly slower rate (Smits et al., 1998). This unique combination of polyps and aggressive fibromatosis tumours provides a useful tool for studying relevant extra-abdominal manifestations of FAP (Fodde and Smits, 2001). The Apc+/Apc1638N serves as a mouse model to study APC-driven aggressive fibromatosis and is used in this study.
1.5 Current Therapeutic Strategies

Current methods to manage aggressive fibromatosis have demonstrated limited success. Therapy available to patients includes surgery, radiotherapy and different streams of pharmacological therapies such as non-steroidal anti-inflammatory drugs and hormonal manipulation.

1.5.1 Surgery and Radiotherapy

Surgical excision remains a treatment of choice for abdominal wall and extra-abdominal tumours but becomes more challenging with larger intra-abdominal tumours as it may lead to sacrificing critical structures and hemorrhaging. Several studies have shown that surgical excision is associated with further complications including a high recurrence rate in the initial years post-operation. Furthermore, wide surgical resection margins may result in significant mutilation for some patients, including amputation and compromised appearance and function.

In attempt to avoid disfigurement, radiotherapy is employed either independently as an alternative to surgery or in conjunction after conservative surgery. A review of reported success rates comparing surgery and radiotherapy indicates that surgery alone is not as effective as surgery in conjunction with radiotherapy or radiotherapy alone (Nuyttens et al., 2000). Still, the addition of radiotherapy for patients has demonstrated a relapse rate of 31% for unresectable tumours (Ballo et al., 1998). Furthermore, studies have reported several radiation-induced malignancies including fibrosarcomas and lymphomas (Acker et al., 1993; Bataini et al., 1988; Goy et al., 1997; Rock et al., 1984).

To address the issue of recurrent tumours, systemic therapies have been investigated as a potential alternative to surgery and radiotherapy. These modalities include anti-inflammatory agents and hormonal therapy.

1.5.2 Non-Steroidal Anti-Inflammatory Drugs

The use of non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, sulindac and colchicine against aggressive fibromatosis has been explored. NSAIDs may act through both cyclooxygenase (COX)-dependent and –independent mechanisms (Abramson and Weissmann, 1989; Ahnen, 1998). COX, also known as prostaglandin H synthase, catalyzes reactions involved in the synthesis of prostaglandins from arachidonic acid. COX exists as two isozymes:
COX-1, which is ubiquitously expressed; and COX-2, which is inducible by growth factors and cytokines. NSAIDs have been shown to inhibit proliferation and induce cell death in cells that do not express COX (Jiang et al., 2003). Potential COX-independent mechanisms include the inhibition of NF-κB signaling pathway (Wong et al., 2003), which regulates cell proliferation and promotes cell survival, and the upregulated expression of apoptosis-related genes such as c-myc, bax, and bak (Zhou et al., 2001, Zhu et al., 1999).

Our lab previously demonstrated that human aggressive fibromatoses tumours express COX-2 and produce prostaglandin E2 at a significantly higher level than normal fibroblastic tissue, and that COX inhibitors decreased cell proliferation of tumours in vitro (Poon et al., 2001b). The Apc\textsuperscript{+}/Apc\textsuperscript{1638N} aggressive fibromatosis mouse model also expressed elevated COX-2 levels. Upon breeding Apc\textsuperscript{+}/Apc\textsuperscript{1638N} mice with Cox-2/- mice, differences were observed only in the size of aggressive fibromatosis tumours but not in the number of tumours formed, suggesting that Cox-2 may play a role in tumour growth rather than tumour initiation (Poon et al., 2001b). Different neoplastic lesions including colonic neoplasia are shown to express COX-2, and neoplastic processes are characterized by elevated level of arachidonic acid metabolites such as prostaglandin E2 (Williams et al., 1999). In mice harbouring germ-line Apc mutations, COX-2 inhibition results in a significant decrease in the number of gastrointestinal tumours (Oshima et al., 1996).

Although in vivo studies on the efficacy of NSAIDs against aggressive fibromatosis have indicated its potential, total regression of tumour cells have yet to be achieved. Similarly, patients have demonstrated limited success as defined mostly by partial response or only stabilized disease, resembling the natural course of the disease (Belliveau and Graham, 1984; Dominguez-Malagon et al., 1992; Klein et al., 1987; Waddell and Kirsch, 1991). As such, NSAIDs alone may not suffice as a stand-alone treatment but may prove to be an effective adjuvant therapy.

### 1.5.3 Hormonal Therapy

The majority of investigations on the role of hormones in aggressive fibromatosis has so far been dedicated to the role of estrogen in disease progression. Aggressive fibromatosis seem to occur more frequently in women with nearly 80% of these tumours occurring in women (McAdam and Goligher, 1970). The speed of growth of tumours appear higher during pregnancy, in
premenopausal compared to postmenopausal women, and in females relative to males (Reitamo et al., 1986). However, conflicting studies on the incidence of aggressive fibromatosis in FAP patients categorized by gender have contradicted these previous findings. While some demonstrated an increased incidence in females (Bertario et al., 2001; Klemmer et al., 1987; Sturt et al., 2004), others have shown no significant differences between genders (Gurbuz et al., 1994; McAdam and Goligher, 1970).

Immunohistochemical analysis of aggressive fibromatoses tumours in FAP patients demonstrated positive staining for estrogen receptors (ERs) (Alman et al., 1992). Although the detected receptor levels were low (Bruzzone et al., 1948; Jadrijevic et al., 1956), the absence of estrogen receptors has also been reported (Fong et al., 1993; Serpell et al., 1999; Sorensen et al., 2002). Guinea pigs with prolonged estrogen administration developed fibrous tumours histologically similar to human aggressive fibromatosis, and the administration of testosterone, progesterone and desoxycorticosterone inhibited the development of these aggressive fibromatosis-like tumours (Bruzzone et al., 1956; Lipschutz, 1950). A small in vitro study of four FAP-associated aggressive fibromatosis tumours demonstrated that proliferation was stimulated by the addition of estrogentic compounds and inhibited by tamoxifen (Tonelli et al., 1994), implicating a role for estrogen. While aggressive fibromatosis may be immunohistologically negative for the presence of estrogen receptors, it has been shown that this does not necessarily preclude that the tumour is insensitive to estrogen and is not affected by estrogen-modifying therapies (Leithner et al., 2005). A number of hormonal agents continue to be tested, including tamoxifen (Kinzbrunner et al., 1983; Procter et al., 1987; Sportiello and Hoogerland, 1991), progesterone (Lanari, 1983), and testolactone (Fujimoto and Hidai, 1990; Waddell and Kirsch, 1991). However, similar to the investigation of the efficacy of other treatments, the rarity of aggressive fibromatosis, the conflicting and partial responses observed in patients, and the general lack of randomized studies neither conclusively show a causative role of estrogen nor support that anti-estrogen agents should be a standardized method of treatment.

1.5.4 Other Therapeutic Strategies

Cytotoxic chemotherapy for aggressive fibromatosis in FAP patients have been reserved and used for inoperable tumours or progressive tumours despite treatment with NSAIDs and hormonal manipulation. Common agents include doxorubicin in combination with others such
as dacarbazine, cyclophosphamide and vincristine (Janinis et al., 2003). 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 (Azzarelli et al., 2001; Weiss and Lackman, 1989).

One avenue currently explored is the use of interferon-gamma (IFN-gamma). IFN-gamma has shown to have the capacity to inhibit proliferation and collagen synthesis of fibroblasts (Duncan and Berman, 1985). Clinical studies demonstrate moderate efficacy of IFN treatment (Acker et al., 1993; Bauernhofer et al., 1996; Geurs and Kok, 1993; Hardell et al., 2000); however, results from IFN treatment are compromised by patient numbers similar to studies using hormonal manipulation and cytotoxic chemotherapy against aggressive fibromatosis.
1.6 High Throughput Screening and Drug Discovery

High throughput screening is the primary tool and the driving force behind drug discovery. This process entails a large number of compounds tested in an automated fashion for activating or inhibiting activity on a particular biological target. As such, high throughput screening begins with a target choice and ends with obtaining a lead. This process first requires a defined target choice, and in deciding potential targets, one should take into account important factors including disease relevance and the appropriate screening assays required. Several methods could be employed to obtain leads or hits for any given target, and they are broadly categorized as cell-free assays and cell-based assays. Ideal assays involve minimal manipulation, are automated, require small volumes, and yield reproducible and robust responses.

1.6.1 Cell-Free Assays

Cell-free high throughput screening assays measure the biological activity of a particular molecular target, usually purified or partially purified target proteins or recombinant proteins. With a well-defined target, such in vitro assays are often used to assess direct biological activity. These include enzymatic activity, such as kinases (Burns et al., 2006) and transferases (Swaney et al., 2006); receptor-ligand binding, such as G-protein coupled receptors (Allen et al., 2000) and ion channels (Xu et al., 2001); and protein-protein interactions. Cell-free assays are often direct and specific to the target of choice, and involve homogeneous reactions to minimize the number of steps. Biological activity is measured by changes in an optical signal as a result of compound-target interaction. This signal is usually in the form of luminescence, fluorescence, absorbance or light emission. While the nature of cell-based assays makes this approach simple and robust, there are several drawbacks. It is important to note that a small molecule in a reconstituted in vitro assay does not necessarily represent the same activity in a cellular or physiological context. Furthermore, issues of membrane permeability, cytotoxicity, compound metabolism and off-target effects, among others cannot be assayed using cell-free systems.

1.6.2 Cell-Based Assays

Cell-based assays have emerged as a more physiological relevant platform. Such in vitro assays have been used to assess a less well-defined biological target and to focus more on identifying modulators of a pathway of interest, expanding the repertoire of targets as compared to cell-free
assays which measure for a single biological reaction. As such, cell-based assays are highly useful for broad targets or biological process targets, which are expressed in the appropriate physiological context with the necessary cellular components and feedback mechanisms already present. The selection of potential cellular systems includes primary cells and cell lines, and would depend on the behaviour and reproducibility of the signal or observation. Generally, this cellular context better mimics the normal biological and physiological situation than cell-free systems. This would negate the determination and further addition of cellular cofactors. Furthermore, this approach could potentially assay for and address the issue of bioavailability and cytotoxicity. However, the challenge lies in unraveling the mechanism of action after having identified the target, which could prove to be potentially costly and lengthy to achieve.

The most commonly employed assays can be categorized into reporter gene assays, second messenger or functional assays, and phenotypic assays. Reporter gene assays (Beck et al., 2005; Li et al., 2007) are commonly used to detect the transcriptional regulation of a particular gene or to monitor cellular responses at the transcriptional level. This is accomplished by linking the promoter, or elements of the promoter, from the gene of interest to the coding region of a reporter protein or a protein with an easily detectable enzymatic activity. Commonly used reporter proteins include luciferase, fluorescent proteins, beta-galactosidase, and secreted alkaline phosphatase. One disadvantage is that reporter gene transcription may be dependent on an indirect and amplified signaling upstream or receptor activation, and as such, it may be more useful to employ screens that detect signaling activities more proximal to the receptor. Second messenger or functional assays (Kariv et al., 1999; Kenny et al., 2003) monitor signaling transduction or pathways following activation of a cell-surface receptor. These events following receptor activation and ion channel activation may include changes in intracellular $\text{Ca}^{2+}$ concentration, membrane potential, pH levels, etc. Specific protocols involving the use of fluorescent dye sensitive to these changes have been designed to detect and measure such events. Phenotypic assays (Eggert et al., 2004; Yarrow et al., 2005) generally assess cellular processes and are uniquely developed based on the processes of interest such as cell migration, cell proliferation, cytokinesis and cytotoxicity. However, overall phenotypic responses may be attributed to modulation, either activation or inhibition, of multiple potential targets. This type of assay renders cell-based assays more elusive than cell-free systems in terms of determining the mechanism of action.
1.7 MicroSource Spectrum Compound Library

Our lab previously conducted a high-throughput drug screen using the MicroSource Spectrum Collection, which contains approximately 2,000 compounds made up of 50% drug components, 30% natural products and 20% other bioactive components, and over two-thirds of this collection is clinically approved and in use (http://www.msdiscovery.com, MicroSource Discovery Systems, Inc.). Drugs were screened under the criteria that candidates of interest would decrease cell viability of primary cells derived from human aggressive fibromatosis tumours but not of normal dermal fibroblasts from the same patients. Cells were treated with compounds in duplicates as well as vehicle control DMSO and a total of five independent screens were performed on the same library to ensure reproducibility. Relative changes in cell viability were measured using the Sulforhodamine B (SRB) assay. The SRB is a pink aminoxanthene dye with two sulfonic groups that bind to basic amino-acid residues in a stoichiometric manner such that the amount of dye staining the cells is directly proportional to cell density. This assay is an efficient and cost-effective method for screening changes in cell viability through measuring cell density as reflected by dye retention proportional to total cell protein content (Vichai and Kirtikara, 2006). The SRB assay has been previously used in drug screens in our lab as well as others to detect changes in cell viability. Using other compound libraries provided by the National Institutes of Health, we performed the SRB assay and, under the same target candidate criteria, identified several currently FDA-approved pharmacological agents that had been prescribed to treat aggressive fibromatosis, including vincristine sulfate, imatinib mesylate, and doxorubicin hydrochloride.

In this particular screen, the SRB assay identified three candidates of interest from the collection: 1) Nefopam, the active ingredient found in analgesic agents (Figure 5); 2) methyl-everminic acid, a synthetic compound; 3) alpha-tocopherol acetate, the metabolized active form of vitamin E. In this thesis, we focus on Nefopam and alpha-tocopherol acetate.

1.7.1 Nefopam

Nefopam (5-methyl-1-phenyl-1,3,4,5,6-tetrahydro-5-methyl-1-phenyl-1h-2,5-benoxazocine) is used as a post-operative analgesic mainly in European countries for the relief of moderate to severe pain. It is a non-opioid alternative and is unrelated chemically and pharmacologically to
other analgesic agents. Clinically, it has so far been proven to be safe with minimal side effects, including nausea, dizziness and sweating, and it has no depressant action on the central nervous system (Pillans and Woods, 1995; Tigerstedt et al., 1979).

Mechanistic studies have suggested that the analgesic activity of Nefopam involves inhibition of catecholamine and serotonin reuptake in the central nervous system (Fuller and Snoddy, 1993; Rosland and Hole, 1990). Increased levels of noradrenaline, dopamine and serotonin in the synaptic cleft are shown to modulate antinociception. Using several rodent models, it has been shown that Nefopam selectively modulates adrenergic, dopaminergic and serotonergic receptor subtypes. In addition, in vitro binding assays have demonstrated that Nefopam may act through direct or indirect mechanisms to modulate such receptors (Girard et al., 2006). Another proposed mechanism of action of Nefopam on neurotransmission is mediated by the excitatory amino acid glutamate. Recent studies in primary cultures of rat cerebellar neurons demonstrated that Nefopam could inhibit sodium influx by blocking membrane voltage-sensitive sodium channels necessary for propagation of action potential down the axon and consequently the synaptic release of glutamate (Fernandez-Sanchez et al., 2001; Verleye et al., 2004). Furthermore, it is thought that Nefopam blocks the three ionotropic glutamate receptor subtypes without direct interactions with the receptors thereby reducing excitability of postsynaptic neurons (Verleye et al., 2004).

Collectively, these studies prompt further investigation to clarify the nature of the interactions between Nefopam and G-protein coupled receptors, which is a large family of receptors encompassing adrenergic receptors as well as glutamate receptors (Girard et al., 2009; Girard et al., 2004; Marazziti et al., 1991). While its exact mechanism of action is poorly understood, its neoplastic potential has yet to be explored.

1.7.2 Alpha-Tocopherol Acetate

alpha-Tocopherol acetate is the metabolized and most biologically active form of vitamin E. Many studies have revealed its protective properties against oxidative damage whereby its chemical structure inhibits free radical chain reactions by absorbing free radicals (Burton, 1994; Packer and Landvik, 1990). More recently, focus has been placed beyond its anti-oxidative properties (Azzi and Stocker, 2000). Studies have shown that it is able to inhibit protein kinase C activity and phospholipase A2 as well as activate protein phosphatase 2A and acylglycerol
kinase (Azzi et al., 2002; Zingg and Azzi, 2004). At the transcriptional level, alpha-tocopherol acetate can modulate downstream gene expression, including MMP-19 and collagenase (Zingg and Azzi, 2004).

It has been suggested that vitamin E decreases the size of hyperplastic wounds, a process also associated with the stabilization of beta-catenin. With the potential to modulate beta-catenin signaling, vitamin E may serve as a potential candidate to treat other fibroproliferative disorders such as aggressive fibromatosis.

### 1.7.3 Androgens

In the Apc^{+/−}/Apc^{1638N} mouse model, aggressive fibromatosis tumours occur with 100% penetrance where male mice develop an average of 45 tumours and females 16 (Fodde and Smits, 2001; Smits et al., 1998), implicating a possible role of androgens. Studies on the incidence of aggressive fibromatosis in FAP patients based on gender have been contradictory in their findings with some studies showing increased incidence in females (Bertario et al., 2001; Klemmer et al., 1987; Sturt et al., 2004) while others demonstrated no significant differences between genders (Gurbuz et al., 1994; McAdam and Goligher, 1970). More recently, immunohistochemical analysis of 27 cases reported positive staining for estrogen, progesterone and androgen receptors (Ishizuka et al., 2006). Perhaps androgens play a role in aggressive fibromatosis and the understanding of this role would provide a novel hormone-based therapeutic strategy.
1.8 Thesis Summary and Rationale

Aggressive fibromatosis is a fibroproliferative tumour that can occur as a sporadic lesion or a manifestation in FAP patients. Mutations frequently occur in either the APC gene or CTTNb1 gene, resulting in the stabilization of beta-catenin, which appears to be a general occurrence in aggressive fibromatosis tumours. Despite the variety of therapeutic strategies available, current therapies have yet to demonstrate total success for primary and recurrent tumours. As such, aggressive fibromatosis remains a clinical challenge and there remains a need for more effective therapeutic strategies.

My research sheds light on the anti-neoplastic properties of Nefopam, a currently approved analgesic agent as a possible adjuvant therapy, as well as the role of androgens as a potential novel target in aggressive fibromatosis.

1.8.1 Hypothesis

Pharmacological modulation by Nefopam can inhibit aggressive fibromatosis tumours, while androgens are able to promote their development. In addition, these agents are able to modulate beta-catenin expression levels in mesenchymal cells.

1.8.2 Research Objectives

The novel anti-neoplastic capacities of analgesic agent Nefopam against human and murine aggressive fibromatosis are detailed in Chapter 2. Specifically, I investigate its ability to modify cell viability, cell proliferation, and beta-catenin levels. Using Apc^+/Apc^{1638N} mouse model for aggressive fibromatosis, I describe the phenotypic effects of Nefopam on the development of aggressive fibromatosis tumours. I demonstrate that Nefopam may serve as a potential novel adjuvant therapeutic strategy for FAP-associated aggressive fibromatosis.

The role of androgens in the development of aggressive fibromatosis is explored in Chapter 3. I use male Apc^+/Apc^{1638N} mice that have undergone orchidectomy to uncover the effects that androgens may have on the development of aggressive fibromatosis tumours. I explore how beta-catenin levels are modulated by androgen manipulation in tumours and begin to describe the effects of testosterone on primary aggressive fibromatosis cell behaviour in vitro.
Conclusions from this research set the stage for further exploration of the anti-neoplastic mechanisms of Nefopam and the role of androgens in aggressive fibromatosis. Such future experimental approaches are outlined in Chapter 4. It will be essential to determine how Nefopam modulates beta-catenin levels, which may prove to be applicable to other fibroproliferative processes mediated by elevated levels of beta-catenin, such as wound healing. Furthermore, research on the role of androgens will shed light on how current hormonal manipulation technology such as androgen-blocking agents could be employed as another potential therapeutic strategy for aggressive fibromatosis.
1.9 References


1.10 Figures

**Figure 1. Canonical Wnt/beta-catenin signaling pathway.** Three pathways can be activated downstream of the Wnt receptors; however, the canonical Wnt/beta-catenin signaling pathway is the most well studied and understood of them all. In the absence of an activating Wnt ligand, multiprotein complex comprised of Axin, APC, GSK3beta and CK1gamma is functional to facilitate the phosphorylation of beta-catenin at specific serine and threonine sites. Phosphorylation of beta-catenin implicates its degradation through an ubiquitin-mediated process. Upon binding of an activating Wnt ligand to Fz receptors and LRP5/6 co-receptors, cytoplasmic protein Dsh becomes phosphorylated, which destabilizes the multiprotein complex by acting as a docking site for Axin. Stabilized beta-catenin is able to accumulate in the cytoplasm and translocate into the nucleus to activate downstream Wnt target genes (Adapted from Silkstone et al., 2008).
Figure 2. Genotype-phenotype correlations at the APC gene in human and mouse studies.

Mouse models containing different mutations throughout the Apc gene were analyzed, demonstrating consistent correlations between the location of mutation and subsequent phenotype. There exist mutational cluster regions whereby the type and location of these mutations confer an increased probability of developing a particular phenotype such as aggressive fibromatosis tumours. One proposed theory to explain the phenotypic differences describes the importance of beta-catenin protein dosage, which is regulated by APC, as well as the importance of subsequent levels of beta-catenin transcriptional activity, which determines the activation of different transcriptional programs (Adapted from Fodde et al., 2001).
Figure 3 Mutational analysis performed on human CTNNb1 gene. Beta-catenin expression levels are post-translationally regulated through sequential phosphorylation at codons 33, 37, 41 and 45 and subsequent ubiquitin-mediated degradation. Aggressive fibromatosis patients have shown a high frequency of mutations at codons 41 and 45, resulting in the inability of beta-catenin to become phosphorylated by GSK3beta and CK1gamma and the stabilization of beta-catenin in the cytoplasm (Adapted from Tejpar et al., 1999).
Figure 4. Immunohistochemical staining of beta-catenin. (A) Human aggressive fibromatosis tumours showing intense staining of cytoplasm in most cells and potentially nuclear staining in others. (B) Normal marginal fascial tissue from the same patient with a marked decreased staining for beta-catenin (Adapted from Tejpar et al., 1999).
The MicroSource Spectrum Collection contains approximately 2,000 compounds made up of 50% drug components, 30% natural products and 20% other bioactive components, two-thirds of this collection are clinically approved and in use. Drugs were screened under the criteria that candidates of interest would reduce cell viability of primary cells derived from human aggressive fibromatosis tumours (n=2) but not of immortalized human fibroblasts. The SRB assay was employed to screen for changes in cell density as a measurement of cell survival. After screening the compounds in duplicates fives times independently, Nefopam was consistently identified with the potential to reduce relative cell survival as compared to treatment with DMSO. Error bars represent 95% confidence intervals and are significant when bars do not cross the mean of the comparison.
2 Targeting beta-catenin as Drug Therapy for Aggressive Fibromatosis

2.1 Abstract

Aggressive fibromatosis (also known as desmoid tumour) is a locally invasive and benign, fibroproliferative tumour. Tumours are associated with somatic mutations resulting in the stabilization of β-catenin and the dysregulation of β-catenin-mediated signaling. Stabilized β-catenin accumulates in the cytoplasm and translocates into the nucleus to activate T-cell factor/lymphoid enhancer binding factor-dependent transcription of downstream target genes. Chemotherapeutic agents selectively targeting this pathway are important candidates for the treatment of aggressive fibromatosis. Using the MicroSource Spectrum Compound library, our lab identified Nefopam, a currently approved analgesic agent with the potential to inhibit cell viability of human aggressive fibromatosis tumours but not normal fibroblasts from the same patients. Nefopam is not an anti-inflammatory cyclooxygenase-2 inhibitor and its mechanism of action is poorly understood. In this study, primary cells derived from human aggressive fibromatosis tumour treated with Nefopam demonstrated a marked decrease in cell viability, cell proliferation, and β-catenin protein levels. Male Apc+/Apc1638N mice predisposed to developing aggressive fibromatosis tumours were treated with Nefopam for 3 months. Compared to littermate controls, tumour assessment indicates that Nefopam has the capacity to significantly reduce the number of tumours with no difference in tumour volumes in vivo. This study offers a novel therapeutic approach for the treatment of aggressive fibromatosis.

(Dec 2009) Nefopam, Provisional Patent, Nefopam as an agent to treat neoplasia and hyperplastic scars, Granted, 61286633, United States, Benjamin Alman, Raymond Poon, Helen Hong
2.2 Introduction

Aggressive fibromatosis (AF) is a locally invasive fibroproliferative tumour comprised of mesenchymal fibroblast-like spindle cells. AF occurs as either a sporadic lesion or a manifestation in familial syndromes, such as familial adenomatous polyposis (FAP). Most cases of sporadic AF are associated with somatic mutations in β-catenin or Adenomatous Polyposis Coli (APC), while familial cases of AF are associated with germline mutations in the APC gene (Alman et al., 1997a; Tejpar et al., 1999). Such mutations are characterized by β-catenin protein stabilization and activation of β-catenin-mediated T-cell factor (TCF)-dependent transcriptional activity (Alman et al., 1997a; Cheon et al., 2002). β-catenin stabilization is sufficient to cause AF lesions as demonstrated using a transgenic mouse model that overexpresses the stabilized form of β-catenin (Cheon et al., 2002). The Apc+/Apc1638N AF mouse model contains germline mutations near the 3’ terminus of the Apc gene and is predisposed to developing AF tumours as well as upper gastrointestinal polyps with full penetrance (Fodde and Smits, 2001).

β-catenin is an important signaling mediator in the canonical Wnt/β-catenin signaling pathway involved in several developmental and regenerative processes as well as neoplasia. The canonical Wnt/β-catenin signaling pathway is initiated when activating Wnt ligands bind to cognate receptor complex comprised of a member of the seven-transmembrane-domain receptor of the Frizzled family and an LDL-related protein 5/6 (LRP5/6) co-receptor (Bhanot et al., 1996; Pinson et al., 2000). In the absence of activating Wnt ligands, cytoplasmic protein Dishevelled (Dsh) remains unphosphorylated, which allows a multiprotein complex composed of scaffolding proteins APC and Axin, casein kinase 1-gamma (CK1gamma) and glycogen synthase kinase 3-β (GSK3β), to degrade β-catenin (Rubinfeld et al., 1996). β-catenin becomes phosphorylated by kinases CK1gamma and GSK3β, which targets it for ubiquitin-mediated degradation (Aberle et al., 1997). In the presence of activating Wnt ligands, Dsh becomes phosphorylated and the multiprotein complex is destabilized. β-catenin accumulates in the cytoplasm and translocates into the nucleus (Daniels and Weis, 2005). Together with TCF/LEF transcription factors, β-catenin regulates the expression of downstream cell-specific target genes (Behrens et al., 1996; van de Wetering et al., 1997).
Taken together, beta-catenin plays an important role in AF tumours and is an attractive therapeutic target. Pharmacological agents with the capacity to modulate beta-catenin signaling would be potential candidates of interest. Here, we report the use of the Sulforhodamine (SRB) assay to identify such candidates with the ability to inhibit cell viability of human AF tumours but not normal dermal fibroblasts of the same patients. Using this assay, several compounds were identified including imatinib mesylate, and doxorubicin hydrochloridesulindac, which have previously been used to treat AF tumours. We also identified Nefopam, a currently approved post-operative analgesic agent. Nefopam is not an anti-inflammatory cyclooxygenase-2 inhibitor and may modulate different G-protein coupled receptors, including adrenergic and serotonergic receptors (Girard et al., 2009; Girard et al., 2004; Marazziti et al., 1991). Its mechanism of action as an analgesic agent is still poorly understood.

The anti-neoplastic properties of Nefopam have yet to be explored. Our preliminary data demonstrate that Nefopam decreases cell viability of primary human AF tumour cells. We thus examined the ability of Nefopam to modulate beta-catenin levels in primary human AF tumour cells and capacity to reduce the development of AF tumours in the Apc+/Apc1638N AF mouse model. This study offers Nefopam as a novel therapeutic adjuvant for the treatment of AF.
2.3 Results

2.3.1 Nefopam decreases the number of aggressive fibromatosis tumours formed in Apc+/Apc\(^{1638N}\) mice.

We investigated whether Nefopam treatment was able to modulate the phenotype of aggressive fibromatosis tumours in vivo using the previously established and well characterized mouse model, Apc\(^{+/}\)/Apc\(^{1638N}\) mice. The number of tumours formed in male mice treated with Nefopam was significantly reduced compared to the number formed in mice provided with No Treatment or treated with 0.1% DMSO as vehicle or carrier control at 6 months of age (8.18±1.77 vs 13.2±2.30 or 12.09±1.31, p<0.05, Figure 6 left). However, there were no significant differences in tumour volumes between the study groups (p>0.05, Figure 6 right). These mice are known to also develop upper gastrointestinal polyps with full penetrance. Interestingly, there were no significant differences in the number of polyps observed at 6 months of age (p>0.05, Figure 6 left).

2.3.2 Nefopam regulates cell proliferation in human aggressive fibromatosis tumour cells.

To determine how Nefopam may modify cell behaviour, we studied primary cell cultures derived from five human aggressive fibromatosis tumours. First, we counted fewer number of live cells in 4 of the 5 human tumours as a result of Nefopam treatment compared to those treated with 0.1% DMSO (p<0.05, Figure 7). Interestingly, there were no significant differences in the number of dead cells counted as a result of Nefopam treatment for all human aggressive fibromatosis tumours (p<0.05). As beta-catenin is able to regulate the rate of proliferation in mesenchymal cells, we investigated the effects of Nefopam on proliferation in primary cell cultures. Using the BrdU incorporation assay, we measured the percentage of BrdU+,DAPI+ cells compared to total DAPI+ cells and found that Nefopam-treated human aggressive fibromatosis tumours displayed a significantly fewer number of cells with incorporated BrdU (p<0.05, Figure 8), suggesting that Nefopam may inhibit tumour cell viability by reducing the rate of proliferation.
2.3.3 Nefopam decreases beta-catenin levels in human aggressive fibromatosis tumour cells.

Aggressive fibromatosis tumours are characterized by an increase in beta-catenin levels or stabilization of beta-catenin. To examine whether Nefopam has the capacity to modulate beta-catenin levels, we studied primary cell cultures derived from several human aggressive fibromatosis tumours. Western Blot analysis using an antibody against total beta-catenin demonstrated a marked decrease in the amount of protein at size 92kDa consistent with total beta-catenin as a result of Nefopam treatment for 5 days (Figure 9). Densitometry analysis quantitatively showed nearly a 5-fold decrease in total beta-catenin levels when cells were treated with Nefopam compared to those that were treated with 0.1% DMSO (p<0.05, Figure 9).

2.3.4 Nefopam moderately decreases cell viability in normal human fibroblasts.

While we observed that Nefopam is able to modulate beta-catenin levels and viability of primary cells derived from human aggressive fibromatosis tumours, we next investigated whether these changes were also observed in normal dermal fibroblasts derived from the same patients. We counted a significant decrease in the number of viable cells upon treatment with Nefopam as compared to those treated with 0.1% DMSO (p<0.05, Figure 10). Interestingly, these changes were considerably mild as compared to changes in the number of viable cells of the aggressive fibromatosis tumour derived from the same patient. Furthermore, there were no significant differences in the number of dead cells counted when treated with Nefopam compared to 0.1% DMSO as well as between normal cells and tumour cells (p<0.05). This suggests that Nefopam is able to decrease cell viability in normal fibroblasts, potentially through affecting proliferative programming in a beta-catenin-mediated manner, similar to human aggressive fibromatosis tumours.
2.4 Discussion

In this study, we demonstrated that Nefopam decreased cell viability, proliferation and levels of beta-catenin in primary cells derived from human aggressive fibromatosis tumours. Furthermore, Nefopam is able to improve the phenotype of aggressive fibromatosis as demonstrated by the decreased number of tumours formed in Apc\(^{+/Apc^{1638N}}\) mouse model.

Aggressive fibromatosis is characterized by an increase in beta-catenin levels (Tejpar et al., 1999). This study revealed that Nefopam is able to decrease cell viability and cell proliferation, which are regulated by beta-catenin-mediated signaling. Beta-catenin dosage is important in determining the expression of certain downstream Wnt target genes and the initiation of different tissue-specific programs (Hecht and Kemler, 2000; Mosimann et al., 2009). The effects of differential beta-catenin dosage are exemplified in Apc\(^{\text{Min}}\) mice, which produces truncated Apc protein lacking all beta-catenin regulatory domains and rarely develop aggressive fibromatosis lesions (Halberg et al., 2000), whereas the Apc\(^{+/Apc^{1638N}}\) mice, which produce residual amounts of Apc protein with minimal beta-catenin regulatory capacity, develop aggressive fibromatosis with full penetrance (Smits et al., 1998).

Apc\(^{+/Apc^{1638N}}\) mice have been well characterized and shown to have high protein levels of beta-catenin (Poon et al., 2001b; Smits et al., 1999). Our lab has previously demonstrated that stabilization of beta-catenin increased mesenchymal proliferation, motility and invasiveness in vitro (Cheon et al., 2002). The effects of beta-catenin-mediated signaling on cell viability in fibroblasts have yet to be fully elucidated. Previous studies using fibroblasts expressing stabilized beta-catenin have demonstrated an increase in proliferation (Soler et al., 1999) as well as an increase in apoptosis (Kim et al., 2000). These differences in observation could be due to the dosage of stabilized beta-catenin as there is evidence of a beta-catenin-mediated TCF-independent mechanism, which regulates apoptosis and is activated only at higher levels of beta-catenin expression (Kim et al., 2000). We have found that Nefopam treatment resulted in a marked decrease in the live cells as well as decreased levels of BrdU incorporation with no differences in the number of dead cells, suggesting that Nefopam may downregulate beta-catenin levels to the critical level required to modulate proliferative programming rather than programmed cell death to regulate overall cell viability.
However, our data does not address the exact mechanism of Nefopam through which it effectively reduces beta-catenin levels. Possible explanations for the observed decrease in total levels of beta-catenin may be that Nefopam modulates the functionality of GSK3beta directly or indirectly through destabilizing the multiprotein complex. Though CK1 and GSK3 are both found in the multiprotein degradation complex, beta-catenin regulation occurs through sequential phosphorylation by CK1 and GSK3, where CK1 merely initiates and primes beta-catenin for further phosphorylation by GSK3. The serine sites phosphorylated by GSK3 are then recognized by the ubiquitin ligase and will indicate the degradation of beta-catenin. As such, GSK3beta is the main regulator in the Wnt pathway (Polakis, 2002). Given that GSK3beta plays a role in cellular processes other than the canonical Wnt/beta-catenin signaling pathway, our data does not exclude the potential involvement of other upstream pathways that may subsequently modulate beta-catenin levels. Furthermore, our methods of protein extraction account for protein found only within the cytoplasm and nucleus but exclude membrane proteins. Beta-catenin is involved in cell adhesion through interaction with the cytoplasmic domain of type I cadherins and plays an essential role in the structural organization and function of cadherins by linking cadherins through alpha-catenin to the actin cytoskeleton (Gumbiner, 2000; Jamora and Fuchs, 2002). Overexpression studies of cadherins have reduced the availability of beta-catenin by sequestering it at the cell membrane and thereby made it unavailable for signaling to the nucleus (Heasman et al., 1994; Sanson et al., 1996). Immunohistochemistry studying the presence of cadherin/catenin adhesion complex in aggressive fibromatosis tumours has been performed and demonstrates positive staining for N-cadherin and alpha-catenin (Ferenc et al., 2009). Perhaps Nefopam is modulating the localization of beta-catenin, which could potentially address the decrease in total beta-catenin levels in our collected protein as observed in our work.

Analysis of gene expression in aggressive fibromatosis tumours treated with Nefopam may provide an understanding of how beta-catenin may modulate transcriptional programmes to affect cell behaviour such as cell viability and proliferation. Previous studies have indicated that aberrant Wnt/beta-catenin signaling and downstream gene expression are associated with aggressive fibromatosis. Known Wnt target gene cyclin D1 is closely associated with an enhanced proliferative capacity in mesenchymal stem cells (Karow et al., 2008). Immunohistochemical evaluation of aggressive fibromatosis tumours revealed that nuclear beta-catenin accumulation correlates with overexpression of cyclin D1 (Saito et al., 2001).
observed changes in cell proliferation could be due to the ability of Nefopam to modulate beta-catenin signaling and expression of certain genes such as cyclin D1. Matrix metalloproteinases (MMPs) are other downstream target genes of the Wnt/beta-catenin signaling pathway and has been shown to play an important role in tumorigenesis and tumour invasion (Matono et al., 2008; Orlichenko and Radisky, 2008). Our lab previously found that aggressive fibromatosis tumours in mice expressed MMP-3 with nearly a 5-fold increase compared with normal fibrous tissue, and that MMP3 expression in tumours increased efficiency to process fibrillar collagens, motility and ability to invade through Matrigel, without effect on cell proliferation or apoptosis (Kong et al., 2004). When MMP activity was inhibited pharmacologically or by overexpressing tissue inhibitors of MMPs, tumours of smaller volumes were found in mice, suggesting the importance of MMPs in the ability of aggressive fibromatosis tumours to invade through local extracellular matrix (Kong et al., 2004). Perhaps Nefopam in conjunction with pharmacological inhibitors of MMPs would allow for a more comprehensive strategy to manage aggressive fibromatosis.

We used the Apc<sup>+/-</sup>/Apc<sup>1638N</sup> mice as our animal model aggressive fibromatosis, which has been well characterized and previously used to study tumour development in the FAP context (Fodde et al., 1994). Similar to FAP patients, Apc<sup>+/-</sup>/Apc<sup>1638N</sup> mice develop several upper gastrointestinal polyps as well as extra-colonic aggressive fibromatosis lesions (Fodde et al., 1994; Fodde and Smits, 2001). We found that Apc<sup>+/-</sup>/Apc<sup>1638N</sup> mice treated with Nefopam developed a significantly fewer number of aggressive fibromatosis lesions compared to littermate controls with no observable differences in the average size of tumours. This suggests that Nefopam is affecting the initiation rather than the progression of tumour development in vivo.

Our lab has previously shown that mesenchymal tumours including aggressive fibromatosis contain a subpopulation of cells with tumour-initiating capacity, referred to as side-population cells (Wu et al., 2007). Furthermore, these cells have the capacity to initiate tumours upon serial transplantation in immunodeficient mice (Wu et al., 2007). One of the challenges in managing aggressive fibromatosis is the high recurrence rate of tumours from currently existing therapies, and the recurrence of tumours may be attributed to the presence of cells with the capacity to self-renew and initiate tumours. Side populations were identified using the Hoescht dye exclusion method whereby this method selects for cells with the capacity to exclude dye through the expression of protein transporters (Zhou 2001, Zhou 2002). The presence of efflux protein transporters in these cells may also contribute to the resistance of tumours to chemotherapies as
demonstrated by the low efficacy of currently existing therapies for aggressive fibromatosis (Dean 2005). Perhaps Nefopam may also have the ability to target these mesenchymal tumour-initiating cells and further analysis of the effect of Nefopam on side populations may help to confirm this and to better characterize the anti-neoplastic capacities of Nefopam.

Upon treating Apc\(^+\)/Apc\(^{1638N}\) mice with Nefopam, we found a marked decrease in the number of aggressive fibromatosis tumours compared to littermate controls with no observable differences in the number of polyps formed. The Alman lab had previously treated human colon cancer SW40 cells with Nefopam and found no differences in cell viability in vitro as we had with our primary cells derived from human aggressive fibromatosis tumours. However, when SW40 colon cancer cells were xenografted into immunodeficient mice and treated with Nefopam, a slight decrease in the size of tumours was observed. A possible explanation for these observations collectively is that the tumour cells were seeded in the dermis or mesenchymal tissues beneath the skin and that Nefopam is able to act upon cells within the mesenchymal niche to indirectly exert its antineoplastic effects. We demonstrated that Nefopam is able to exert a mild effect on normal fibroblasts. Primary cell cultures derived from normal dermal tissue of aggressive fibromatosis patients treated with Nefopam demonstrated a moderate decrease in beta-catenin expression levels as compared to its effect on tumour cells. Changes in beta-catenin levels in turn could activate different transcriptional programs, resulting in the modulation of cell behavior such as cell viability and proliferation as was observed. However, other transcriptional programmes activated could also dictate the interaction between cells, which would address the observed effect of Nefopam on colon cancer cells seeded in the dermis layer of TCF mice and no effect on the gastrointestinal polyps in Apc\(^+\)/Apc\(^{1638N}\) mice.

This preference for mesenchymal cells demonstrated by Nefopam prompted our study of its application in other fibroproliferative processes. Fibroproliferative processes are a group of disorders characterized by an excessive proliferation of mesenchymal fibroblast-like spindle cells (Chaudhuri and Das Gupta, 1998; Lattes, 1980). Aggressive fibromatosis tumours are monoclonal proliferations that fall within the broad spectrum of these processes (Alman et al., 1997b), which could range from hypertrophic wounds to the development of neoplasms. During normal wound healing, different cell types and signaling pathways are activated to reconstitute the epithelial and dermis layers of the skin. Following cutaneous injury, three sequentially distinct but overlapping processes are initiated: inflammatory, proliferation, and remodeling
During the proliferative phase, mesenchymal fibroblast-like cells accumulate in the dermal component of the skin while the epithelial cell barrier layer is reformed (Martin, 1997; McClain et al., 1996; Singer and Clark, 1999). Beta-catenin has been shown to mediate both epithelial and mesenchymal cell activity in the proliferative phase, whereby it is able to increase proliferation and differentiation of dermal mesenchymal cells, and decrease migration of epithelial keratinocytes (Cheon et al., 2002). Mouse models have demonstrated that beta-catenin modulates wound sizes and that induced stabilization and increased expression of beta-catenin by lithium treatment led to larger wounds (Cheon et al., 2006). We had previously generated a transgenic mouse in which stabilized β-catenin is expressed under control of a tetracycline-regulated promoter (Cheon et al., 2002). Wounded mice healed with hyperplastic cutaneous wounds as compared to littermate control mice, implicating the importance of beta-catenin in mesenchymal cells and its involvement in wound healing (Cheon et al., 2002).

Hyperplastic wounds are characterized by a marked elevation in beta-catenin levels during the proliferative phase (Cheon et al., 2005; Cheon et al., 2002). Unpublished data from the Alman lab so far suggests that Nefopam has the capacity to modulate beta-catenin levels specifically in mesenchymal-derived cells. As expected, we found that Nefopam could modulate beta-catenin levels in mesenchymal cells demonstrated by a marked decrease in total beta-catenin levels in immortalized human fibroblast cells. To examine the effects of Nefopam on beta-catenin levels during wound healing, we studied wounded tissue harvested from Tcf reporter mice. Preliminary data show that Nefopam had not only decreased total beta-catenin levels during the proliferative phase in wounded Tcf mice as compared to the control group, but Nefopam treatment resulted in a significant decrease in wound sizes. This further suggests that Nefopam may be able to improve wound healing through a beta-catenin-mediated mechanism.

Our study shows for the first time the anti-neoplastic properties of Nefopam. Nefopam may serve as a novel therapeutic approach to aggressive fibromatosis. An understanding of how these pathways are modulated could facilitate the potential application of Nefopam in other fibroproliferative disorders, such as hypertrophic scarring.
2.5 Materials and Methods

2.5.1 MicroSource Spectrum Compound Library

The MicroSource Spectrum Collection contains approximately 2,000 compounds made up of 50% drug components, 30% natural products and 20% other bioactive components. Over two-thirds of this collection are clinically approved and in use (Microsource website). Drugs were screened under the criteria that candidates of interest would decrease cell viability of primary human AF cell cultures but not of normal dermal fibroblasts from the same patients.

2.5.2 Nefopam

Nefopam (5-methyl-1-phenyl-1,3,4,5,6-tetrahydro-5-methyl-1-phenyl-1h-2,5-benzoazocine) is used as a post-operative analgesic mainly in European countries for the relief of moderate to severe pain. It is a non-opioid alternative and unrelated chemically and pharmacologically to any other analgesic agents. Clinically, it has so far proven to be safe with minimal side effects, including nausea, dizziness and sweating, and it has no depressant action on the central nervous system (Tigerstedt 1979, Pillans 1995). It has been shown to be able to modulate serotonin and histamine receptors (Marazziti 1991, Girard 2009), which are part of the family of G-protein coupled receptors (Girard 2004).

2.5.3 Apc+/Apc\textsuperscript{1638N} AF mouse model and treatment strategy

The generation and phenotype of Apc\textsuperscript{+/Apc\textsuperscript{1638N}} mice have been well characterized. These mice harbour a targeted mutation at codon 1638 in the Apc gene as a result of a neomycin insertion in antisense orientation at exon 15. Male mice develop an average of 45 aggressive fibromatosis tumours and 6 gastrointestinal polyps by the age of 6 months, while female mice develop significantly fewer numbers of tumours.

Male Apc\textsuperscript{+/Apc\textsuperscript{1638N}} mice were divided into three study groups: 1) No Treatment (n=11), 2) 0.1% DMSO (n=10), and 3) Nefopam at 40mg/kg body weight (n=10). Treatment by daily oral gavaging began 2 months after Apc\textsuperscript{+/Apc\textsuperscript{1638N}} mice were weaned and continued for 3 months up to approximately 6 months of age. At autopsy, tumours and intestinal polyps were scored macroscopically. Tumours and normal tissue were harvested for protein extraction and fixed for
histological examination. High performance liquid chromatography was performed on collected serum samples to confirm drug uptake in mice.

2.5.4 Human AF tumor and normal fascial tissue samples

Samples of human aggressive fibromatosis tumors were obtained at the time of surgery from the Hospital for Sick Children (Toronto, Ontario). Tumor tissue and surrounding normal fascial tissue from the same patient were harvested and processed immediately after surgical excision. Tissues were cryopreserved and stored in liquid nitrogen.

2.5.5 Cell culture studies

Primary cell cultures from the human aggressive fibromatosis tumour and normal fascial tissue samples were established. Monolayer cultures were cultured in DMEM supplemented with 10% fetal bovine serum and maintained at 37°C in 5% CO₂. Cells were divided when confluent and experiments were performed only between the first and fifth passages. Prior to experimental studies, cells were seeded overnight and treatment began the following day where cells were treated with vehicle control 0.1% DMSO with or without 250µm Nefopam prepared in DMEM media.

Cell viability assay and proliferation assay were performed after 2x10⁴ cells were seeded and treated with Nefopam for 5 days. Cell viability was measured using the Trypan Blue Dye Exclusion method. Adhered cells and detached cells were collected at once and stained with Trypan Blue Dye at a 1:1 ratio. Both live (clear) and dead (blue) cells were counted using the hemocytometer. Proliferation was measured using 5-bromo-2-deoxy-uridine (BrdU) Incorporation assay. After BrdU incubation for 12 hours, cells with incorporated BrdU were identified using rabbit monoclonal anti-BrdU antibody and horse anti-mouse antibody conjugated to Alkaline Phosphatase. Presence of BrdU was detected using Alkaline Phosphatase substrate. Percentage of positively stained nuclei (BrdU+,DAPI+ cells) out of total nuclei (DAPI+ cells) was analyzed over 10 high-powered fields.

2.5.6 Protein extraction and Western Blot Analysis

Human aggressive fibromatosis tumours and fibroblasts from normal tissue samples were washed twice with PBS and lysed with Reporter Gene Assay Lysis Buffer (Roche). Lysates
were centrifuged at 16,000 x g 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, transferred to a nitrocellulose membrane (Amersham), and immunoblotted overnight at 4 °C with primary antibodies against phospho-GSK3beta (Ser 9, rabbit polyclonal, New England Biolabs), total GSK3beta (mouse monoclonal, Transduction Laboratories), active beta-catenin (mouse polyclonal, Upstate Biotechnology), total beta-catenin (mouse monoclonal, Upstate Biotechnology), and GAPDH (mouse monoclonal, Upstate Biotechnology). Horseradish peroxidase-tagged secondary antibodies and Enhanced ChemiLuminescence (Amersham) were used to detect hybridization. Densitometry was performed using the AlphaEaseFC software (Alpha Innotech). Western blotting was performed in triplicates to ensure reproducibility.

2.5.7 Statistical analysis

Power calculations were used to determine the minimum number of mice required in study groups for biological significance. Data in this work were calculated as mean ± 95% confidence intervals. Studies were performed in triplicates to ensure reproducibility.
2.6 References


2.7 Figures

![Graph showing average number of tumours and gastrointestinal polyps formed in Apc\(^+\)/Apc\(^{1638N}\) male mice treated with different treatments. (Left) The means and 95% confidence intervals of the average number of tumours and gastrointestinal polyps formed in Apc\(^+\)/Apc\(^{1638N}\) male mice are shown. While DMSO-treated mice showed a comparable number of tumours to mice that received no treatment, mice treated with Nefopam developed significantly fewer tumours. Differences in the number of polyps formed were not observed amongst the study groups. (Right) Average volume (mm\(^3\)) per tumour formed in Apc\(^+\)/Apc\(^{1638N}\) mice treated with DMSO and Nefopam were analyzed. Differences in average size of tumours were not observed. Statistically significant differences (p<0.05) compared to DMSO and No Treatment control groups are indicated with an asterisk above the bar.

**Figure 6. Apc\(^+\)/Apc\(^{1638N}\) mice treated with Nefopam developed fewer aggressive fibromatosis tumours.** Male Apc\(^+\)/Apc\(^{1638N}\) mice were divided into three study groups: 1) No Treatment (n=11), 2) 0.1% DMSO (n=10), and 3) Nefopam at 40mg/kg body weight (n=10). (Left) The means and 95% confidence intervals of the average number of tumours and gastrointestinal polyps formed in Apc\(^+\)/Apc\(^{1638N}\) male mice are shown. While DMSO-treated mice showed a comparable number of tumours to mice that received no treatment, mice treated with Nefopam developed significantly fewer tumours. Differences in the number of polyps formed were not observed amongst the study groups. (Right) Average volume (mm\(^3\)) per tumour formed in Apc\(^+\)/Apc\(^{1638N}\) mice treated with DMSO and Nefopam were analyzed. Differences in average size of tumours were not observed. Statistically significant differences (p<0.05) compared to DMSO and No Treatment control groups are indicated with an asterisk above the bar.
Figure 7. Nefopam on cell viability of human aggressive fibromatosis tumour cells. The means and 95% confidence intervals of cell viability are shown for primary cells derived from human aggressive fibromatosis tumours (n=5) treated with DMSO and Nefopam for 5 days. Cell viability is measured by staining cells with Trypan Blue Dye and counted for both live (clear) and dead (blue) cells. Nefopam significantly decreased the number of live cells while the number of dead cells did not change. Statistically significant differences (p<0.05) compared to controls are indicated with an asterisk above the bar.
**Figure 8. Nefopam modulates cell proliferation in vitro.** Primary cells derived from human aggressive fibromatosis tumours (n=2) treated with DMSO and Nefopam in triplicate for 5 days, and cell proliferation as measured by the number of BrdU+, DAPI+ cells compared to total DAPI+ cells. Nefopam significantly reduces the incorporation of BrdU into cells. The means and 95% confidence intervals are shown. Statistically significant differences (p<0.05) compared to the control are indicated by asterisk above the bar.
Figure 9. Modulation of beta-catenin expression levels by Nefopam in human aggressive fibromatosis tumour cells. Western Blot analysis of protein extracted from primary cells derived from human aggressive fibromatosis tumours (n=5) treated in triplicates with DMSO and DMSO, showing a significant decrease in total beta-catenin protein levels in cells treated with Nefopam and equal loading from Actin staining. The means and 95% confidence intervals are shown. Statistically significant differences (p<0.05) compared to the control are indicated by asterisk above the bar.
Figure 10. Nefopam on cell viability of aggressive fibromatosis tumour and normal dermal tissue harvested from the same patient. Primary cells derived from tumours and normal tissue of the same patient (n=2) treated in triplicate with Nefopam and DMSO for 5 days, and cell viability as measured by staining with Trypan Blue Dye. Number of cells is shown as a relative percentage to vehicle control DMSO. Nefopam decreased the percentage of live cells of tumours more than that of normal dermal tissues while the number of dead cells did not change. The means and 95% confidence intervals are shown. Statistically significant differences (p<0.05) compared to the control are indicated by asterisk above the bar.
3 Hormonal Manipulation as Therapy for Aggressive Fibromatosis

3.1 Abstract

Aggressive fibromatosis is a benign fibroproliferative tumour characterized by the stabilization of beta-catenin levels. Tumours lack the ability to metastasize systemically but are able to locally invade into nearby tissue and organs, potentially leading to significant morbidity and mortality. Current therapies include surgery and radiotherapy but are often characterized by only a partial response associated with a high recurrence rate. Research focus has recently been placed on systemic chemotherapeutic strategies such as hormonal manipulation. When mice predisposed to developing aggressive fibromatosis underwent orchidectomy, the number of tumours was significantly reduced as compared to littermate controls, suggesting that androgens play a role in the development of tumours. Upon administering testosterone in orchidectomized aggressive fibromatosis mice, there was a rescued phenotype as indicated by the number of tumours formed comparable to non-orchidectomized mice. Testosterone treatment in orchidectomized mice resulted in a marked increase in beta-catenin levels in tumours. In vitro studies using primary cells derived from human aggressive fibromatosis tumours show that testosterone increases the number of viable cells. Together, our data demonstrates that testosterone increases beta-catenin levels and suggests that it promotes the development of aggressive fibromatosis. This work supports the current exploration of androgens as a possible therapeutic strategy against aggressive fibromatosis.

“Testosterone regulates cell proliferation in aggressive fibromatosis” submitted to British Journal of Cancer (Sept 2010) Hong, Helen; Nadesan Puvvidran; Poon, Raymond; Alman, Benjamin.
3.2 Introduction

Aggressive fibromatosis (also known as desmoids) is a locally invasive and benign, fibroproliferative tumour. Although they lack the ability to metastasize systemically, they are able to invade locally into nearby tissue and organs. This potentially leads to significant deformity, morbidity and mortality due to pressure effects and obstruction of vital structures and organs. Aggressive fibromatosis tumours may occur as a sporadic lesion or as a manifestation of familial disorders such as FAP. Most cases of sporadic tumours are associated with somatic mutations in genes coding for beta-catenin or APC, while familial cases are associated with germline mutations in the APC gene (Alman et al., 1997a; Tejpar et al., 1999). Tumours harboring such mutations are characterized by beta-catenin protein stabilization and activation of beta-catenin mediated TCF-dependent transcriptional activity (Alman et al., 1997a; Cheon et al., 2002).

Beta-catenin is an important signaling mediator in the canonical Wnt/beta-catenin signaling pathway, which is involved in several developmental and regenerative processes as well as neoplasia. The canonical Wnt/beta-catenin signaling pathway is initiated when activating Wnt ligands bind to cognate receptor complex comprised of a member of the seven-transmembrane-domain receptor of the Fz family and LRP5/6 co-receptor (Bhanot et al., 1996; Pinson et al., 2000). In the absence of activating Wnt ligands, cytoplasmic protein Dsh remains unphosphorylated and a multiprotein complex composed of scaffolding proteins APC, Axin, CK1gamma and GSK3beta is functional (Rubinfeld et al., 1996). Beta-catenin becomes phosphorylated by kinases CK1gamma and GSK3beta, which target beta-catenin for ubiquitin-mediated degradation (Aberle et al., 1997). In the presence of activating Wnt ligands, Dsh becomes phosphorylated and the multiprotein complex is destabilized. Stabilized beta-catenin is able to accumulate in the cytoplasm and translocate into the nucleus (Daniels and Weis, 2005). Together with TCF/LEF transcription factors, beta-catenin regulates the expression of downstream cell-specific target genes (Behrens et al., 1996; van de Wetering et al., 1997).

The role of sex hormones has been suggested in aggressive fibromatosis but its exact mechanism has yet to be elucidated. A recent study reported that 27 cases stained positive for not only estrogen and progesterone receptors but androgen receptors as well (Ishizuka et al., 2006). The well-established aggressive fibromatosis Apc+/Apc\textsuperscript{1638N} mouse model demonstrates that male
mice consistently develop an average of 46 tumours while females an average of 16 (Poon et al., 2001a; Smits et al., 1998).

These observations suggest that aggressive fibromatosis tumour development may be regulated by hormonal mechanisms. Studies of other beta-catenin-mediated tumours, most notably prostate cancer, have demonstrated interplay and communication between beta-catenin and androgen pathways. It is possible that beta-catenin activity may be modulated by androgen signaling during tumourigenesis. Thus, we examined the role of androgens in beta-catenin signaling in the development of aggressive fibromatosis tumours, using primary cultures derived from human AF tumours as well as the Apc^+/Apc^{1638N} AF mouse model.
3.3 Results

3.3.1 Castrated Apc⁺/Apc¹⁶³⁸N mice develop fewer aggressive fibromatosis tumours.

First we examined whether testosterone plays a role in and investigated the nature of this role in the development of aggressive fibromatosis. A group of male Apc⁺/Apc¹⁶³⁸N mice were castrated and tumour numbers were compared to those in non-castrated littermate control mice. The number of tumours formed in castrated Apc⁺/Apc¹⁶³⁸N mice was significantly reduced as compared to non-castrated mice (8.63±2.25 vs. 13.6±2.30, p<0.05, Figure 11 left) and similar to the number developed in female mice (8.63±2.25 vs 6.23±2.45, p<0.05, Figure 12). There were no significant differences in the number of upper gastrointestinal polyps formed between the study groups (p<0.05). These observations suggest that testosterone may play a role in aggressive fibromatosis and may promote the development of mesenchymal tumours.

3.3.2 Testosterone administration rescues aggressive fibromatosis phenotype in castrated Apc⁺/Apc¹⁶³⁸N mice.

To confirm that testosterone may contribute to the development of aggressive fibromatosis, castrated male Apc⁺/Apc¹⁶³⁸N mice were administered dihydrotestosterone to restore serum levels of testosterone comparable to that of littermate control mice. We found that testosterone produced a rescue effect from the castration in these mice. In comparing the development of tumours in castrated mice between those that were treated with Testosterone and those with No Treatment, we observed a marked increase the number of tumours as result of Testosterone treatment (12.63±2.46 vs. 8.63±2.25, p<0.05, Figure 11 left). As a vehicle control, a separate group of castrated male Apc⁺/Apc¹⁶³⁸N mice were treated with Olive Oil carrier. As expected, tumour load was comparable to that in castrated mice provided with No Treatment (6.63±2.16 vs. 8.63±2.25, p>0.05, Figure 11 left). In addition, the average volume (mm³) per tumour derived from orchidectomized Apc⁺/Apc¹⁶³⁸N mice treated with Olive Oil and Testosterone was determined. Mice treated with testosterone developed larger tumours than those treated with Olive Oil (9.12±3.07 vs. 4.15±2.08, p<0.05, Figure 11, right). This data strongly attributes the observed effect of induced aggressive fibromatosis tumour growth in castrated mice to Testosterone treatment.
3.3.3 Testosterone increases beta-catenin levels in tumours derived from castrated Apc\textsuperscript{+/Apc\textsuperscript{1638N}} mice.

Aggressive fibromatosis is characterized by an increase and stabilization of beta-catenin levels. Our lab has previously shown that stabilized beta-catenin alone is sufficient to induce the development of aggressive fibromatosis tumours (Cheon et al., 2002). To investigate whether beta-catenin levels are modulated amongst our castrated Apc\textsuperscript{+/Apc\textsuperscript{1638N}} mice, tumours from castrated mice treated with Olive Oil carrier and with Testosterone were harvested for protein extraction. Western Blot analysis using an antibody against total beta-catenin demonstrated a marked increase in the amount of protein at size 92kDa consistent with total beta-catenin in castrated mice treated with Testosterone compared to those treated with Olive Oil (Figure 13A). Beta-catenin levels from six primary cell cultures derived from human AF tumours increased with the level of dihydrotestosterone in a dose-dependent manner (Figure 13B).

3.3.4 Testosterone regulates cell proliferation in human aggressive fibromatosis tumour cells in vitro.

To examine how testosterone may be affecting cell behaviour, we used primary cells derived from human aggressive fibromatosis tumours. Using Trypan Blue Dye Exclusion assay, we assessed the number of live and dead cells as a result of treatment with Testosterone or DMEM media alone. In a dose response to increasing concentrations of dihydrotestosterone (1ng/ml to 100ug/ml), we found that there was a corresponding increase in the number of live cells of statistical significance (p<0.05, Figure 14A). We also noted that there were no significant differences in the number of dead cells as a result of treatment with Testosterone (p>0.05, Figure 13A). Dihydrotestosterone enhanced cell proliferation in a dose-dependent manner as indicated by the ratio of BrdU incorporation in cells (Figure 14B), and had no effect on apoptotic rates as measured by the level of positive TUNEL staining (Figure 14B). These results suggest that testosterone may be modulating proliferative programming instead of apoptosis to affect overall cell viability of aggressive fibromatosis primary cells.
3.4 Discussion

Aggressive fibromatosis is a fibroproliferative process mediated by dysregulated beta-catenin-mediated signaling, where mutations found in genes coding for APC and beta-catenin implicate a role of the canonical Wnt signaling pathway. However, mounting evidence suggest that sex hormone signaling pathways may also modulate beta-catenin-mediated transcription. Frequency of aggressive fibromatosis tumours was reported to be greater in women (McAdam and Goligher, 1970). Furthermore, the speed of growth of tumours is higher during pregnancy, in premenopausal compared to postmenopausal women, and in females relative to males (Reitamo et al., 1986). However, these observations have been greatly challenged by studies demonstrating the opposite, that there are no significant differences in tumour development between genders (Gurbuz et al., 1994; McAdam and Goligher, 1970). Apc+/Apc1638N mice have been well established as the mouse model to study FAP-driven aggressive fibromatosis. Male Apc+/Apc1638N develop an average of 46 tumours while female mice develop an average of 16 (Smits et al., 1998). To elucidate the potential role of sex hormones in aggressive fibromatosis, a recent study reported that 27 cases stained positive for not only estrogen and progesterone receptors but androgen receptors as well (Ishizuka et al., 2006). The exact mechanism of androgens in the development of aggressive fibromatosis remains unclear; however, the preliminary work presented here provides greater insight and implicates a pathogenic role of androgens. In this study, we showed that testosterone increased levels of beta-catenin and cell viability in primary cells derived from human aggressive fibromatosis tumours.

Androgens play an important role in the normal differentiation of reproductive organs and for the development and progression of prostate cancer (Verras and Sun, 2006). Androgen signaling is mediated through the androgen receptor, which is ligand-dependent nuclear hormone receptor (Gelmann, 2002). In the absence of an activating ligand, androgen receptors remain bound to heat shock proteins in the cytoplasm. In the presence of a ligand, androgen receptors translocate into the nucleus to bind androgen responsive elements and activate downstream transcription and expression of genes including prostate-specific antigen (Mulholland et al., 2002). Prostate cancer progression despite androgen ablation therapies has suggested the presence of other signaling pathways that could modulate the activity of androgen receptors aside from androgens themselves (Balk, 2002; Feldman and Feldman, 2001).
One identified co-regulator of the androgen receptor is beta-catenin, which has been suggested as one of the underlying causes to hormone refractory or androgen-independent prostate cancer (Chesire and Isaacs, 2003; Mulholland et al., 2002; Truica et al., 2000). Mounting evidence indicates that crosstalk between the androgen receptor and canonical Wnt pathways occur at several levels. Wnt ligands including Wnt3a have been shown to induce downstream androgen receptor-mediated transcription through an androgen-independent, beta-catenin-dependent manner (Verras et al., 2004; Verras and Sun, 2006). Furthermore, GSK3beta negatively regulates androgen receptor-mediated transcription (Salas et al., 2004; Sharma et al., 2002; Wang et al., 2004). It has been demonstrated that beta-catenin interacts with the androgen receptor to increase androgen receptor-mediated transcriptional activity (Masiello et al., 2004; Pawlowski et al., 2002; Truica et al., 2000; Yang et al., 2002). With an additional coactivator, competition for beta-catenin is created between androgen receptor and TCF/LEF transcription factors (Mulholland et al., 2003). This is demonstrated when ligand-bound androgen receptor represses Tcf-mediated transcription in prostate cancer cells (Chesire and Isaacs, 2002), neuronal cells (Pawlowski et al., 2002), and colon cancer cells (Mulholland et al., 2003). In contrast, androgen ablation renders beta-catenin available for activation of TCF/LEF target genes (Mulholland et al., 2003; Verras and Sun, 2006).

While these studies demonstrate that androgen receptor-mediated transcriptional activity could be induced in a Wnt or beta-catenin mediated manner, others have shown that TCF/LEF-mediated transcription could be induced by androgen receptor. It has been shown that androgen receptors could potentiate Wnt signaling in prostate cancer cells. Chromatin immunoprecipitation revealed that Wnt ligands induced the recruitment of the androgen receptor-beta-catenin complex to promoter regions of Wnt target genes including myc and cyclin D1. In contrast, addition of dihydrotestosterone significantly reduced LEF-dependent transcription, suggesting that androgens recruit beta-catenin to the androgen receptor-mediated signaling pathway, an observation further alluding to the possible competition for beta-catenin that exists between androgen receptor and TCF/LEF transcription factors (Schweizer et al., 2008). In mesenchymal multipotent C3H 10T1/2 cells, testosterone and dihydrotestosterone promoted nuclear translocation of the androgen receptor-beta-catenin complex as well as the physical interaction of androgen receptor, beta-catenin and TCF-4, resulting in the upregulated expression of several Wnt target genes such as myoD, myosin heavy chain II proteins and...
follistatin. These effects leading to myogenic differentiation were blocked upon addition of bicalutamide, an androgen receptor antagonist, suggesting that ligand bound androgen receptor has the capacity to form a complex with beta-catenin and TCF4. In rodents, orchidectomy lowered the expression of follistatin, an effect that was reversed by testosterone supplementation (Singh et al., 2009). While these studies are contingent on the idea of androgen receptor interacting directly with beta-catenin to activate Wnt target genes, evidence for a beta-catenin-independent, androgen-dependent TCF/LEF transcription has also been provided. Binding of androgen receptors to DNA could be enhanced by high mobility group box domains, which are found in TCF/LEF transcription factors. Functional assays have shown a direct interaction between the androgen receptor DNA binding domain and TCF4 (Amir et al., 2003). Furthermore, pull-down and chromatin immunoprecipitation assays demonstrated that androgen receptor associates with Tcf4 binding sites on the c-myc promoter independently of beta-catenin (Amir et al., 2003). Together, these studies indicate dynamic and direct interactions that may occur amongst beta-catenin, androgen receptor and TCF/LEF transcription factors to regulate downstream androgen receptor- and TCF/LEF-mediated transcription.

Using the castrated Apc⁺/Apc¹⁶³⁸N mouse model, we demonstrated that androgens could play a role in aggressive fibromatosis. Castrated Apc⁺/Apc¹⁶³⁸N mice developed significantly fewer tumours as compared to littermate controls while testosterone treatment in these mice restored tumour load or number of tumours as comparable to non-castrated Apc⁺/Apc¹⁶³⁸N mice. While studies in prostate cancer cells would have led us to believe that testosterone treatment diverted beta-catenin towards androgen-receptor-mediated signaling and away from TCF/LEF-mediated transcription to result in fewer prostate tumours, we observed the opposite effect in aggressive fibromatosis. This could be due to differences in cell types to explain the possibility that certain promoter regions or genes are inaccessible or silenced. Literature to date has yet to indicate any gene expression downstream of androgen receptor-mediated transcription in aggressive fibromatosis. Although previous studies have indicated a positive staining for the presence of androgen receptors (Leithner et al., 2005), expression of the androgen receptor gene is not regulated by androgen receptors in a positive feedback mechanism as androgen ablation is often accompanied by increased expression of the androgen receptor gene. Studies of the promoter region have identified that it contains long homopurine and homopyrimidine stretches, which binds to SP1 activator as well as cAMP-response element binding protein factor and AP2 to
mediate transcriptional activity (Faber et al., 1993; Mizokami et al., 1994). Therefore, it is possible that androgen response elements are inaccessible in aggressive fibromatosis tumour cells. As such, while androgens could induce the nuclear translocation of the androgen-receptor-beta-catenin complex, the complex would interact with TCF/LEF coactivators by default and activate the expression of downstream Wnt target genes, contributing to dysregulated beta-catenin signaling described in aggressive fibromatosis.

Androgen receptors are ligand-dependent nuclear hormone receptors (Gelmann, 2002), which comprises two functions: its transcriptional activity as well as its capacity to translocate the androgen ligand into the nucleus. Androgen ablation through castration in Apc⁺/Apc¹⁶³⁸N mice resulted only in a reduction of tumour numbers as opposed to complete elimination of tumours. This perhaps could be explained by the supplementary role of androgen receptors as a chaperone to shuttle beta-catenin into the nucleus. In the absence of androgens in castrated Apc⁺/Apc¹⁶³⁸N mice, aggressive fibromatosis tumours develop as cytoplasmic beta-catenin is shuttled into the nucleus by APC to activate TCF/LEF-mediated Wnt target genes. However, the presence or restoration of androgens, either endogenous or exogenous, provided beta-catenin with an additional chaperone to supplement translocation into the nucleus more effectively and further activate downstream Wnt target genes. Such interactions between ligand-bound androgen receptors and beta-catenin have previously been observed in prostate cancer cell lines where by addition of androgen analogue enhanced nuclear translocation of the androgen receptor-beta-catenin complex (Mulholland et al., 2002).

We demonstrated that testosterone treatment increased beta-catenin levels in tumours from castrated Apc⁺/Apc¹⁶³⁸N mice as compared to tumours from vehicle control castrated mice. This data does not address the exact mechanism of androgens through which it may effectively modulate beta-catenin levels and further investigation will be needed to clarify this. It should be noted that our methods of protein extraction account for protein found only within the cytoplasm and nucleus but exclude membrane proteins. Beta-catenin is involved in cell adhesion through interaction with the cytoplasmic domain of type I cadherins and plays an essential role in the structural organization and function of cadherins by linking cadherins through alpha-catenin to the actin cytoskeleton (Gumbiner, 2000; Jamora and Fuchs, 2002). Overexpression studies of cadherins have reduced the availability of beta-catenin by sequestering it at the cell membrane and thereby made it unavailable for signaling to the nucleus (Heasman et al., 1994; Sanson et al.,
1996). Immunohistochemistry studying the presence of cadherin/catenin adhesion complex in aggressive fibromatosis tumours have been performed and demonstrate positive staining for N-cadherin and alpha-catenin (Ferenc et al., 2009). As in the nucleus, perhaps androgen signaling could modulate the availability of beta-catenin in the cytoplasm through modulating the interactions between beta-catenin and cell membrane proteins. We also observed increased beta-catenin levels accompanied by increased tumour load in our Apc+/Apc\(^{1638N}\) mice in the presence of androgens. As a result, we expect that beta-catenin would activate the expression of TCF/LEF-mediated Wnt target genes, which have been shown to be involved in cell proliferation, providing further explanation to our in vitro data where we demonstrated that testosterone increased the number of viable cells while having a mild to no effect on the number of dead cells in primary cells derived from human aggressive fibromatosis tumours.

Taken together, our study provides greater insight to the dynamic role of androgens and androgen signaling in the development of aggressive fibromatosis tumours. Our data demonstrates that androgen receptor-blocking agents could serve as a novel therapeutic approach to aggressive fibromatosis. Future studies will include investigations of how androgens are able to upregulate the expression of beta-catenin or whether other signaling pathways upstream are able to modulate the crosstalk between androgen receptors and the canonical Wnt pathway. An understanding of how this could further underscore the importance of modulating beta-catenin levels as well as identify other pathways potentially involved in aggressive fibromatosis tumours.
3.5 Materials and Methods

3.5.1 Apc+/Apc\textsuperscript{1638N} AF mouse model and treatment strategy

The generation and phenotype of Apc\textsuperscript{+}/Apc\textsuperscript{1638N} mice have been well characterized. These mice harbour a targeted mutation at codon 1638 in the Apc gene as a result of a neomycin insert in antisense orientation at exon 15. Male mice develop an average of 45 aggressive fibromatosis lesions and 6 gastrointestinal polyps by the age of 6 months, while female mice develop significantly fewer tumours. At 6 weeks of age, male mice begin sexual maturation and testosterone levels range between 9.0-14.5 nM and remain at these levels for up to 1 year of age (Jones et al., 2003).

Male Apc\textsuperscript{+}/Apc\textsuperscript{1638N} mice underwent orchidectomy at 6 weeks of age as to abrogate normal androgen production prior to the establishment of testosterone serum levels. Mice were divided into four study groups: 1) Non-castrated with No Treatment (n=12), 2) Castrated with No Treatment (n=12), 3) Castrated with Olive Oil vehicle control (n=12) and 4) Castrated with Testosterone at 50 ug/g body weight. Drugs were administered by intra-muscular injections every 14 days as previously reported (Snyder et al., 2009). At the time of autopsy, aggressive fibromatosis tumours and upper gastrointestinal polyps were scored macroscopically. Tumours and normal tissue were harvested for protein extraction. High performance liquid chromatography was performed on collected serum samples to confirm drug uptake in mice.

3.5.2 Human aggressive fibromatosis tumour and normal tissue samples

Samples of human aggressive fibromatosis tumors were obtained at the time of surgery from the Hospital for Sick Children (Toronto, Ontario). Tumours and surrounding normal fascial tissue from the same patient were harvested and processed immediately after surgical excision. Tissues were cryopreserved and stored in liquid nitrogen vapour.

3.5.3 Cell culture studies

Primary cell cultures from the human aggressive fibromatosis tumours and normal fascial tissue samples were established. Monolayer cultures were cultured in DMEM supplemented with 10% fetal bovine serum and maintained at 37°C in 5% CO\textsubscript{2}. Cells were divided when confluent and experiments were performed only between the first and fifth passages. Prior to experimental
studies, $2 \times 10^4$ cells were seeded overnight and treatment began the following day where cells were treated with DMEM media or Testosterone. Cell viability was measured using the Trypan Blue Dye Exclusion method. Cells were stained with Trypan Blue Dye at a 1:1 ratio, and both live (clear) and dead (blue) cells were tallied. Proliferation was measured using bromodeoxyuridine (BrdU) incorporation and apoptosis measured using the TUNEL assay, using previously reported techniques (Li et al., 2001). The percent BrdU incorporation and number of TUNEL stained cells were counted over ten high-powered fields from three independent culture flasks for each patient sample.

3.5.4 **Protein extraction and Western Blot Analysis**

Samples were washed twice with PBS and lysed with Reporter Gene Assay Lysis Buffer (Roche). Lysates were centrifuged at 16,000 x g 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, transferred to a nitrocellulose membrane (Amersham), and immunoblotted overnight at 4°C with primary antibodies against phosphoGSK3β (Ser 9, rabbit polyclonal, New England Biolabs), total GSK3β (mouse monoclonal, Transduction Laboratories), active beta-catenin (mouse polyclonal, Upstate Biotechnology), total beta-catenin (mouse monoclonal), and GAPDH (mouse monoclonal, Upstate Biotechnology). Horseradish peroxidase-tagged secondary antibodies and Enhanced ChemiLuminescence (Amersham) were used to detect hybridization. Densitometry was performed using the AlphaEaseFC software (Alpha Innotech).

3.5.5 **Statistical analysis**

Power calculations were used to determine the minimum number of mice required in study groups for biological significance. Data in this work were calculated as mean ± 95% confidence intervals. Studies were performed in triplicates to ensure reproducibility.
3.6 References


catenin dysregulation in sporadic aggressive fibromatosis (desmoid tumor). Oncogene 18, 6615-6620.


3.7 Figures

Figure 11. The number and volume of aggressive fibromatosis tumours in Apc<sup>+/−</sup>/Apc<sup>1638N</sup> mice. Non-castrated Apc<sup>+/−</sup>/Apc<sup>1638N</sup> mice (n=12) and castrated Apc<sup>+/−</sup>/Apc<sup>1638N</sup> mice treated with No Treatment (n=12), Olive Oil Carrier (n=12), or Testosterone (n=12) were analyzed for number and volume of tumours. (Left) Average number of tumours and (Right) average volume (mm<sup>3</sup>) per tumour derived from Apc<sup>+/−</sup>/Apc<sup>1638N</sup> mice. The means and 95% confidence intervals are shown. Statistical significant differences (p<0.05) compared to controls are indicated with an asterisk above the bar.
Figure 12. The number of aggressive fibromatosis tumors in five-month-old Apc1638N mice. Castrated mice develop a similar number of tumors as female mice. Data is given as means and 95% confidence intervals. An asterisk above data shows a significant difference from castrated male mice.
Figure 13. Testosterone modulates beta-catenin levels in aggressive fibromatosis tumours. 
(A) Western Blot analysis from tumours harvested from castrated Apc\textsuperscript{+}/Apc\textsuperscript{1638N} mice treated with Testosterone (n=3) and Carrier (n=3), showing a marked increase in total beta-catenin protein levels in tumours harvested from mice treated with increasing concentration of Testosterone. GAPDH staining indicates equal loading.  
(B) Dose-dependent increase of beta-catenin levels in primary cells derived from AF tumours treated with Testosterone. The means and 95% confidence intervals are shown. Statistical significant differences (p<0.05) compared to controls are indicated with an asterisk above the bar.
**Figure 14. Testosterone increases cell viability of human aggressive fibromatosis tumours in vitro.** Primary cells derived from human aggressive fibromatosis tumour (n=1) treated in triplicates with increasing concentration of dihydrotestosterone or DMEM media alone for a period of 5 days. (A) Cell viability as measured by staining cells with Trypan Blue Dye and counted for both live and dead cells. Testosterone significantly increased the number of live cells while the number of dead cells did not change. (B) Proliferation as measured by the proportion of cells with positive BrdU incorporation. (C) Apoptosis as measured by the proportion of cells with positive TUNEL staining. The means and 95% confidence intervals are shown. Statistical significant differences (p<0.05) compared to controls are indicated with an asterisk above the bar.
Chapter 4

4 Summary, Conclusion and Future Experiments

4.1 Summary

Aggressive fibromatosis is a benign fibroproliferative tumour characterized by the stabilization of beta-catenin levels. Tumours occur either as sporadic lesions or as a manifestation in familial syndromes. Genetic analysis of aggressive fibromatosis tumours implicates a tumourigenic role of the canonical Wnt/beta-catenin signaling pathway. We were interested in pharmacological agents with the capacity to modulate beta-catenin levels and inhibit the development of aggressive fibromatosis tumours.

Using the MicroSource Spectrum Compound library, our lab identified Nefopam with the potential to inhibit cell viability of human aggressive fibromatosis tumours but not normal fibroblasts from the same patients. In Chapter 2, primary cells derived from a number of human aggressive fibromatosis tumours were treated with the analgesic agent. We demonstrated that Nefopam inhibits cell viability and proliferation, as well as reduces beta-catenin protein levels in vitro. Tumour counts from male Apc\(^+\)/Apc\(^{1638N}\) mice treated daily for 3 months indicate that Nefopam has the capacity to significantly reduce the number of tumours formed in vivo.

Aggressive fibromatosis tumours have been shown to be associated with sex hormones. In Chapter 3, we found that orchidectomized male Apc\(^+\)/Apc\(^{1638N}\) mice displayed a significantly smaller number of tumours, suggesting that androgens play a role in the development of tumours. We demonstrated that upon administering testosterone in orchidectomized Apc\(^+\)/Apc\(^{1638N}\) mice, there was a rescued phenotype with a tumour count similar to non-orchidectomized mice. Furthermore, testosterone treatment resulted in a marked increase in beta-catenin levels in tumours. In vitro studies using primary cells derived from human AF show that testosterone increases the number of viable cells. Our data suggests that testosterone plays a role in the development of aggressive fibromatosis and can modulate beta-catenin levels in mesenchymal cells.
4.2 Future Experiments

Our work has demonstrated that Nefopam and androgens have the capacity to modulate total beta-catenin levels; however, several questions remain unanswered and it is unclear as to how exactly these pharmacological agents elicit their biological effects.

4.2.1 Mechanism of beta-catenin regulation

Aggressive fibromatosis is characterized by the stabilization and elevated levels of beta-catenin. Our studies demonstrated that Nefopam decreases beta-catenin levels while androgens increase beta-catenin levels. In the canonical Wnt pathway, beta-catenin is primarily regulated through phosphorylation at specific serine and threonine residues by GSK3beta, leading to its ubiquitination and degradation. As such, the investigation of the mechanism by which beta-catenin is regulated could begin by performing Western Blot analysis blotting for the phosphorylation status of GSK3beta. Like beta-catenin, GSK3beta activity is determined by its phosphorylation at particular sites including serine 9 and 21. Given the role of GSK3beta, beta-catenin stability and activity could also be determined through Western Blot analysis blotting for phosphorylation at specific serine or threonine sites, which directs beta-catenin to ubiquitin-mediated degradation. Alternatively, regulation of beta-catenin levels could also be mediated through its subcellular localization. In epithelial tissues, E-cadherin complexes with the actin cytoskeleton through interaction with cytoplasmic catenins. The cadherin-bound pool of beta-catenin could be released to participate in downstream signaling. As such, the localization may account for changes observed in the levels of beta-catenin and could be detected by performing immunohistochemical analysis. It is unclear as to whether such cadherin-catenin complexes play a role in aggressive fibromatosis, though previous studies have demonstrated positive staining for N-cadherin in tumour cells. Beta-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, beta-catenin levels could be regulated through ubiquitination and degradation, which could be detected by performing ubiquitination assays, as well as through its production at the transcriptional levels, which could be detected by measuring CTNNB1 mRNA transcript levels using quantitative polymerase chain reaction.
4.2.2 Regulation and expression of Wnt target genes

Several groups including our lab have previously demonstrated a general localization of beta-catenin in the nucleus in aggressive fibromatosis tumours. Stabilized beta-catenin is able to translocate into the nucleus and together with TCF/LEF transcription factors activate the expression of downstream cell-specific Wnt target genes. In our study, we found that Nefopam decreased while testosterone increased total beta-catenin levels in aggressive fibromatosis tumours. To determine whether these pharmacological agents have an effect on the subsequent transcriptional function of beta-catenin, a TCF-reporter assay could be performed. Primary cells could be transiently transfected with TCF-LEF luciferase reporter construct pTOPFLASH or the control reporter pFOPFLASH, which contains mutant TCF-LEF consensus binding sequence. To control for transfection efficiency, cells would also be transfected with a beta-galactosidase expression vector. Doubly transfected cells treated with the pharmacological agents of interest would be measured for luciferase enzyme activity detected by a luminometer and normalized to beta-galactosidase activity. Relative luminescence activity would indicate potential effects these pharmacological agents may have on transcriptional activity as a result of modulating total beta-catenin protein expression levels.

As described by the “just right” hypothesis about beta-catenin signaling, various transcriptional programs are activated as a result of different levels of nuclear beta-catenin levels present. This is evident in several mouse models with mutations in the Apc gene producing an array of beta-catenin regulatory capacities of the Apc protein and resulting consistently with certain phenotypic manifestations. We observed that Nefopam decreased total beta-catenin levels and proliferation of primary cells derived from human aggressive fibromatosis tumours, and that testosterone increased total beta-catenin levels and the number of viable cells. Such biological changes would be better understood upon establishing differential gene expression profiles of treated tumours. To determine the gene expression profile modified by these pharmacological agents, real-time polymerase chain reaction could be performed on extracted RNA. Differential gene expression profiles would be established and indicate downstream genetic consequences of these pharmacological agents to elicit such phenotypic observations both in vitro and in vivo.
4.3 Concluding Remarks

Aggressive fibromatosis is a fibroproliferative tumour that can occur as a sporadic lesion or a manifestation in familial syndromes such as FAP. Tumours are characterized by the stabilization and elevated levels of beta-catenin. Despite the variety of therapeutic strategies available, current therapies have yet to demonstrate total success for primary and recurrent tumours. As such, aggressive fibromatosis remains a clinical challenge and there remains a need for more effective therapeutic strategies. This research sheds light on the anti-neoplastic properties of Nefopam as a possible adjuvant therapy, the potentially pathogenic role of androgens, and the implication of androgen signaling as a novel therapeutic target in aggressive fibromatosis.
Appendices

5 Alpha-tocopherol Acetate

5.1 Summary

Fibroproliferative processes are a group of disorders characterized by an excessive proliferation of mesenchymal fibroblast-like spindle cells, ranging from hypertrophic wounds to neoplasms such as aggressive fibromatosis. During wound healing, several cell types and signaling pathways are activated to reconstitute the epithelial and dermis layers of the skin. Following cutaneous injury, three sequentially distinct but overlapping processes are initiated: inflammation, proliferation and remodeling. During the proliferative phase, mesenchymal cells accumulate in the dermal component of the skin while the epithelial cell barrier is reformed (Singer et al., 1999, Martin et al., 1997, McClain et al., 1996). Beta-catenin has been shown to mediate epithelial and mesenchymal cell activity; it increases proliferation and differentiation in dermal mesenchymal cells and decreases migration in epithelial keratinocytes (Cheon et al., 2002). Mouse models have demonstrated that beta-catenin can modulate wound sizes. Induced levels of beta-catenin by lithium treatment result in wound healing of larger size (Cheon et al., 2006). A transgenic mouse in which stabilized beta-catenin is expressed in mesenchymal cells under control of a tetracycline-regulated promoter was generated. Wounded mice healed with hyperplastic scars compared to wildtype control (Cheon et al., 2002). Furthermore, stabilized beta-catenin is sufficient for these mice to spontaneously develop aggressive fibromatosis tumours, indicating that hypertrophic wounds and aggressive fibromatosis are both characterized by elevated levels of beta-catenin. Alpha-tocopherol acetate is the metabolized and biologically active form of vitamin E. The effect of vitamin E on wound healing is complex. It is shown to penetrate into the dermis and reduce the formation of oxygen radicals that impede healing and damage DNA, cellular membranes and lipids (Zurada et al, 2006). Vitamin E is thought to be a membrane-stabilizing agent, as demonstrated by its ability to alter collagen and glycosaminoglycan production and inhibit the spread of peroxidation of lipids in cellular membranes (Zurada et al., 2006). As aggressive fibromatosis and hypertrophic scars are both characterized by elevated levels of beta-catenin, we investigated whether vitamin E would prove to be useful against aggressive fibromatosis tumours.
5.2 Figures

Figure 14. Alpha-tocopherol acetate treatment does not affect the number of tumours developed in Apc\textsuperscript{+}/Apc\textsuperscript{1638N} mice. Average number of tumours and gastrointestinal polyps formed in Apc\textsuperscript{+}/Apc\textsuperscript{1638N} male mice were counted after 3 months of daily oral gavaging of No Treatment (n=11), 0.1% DMSO (n=10), and alpha-tocopherol acetate (n=10). Mice treated with alpha-tocopherol acetate developed number of tumours comparable to that found in mice provided with No Treatment or DMSO. Differences in the number of polyps formed were not observed amongst the study groups. The means and 95% confidence intervals are shown. Statistical significant differences (p<0.05) compared to controls are indicated with an asterisk above the bar.
Figure 15. Alpha-tocopherol acetate deficiency in Apc\(^+\)/Apc\(^{1638N}\) male mice display similar number of aggressive fibromatosis tumours as compared to littermate controls. To investigate whether deficiency in alpha-tocopherol acetate would affect the development of aggressive fibromatosis tumours, Apc\(^+\)/Apc\(^{1638N}\) mice were crossed with Ttpa\(^-\)/- mice, which are homozygous null for the tocopherol (alpha) transfer protein required for the systemic maintenance of alpha-tocopherol levels. Ttpa\(^-\);Apc\(^+\)/Apc\(^{1638N}\) mice were evaluated for tumours and gastrointestinal polyps formed as compared to littermate controls, Ttpa\(^+\)/+;Apc\(^+\)/Apc\(^{1638N}\) and Ttpa\(^-\);Apc\(^+\)/Apc\(^{1638N}\) mice. There were no differences in the number and size of aggressive fibromatosis tumours and number of polyps formed amongst the study groups. The means and 95% confidence intervals are shown. Statistical significant differences (p<0.05) compared to controls are indicated with an asterisk above the bar.
5.3 References


