EPIGENETIC MODIFICATION OF PHENOTYPE IN INTESTINAL SMOOTH MUSCLE CELLS

by

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Abstract

Inflammation causes proliferation of intestinal smooth muscle cells (ISMС), contributing to the thickened intestinal wall and the dysmotility symptoms observed in inflammatory bowel disease (IBD). A unique consequence of Crohn’s disease (CD), a subset of IBD characterized by chronic, transmural intestinal inflammation, is the formation of obstructive strictures attributable to ISMC hyperplasia. *In vitro*, prolonged proliferation of ISMC results in loss of their normal contractile phenotype, including decreased expression of contractile markers and the neurotrophin glial cell-line derived neurotrophic factor (GDNF). In other diseases of smooth muscle hyperplasia, epigenetic modifications, including DNA methylation and histone acetylation, affect smooth muscle phenotype and contribute to impaired tissue function. Any epigenetic role in regulating gene expression and the contractile phenotype of ISMC is unknown.

Repeated passage ISMC were treated with trichostatin A (TSA) and 5-azacytidine (AZA), which inhibit enzymes that deacetylate histones and methylate DNA, respectively, and the outcomes on phenotype were assessed. TSA and AZA treatment decreased the enhanced growth response of repeated passage ISMC, and increased the expression of contractile markers, SMA and SM22. Repeated passage cells treated with TSA and AZA increased expression of muscarinic receptor 3, myosin light chain kinase 2, and showed an increased response to carbachol stimulation. TSA and AZA treatment also increased GDNF expression, and improved the neurotrophic function of repeated passage ISMC. Compared to low passage ISMC, repeated passage cells had increased DNA methyltransferase 1 (DNMT1) and histone deacetylase 2 (HDAC2) expression. ISMC from human strictures showed a decreased contractile phenotype, with increased levels of DNMT1 and HDAC5. Repeated passage human ISMC also had decreased expression of contractile markers SMA and SM22, mimicking the rat model. These findings suggest that the altered contractile phenotype of ISMC is associated with epigenetic changes, including increased DNA methylation and histone deacetylation. Furthermore, this evidence supports a potential therapeutic role for improving the contractility of ISMC, and restoring proper motility in CD patients.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ASMC</td>
<td>airway smooth muscle cells</td>
</tr>
<tr>
<td>AZA</td>
<td>5-azacytidine</td>
</tr>
<tr>
<td>CArG</td>
<td>CC(A/T)$_6$GG</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNBS</td>
<td>2,4-dinitrobenzene sulfonic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ENS</td>
<td>enteric nervous system</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial cell-line derived neurotrophic factor</td>
</tr>
<tr>
<td>H/H</td>
<td>Hank’s balanced salt solution/4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetyltransferases</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid</td>
</tr>
<tr>
<td>HPRT</td>
<td>hypoxanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
</tr>
<tr>
<td>IOD</td>
<td>integrated optical density</td>
</tr>
<tr>
<td>ISMC</td>
<td>intestinal smooth muscle cells</td>
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</table>
M2 muscarinic receptor 2
M3 muscarinic receptor 3
MLCK myosin light chain kinase
MMP-9 matrix metalloproteinase 9
NM-ISM C normal margin intestinal smooth muscle cells
PAGE polyacrylamide gel electrophoresis
pAKT phosphorylated protein kinase B
PBS phosphate buffered saline
PBS-T phosphate buffered saline with 0.2% Tween-20
PCNA proliferating cellular nuclear antigen
PDGF-BB platelet-derived growth factor-BB
PDGF-Rβ beta type platelet-derived growth factor receptor
qPCR quantitative polymerase chain reaction
RPM revolutions per minute
SDS sodium dodecyl sulfate
SM22 SM22-α
SMA smooth muscle α-actin
SMC smooth muscle cell
SNAP-25 synaptosomal-associated protein 25
SX-ISM C strictured intestinal smooth muscle cells
TBS tris-buffered saline
TBS-T tris-buffered saline with 0.1% Tween-20
TCA trichloroacetic acid
TH T helper cell
TNBS 2,4,6-trinitrobenzene sulfonic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>UC</td>
<td>ulcerative colitis</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cells</td>
</tr>
<tr>
<td>Wst-8</td>
<td>water soluble tetrazolium salt-8</td>
</tr>
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Chapter 1

Introduction

1.1 The Neuromuscular Layer of the Gastrointestinal Tract

1.1.1 General Structure of the Gastrointestinal Tract

The gastrointestinal tract is a long hollow tube comprised of functionally distinct layers, each carrying out specific digestive functions. The innermost layer, termed the mucosa, is lined with epithelial cells which provide a physical barrier from luminal contents, separating the gastrointestinal tract from the external environment. Functionally, epithelial cells absorb nutrients, secrete fluids and antimicrobial agents into the lumen, and release a variety of hormones important in digestion. Below the epithelia lies the lamina propria, which contains immune cells and the muscularis mucosae; a thin muscular layer that moves the epithelia towards the lumen. The submucosa, a layer of connective tissue enriched with blood and lymphatic vessels, separates the mucosa from the outer muscular layers, termed the muscularis externa (Fig. 1). The muscularis externa is comprised of smooth muscle cells that contract and relax to mix luminal contents, and propel contents in an aboral direction.

All aspects of digestion are controlled by the enteric nervous system (ENS); an intrinsic neural network that runs throughout the gastrointestinal tract. The ENS is made up of two layers, each consisting of interconnected neurons housed in ganglia. The submucosal plexus, situated in the submucosa, is mostly responsible for mucosal absorption, secretion, and muscularis mucosae contractility (Barrett et al., 2006). The myenteric plexus is situated between the inner circular and outer longitudinal smooth muscle layers of the muscularis externa (Fig. 1), and principally innervates the surrounding smooth muscle cells.
Figure 1. Schematic illustration of the layers of the gastrointestinal tract. This image represents a cross-section through the small intestine, revealing the circular smooth muscle layers of the muscularis externa, and the myenteric and submucosal plexus of the ENS. (Modified from Smout and Akkermans, 1992).
Innervation of the muscularis externa regulates contraction and relaxation of smooth muscle cells, and thus accomplishes motility of the gastrointestinal tract. Circular smooth muscle cells are oriented circumferentially, and decrease the diameter of the lumen when they contract. Longitudinal smooth muscle cells are positioned parallel to the lumen, and shorten the length of the intestine when they contract.

1.1.2 Regulation of Intestinal Smooth Muscle Contraction

Since Bayliss and Starling (1899) originally described the motor patterns of the intestine, it is well-accepted that intestinal smooth muscle cells (ISMС) are excitable and subject to neural and myogenic inputs. Although traditionally referred to as myogenic, non-neural rhythmic contractions of ISМС are coordinated by the pacemaker cells of the gut, called interstitial cells of Cajal. Interstitial cells of Cajal generate slow waves which propagate to other cells and spread to adjacent ISМС by gap junctions. ISМС are also inter-connected by gap junctions, allowing Interstitial cells of Cajal-derived electrical events to spread to many ISМС, causing rhythmic and synchronous smooth muscle contractions.

Smooth muscle contractions are also stimulated by ENS innervation from the myenteric plexus. Neurotransmitters released by myenteric neurons are recognized by receptors on ISМС surface, which activate intracellular signaling pathways that lead to contraction. Acetylcholine, the major excitatory neurotransmitter in the intestine, is recognized by muscarinic receptors 2 and 3 (M₂, M₃), although only M₃ receptors are involved in mediating ISМС contraction (Murthy, 2006). Coupled to membrane bound Gα₉ protein, M₃ receptor activation leads to hydrolysis of phosphatidylinositol 4,5-biphosphate by phospholipase C, producing inositol triphosphate and diacylglycerol. Inositol triphosphate then activates its receptors on the sarcoplasmic reticulum, leading to the release of stored Ca²⁺, activating calmodulin. Downstream signaling of Ca²⁺-activated calmodulin activates myosin light chain kinase (MLCK) phosphorylation of the 20-kDa
regulatory light chain of myosin II, initiating actin and myosin cross-bridge cycling, ultimately leading to ISMC contraction.

Contraction of ISMC thus relies on the coordinated activity of receptors, intracellular signaling proteins, and contractile elements. Consequently, expression of these contractile proteins is imperative to proper ISMC function. Factors which alter expression of these proteins, such as inflammation of the gut, can compromise the contractile ability of ISMC, and therefore disrupt normal intestinal motility.

1.2 Inflammatory Bowel Disease

Activation of the immune system housed in the gut, which might arise from an invading pathogen, can result in an inflammatory response. Inflammation can be superficial and only affect the mucosa, or transmural affecting each layer of the intestine, including the neuromuscular layer. Dysmotility of the intestine can arise from altered structure and function of smooth muscle during transmural inflammation. Symptoms of dysmotility are apparent in diseases of intestinal inflammation, emphasizing the importance of understanding how inflammation affects the smooth muscle layers (Bernardini et al., 2012).

Chronic intestinal inflammation is characteristic of inflammatory bowel disease (IBD), an idiopathic disease which affects approximately 1 in every 1,000 individuals in the western world (Molodecky et al., 2012). IBD consists of two main subtypes; ulcerative colitis (UC) and Crohn’s disease (CD). The incidence rates of IBD are 19.2 and 20.2 per 100,000 people for UC and CD in Canada, respectively, which are expected to increase since the disease is usually diagnosed at a young age (Molodecky et al., 2012). The causes of IBD are currently unknown, although a combination of genetics, environmental exposures, the immune system, or the microbiota is thought to underlie its etiology.
1.2.1 The Pathogenesis of Crohn’s Disease

The two main forms of IBD differ in their location of disease and regions affected by inflammation (Venema et al., 2017). In UC, inflammation largely affects the mucosal layer, and the disease is always limited to the colon and rectum. In CD, inflammation affects all layers of the gut and can arise anywhere throughout the gastrointestinal tract. CD patients typically present with symptoms suggestive of dysfunction to each layer of the gut, including abdominal pain, fever, diarrhea, bloody stool, and weight loss (Baumgart & Sandborn, 2012; Kim & Cheon, 2017; Venema et al., 2017). The inflammatory response in CD is thought to be Th1 mediated, as evidenced by increased Th1-released IFN-γ (Breese et al., 1993; Fuss et al., 1996) and IL-2 (Breese et al., 1993) levels in CD patients. Recent work also suggests a role for Th17 cells in driving the inflammation of CD (reviewed in Antoniou et al., 2016).

Although CD can affect the whole gastrointestinal tract, the most common site for inflammation is the terminal ileum, followed by the colon and ileocolon region. A unique consequence of chronic inflammation in CD is the development of intestinal strictures, which are non-compliant regions of the intestine that cause obstruction. In a 2001 study from the University of British Columbia, 33.6% of patients assessed over a 20 year period had stricturing CD (Freeman, 2001). Similarly, a Norwegian study found that 28% of CD patients had strictures at the time of diagnosis (Henriksen et al., 2007). Additionally, patients initially free of strictures might go on to develop strictures during the course of their disease. Progression to stricturing behavior of CD increased from 11% to 48-52% 5 years after diagnosis, and to 69-70% 10 years after diagnosis in another study, suggesting that virtually all CD patients will present with stricturing complications if tracked over time (Louis et al., 2001).

Stricture formation arises from improper activation of mesenchymal cells following inflammatory insults in CD patients (reviewed in Li and Kuemmerle, 2014). Activated ISMC contribute to stricture development in three ways; excessive production and deposition of extracellular matrix (Graham et al., 1987; Severi et al., 2014), proliferation and infiltration
throughout the submucosa and mucosa (Koukoulis et al., 2001), and hypertrophy, contributing to the thickened intestinal wall (Gelbmann et al., 1999). Although all three events occur in stricture formation, ISMC hyperplasia and hypertrophy are more prominent features than fibrosis, suggesting that strictures are developed from inflammation-induced ISMC changes (Chen et al., 2017). The mechanisms behind the hyperplastic and hypertrophic response of ISMC in stricture formation are not well understood, emphasizing the importance of research in animal models.

Given the prevalence of stricture formation in CD, the development of effective treatment options and therapies for strictures is of high interest. Unfortunately to date, no specific therapies exist for treating intestinal strictures, although some preliminary data suggests a beneficial role of anti-TNF-α biologics (Feagan et al., 2008). Other preliminary results suggest that mechanical manipulations including stricturoplasty and endoscopic dilation with a balloon might be effective for treating strictures (reviewed in Rieder et al., 2013). However, the most common treatment option is surgical resection of the strictured region, which is required in up to 80% of CD patients 10 years after their disease diagnosis (Farmer et al., 1985). In fact, intestinal strictures are the leading cause of surgery for CD patients (Chang et al., 2015). Unfortunately, surgery is not a cure for stricture formation in CD; more than 1/3 of patients have stricture recurrence post-surgery (Buisson et al., 2012; Ng et al., 2007), and 85% of patients have endoscopically identified recurrence (Ng et al., 2007). Repeated surgeries to remove multiple strictures can lead to further disease complications, including short bowel syndrome.

Understanding the cellular and molecular pathways involved in stricture formation and recurrence will be important in developing appropriate treatments, and prevent the need for repeated surgeries. Since strictures are defined by ISMC hyperplasia and hypertrophy (Chen et al., 2017), it is important to understand the effects of intestinal inflammation on smooth muscle structure. Our understanding of inflammation-induced changes to ISMC have mostly derived from
animal models of intestinal inflammation (Blennerhassett et al., 1992; Lourenssen et al., 2005; Marlow & Blennerhassett, 2006).

1.3 Effects of Intestinal Inflammation on the Neuromuscular Layer in Animal Models

1.3.1 Animal Models of Intestinal Inflammation

Chemically induced colitis is the most commonly used animal model of intestinal inflammation, since it is easy to control and reproduce (Alex et al., 2009; Perše & Cerar, 2012). Alternative experimental models include bacterial induction of colitis, genetically modified animals, and adoptive cell transfer.

The main inducible models of intestinal inflammation involve the administration of dextran sulfate sodium or 2,4,6-trinitrobenzene sulfonic acid (TNBS), respectively. Although both induce colitis, TNBS treatment induces transmural inflammation of the intestinal wall, and is therefore a better model to assess changes to the neuromuscular layer.

TNBS triggers colitis by binding to high molecular weight proteins and making them antigenic, eliciting an immune response. TNBS is co-administered with ethanol intra-rectally to disrupt the epithelial barrier, allowing TNBS to reach the mucosa. TNBS-induced transmural inflammation involves infiltration of leukocytes into the mucosa, submucosa, and muscularis externa (reviewed in Antoniou et al., 2016). Both acute and chronic TNBS-induced colitis are mediated by Th1/Th17 immune responses as evidenced by increased IFN-γ, IL-12, and IL-17 levels (Alex et al., 2009). The use of 2,4-dinitrobenzene sulfonic acid (DNBS) was an earlier alternative for TNBS, and induces colitis in a similar fashion.

1.3.2 Early Effects of Intestinal Inflammation on the Neuromuscular Layer

Experimental models of intestinal inflammation that cause transmural inflammation, such as TNBS, affect the structure and function of the underlying neuromuscular layer, which might be responsible for the dysmotility symptoms observed in these animals. In a DNBS model of induced
colitis, significant loss of myenteric and submucosal neurons occurred as early as 24 hr. post DNBS-treatment (Sanovic et al., 1999). Later work confirmed that loss of myenteric neurons was an early event in DNBS-treated mice (Boyer et al., 2005), and in TNBS-treated guinea-pigs (Linden et al., 2005; Nurgali et al., 2011). Although the mechanisms of inflammation-induced neuron death are not completely known, immune cells infiltrate the myenteric ganglia 24 hr. post DNBS (Sanovic et al., 1999) and TNBS treatment (Linden et al., 2005), and are thought to produce nitric oxide which can directly kill neurons (Venkataramana et al., 2015). Other evidence suggests that the pro-inflammatory milieu can stimulate caspase-3 mediated apoptosis in neurons (Boyer et al., 2005; De Giorgio et al., 2003). Since myenteric neurons innervate the smooth muscle layers to coordinate contraction, inflammation-induced changes to the ENS will also affect ISMC function.

In addition to damaging the ENS, transmural inflammation also causes structural changes to the muscularis externa. The circular and longitudinal smooth muscle layers were thickened 6 days post infection of *Trichinella spiralis*, which was associated with hyperplasia and hypertrophy of intestinal smooth muscle cells (Blennerhassett et al., 1992; Hogaboam et al., 1995). Proliferation of ISMC was apparent 24 hr. post TNBS-treatment, and peaked 6 days post treatment (Lourenssen et al., 2005). The mechanisms behind this hyperplastic response of ISMC are not fully understood, although it is suggested that pro-inflammatory cytokines increase ISMC growth response to platelet-derived growth factor, a mitogen present in the inflamed milieu (Nair et al., 2014; Stanzel et al., 2010). It is also suggested that innervation of ISMC by myenteric neurons sustains their quiescent, non-proliferative state, and that loss of innervation might promote ISMC growth (Blennerhassett & Lourenssen, 2000; Pelletier et al., 2010).

Decreased axon density in the smooth muscle layers occurred as early as 1 day post TNBS-induced colitis in the rat (Lourenssen et al., 2005). Additionally, axons from inflamed regions appeared damaged and fragmented 1 day post TNBS-treatment (Lourenssen et al., 2005; Nurgali et al., 2011). Loss of smooth muscle innervation might prevent neurotransmitter-coordinated
contractions, or induce ISMC growth, both of which can lead to intestinal dysmotility. While ISMC hyperplasia, neuronal death, and decreased innervation are early events in experimental models of colitis, it is important to assess the long-term consequences on tissue structure and function, since IBD is a chronic, inflammatory disease.

1.3.3 Permanent Effects of Intestinal Inflammation on the Neuromuscular Layer

IBD is characterized by life-long, chronic intestinal inflammation. (Jimenez et al., 2015). Although it is difficult to model chronic inflammation in animals, TNBS-induced colitis is resolved 16 days post-treatment in rats and 28 days post-treatment in the guinea-pig (Krauter et al., 2007; Wells & Blennerhassett, 2004). Structural and functional changes to the neuromuscular layer provide insights into the lasting consequences that are present at these time points, and therefore persist after the resolution of intestinal inflammation.

Neuronal loss persists following the resolution of DNBS and TNBS-induced colitis in rats (Lin et al., 2005; Sanovic et al., 1999) and TNBS-treated guinea-pigs (Linden et al., 2005), showing that chronic inflammation can decrease myenteric neuron number. Colitis-induced increases in ISMC peaked at 6 days post TNBS treatment, and was maintained at that level up to 35 days post TNBS-treatment, suggesting increased ISMC number is a lasting consequence of intestinal inflammation (LourensSEN et al., 2005).

Decreased axon density in the smooth muscle layers was reversed by 6 days post TNBS treatment, and maintained following the resolution of inflammation (LourensSEN et al., 2005). These findings are consistent with the notion that myenteric innervation of ISMC suppresses their growth response, since re-innervation prevented any further hyperplasia. The ability of surviving neurons to undergo axonal outgrowth during acute inflammation is thought involve glial cell-line derived neurotrophic factor (GDNF), secreted from proliferating ISMC (Han et al., 2015). The pro-inflammatory cytokines TNF-α and IL-1β, which are implicated in TNBS-induced colitis, increase GDNF expression by ISMC, which can induce axon outgrowth in vitro (Gougeon et al., 2013;
Rodrigues et al., 2011). This suggests that the pro-inflammatory environment induces GDNF-mediated axonal outgrowth in early colitis, which restores innervation to normal levels in the smooth muscle layers.

Despite restored innervation to the smooth muscle layers, symptoms of dysmotility persist in the chronically inflamed intestine (reviewed in Scirocco et al., 2016), suggesting permanent alterations to the ISMC. ISMC isolated from rats treated with TNBS for 35 days appeared phenotypically altered compared to ISMC isolated from non-inflamed controls (Nair et al., 2011). These ISMC have decreased expression of contractile proteins and an increased growth response to the mitogen platelet-derived growth factor (PDGF-BB), both of which contribute to improper motility in the chronically inflamed intestine (Nair et al., 2011). Therefore, in addition to structural changes to the smooth muscle, inflammation also alters expression of key elements involved in normal ISMC function; contraction and cellular quiescence. Understanding the mechanisms of this altered ISMC phenotype and dysfunction during chronic inflammation will be important for improving our knowledge of the pathogenesis of chronic inflammatory diseases, including IBD.

1.3.4 Stricture Formation in Animal Models of CD

TNBS-induced colitis is the most commonly used model for generating CD-like strictures in animals, reflecting human strictures with ISMC infiltration to the submucosa and mucosa (Chen et al., 2017; Lawrance et al., 2003). Approximately 50% of rats treated with TNBS for 35 days developed intestinal strictures, imitating the prevalence of strictures in human CD (Marlow & Blennerhassett, 2006). Strictures also persisted 90 days post TNBS treatment, suggesting a permanent structural change (Marlow & Blennerhassett, 2006).

Features of TNBS-induced strictures include thickening of the intestinal wall mostly attributable to ISMC hyperplasia, with minor increases in collagen content, along with complete submucosal and myenteric neuron loss, and decreased innervation to the muscularis externa (Marlow & Blennerhassett, 2006). Additionally, most of the protein content within strictured
regions were from ISMC, suggesting smooth muscle hyperplasia is the most predominant feature in the TNBS model (Marlow & Blennerhassett, 2006), paralleling the tissue changes seen in human strictures (Chen et al., 2017). Although some work suggests that insulin-like growth factor-1 regulates ISMC hyperplasia in intestinal strictures (Mahavadi et al., 2011), the mechanisms of inflammation-induced smooth muscle growth are largely unknown.

It is well characterized that smooth muscle cells (SMC) can modulate their phenotype from a contractile quiescent state into a synthetic, proliferative one (reviewed in Owens et al., 2004). Evidence from both CD patients and animal models suggest that ISMC undergo a similar phenotypic switch within intestinal strictures, characterized by decreased expression of contractile proteins (Marlow & Blennerhassett, 2006; Suekane et al., 2010). However, the factors which regulate ISMC phenotypic switching are unknown. Research from other diseases of SMC hyperplasia, including atherosclerosis and asthma, provide insight into mechanisms that regulate SMC phenotype, which might also affect ISMC growth and phenotype in intestinal strictures.

### 1.4 Phenotypic Switching of Smooth Muscle Cells

Smooth muscle cells (SMC) are not terminally differentiated, but rather display phenotypic plasticity in response to proliferative stimuli as well as different environmental factors (reviewed in Owens, 1995). The ability of SMC to change phenotypic characteristics, termed phenotypic switching, is very clear in dedifferentiation from a contractile, quiescent state into a non-contractile, synthetic, and proliferative phenotype (Chamley-Campbell et al., 1979). Further, it is well accepted that SMC can display a wide variety of phenotypes, including intermediate states between the traditional contractile and synthetic ones (reviewd in Owens, 1995; Owens et al., 2004). Phenotypic switching of SMC is implicated in the pathogenesis of many diseases, including CD, atherosclerosis, and asthma, where it is known to contribute to disease pathogenesis (reviewed in Bennett et al., 2016; Gomez & Owens, 2012; Hirst et al., 2000; Wright et al., 2013).
1.4.1 Defining the contractile and synthetic SMC phenotypes

The contractile SMC phenotype is generally characterized by the absence of proliferation, migration, and low protein synthetic activity, while there is high expression of ion channels, contractile proteins, and signaling molecules required for carrying out contraction (reviewed in Owens et al., 2004; Scirocco et al., 2016). Since SMC exhibit phenotypic plasticity, it is important to assess more than one marker of contractile phenotype when defining the differentiated state. Contractile proteins are logical markers of defining this mature, contractile phenotype, which include smooth muscle α-actin (SMA) and SM22-α (SM22). SMA is the most abundantly expressed protein in SMC, and plays a role in contraction and motility (Fatigati & Murphy, 1984). SM22 is a calponin-like protein whose function is currently unknown, although it is also thought to play a role in maintaining structural integrity during contraction (Zhang et al., 2001).

In contrast, the synthetic SMC phenotype involves high rates of proliferation and migration, increased synthetic activity accompanied with increased number of organelles, and decreased expression of contractile markers (reviewed in Owens et al., 2004; Scirocco et al., 2016). Dedifferentiated SMC are known to contribute to pathogenesis of many diseases, including CD, emphasizing the importance of understanding the environmental factors and molecular mechanisms which control SMC phenotypic switching (reviewed in Bennett et al., 2016; Gomez & Owens, 2012; Hirst et al., 2000; Wright et al., 2013).

1.4.2 Phenotypic Switching of Intestinal Smooth Muscle Cells

As mentioned earlier, there is evidence in both human and animal models of CD, including in strictured regions of TNBS-treated rats, that ISMC develop an altered phenotype (Marlow & Blennerhassett, 2006; Nair et al., 2011; Suekane et al., 2010). The factors that control ISMC phenotypic switching remain largely unknown, although recent work by our lab has provided some insights.
It is well established that the onset of proliferation causes SMC to dedifferentiate into synthetic, proliferative cells (reviewed in Owens et al., 2004). Culturing ISMC from adult rat colon also decreased the contractile phenotype, characterized by decreased expression of marker proteins SMA, SM22, and desmin, compared to freshly isolated ISMC (Nair et al., 2011). However, loss of the ISMC contractile phenotype was reversible, whereby depriving cultured cells of serum, and therefore preventing proliferation, restored expression of these contractile markers (Nair et al., 2011). Additionally, expression of SM22 was inversely associated with expression of cyclin D1, a marker of cell cycle progression, in cultured ISMC (Nair et al., 2011). Proliferating ISMC isolated from TNBS-treated rats also had decreased expression of contractile markers, and decreased responsiveness to contractile stimuli (Nair et al., 2011; Wells & Blennerhassett, 2004). Overall, this suggests that serum-induced proliferation of ISMC decreases their contractile phenotype.

Earlier work identified PDGF-BB as the mitogen found in fetal calf serum (FCS) responsible for ISMC-induced proliferation, suggesting PDGF-BB might directly modulate ISMC phenotype (Stanzel et al., 2010; Nair et al., 2014). However, the molecular mechanisms for how PDGF-BB might affect expression of contractile markers in ISMC remain unknown.

In addition to decreased contractile marker expression and enhanced growth response, proliferating ISMC in vitro and in vivo also have increased expression of GDNF (Han et al., 2015). However, serum deprivation reversibly decreased GDNF expression in cultured ISMC (Han et al., 2015). Since GDNF was found to support neuronal survival and induce axonal outgrowth (Gougeon et al., 2013; Rodrigues et al., 2011), proliferating ISMC in early colitis might restore axonal innervation from surviving neurons through secretion of GDNF.

Although serum deprivation restored ISMC contractile phenotype, it is important to assess the consequences of prolonged proliferation of ISMC on phenotype, since increased ISMC number is a permanent consequence of intestinal inflammation (Blennerhassett et al., 1992; Lourensssen et al., 2005). High passage ISMC, which are passaged 10 times or more upon reaching confluency
and have thus undergone numerous rounds of proliferation, have non-reversible decreased expression of GDNF and contractile markers SMA and SM22 (Han et al., 2015; Nair et al., 2011). Consequently, repeated proliferation of ISMC decreases their normal contractile phenotype and ability to express GDNF or support axonal outgrowth. This suggests that high passage ISMC have decreased contractile and neurotrophic functions.

These high passage cells therefore might represent an in vitro model of strictured ISMC, since loss of contractile phenotype and smooth muscle innervation are characteristic of strictures from TNBS-treated rats (Marlow & Blennerhassett, 2006). Therefore, understanding the mechanisms which regulate permanent loss of contractile phenotype in high passage ISMC can provide insight into smooth muscle dysfunction in strictures. However, the mechanisms which underlie ISMC phenotypic switching are unknown. Research focused on vascular and airway smooth muscle phenotype modulation can provide insight into the molecular mechanisms affecting ISMC phenotypic switching.

1.4.3 Phenotypic Switching of Vascular and Airway Smooth Muscle Cells

Vascular smooth muscle cells (VSMC) contract to regulate the flow of blood passing through blood vessels, ultimately affecting blood flow and pressure (reviewed in Gomez & Owens, 2012). Normal VSMC are thus contractile, and exhibit low or absent levels of proliferation, migration, and synthetic activity. However, there is evidence that VSMC are phenotypically altered in atherosclerosis, and contribute to disease pathogenesis by proliferating and migrating to the arterial intima (reviewed in Raines and Ross, 1993). VSMC and ISMC therefore share a similar pathogenic response; hyperplasia which leads to phenotypic modulation.

A multitude of environmental factors which can influence VSMC phenotype, including growth factors, pro-inflammatory cytokines, extracellular matrix protein and cell-cell interactions have been identified (reviewed in Owens et al., 2004). The most well-defined factor in modulating VSMC phenotype is the mitogen, PDGF-BB, which has been shown to directly decrease mRNA
expression and protein synthesis of SMA (Corjay et al., 1989), smooth muscle myosin heavy chain and smooth muscle α-tropomyosin (Holycross et al., 1992). PDGF-BB treatment also decreased expression of SMA, smooth muscle myosin heavy chain and the contractile marker calponin in cultured airway smooth muscle cells (ASMC; Dekkers et al., 2007).

Research aimed at elucidating the molecular mechanisms of VSMC phenotypic modulation identified switching occurs at the transcriptional level. In particular, expression of all smooth muscle marker genes in VSMC are regulated by binding of serum response factor and its coactivator myocardin to CC(A/T)6GG (CArG) elements in promoter regions (reviewed in Owens et al., 2004). PDGF-BB modulates SMC phenotype by affecting this transcriptional process; PDGF-BB induces expression of different transcription factors which decrease binding affinity of serum response factor and myocardin to these CArG sites, ultimately preventing transcription of SMC markers (Liu et al., 2005). However, the complete molecular mechanism of PDGF-BB-induced suppression of SMC marker expression is still unknown. Recent work has suggested that PDGF-BB induces epigenetic changes and that epigenetic modifications can account for altered phenotype of SMC. This is a novel and important aspect of smooth muscle phenotype that is reviewed below.

1.5 Epigenetic Regulation of Smooth Muscle Phenotype

Epigenetics, defined as heritable patterns of gene expressions without changes to the DNA sequence (Berger et al., 2009), has transpired as a key regulatory process for affecting SMC phenotypic switching (reviewed in Alexander and Owens, 2012; Clifford et al., 2014; Liu et al., 2014). The two most well-defined epigenetic mechanisms of altered gene expression are changes in DNA methylation and histone acetylation, and these have garnered interest in affecting the phenotype of SMC (reviewed in Sawan et al., 2008). It is important to understand how these processes work and to assess the evidence that DNA methylation and histone acetylation affect
gene expression in order to understand how these epigenetic changes might play a role in SMC phenotypic switching.

1.5.1 DNA Methylation Decreases Gene Expression

DNA methylation is one of the most widely-studied epigenetic modifications that can affect gene expression. The addition of a methyl group to cytosine residues typically occurs in areas abundant with cytosine and guanine bases, termed CpG sites, which are often located within the promoter regions of genes (reviewed in Clifford et al., 2014). Methylated CpG sites inhibit gene expression by sterically blocking transcription factor or RNA polymerase binding to promoters, or through increased affinity for transcription repressors (reviewed in Bird, 2002). DNA methylation is carried out by DNA methyltransferases (DNMT), which consists of two families: DNMT3 and DNMT1. Although both families are capable of methylating CpG sites, the DNMT3 family isoforms establish methylation patterns, while the DNMT1 family maintains this pattern throughout cell division (Cheng & Blumenthal, 2008).

Early work on the role of DNA methylation in regulating gene expression comes from studies on cancer cell lines and the advent of tumorigenesis. A wide-variety of anti-tumor genes including tumor suppressors, cell cycle regulators, and cell-cell interaction mediators are hypermethylated in various tumors (reviewed in Christman, 2002). This suggests that inappropriate silencing of key genes can contribute to or cause tumorigenesis, and sparked interest in developing agents which could reverse hypermethylation. 5-azacytidine (AZA) remains one of the most well-studied DNMT inhibitors. AZA incorporates into CpG sites, irreversibly binding the active site of DNMT and inhibiting its activity (Issa, 2003). Previously silenced genes can be restored through AZA treatment and subsequent demethylation of their promoters. For example, AZA treatment increased expression of the tumor suppressor gene BRCA1 in three different cancer cell lines (Magdinier et al., 2000). In fact, clinical trials for decitabine, an AZA analogue, have shown therapeutic promise in a number of different cancers (reviewed in Nie et al., 2014).
1.5.2 Histone Hypoacetylation Decreases Gene Expression

The role of chromatin structure, comprised of DNA and protein, in regulating smooth muscle marker expression is an emerging topic (reviewed in Alexander and Owens, 2012). DNA wrapped around an octamer of histone proteins form the nucleosomes, the basic unit of chromatin, which are interconnected by linker histone proteins. Histones have exposed N-terminus tails that are a site of various post-translational modifications, including acetylation, which can affect the structure of the proteins and surrounding DNA base pairs (Suto et al., 2000). Briefly, acetyl groups added to lysine residues neutralize their positive charge, rendering the chromatin into an open and relaxed structure, permitting access for the transcription machinery (Suto et al., 2000). The acetylation status of histones, and therefore their transcriptional accessibility, is a balance of the activity of two enzymes: histone acetyltransferases (HAT) and histone deacetylases (HDAC).

Similarly to DNMT, there is evidence that HDAC are upregulated in various cancers and can participate in tumorigenesis through silencing of important tumor suppressor and cell cycle regulatory genes (reviewed in Chen et al., 2015). HDAC are subdivided into 4 families, which are all capable of removing acetyl groups from histones, and differ in their tissue-specific expression patterns. The advent of agents to prevent HDAC-mediated gene silencing, including the pan-HDAC inhibitor trichostatin A (TSA), has showed therapeutic promise in preventing tumor development and progression (reviewed in Chen et al., 2015). For example, TSA treatment increased expression of a multitude of genes in three different cancer cell lines, including the tumor suppressor p21 (Glaser et al., 2003). Vorinostat, a structural analogue to TSA, is now on the market as a treatment option for T-cell lymphoma patients (Duvic & Vu, 2007).

The roles of DNA methylation and histone acetylation in affecting SMC phenotype are less well-understood, but are largely derived from earlier work in cancer cell lines. Some research also highlights a role for these two epigenetic processes in affecting CD pathogenesis, which suggests that there is the potential for DNA methylation and histone acetylation to influence ISMC phenotype.
1.5.3 Epigenetic Changes During Intestinal Inflammation

The role of epigenetic modifications is not exclusive to tumorigenesis and cancer progression; there is some evidence showing DNA methylation and histone acetylation changes in the inflamed gut. Four different studies have identified a number of genes which show methylation changes in IBD patients compared to healthy controls, although the majority of work has focused on UC and not CD (reviewed in Ventham et al., 2013). Of the work looking at CD patients, these studies have only considered gene-methylation changes in the mucosa, and therefore the role of epigenetic regulation within the neuromuscular layer in CD remains unknown (Cooke et al., 2012). Although some evidence suggests DNA methylation can affect epithelial cell differentiation and growth in the context of colorectal cancer, there is little overall work revealing a role in gene silencing or cell phenotype (Elliott & Kaestner, 2015).

There is also work that shows a putative role for histone acetylation in regulating aspects of CD pathogenesis, including regulating the inflammatory response (reviewed in Ventham et al., 2013). For example, increased histone acetylation levels were detected in Peyer’s patches from TNBS-treated rats and human IBD patients (Tsaprouni et al., 2011). Other work shows a regulatory role of HDAC activity in controlling the epithelial and T-cell response to inflammation (Turgeon et al., 2013; Zimmerman et al., 2012).

There is an overall lack of work aimed at epigenetic changes to the neuromuscular layer during intestinal inflammation, and how these modifications might contribute to CD pathogenesis. However, some insight into the potential role of epigenetic regulation of intestinal smooth muscle gene expression can be derived from studies in vascular and airway smooth muscle cells.

1.5.4 Epigenetic Control of Vascular and Airway Smooth Muscle Phenotype

In addition to the multitude of environmental factors which control SMC phenotypic switching, epigenetic control of these processes is emerging as an important mediator. Both changes in DNA methylation and histone acetylation, and the processes which control these
epigenetic events have been implicated in controlling SMC phenotype (reviewed in Liu et al., 2014; Alexander & Owens, 2012).

The role of DNA methylation in controlling SMC phenotype is less well defined compared to histone modifications (Liu et al., 2014), and most work involves the use of DNMT inhibitors. Loss of expression of smooth muscle markers is a key aspect in phenotypic switching in both vascular and airway SMC (reviewed in Clifford et al., 2014; Owens et al., 2004). Hypermethylation at the promoter of SM22 was a key step in phosphate-induced VSMC phenotypic switching (Oca et al., 2010). This suggests that DNA methylation might mediate phenotypic switching for environmental factors, including PDGF-BB. Indeed, AZA treatment of cultured ASMC and VSMC prevented PDGF-BB induced suppression of SMA and SM22 (Ning et al., 2013; Zhuang et al., 2016), along with the contractile protein MLCK (Ning et al., 2013). In both cases, PDGF-BB induced expression of DNMT1, suggesting inhibition of this epigenetic enzyme was responsible for restored contractile phenotype.

Dedifferentiation of SMC also involves increased proliferation and migration (reviewed in Clifford et al., 2014; Owens et al., 2004). AZA treatment inhibited PDGF-BB-induced proliferation and migration of airway and vascular SMC (Ning et al., 2013; Zhuang et al., 2016). Furthermore, AZA treatment prevented neointimal formation and decreased arterial plaque area in two different in vivo models of atherosclerosis (Zhuang et al., 2016). Overall, this suggests that DNA methylation can account for the switch from contractile to synthetic phenotypes in SMC. Moreover, PDGF-BB might modulate SMC phenotype by silencing marker gene expression through enhanced DNA methylation.

Early work highlighting a role of histone modifications in affecting SMC phenotype showed that binding of the transcription factor serum response factor to its CArG boxes in smooth muscle marker genes depended on relaxed chromatin structure, which was in part due to acetylation at histone 4 (Manabe & Owens, 2001). Later work revealed that PDGF-BB suppressed SMA and
smooth muscle myosin heavy chain expression *in vitro* and *in vivo* by decreasing acetylation of histone 4 at these marker gene promoters (McDonald et al., 2006), carried out by increased activity of HDAC2, HDAC4, and HDAC5 (Yoshida et al., 2007). This suggests that expression of smooth muscle markers relies on appropriate acetylation levels, which can be modified by PDGF-BB. PDGF-BB-mediated phenotypic switching also involves increased SMC proliferation and migration, which appears to be mediated by increased HDAC4 expression (Usui et al., 2014). Similarly to AZA, TSA treatment prevented PDGF-BB-induced proliferation of VSMC in culture, and also decreased neointimal formation *in vivo* (Jin et al., 2011; Okamoto et al., 2006). Overall, this suggests that PDGF-BB might also affect SMC phenotype through decreased histone acetylation.

Since serum, which contains PDGF-BB, can induce phenotypic switching of ISMC (Nair et al., 2011), DNA methylation and histone acetylation might also account for altered high passage phenotype. However, there is very little work on the role of epigenetics in controlling ISMC phenotype. One study by Shi and Sharna (2013) showed that decreased expression of the protein phosphatase 1 regulatory subunit, a protein involved in mediating contraction, might be due to promoter hypermethylation. Although this paper suggests the expression of this one protein can be epigenetically controlled in ISMC, no future work on this concept has arisen.

1.6 Hypothesis and Goals

ISMCP hyperplasia and phenotypic switching underlie stricture formation and symptoms of dysmotility in CD patients. An understanding of the mechanisms which alter the phenotype of ISMC is important, as it might provide insights into novel therapeutic options for restoring proper motility in IBD.

It is unknown if epigenetic changes, including DNA methylation and histone acetylation, occur in high passage rat ISMC, and if these changes can affect the altered phenotype of these cells. It is also unknown if ISMC from human strictures have an altered phenotype, and if epigenetic
modifications alter their gene expression. Overall, this thesis will provide insight into the mechanisms which control phenotypic switching of rat ISMC at high passage, if smooth muscle cells from human strictures have an altered contractile phenotype, and if epigenetic modifications also play a role in modulating human ISMC gene expression. The goals of this project are:

- Use molecular techniques to determine if inhibition of HDAC and DNMT through TSA and AZA treatment, respectively, can restore the contractile phenotype of rat ISMC at high passage
  - Assess if TSA and AZA treatment can suppress the enhanced growth response of high passage cells
  - Determine if TSA and AZA treatment can increase expression of contractile markers that are decreased in high passage cells
  - Test if TSA and AZA treatment can increase expression of elements involved in excitation and contraction in high passage cells
  - Determine if TSA and AZA treatment can increase expression of GDNF, and subsequent neurotrophic function of high passage cells
- Determine if ISMC from human strictures have an altered contractile phenotype
- Elucidate the putative epigenetic mechanisms which control gene expression in high passage rat cells and ISMC from human strictures
- Generate repeated passage human ISMC to determine if protracted growth of human cells mimics the changes in phenotype and epigenetic modifications observed in the high passage rat model
Chapter 2

Methods

2.1 Animals

Sprague-Dawley rats were obtained from Charles River Laboratories (Montreal, QC) and housed in pairs in microfilter-isolated cages. Animals had free access to water and food (Purina #5008). Adult male rats were used for isolation of circular smooth muscle cells, and neonatal rat pups bred in the GIDRU animal facility were used to generate co-cultures. A course in animal care (QACS 799) was completed at the start of the project, and all experiments were approved by the Queen’s University Animal Care Committee and adhered to the Canadian Council of Animal Care’s policies.

2.2 Isolation of Rat and Human Circular Smooth Muscle

2.2.1 Rat circular smooth muscle cells

Kurtis Miller (lab technician) performed the following dissection and dissociation procedures. To generate primary intestinal smooth muscle cultures, the colons of adult male rats were isolated in Hank’s balanced salt solutions (1 mM CaCl₂, 0.25 mM EDTA, 10 mM HEPES, 1 mM MgCl₂, 10 mM glucose, 4 mM KCl, 125 mM NaCl, 10 mM Taurine, pH 7.8). The circular smooth muscle layer was isolated with forceps and strips were placed into Hank’s balanced salt solutions with 5 mg papain, 50 µM of CaCl₂, 10 mg of bovine albumin serum (Bioshop), 10 µL of 1 M dithiothreitol (Sigma), and 2.5 mg of type F collagenase (Sigma) for 2 hr. at 4°C. The tissue was warmed at room temperature for 1 hr., and then at 37°C for 1 hr. with light agitation. Pellets were removed from Hank’s balanced salt solutions, washed in Dulbecco’s modified eagle medium (DMEM, GIBCO), and plated into 60 mm culture dishes with 5% fetal calf serum (FCS; Invitrogen) containing DMEM.
To generate low and high passage cells, primary cultures of ISMC were washed and replenished with fresh 5% FCS every 3-4 days, until they reached 80-95% confluency. Cells were then removed from their plates with a 1:1 mix of 0.25% trypsin II (Sigma) and 2 mM EDTA (Sigma) in Hank’s balanced salt solutions/HEPES (H/H), and passed into 2 new 60 mm dishes in 5% FCS at sub-confluency. This process was repeated 1-3 times to generate low passage cultures, or 10 or more times to achieve high passage cultures. Cultures destined for immunocytochemistry experiments were passaged onto collagen-coated glass cover slips in 24-well plates.

2.2.2 Human circular smooth muscle cells

Prior to the use of human tissue, our experiments were approved by the Queen’s University Health Sciences Research Ethics Board. Michael Blennerhassett (supervisor) performed the following dissociation procedures. Circular smooth muscle cells (CSMC) were isolated from specimens of human strictured ileum resected for obstructive symptoms of Crohn’s disease. Human CSMC were isolated separately from the proximal normal margin or from the strictured region. In addition, normal human CSMC were isolated from specimens of terminal ileum obtained at time of resection for colon cancer, and in some cases, from proximal normal colon.

In all cases, intact tissue was dissected to yield strips of circular smooth muscle, 2-4 mm in length. These were incubated in the enzyme solution described above for rat tissue overnight at 4°C, then at room temperature for 60 min., and finally placed in 37°C water bath for 10 min. The enzyme solution was then replaced with growth medium and gentle trituration was used to liberate the smooth muscle cells. Initially, these appeared as long bipolar spindle-shaped cells with high viability (>95%), which then rounded up to form spheres over the next several hours. For cell suspensions from strictured regions where immune cells were prominent, differential plating was used to reduce immune cell numbers without noticeable effect on culture success.

Low and high passage cultures were also generated for both strictured and normal human smooth muscle cells, as described above.
2.3 Primary Co-Culture Preparation

To generate primary co-cultures of neurons and ISMC, the small intestine of neonatal rat pups (aged 2-6 days) was dissected and incubated in H/H (pH 7.35) at 24°C. The mesentery was removed and the mucosa was gently separated from the underlying smooth muscle layers and myenteric plexus. The smooth muscle/myenteric plexus was cut into small pieces and incubated in 0.25% trypsin II at 37°C for 75 min., with periodic light agitation. The supernatant was removed and the cells were resuspended in DMEM with 10% FCS and triturated to generate a single cell suspension. Cells were counted with a hemocytometer and placed in 24-well plates on collagen-coated glass cover slips at a cell density of 3.5-4.0x10^5 cells/mL in 5% FCS. 2 days later, the cultures were deprived of serum for 4 hr. before exposure to experimental conditions.

2.4 Treatment of Cell Cultures

To assess the outcome of phenotype in cultured high passage smooth muscle cells, the following treatments were applied in DMEM for 18 hr., 24 hr., or 3 days:

Inhibitors: DNMT inhibitor 5-azacytidine (2 µM and 10 µM in water; Sigma), HDAC inhibitor trichostatin A (0.1 µM in water; Cell Signaling Technologies).

AZA and TSA were dissolved in water, diluted at 1:000 in DMEM to form working solutions, and diluted again in DMEM to the appropriate concentrations. At the time of treatment, ISMC cultures were washed in fresh DMEM, which was then aspirated, and the cultures were cultured with DMEM containing AZA or TSA.

2.5 Conditioned Media Preparation and Trichloroacetic Acid Protein Precipitation

To detect secreted GDNF protein, conditioned media (CM) from TSA and AZA treated high passage cultures was collected as 2.0 mL aliquots. CM was immediately centrifuged for 10 min. at 10000 RPM at 4°C, and the supernatant was stored at -80°C.
To precipitate the proteins from the supernatants, CM was thawed on ice, and 500 μL was mixed with 5 μL of 2% sodium deoxycholate (Sigma), vortexed, and stored on ice for 30 min. 50 μL of trichloroacetic acid (TCA; Sigma) was added, vortexed, and stored on ice for 2 hr. Samples were then centrifuged at 13000 RPM for 10 min. at 4°C, washed with 400 μL of chilled acetone (Sigma), and re-centrifuged. Supernatant was discarded, and the pellet was dissolved in 40 μL of 1x reducing sample buffer 20 mM Tris/HCl, 6% glycerol, 0.5% SDS, 2% β-mercaptoethanol, 0.001% bromophenol blue), then stored at -80°C.

2.6 Western Blot Analysis

2.6.1 SMA and SM22 protein expression

High passage smooth muscle cells were cultured in a 6-well plate at 2.0x10^5 cells/well in 5% FCS, then exposed to treatment conditions for 3 days. Cells were washed in DMEM then counted for total cell number using a hematocytometer. Cells were dissolved in sample buffer at 1000 cells/μL, and stored at -80°C.

At the time of immunoblotting, samples were thawed on ice, heated at 95°C for 5 min., and 10,000 cells were loaded into a 0.75 mm thick 12% SDS-PAGE gel. Samples were electrophoresed (200 V for 5 min., 120 V for 1 hr.), then transferred onto a polyvinylidene fluoride membrane (15 V for 1.5 hr.; Biorad semi-dry transfer apparatus). Post-transfer, membranes were washed in distilled water, blocked in Tris-buffered saline containing 0.1% Tween-20 (TBS-T; Bioshop) and 5% fat-free milk for 1 hr., then placed in primary antibodies overnight at 4°C with agitation. Anti-SMA antibody (1:1000; mouse; Novus) and anti-SM22 (1:5000; rabbit; Abcam) antibody were prepared in TBS-T with 5% milk and 0.005% azide. Membranes were washed 3x for 10 min. at room temperature with agitation, then placed in appropriate horseradish peroxidase linked secondary antibodies in TBS-T for 2 hr.: goat anti-mouse horseradish peroxidase (1:20000, PI-31430, Thermo Scientific), or goat anti-rabbit horseradish peroxidase (1:4000, 7074S, Millipore). All antibodies were tested for species specification by mismatching secondary antibodies.
Membranes were washed 3x over 30 min. at room temperature with agitation, then exposed to 300 μL chemiluminescent substrate (Millipore). Membranes were imaged using a ChemiDoc MP System (Bio-Rad), exposed until just below band saturation, and the integrated optical densities (IOD) were quantified using the Image Lab software (Bio-Rad).

2.6.2 pAKT and AKT protein expression

High passage smooth muscle cells were seeded in a 24-well plate at a density of 3x10⁴ cells/well in 5% FCS, exposed to AZA and TSA for 3 days, then treated with 100 μM carbachol for 7 or 15 min. Cells were collected via scraping in 100 μL of 1x sample buffer, duplicates were combined and stored at -80°C.

Samples were electrophoresed with a 10% SDS-PAGE gel and semi-dry transferred as above. Polyvinylidene fluoride membranes were blocked in TBS-T with 5% milk for 1hr. at room temperature with agitation, then incubated in primary antibodies overnight at 4°C. Both pAKT (Cell Signaling; rabbit) and AKT (Cell signaling; rabbit) antibodies were diluted at 1:1000 in TBS-T with 5% bovine serum albumin and 0.005% azide. Membranes were washed, treated with secondary antibodies, imaged, and quantified as described earlier.

2.6.3 GDNF protein expression

TCA-precipitated CM samples and 4 ng of GDNF peptide (PeproTech) were thawed on ice, combined with 1 M dithiothreitol at 1/10th the total volume, and boiled for 5 min. at 95°C. The samples were then loaded into a 15% SDS-PAGE gel, and electrophoresed and transferred as described above. Membranes were dried post-transfer for 1hr. at 37°C, re-activated in methanol for 1min., washed with distilled water, then blocked in TBS-T with 5% milk for 1hr. at room temperature. Membranes were incubated with primary GDNF antibody (R&D; goat) diluted at 1:1000 in TBS-T with 5% milk and 0.005% azide, washed, then placed in 1:15000 diluted donkey
anti-goat horseradish peroxidase (R&D). Band visualization and quantification was carried out as described earlier.

2.7 Quantitative PCR Analysis

RNA was isolated from cultured low and high passage ISMC pellets using an RNeasy kit (Qiagen) according to manufacturer’s instruction, with modifications as previously described (Han et al., 2015). 1 µg of RNA was converted into cDNA using an iScript cDNA synthesis kit (Bio-Rad) in an Eppendorf 5331 Mastercycler Gradient (Marshall Scientific). Following generation of cDNA, samples were stored at -20°C.

qPCR was performed to assess expression of SMA, SM22, MLCK, M3, and GDNF following 18 or 24 hr. of TSA or AZA treatment in high passage cells. Expression of DNMT1, HDAC2, and HDAC5 were also assessed in low and high passage rat ISMC, along with low and high normal or strictured human ISMC, cultured in DMEM with and without 5% FCS. The efficiencies of new primers were assessed prior to testing, to confirm appropriate changes in gene expression were being measured. All qPCR reactions were performed in MicroAMP optical 96 well reaction plates (Bio-Rad) containing 5 µL of iTaq™ SYBR® Green Supermix, 0.5 µL of cDNA, 3.5 µL of RNase DNase free water (GIBCO), and 0.5 µL of each forward and reverse primer (Table 1). New primers were tested for efficiency in serial dilutions of sample cDNA.

qPCR reactions were carried out in a Step One Plus Real Time PCR System (Applied Biosystems). Samples were cycled for 3 sec. at 95°C and 30 sec. at 60°C for all primers, except GDNF which included an additional 30 sec. 72°C step. Changes in expression were quantified by determining the ddCT, which was detected as number of cycles performed to reach a fluorescence cut-off. Expression of all genes in both rat and human samples were normalized to expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) and β-actin by determining their respective ddCT ratios.
Table 1. qPCR primer sequences.

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2.8 Immunocytochemistry and Immunohistochemistry

2.8.1 Immunocytochemistry of smooth muscle cultures and co-cultures:

Immunocytochemistry was used to detect expression of proliferating cell nuclear antigen (PCNA) in low and high passage rat ISMC, as well as changes in SMA and SM22 expression high passage cells following TSA or AZA treatment. Immunocytochemistry was also used to assess
axon outgrowth of co-cultures in response to conditioned media isolated from TSA or AZA treated high passage rat ISMC.

At the end of each treatment, cells were washed in DMEM and fixed with neutral buffered formalin for 10 min. at room temperature. Cover slips were washed with phosphate buffered saline (PBS) three times over a 30 min. period, then incubated in appropriate primary antibodies diluted in PBS containing 0.2% Tween-20 (PBS-T) and 0.005% azide. Smooth muscle cultures were exposed to anti-PCNA (1:2000; rabbit; Cell Signaling), anti-SMA (1:500; mouse), anti-SM22 (1:2000; rabbit). To assess changes in axon growth, co-cultures were exposed to the neural marker anti-HuD (1:500; mouse; Invitrogen) and anti-SNAP25 (1:4000; rabbit; Sigma). Cover slips were incubated in primary antibodies overnight at 4°C. The next day, cover slips were washed in PBS 3x for 10 min., then placed in appropriate Alexa Fluor secondary antibodies: 488 goat-anti mouse (1:1000; Invitrogen), and 555 goat anti-rabbit (1:5000; Invitrogen). Cover slips were washed in PBS-T, treated with Hoechst 333258 (0.1 µL/Ml; Sigma) for 1 min. to stain all nuclei, then washed again in PBS-T. To visualize protein expression, cover slips were mounted on glass slides using approximately 10 µL of 60% glycerol in water. Representative images were captured at 40x magnification with a Retiga 2000R CCD camera (QImaging) at 1600x1200 pixels, using appropriate filters adjusted for antibody visualization.

2.8.2 Immunohistochemistry of human tissue

Immunohistochemistry was used to detect SMA and SM22 protein expression in sections of strictured and normal margin human tissue from CD patients. Sandra Lourenssen (research associate) performed the following tissue section preparations. Sections of human terminal ileum were fixed in neutral buffered formalin for 24-48 hr. with gentle agitation and then embedded in paraffin. Wax-embedded tissues were cut into 4 µm sections using a Shandin-Finesse cryotome and stored at room temperature.
Sections were dewaxed, placed in Target Retrieval Solutions, pH 9.0 (Dako) at 95°C for 20 min., then cooled to room temperature. Sections were then blocked in 1% goat serum (Invitrogen) in PBS-T for 1 hr., then treated with anti-SMA antibody (1:1000 in PBS-T; mouse) and anti-SM22 antibody (1:2000 in PBS-T; rabbit). Samples were washed in PBS-T, then treated with appropriate Alexa Fluor secondary antibodies: 488 goat anti-mouse (1:1000) and 555 goat anti-rabbit (1:2000). Slides were washed again in PBS-T, then treated with Hoechst 333258. Slices were imaged using a 40x objective on an Olympus BX51 microscope.

2.9 Neuron and Axon Quantification

To assess the outcome on axon outgrowth in co-cultures following CM treatment, neurons and axon numbers were quantified using an Olympus BX51 with a 40x objective, as described before (Gougeon et al., 2013). To determine neuron number, HuD-positive neurons were counted in every third field of view in both a horizontal and vertical strip, accounting for approximately 4% of the total area of the cover slip. To determine axon number, SNAP-25-positive axons which intersected a midline across consecutive visual fields in both horizontal and vertical axes were counted. Axon density was determined by dividing the square of total axons by the total number of neurons for each cover slip (Lourensens et al., 2010). Duplicates for each condition were averaged together.

2.10 Smooth Muscle Marker Expression Quantification

Images at 40x magnification of SMA or SM22 labelled cells were quantified using Image Pro Plus (Media Cybernetics). Each image was converted to grayscale, and the average optical density of three cells which intersected an arbitrary vertical midline were quantified, excluding the nucleus. IOD were averaged across all cells in each condition, and normalized to untreated high passage controls.
2.11 Wst-8 Assay

To assess the outcome of TSA and AZA treatment on ISMC growth response, high passage cells were plated at 7.5x10³ cells/well in a 96-well plate with 5% FCS. TSA and AZA treatments were applied in fresh 5% FCS media for 3 days, and treatments were replenished in half of the wells every 24 hr. Post treatments, the cells were washed in phenol red-free DMEM (Invitrogen) warmed to 37°C, and cell number was assessed using the Wst-8 assay (Sigma) according to the manufacturer’s instructions. Briefly, Wst-8 in clear DMEM was applied to cells, and the absorbance of a colorimetric dye produced from the Wst-8 reaction was measured at 450 nm using the Spectra Max M3 plate reader (Molecular Devices). Absorbance was measured immediately after applying the Wst-8 solution to determine initial values, and at 1, 2, and 18 hr. post treatment.

2.12 Statistical Analysis

Data analysis was performed using Microsoft Excel. All values are expressed as the average ± standard error of n independent cell lines or human tissues. Differences between control and multiple treatment groups were statistically significant for p≤0.05, using a one-way analysis of variance (ANOVA) with Dunnett’s post-test. Differences between two groups were statistically significant for p≤0.05, using a two-tailed Student’s t-test.
Chapter 3

Results

3.1 The onset of proliferation of ISMC is associated with a decrease in the contractile phenotype

It is well-accepted that proliferation alters SMC phenotype from a contractile state to a synthetic, proliferative one (reviewed in Owens et al., 2004). Indeed, the contractile marker protein SM22 and proliferation marker PCNA were inversely expressed in low passage cultured ISMC, suggesting that proliferation decreases the contractile phenotype of ISMC (Nair et al., 2011). To confirm these findings, cultures of low passage ISMC were generated by subculturing cells 1-3 times. Cultures of ISMC at high passage (passaged 10 or more times) were also generated by repeated subculturing.

Previous work showed that the purity of ISMC cultures is 99.9% (Stanzel et al., 2010). Low passage ISMC appeared elongated with extended processes, and resembled the typical hill-and-valley formation of cultured smooth muscle cells (Fig. 2A). However, high passage ISMC were smaller in size, and did not form the overlapping, hill-and-valley appearance (Fig. 2B). High passage ISMC also grew faster in culture compared to low passage cells.

To confirm that proliferation is associated with a decrease in the contractile phenotype, low passage ISMC were grown in DMEM with or without 5% FCS, a known mitogen for ISMC (Stanzel et al., 2010). Cells were then fixed to collagen-coated glass cover slips, and stained for SM22 and PCNA expression.

Analysis of low passage ISMC cultured in serum-free media (0%) showed intense SM22 labeling, with few cells positive for the proliferation marker PCNA (Fig. 2C). However, low passage cells cultured in 5% FCS for 3 days showed bright PCNA staining with less intense SM22 staining (Fig. 2D). Staining of SM22 and PCNA in either condition appeared to be
Figure 2. Proliferation of ISMC is associated with decreased expression of contractile marker proteins. A-B. Phase contrast images of ISMC at low passage (P2) and high passage (P13). C-D. Immunofluorescent images of low passage ISMC cultured in serum-free media (C) or 5% FCS (D). Cells grown in serum-free media had low PCNA and high SM22 staining, which was reversed in 5% FCS conditions (arrows). E-F. Immunofluorescent images of high passage ISMC cultured in serum-free media (E) or 5% FCS (F). In either condition, high passage cells showed high PCNA and low SM22 staining (arrows). Scale bars, 100 μm. G. Representative western blot showing no differences in SMA or SM22 protein expression between high passage ISMC cultured in serum-free media or 5% FCS. H. Quantification of the average band density for SMA and SM22 protein expression in cultured high passage ISMC grown in serum-free media or 5% FCS. Experiments were normalized by loading equal cell number in western blots (n=4).
inversely related, agreeing with previous findings that suggest proliferation alters the phenotype of ISMC (Nair et al., 2011). This confirms that ISMC cultured in serum undergo a phenotypic change relative to cells deprived of serum, including loss of contractile marker expression and increased proliferation.

3.1.1 Protracted growth of ISMC in vitro irreversibly alters smooth muscle phenotype

To assess if prolonged proliferation is associated with a further decrease in the contractile phenotype, high passage cells were cultured with or without serum. Unlike low passage cells, high passage ISMC grown in either serum-free media or 5% FCS expressed low levels of SM22 and high levels of PCNA (Fig. 2E-F). This suggests that high passage cells proliferate in both serum-deprived and serum-enriched media. Furthermore, expression of SM22 is not reversible in high passage ISMC after serum-deprivation for 3 days, suggesting a permanent alteration in the contractile phenotype.

To verify that high passage ISMC have a non-reversible altered phenotype, high passage cells were grown in culture with or without serum for 3 days, and expression of marker proteins SMA and SM22 were assessed by western blotting. The same number of cells were loaded to normalize for potential variation in cell size and consequent protein levels (Han et al., 2015; Nair et al., 2011). There were no significant differences in SMA and SM22 protein expression between high passage cells grown in serum-free media and 5% FCS (Fig. 2G). The integrated optical density (IOD) of the 42 kDa SMA and 22 kDa SM22 bands were quantified and averaged across multiple cell lines (n=4), confirming no significant changes in protein expression between conditions (Fig. 2H). This verifies that high passage ISMC have a permanently altered phenotype that is not reversible through growth manipulation.

Proliferation also induces phenotypic switching of VSMC and ASMC (reviewed in Hirst et al., 2000; Owens et al., 2004; Wright et al., 2003). The phenotypes of these cells also appear to be reversible, but through manipulating epigenetic processes that affect transcription. Specifically,
histone hypoacetylation and DNA hypermethylation appear to play a role in influencing the non-contractile and synthetic phenotype of smooth muscle cells (Yoshida et al., 2007; Ning et al., 2013; Zhuang et al., 2016). Therefore, we tested whether similar epigenetic mechanisms might account for the altered phenotype of high passage ISMC.

3.2 TSA and AZA suppress serum-induced growth response in high passage ISMC

Since low passage cells express higher levels of SMA and SM22 than high passage cells (Nair et al., 2011), we predict that histone hypoacetylation and DNA hypermethylation might account for decreased expression of these contractile markers. To determine if these epigenetic changes are responsible for the altered phenotype of high passage cells, we used two common compounds known to cause epigenetic modifications: trichostatin A (TSA) and 5-azacytidine (AZA). TSA inhibits HDAC activity, preventing the removal of acetyl groups from histones and rendering the associated DNA accessible to transcriptional machinery. AZA inhibits DNMT activity, preventing the addition of methyl groups to DNA which also increases gene expression. Before applying these agents to cultures, we first determined appropriate, non-cytotoxic concentrations of both drugs.

3.2.1 Effect of TSA and AZA on high passage cell number

High passage cells were cultured in 96-well plates and treated with a range of TSA and AZA concentrations for 3 days in serum-free media. Following treatments, the number of living cells was detected with the Wst-8 assay; a colorimetric assay of cell viability. Results were normalized to cells grown in serum-free media, and cells grown in 5% FCS were used as a positive control.

After 3 days in culture, untreated cells showed a slight increase in relative cell number, compared to the initial seeding density. Concentrations of TSA decreased Wst-8 values, while concentrations above 0.1 μM were statistically lower than serum-free controls (Fig. 3A). Treatment with 0.1 μM TSA led to an approximate 23% ± 0.06% decrease compared to untreated
Figure 3. Serum-induced growth of high passage ISMC is diminished by TSA and AZA treatment. 
A-B. Cell number determined by Wst-8 assay of high passage ISMC treated with TSA (A) or AZA (B) for 3 days in serum-free media. The relative amount of living cells was assessed with the Wst-8 proliferation assay, normalized to untreated cells, and averaged across cell lines (n=4 ± sem; p<0.05). *p<0.05 for one-way ANOVA compared with control.
C. Growth assay using high passage ISMC cultured in 5% FCS and treated with TSA or AZA, applied for 3 days or replenished every 24 hr. for 3 days. TSA and 10 μM AZA decreased the growth response in non-replenished and replenished conditions, while 2 μM AZA only decreased the growth response in replenished conditions. Results were normalized to control cells grown in 5% FCS alone, and averaged across cell lines (n=4 ± sem; p<0.05). *p<0.05 and **p<0.01 for one-way ANOVA compared with control.
controls, and was the highest concentration that did not significantly affect cell number (Fig. 3A). Therefore, 0.1 μM TSA did not cause non-specific cytotoxicity to high passage ISMC. Elsewhere in a study assessing VSMC proliferation, 0.1 μM TSA was similarly found to be the highest non-cytotoxic concentration that could elicit an anti-proliferative response (Jin et al., 2011). We therefore chose 0.1 μM TSA as the optimal, non-cytotoxic concentration for treating high passage ISMC.

High passage cells appeared to be more resistant to cytotoxicity in the presence of AZA than to addition of TSA, with only 35 μM and 50 μM concentrations inducing a significant decrease in cell number (Fig. 3B). Interestingly, concentrations of 2 μM and 10 μM AZA resulted in similar effects on cell number (Fig. 3B). Most cell lines were resistant in the presence of 10 μM AZA, but to account for the few cell lines that were less resistant, we decided that both 2 μM and 10 μM AZA were optimal concentrations for treating high passage ISMC.

3.2.2 TSA and AZA prevent serum-induced growth of ISMC

In addition to decreased contractile marker expression, phenotypic switching of smooth muscle also renders cells to be hyperproliferative (reviewed in Owens et al., 2004). Indeed, high passage ISMC had a higher proportion of proliferating cells (Nair et al., 2011) and grew faster in culture (Han et al., 2015) compared to low passage cells, suggesting a heightened growth response. In other systems, TSA (Kee et al., 2011; Okamoto et al., 2006) and AZA (Ning et al., 2013; Zhuang et al., 2016) treatment prevented PDGF-BB-induced proliferation of SMC. Therefore, we tested whether TSA and AZA treatment on high passage cells might reduce their growth response to serum, which contains the ISMC mitogen PDGF-BB (Stanzel et al., 2010).

To determine if TSA and AZA can prevent serum-induced growth of high passage ISMC, cells were plated in a 96-well plate, and treated with both agents in 5% FCS. Cells grown in serum-free media and 5% FCS alone acted as negative and positive controls, respectively. Media containing TSA or AZA was replenished every 24 hr. in half of the conditions. Post-treatment, the
growth response of plated cells was assessed with the Wst-8 assay, and results were averaged and normalized to the 5% FCS control.

Treatment with TSA significantly decreased the growth response of high passage ISMC to less than half that of 5% FCS alone in both non-replenished (0.42 ± 0.04%, n=4, p<0.01; Fig. 3C) and replenished conditions (0.23 ± 0.06%, n=4, p<0.01; Fig. 3C). Similarly, 10 μM AZA appeared to decrease the growth response in both non-replenished (0.70 ± 0.15%, n=4, p>0.05; Fig. 3C) and replenished conditions (0.34 ± 0.07%, n=4, p<0.01; Fig. 3C) compared to cells grown in 5% FCS alone. Interestingly, 2 μM AZA treatment for 3 days showed a non-significant but slight increase in the absorbance measured in the presence of Wst-8 compared to 5% FCS alone (1.05 ± 0.10%, n=4, p>0.05; Fig. 3C), while replenished 2 μM AZA appeared to decrease the growth response (0.74 ± 0.11%, n=4, p>0.05; Fig. 3C). Overall, this suggests that TSA and AZA treatment can prevent serum-induced growth of high passage ISMC, and can therefore induce a less-proliferative phenotype.

3.3 TSA and AZA increase contractile marker expression

In addition to an enhanced growth response, high passage ISMC also have decreased expression of contractile markers compared to low passage cells (Nair et al., 2011). Since TSA and AZA treatment restored expression of SMA and SM22 in VSMC and ASMC (Jin et al., 2011; Ning et al., 2013; Okamoto et al., 2006; Zhuang et al., 2016), we hypothesized that these agents might also restore contractile protein expression in high passage ISMC. To determine if TSA and AZA treatment could restore expression of markers SMA and SM22, we cultured high passage cells in 6-well plates and treated them with either TSA or AZA in serum-deprived media. Cells were collected and assessed for changes in gene expression through qPCR, western blotting, and immunocytochemistry.
TSA treatment increased SMA (4.72 ± 0.62, n=5, p<0.05; Fig. 4A) and SM22 mRNA expression (3.56 ± 0.62, n=3, p<0.05; Fig. 4A) compared to untreated high passage ISMC. Similarly, 2 μM AZA and 10 μM AZA increased SMA (2 μM AZA: 2.4 ± 0.50, n=4, p>0.05; Fig. 4A; 10 μM AZA: 4.44 ± 0.99, n=5, p<0.05; Fig. 4A) and SM22 (2 μM AZA: 2.31 ± 0.36, n=4, p<0.05; Fig. 4A; 10 μM AZA: 2.28, n=6, p<0.05; Fig. 4A) mRNA expression compared to untreated cells. This suggests that both histone hypoacetylation and DNA hypermethylation account for decreased expression of contractile markers in high passage ISMC, since TSA and AZA treatment increased their expression.

To assess if these changes in mRNA levels were consistent in protein expression, the amount of SMA and SM22 were assessed by western blotting following TSA and AZA treatments. Equal numbers of cells were loaded into each lane in case of variation in cell size or TSA- or AZA-induced changes to housekeeper gene expression. Western blot analysis revealed an increase in SMA and SM22 protein expression compared to untreated cells (Fig. 4B).

Western blots were repeated for different cell lines and bands representing SMA and SM22 protein expression were quantified and averaged. Compared to untreated cells, TSA increased SMA (2.10 ± 0.36, n=5, p<0.05; Fig. 4C) and SM22 (2.88 ±0.36, n=5, p<0.05; Fig. 4C) protein expression. 2 μM AZA also increased SMA (2.30 ± 0.33, n=5, p<0.05; Fig. 4C) and SM22 protein expression (2.76 ± 0.41, n=5, p<0.05; Fig. 4C), compared to untreated cells. These results showed similar outcomes to the qPCR experiments and suggest that TSA and AZA treatment also increases SMA and SM22 protein expression in high passage cells.

Interestingly, there appeared to be two distinct cell populations which varied in their response to TSA and AZA treatment. While 5 cell lines showed a significant increase in SMA and SM22 protein expression following TSA treatment (Fig. 4C), 2 other cell lines showed dramatic increases in SMA (100.94 ± 7.36, n=2, p<0.05) and SM22 (399.66 ± 358.01, n=2, p>0.05) protein levels. Likewise, 3 other cell lines showed substantial increases in SMA
Figure 4. TSA and AZA increase SMA and SM22 expression in high passage ISMC.

A. Quantitative PCR analysis for SMA and SM22 mRNA in TSA- and AZA-treated high passage ISMC. mRNA for SMA and SM22 increased in TSA- and AZA-treated cells, compared to untreated controls. Relative changes in mRNA expression were averaged across different cell lines (n=3-6 ± sem, p<0.05). *p<0.05 for one-way ANOVA compared with controls.

B. Representative western blot indicating increased SMA and SM22 protein expression in TSA- and AZA-treated high passage ISMC.

C. Quantification of the average changes in SMA and SM22 protein expression in TSA- and AZA-treated high passage ISMC, compared to untreated controls (n=5 ± sem, p<0.05). Experiments were normalized by loading equal cell numbers. *p<0.05 for one-way ANOVA compared with control.
(301.06 ± 212.48, n=3, p>0.05) and a significant but smaller increase in SM22 (28.75 ± 2.73, n=3, p<0.01) protein expression. This suggests that different high passage cell lines have variable responses to TSA and AZA treatment, and therefore might possess variable levels of histone hypoacetylation, DNA hypermethylation, or resistance to drug-induced cytotoxicity.

To confirm our western blot findings that showed TSA- and AZA-induced increases in SMA and SM22 expression, TSA- or AZA-treated high passage ISMC were labeled with antibodies to detect SMA and SM22, and protein expression was assessed on a per cell basis. TSA and both concentrations of AZA appeared to increase SMA and SM22 protein levels (Fig. 5A-C). Protein expression was quantified by measuring the density of cytoplasmic staining in triplicate conditions, and averaged across different cell lines. TSA appeared to increase SMA (1.70 ± 0.17, n=4, p>0.05; Fig. 5D) and SM22 (1.73 ± 0.02, n=3, p<0.01; Fig. 5D) protein levels, compared to untreated controls. Similarly, there was an increase in SMA (2 µM AZA: 1.63 ±0.09, n=4, p>0.05; Fig. 5D; 10 µM AZA: 1.83, n=4, p>0.05; Fig. 5D) and SM22 (2 µM AZA: 1.73 ±0.09, n=3, p<0.01; Fig. 5D; 10 µM AZA: 1.87, n=3, p<0.01; Fig. 5D) protein expression in AZA treated cells. These results confirm the changes in protein expression measured from western blotting, and further suggest that histone hypoacetylation and DNA hypermethylation account for the decreased expression of SMA and SM22 in high passage cells.

3.4 TSA and AZA increase the capacity for excitation and contraction in high passage ISMC

Since incubation with TSA and AZA restored expression of contractile markers in high passage cells, we next wanted to assess if inhibition of histone hypoacetylation and DNA methylation could affect expression of elements involved in carrying out smooth muscle contraction. In cultured ASMC, AZA increased MLCK2 expression, which encodes for a protein involved in regulating the final steps in the contractile process of smooth muscle cells.
Figure 5. TSA and AZA increase contractile marker expression in high passage ISMC. A-C. Immunofluorescent images for SMA (A) and SM22 (B) protein expression in TSA- and AZA-treated high passage ISMC. TSA and AZA treatment increased SMA and SM22 protein expression compared to untreated controls. Nuclei were stained with Hoechst (C). Scale bars, 50 µm. D. Quantification of the cytoplasmic density of SMA and SM22 protein expression in TSA- and AZA-treated high passage ISMC. Densities were normalized to untreated controls and averaged across different cell lines (n=3-4 ± sem, p<0.05). *p<0.05 and **p<0.01 for one-way ANOVA compared with controls.
(Ning et al., 2013). We therefore tested the effects of TSA and AZA treatment on MLCK2 expression in high passage cells.

High passage cells were cultured in 6-well plates with TSA or AZA in serum-free media for 24 hr., then cells were collected and prepared for qPCR. We first wanted to assess if high passage cells expressed lower levels of MLCK2 compared to low passage cells. Low passage ISMC had variable and relatively higher levels of MLCK2 mRNA expression (1.18 ± 0.47, n=3, p>0.05; Fig. 6A) compared to high passage cells. However, TSA appeared to increase MLCK2 expression (3.12 ± 1.31, n=5, p>0.05; Fig. 6A) in high passage cells, compared to untreated controls. TSA treatment rendered variable changes in MLCK2 mRNA expression in high passage cells lines: 1 cell line showed a slight decrease, 2 cell lines showed a slight increase, and 2 cell lines showed a much larger increase. This might suggest that the gene encoding for MLCK2 is not hypoacetylated in some high passage cells, or that TSA is unable to restore expression in some cell lines. In contrast, both concentrations of AZA increased MLCK2 expression compared to untreated cells (2 μM AZA: 1.56 ± 0.08, n=3, p>0.05; Fig. 6A; 10 μM AZA: 2.65 ± 0.25, n=4, p<0.05; Fig. 6A). This suggests that DNA methylation but not histone hypoacetylation in high passage ISMC plays a role in silencing MLCK2 expression.

We then wanted to determine the effect of TSA and AZA treatment on M₃ expression, a muscarinic receptor involved in receiving excitatory cholinergic stimuli. Previous work from our lab showed that M₃ expression was decreased in proliferating smooth muscle cells isolated from the inflamed rat intestine, compared to healthy controls (Nair et al., 2011). Since high passage cells are a model of prolonged proliferation, we predicted that TSA and AZA treatment might restore M₃ expression.

We first wanted to determine the effect of TSA and AZA treatment on M₃ expression levels between low and high passage ISMC, to confirm that high passage cells have an altered contractile phenotype. Low passage cells expressed higher but variable levels of M₃ mRNA
Figure 6. TSA and AZA increase expression of contractile elements in high passage ISMC.  

A. Quantitative PCR analysis for MLCK2 mRNA expression in low passage ISMC and TSA- (0.1 μM) and AZA-treated (2 μM and 10 μM) high passage cells. Both concentrations of AZA increased MLCK2 expression compared to untreated high passage controls.  

B. Quantitative PCR analysis for M₃ expression in low passage and TSA- and AZA-treated high passage cells. Both concentrations of AZA increased M₃ mRNA expression compared to untreated high passage controls.  

*p<0.05 for one-way ANOVA compared with controls.
expression of M₃ was due to histone hypoacetylation or DNA hypermethylation in high passage ISMC, cells were treated with TSA or AZA for 24 hr. and the outcome of gene expression was assessed. Compared to untreated cells, TSA treatment showed a non-significant increase (5.56 ± 3.18, n=5, p>0.05; Fig. 6B) in M₃ expression. Similarly to MLCK2 expression, TSA treatment induced variable changes of M₃ expression across cell lines: 2 cell lines showed a slight decrease, 1 cell line showed a slight increase, and 2 cell lines showed over a 7-fold increase. On the other hand, 2 μM AZA (5.13 ± 1.46, n=5, p<0.05; Fig. 6B) and 10 μM AZA (2.40 ± 0.33, n=4, p<0.05; Fig. 6B) increased M₃ mRNA expression, compared to control cells. This suggests that decreased M₃ expression in high passage ISMC is due to DNA methylation, and is reversible through AZA treatment.

Activation of M₃ receptors by acetylcholine activates intracellular pathways, including activation of AKT, which leads to contraction of smooth muscle cells (Murthy, 2006). Carbachol-induced phosphorylation of AKT was decreased in proliferating ISMC from the inflamed colon of rats compared to healthy controls (Nair et al., 2011). We therefore predicted that TSA and AZA treatment could increase phosphorylated AKT (pAKT) levels in response to carbachol in the high passage cell model. High passage ISMC were plated in 24-well plates, treated with TSA or AZA in serum-free media for 3 days, then supplemented with carbachol for 7 and 15 min. Cells were then collected and prepared for western blotting.

To assess the outcome on carbachol signaling, the baseline ratio (0 min.) of phosphorylated to unphosphorylated (pAKT/AKT) was compared to the ratios at 7 and 15 min. following 100 μM carbachol supplementation. TSA and AZA treatment appeared to increase the ratio of pAKT/AKT at 7 and 15 min. post-carbachol supplementation, compared to untreated cells (Fig. 7A). Westerns were repeated for multiple cell lines, and the ratio of pAKT/AKT at each time point were averaged. Indeed, TSA increased the pAKT/AKT ratio at 7 min. post-carbachol
Figure 7. TSA and AZA increase downstream events following receptor activation in high passage ISMC. A. Representative western blot of phosphorylated AKT (pAKT) and AKT protein in TSA- and AZA-treated high passage cells, following 7 or 15 min. of carbachol exposure. B. Quantification of the ratio of pAKT: AKT in TSA- and AZA-treated high passage cells following carbachol addition. The pAKT: AKT ratios were normalized to non-carbachol treated controls, and averaged across multiple cell lines (n=6 ± sem, p<0.05). TSA treatment increased pAKT: AKT at 7 min. but not 15 min. post-carbachol addition, while both concentrations of AZA increased pAKT: AKT at either time point. *p<0.05 for one-way ANOVA compared with controls.
addition (2.21 ± 0.53, n=6, p<0.05; Fig. 7B), and showed a non-significant increase in pAKT phosphorylation 15 min. post-carbachol supplementation (2.74 ± 1.32, n=6, p>0.05; Fig. 7B). Likewise, 2 μM AZA (1.57 ± 0.22, n=6, p>0.05; Fig. 7B) and 10 μM AZA (2.25 ± 0.19, n=6, p<0.05; Fig. 7B) increased AKT phosphorylation 7 min. post carbachol addition, and showed a non-significant increase 15 min. post-carbachol treatment (2 μM AZA: 1.58 ± 0.32, n=6, p>0.05; Fig. 7B; 10 μM AZA: 3.32 ± 1.67, n=6, p>0.05; Fig. 7B). This suggests that histone hypoacetylation and DNA hypermethylation might decrease the appropriate carbachol-induced signaling cascade, including phosphorylation of AKT, preventing high passage cells from exhibiting smooth muscle contraction properties. However, increased expression of intracellular elements downstream of carbachol signaling through TSA and AZA treatment might restore proper contraction in these cells.

3.5 TSA and AZA increase GDNF expression in high passage ISMC

In addition to being the contractile units of the intestine, smooth muscle cells also function in supporting the survival of myenteric neurons and growth of their neurites through expression of the key neurotrophin GDNF (Gougeon et al., 2013). However, high passage cells have decreased expression of GDNF compared to low passage cells (Han et al., 2015). We predicted that loss of GDNF expression might also be due to epigenetic changes in high passage ISMC. Therefore, the actions of TSA or AZA on expression of GDNF were tested on high passage ISMC using qPCR, western blotting, and a functional bioassay.

Compared to untreated cells, ISMC treated with TSA for 24 hr. showed an increase in GDNF mRNA expression (1.65 ± 0.14, n=4, p<0.05; Fig. 8A). Likewise, both concentrations of AZA increased GDNF mRNA expression, compared to untreated high passage ISMC (2 μM AZA: 1.96 ± 0.21, n=3, p<0.05; Fig. 8A; 10 μM AZA: 2.12 ± 0.19, n=4, p<0.05; Fig. 8A). This suggests that histone hypoacetylation and DNA hypermethylation account for decreased GDNF expression in high passage ISMC, which can be restored by reversing these epigenetic changes.
Figure 8. TSA and AZA increase GDNF expression in high passage ISMC. A. Quantitative PCR analysis for GDNF mRNA expression in TSA- (0.1 μM) and AZA-treated (2 μM and 10 μM) high passage cells. TSA and AZA increased GDNF expression compared to untreated high passage controls. B-C. Representative western blots showing GDNF protein expression in TSA- and AZA-treated high passage cell pellets. High passage cell lines exhibited variable responses to TSA and AZA treatment, where TSA and AZA increased GDNF protein in some cell lines (B) but not others (C). D. Representative western blot of the 40-50 kDa GDNF protein precipitated from conditioned media isolated from TSA and AZA treated high passage ISMC. TSA and AZA treatment increased secreted GDNF protein expression, compared to untreated high passage cells. E. Quantification of secreted GDNF protein expression in TSA and AZA-treated high passage ISMC. The control-normalized band density of secreted GDNF protein was averaged across multiple cell lines (n=6-7 ± sem, p<0.05). TSA and AZA treatment increased secreted GDNF protein expression compared to untreated controls. *p<0.05 for one-way ANOVA compared with controls.
To determine whether inhibiting histone deacetylation and DNA methylation can increase GDNF protein expression, high passage cells were treated with TSA and AZA, then collected and prepared for protein expression by western blotting. A GDNF peptide of 15 kDa was used as a positive control, and represents biologically active GDNF (Cirella, 2016, Thesis). Across 5 different cell lines used, TSA and AZA appeared to induce variable expression of the 15 kDa GDNF product. While some cell lines showed a slight increase in GDNF protein expression following TSA and AZA treatment (Fig. 8B), other cell lines showed no clear changes (Fig. 8C).

To determine if GDNF protein was secreted in high passage cells, the media of cultured cells were collected, and precipitated with TCA. Media was also collected from high passage ISMC treated with TSA or AZA to assess potential changes in secreted GDNF protein. In all conditions, a 40-50 kDa immunoreactive product was detectable (Fig. 8D), while the 15 kDa mature GDNF protein was only detected in 2 cell lines. Changes in the 40-50 kDa product following TSA and AZA treatment were averaged across cell lines, and normalized to untreated cells. TSA (2.40 ± 0.47, n=7, p<0.05; Fig. 8E) and both concentrations of AZA (2 μM AZA: 2.06 ± 0.47, n=6; Fig. 8E; 10 μM AZA: 3.15 ± 0.87, n=6, p<0.05; Fig. 8E) increased secreted GDNF protein compared to untreated conditioned media. This suggests that TSA and AZA can increase secreted GDNF levels, and further suggest that decreased GDNF expression in high passage cells is due to histone hypoacetylation and DNA hypermethylation.

3.5.1 Conditioned media from TSA- and AZA-treated high passage ISMC increase axon density in a neonatal rat co-culture model

Since TSA and AZA treatment increased GDNF mRNA and protein expression in high passage ISMC, we wanted to test if these compounds could also improve the neurotrophic function of high passage cells using a well-established bioassay. The use of a conditioned media co-culture model in our lab has shown that media from low but not high passage cells increased axonal outgrowth (Han et al., 2015). Since TSA and AZA treatment appeared to increase GDNF
expression (Fig. 8A-E), we sought to determine whether these agents might also allow media from treated high passage cells to support neurite outgrowth. Conditioned media from TSA- and AZA-treated high passage ISMC were collected, diluted in half with fresh DMEM, and applied to established co-cultures for 48 hr. Co-cultures were labelled with antibodies to detect HuD (a pan-neuronal marker) and SNAP-25 protein (a SNARE protein involved in neurotransmitter release). Changes in neurite outgrowth were assessed by counting the number of SNAP-positive axons and HuD-positive neurons, then calculating the axon density. Co-cultures treated with serum-free DMEM and 5% FCS were used as negative and positive controls, respectively. TSA or AZA treatment applied directly to co-cultures did not cause an increase in axon outgrowth, and appeared to cause some neuronal cytotoxicity.

Conditioned media isolated from TSA- and AZA-treated high passage cultures increased axon density compared to media from untreated high passage cells (Fig. 9A). Changes in axon density from conditioned media isolated from multiple cell lines were averaged and normalized to untreated controls. Media from TSA-treated high passage ISMC increased axon density (1.73 ± 0.25, n=4, p<0.05; Fig. 9B) compared to controls. Likewise, media from 2 μM AZA (1.68 ± 0.24, n=6, p<0.05; Fig. 9B) and 10 μM AZA (1.59 ± 0.07, n=6, p<0.01; Fig. 9B) treated high passage cultures increased axon density compared to untreated control conditioned media. These results confirm that TSA and AZA treatment increases GDNF expression in high passage ISMC.

3.6 Proliferation drives epigenetic changes in rat ISMC by increasing expression of DNMT1 and HDAC2

Since decreased expression of contractile markers, contractile functional elements, and GDNF expression/function appear to involve DNA hypermethylation and histone hypoacetylation in high passage ISMC, we wanted to investigate the mechanisms that cause these epigenetic changes. Elsewhere, increased DNMT1, HDAC2, and HDAC5 expression and activity are associated with altered expression of contractile markers in cultured ASMC and VSMC
Figure 9. TSA and AZA increase neurotrophic function of high passage ISMC. A. Representative images of neonatal rat co-cultures incubated with media from TSA- (0.1 μM) and AZA-treated (2 μM and 10 μM) high passage ISMC for 48 hr. Conditioned media from TSA- and AZA-treated high passage cultures increased axonal outgrowth on a per neuron basis, compared to media from untreated controls. Scale bar, 20 μm. Neuronal cell bodies were labelled with anti-HuD (red; arrows), and axons were labelled with anti-SNAP-25 (green; triangles). B. Quantification of axon density of conditioned media treated co-cultures. Compared to media from untreated controls, media from TSA- and AZA-treated high passage cultures increased axon density of co-cultures (n=4-6). *p<0.05 for one-way ANOVA compared with controls.
We predicted that growth of ISMC might also induce expression of DNMT1, HDAC2, and HDAC5.

To determine if serum-induced growth is associated with increased expression of these epigenetic modification enzymes, low passage ISMC were cultured in 35 mm dishes in serum-free or 5% FCS for 24 hr., then collected for qPCR. Low passage ISMC grown in 5% FCS appeared to increase DNMT1 (2.02 ± 0.70, n=4, p>0.05; Fig. 10A), HDAC2 (4.61 ± 2.12, n=3, p>0.05; Fig. 10B), and HDAC5 expression (1.33 ± 0.35, n=3, p>0.05; Fig. 10C) compared to cells grown in serum-free media. This suggests that proliferation of low passage ISMC has the potential to induce the expression of enzymes involved in DNA methylation and histone hypoacetylation. To assess if prolonged proliferation of ISMC can further upregulate expression of these epigenetic enzymes, high passage cells were also grown in serum-free media or 5% FCS, and prepared for qPCR.

Compared to low passage cells grown in serum-free media, high passage ISMC in serum-free media had increased DNMT1 (5.56 ± 1.37, n=4, p<0.01; Fig. 10A), and HDAC2 expression (52.43 ± 11.02, n=4, p<0.05; Fig. 10B). Interestingly, HDAC5 mRNA expression was much more variable in high passage ISMC, and there was no significant change compared to low passage cells (Fig. 10C). Similarly, high passage cells grown in 5% FCS had increased DNMT1 (4.63 ± 0.16, n=3, p<0.05; Fig. 10A), and appeared to have increased HDAC2 mRNA expression (46.04 ± 14.81, n=4, p>0.05; Fig. 10B), but not HDAC5 mRNA expression (Fig. 10C). This suggests that cellular growth has the potential to increase DNMT1, HDAC2 and HDAC5 expression in ISMC, but prolonged growth will further increase expression of DNMT1 and HDAC2. There were no significant changes in DNMT1, HDAC2, or HDAC5 mRNA expression when comparing high passage ISMC grown without serum to cells grown in 5% FCS for 24 hr. This suggests that expression of these enzymes is irreversibly upregulated in high passage ISMC. Overall, increased DNMT1 and HDAC2 expression in high passage cells supports the hypothesis that these enzymes might induce DNA
Figure 10. Proliferation increases expression of inhibitory epigenetic machinery in rat ISMC. A. Compared to low passage ISMC cultured in serum-free media (0%), cells grown in 5% FCS appeared to increase DNMT1 mRNA expression, while high passage cells cultured with and without serum showed an increase in DNMT1 mRNA expression (n=4 ± sem, p<0.05). B. HDAC2 mRNA expression slightly increased in low passage cells grown in 5% FCS compared to serum-free media. High passage cells grown in serum-free media or 5% FCS had increased HDAC2 mRNA expression compared to low passage cells grown in serum-free media (n=3-4 ± sem, p<0.05). C. There were no significant differences in HDAC5 mRNA expression between low passage cells grown in serum-free media, 5% FCS, or high passage ISMC grown in either condition (n=3-4 ± sem, p<0.05). *p<0.05 and **p<0.01 for one-way ANOVA compared with controls.
methylation and histone hypoacetylation, respectively, which account for decreased expression of contractile markers, contractile functional elements, and GDNF in high passage cells.

3.7 ISMC isolated from human strictures have decreased expression of SMA and SM22

Prolonged proliferation of rat ISMC in vitro leads to decreased expression of the contractile markers SMA and SM22 (Nair et al., 2011). Excessive growth of ISMC is characteristic of intestinal strictures in both animal models and human CD, and therefore prolonged proliferation of ISMC is thought to lead to stricture formation (Chen et al., 2017; Koukoulis et al., 2001; Marlow & Blennerhassett, 2006). In agreement with the notion that smooth muscle proliferation induces phenotypic switching, ISMC in strictures appear to have decreased expression of contractile markers (Marlow & Blennerhassett, 2006; Suekane et al., 2010). However, there is no direct evidence showing that smooth muscle cells from human strictures develop an altered phenotype.

To assess if human strictures mimic the phenotypic changes of high passage cells, ISMC were isolated from strictured regions (SX-ISM C) of the terminal ileum in 5 CD patients. Smooth muscle cells were also isolated from proximal normal margin tissue to the stricture (NM-ISM C), serving as patient-matched controls. Cells were cultured in 60 mm dishes with 5% FCS, then passaged twice to generate low passage cells.

To assess the phenotype of SX-ISM C and NM-ISM C, cells were collected and prepared for western blotting. Samples were normalized by loading equal cell number and assessing β-actin expression. Compared to NM-ISM C, SX-ISM C showed decreased SMA and SM22 protein expression (Fig. 11A). Western blots were repeated for the strictured and normal margin cells from each patient, and the integrated optical densities (IOD) were averaged. NM-ISM C had increased SMA (5.97 ± 1.38, n=5, p<0.05; Fig. 11B) and SM22 protein expression (5.84 ± 1.03, n=5, p<0.05; Fig. 11B) compared to SX-ISM C (SMA: 2.38 ± 1.2, n=5; Fig. 11B; SM22: 2.27 ±
Figure 11. ISMC isolated from human strictures have decreased expression of contractile markers. A. Representative western blot of SMA, SM22, and β-actin protein expression in ISMC from human strictures and adjacent normal margin tissue. ISMC from strictures had decreased SMA and SM22 protein expression, compared to cells from normal margin regions. Equal number of cells were loaded per western blot, although there were no changes in the housekeeper protein β-actin between ISMC from strictures and normal margin regions. B. Quantification of the band density of SMA and SM22 protein from ISMC isolated from human strictures and normal margin regions. Compared to ISMC isolated from strictures, normal margin ISMC have increased SMA and SM22 protein expression (n=5 ± sem, p<0.05). *p<0.05 for paired student’s t-test compared with control normal margin ISMC.
0.81, n=5; Fig. 11B). This suggests that SX-ISMС have a decreased contractile phenotype compared to NM-ISMС, paralleling the rat high passage ISMC model.

To confirm our western blot findings, sections of strictured and normal margin tissue were labeled with antibodies to detect SMA and SM22 protein. Unlike our western blot findings, there appeared to be no obvious changes in SMA or SM22 protein expression in ISMC from normal and strictured smooth muscle (Fig. 12A-B). Furthermore, there appeared to be differences in accessibility of the antibodies to properly penetrate and stain the tissues. For example, SMA stained the perimeter intensely in some sections, but did not label more internal cells (Fig. 12A). The use of immunohistochemistry might be a less sensitive technique than western blotting in attempting to show changes in protein expression between strictured and normal margin tissue.

3.8 ISMC isolated from human strictures have increased DNMT1, HDAC2, and HDAC5 expression

Since the decreased contractile phenotype in high passage cells might be due to enhanced DNA methylation and diminished histone acetylation, we sought to assess if the same changes also account for SX-ISMС decreased marker expression. Low passage SX-ISMС and NM-ISMС were assessed for DNMT1, HDAC2 and HDAC5 mRNA expression by qPCR.

Interestingly, the 5 strictured and normal margin cell lines generated from different patients presented variable expression of these inhibitory epigenetic enzymes. 3 cell lines (KH23, KH39, and KH42) showed increased DNMT1 and HDAC5 mRNA expression, while the remaining 2 cell lines (KH37 and KH38) only showed increased HDAC2 mRNA expression (Fig. 13A). This variation in the epigenetic changes acquired by strictured smooth muscle cells might arise from differences in the stricture environment or disease state of each patient. Cell lines that showed similar patterns of change in mRNA expression were grouped together, and the increases in DNMT1, HDAC2 and HDAC5 mRNA expression were averaged. DNMT1 (2.93 ± 0.4, n=3, p<0.05; Fig. 13B) and HDAC5 mRNA expression (3.67 ± 0.8, n=3, p<0.05; Fig. 13B) increased
Figure 12. Immunohistochemistry to detect expression of SMA and SM22 in sections of human stricture and normal margin tissue. A-C. Representative images of ISMC labelled with antibodies to detect SMA (A) and SM22 (B) protein. No obvious changes in SMA and SM22 expression were observed between ISMC from strictured and normal margin regions. SMA staining was more intense on ISMC that bordered the tissue, suggesting potential limitations in the accessibility of the antibody to properly coat the whole section. Nuclei in each section were stained with Hoechst (C). Scale bar, 20 µm.
in SX-ISM C, compared to NM-ISM C. HDAC2 mRNA expression in SX-ISM C appeared to increase in the remaining two cell lines (n=2), compared to NM-ISM C. This suggests that SX-ISM C have increased DNA methylation and decreased histone acetylation compared to NM-ISM C, which might account for their decreased expression of SMA and SM22. Interestingly, human SX-ISM C appeared to present with two distinct mechanisms of acquired epigenetic change: 1) increased DNMT1 and HDAC5 expression or 2) increased HDAC2 expression.

3.9 Prolonged growth decreases the contractile phenotype of human ISMC and induces epigenetic changes

Protracted growth through repeated passaging decreases the contractile phenotype of rat ISMC and increases their proliferative capacity (Nair et al., 2011). This in vitro model mimics changes seen in the strictured rat, where smooth muscle cells undergo hyperplasia and have decreased expression of contractile markers (Marlow & Blennerhassett, 2006). ISMC isolated from human strictures also have a decreased contractile phenotype (Fig. 11), which mimics our rat high passage in vitro model.

To further verify this protracted growth model, we wanted to test the effects of repeated passage on human ISMC. Two independent human models were employed: 1) ISMC isolated from human strictures and adjacent normal margin regions in CD patients and 2) ISMC isolated from resection specimens from normal ileum obtained from right hemicolectomy for colon cancer.

3.9.1 High passage ISMC from human strictures and normal margin regions develop an altered contractile phenotype, associated with epigenetic changes

The contractile phenotype of high passage rat ISMC mimics the phenotype of low passage ISMC from human strictures; both cell types have decreased expression of SMA and SM22 (Nair et al., 2011, and Fig. 11). Additionally, both cell types have increased expression of epigenetic enzymes (Fig. 10 and Fig. 13) which act to inhibit gene transcription, suggestive of a
Figure 13. ISMC isolated from human strictures have increased expression of inhibitory epigenetic enzymes. A. Quantitative PCR analysis of DNMT1, HDAC2, and HDAC5 mRNA expression for ISMC isolated from 5 different human strictures, normalized to normal margin ISMC. Structured ISMC displayed two distinct patterns of increased mRNA expression: 3 cell lines (KH23, KH39, and KH42) had increased DNMT1 and HDAC5 expression, while 2 cell lines (KH37 and KH38) had increased HDAC2 expression. B. Cell lines which shared similar patterns of changes in mRNA expression in structured ISMC were grouped together, normalized to normal margin ISMC, and averaged (DNMT1 and HDAC5: n=3 ± sem, p<0.05; HDAC2: n=2 ± sem). DNMT1 and HDAC5 mRNA expression was increased in structured ISMC, compared to normal margin ISMC. *p<0.05 for paired student’s t-test compared with normal margin controls.
mechanism for this altered phenotype. In contrast, low passage rat ISMC and cells from human normal margin regions do not have a decreased contractile phenotype (Nair et al., 2011, and Fig. 11).

To confirm that repeated passaging of smooth muscle cells leads to the development of an altered phenotype, high passage cultures of normal margin and strictured ISMC were generated from 3 different humans. Unlike rat ISMC, human cells did not grow quickly or consistently with subculturing, rendering some cell lines untestable for each experiment.

To determine if repeated passage decreased the contractile phenotype of human cells, expression of SMA and SM22 protein were assessed by western blotting in high passage strictures and normal margin ISMC (n=2). Western blots were normalized by loading equal cell number, and compared to expression of the protein β-actin. High passage SX-ISMC and NM-ISMC from both cell lines had decreased expression of SMA, compared to low passage NM-ISMC (Fig. 14A-B). The same trend was observed for SM22 protein expression in one cell line (KH42; Fig. 14A). In contrast, only high passage strictured cells appeared to have decreased SM22 protein expression in the other cell line (KH37), compared to low passage NM-ISMC (Fig. 14B). Overall, repeated passage of human ISMC decreased expression of SMA, and induced variable changes in SM22 protein expression.

Since increased expression of epigenetic enzymes account for the decreased contractile phenotype of high passage rat ISMC, we tested expression of these genes in high passage human ISMC (n=2). In one cell line (KH23), high passage SX-ISMC and NM-ISMC had increased expression of DNMT1, HDAC2, and HDAC5, compared to low passage NM-ISMC (Fig. 14C). In another cell line (KH42), only high passage strictured cells had increased DNMT1 expression (Fig. 14D). This suggests that repeated passage induces changes in the epigenetic machinery of human ISMC, but expression of these enzymes is variable across cell lines generated from different humans.
Figure 14. Protracted growth decreases the contractile phenotype and induces epigenetic changes in ISMC from human strictures and normal margin tissue. A-B. Western blot analysis of SMA and SM22 protein expression in low and high passage ISMC from human strictures and paired normal margin regions, isolated from two different humans (A: KH42; B: KH37). High passage ISMC from strictures and normal margin regions have decreased SMA expression, compared to low passage normal margin cells. High passage cells from KH23 have decreased SM22 expression, compared to low passage normal margin cells (A). High passage strictured ISMC have decreased SM22 expression, compared to low passage normal margin cells (B). Experiments were normalized by loading equal cell number, and confirmed with expression of β-actin. C-D. Quantitative PCR analysis of DNMT1, HDAC2, and HDAC5 mRNA expression for low and high passage ISMC from human strictures and normal margin regions. Compared to normal margin cells at low passage, high passage ISMC had increased expression of DNMT1, HDAC2, and HDAC5 in KH23 cells (C). In cells isolated from KH42, only high passage strictured cells had increased DNMT1 expression compared to normal margin ISMC at low passage (D).
3.9.2 High passage ISMC from normal ileum of tissue resected for colon cancer develop an altered contractile phenotype, associated with epigenetic changes

In our rat high passage model, ISMC are isolated from healthy and non-inflamed adult rats. Since ISMC from human strictures and normal margin regions are exposed to intestinal inflammation in CD patients, we wanted to test if repeated passage of ISMC from normal, non-inflamed humans would mimic our rat model. ISMC were isolated from the non-inflamed terminal ileum or colon of patients undergoing colorectal surgery. ISMC were cultured in 60 mm dishes with 5% FCS, then passaged twice or ten times to generate low and high passage cells, respectively.

To test if repeated passage decreased the contractile phenotype of normal human ISMC, expression of SMA and SM22 protein were assessed in low and high passage cells with western blotting. Equal cell number were loaded for each experiment, and confirmed with expression of β-actin protein. Compared to low passage cells, human ISMC at high passage have decreased expression of SMA and SM22 (Fig. 15A). Western blots were repeated for low and high passage cells generated from four different patients. High passage human cells had decreased expression of SMA (0.61 ± 0.05646, n=4, p<0.05; Fig. 15B) and appeared to have decreased SM22 protein expression (4.44 ± 0.09, n=4, p>0.05; Fig. 15B) compared to low passage ISMC. This suggests that repeated passage of ISMC from normal, non-inflamed human ileum decreases the contractile phenotype of these cells, as observed in the rat model (Nair et al., 2011).

In high passage rat ISMC, increased expression of DNMT1 and HDAC2 account for decreased expression of contractile markers (Fig. 10). Therefore, we wanted to test if increased expression of these epigenetic enzymes might also account for the decreased contractile phenotype of normal human ISMC at high passage. However, only one cell line (KH29) showed increased DNMT1, HDAC2, and HDAC5 mRNA expression in high passage cells, compared to low passage ISMC (Fig. 15C). High passage ISMC from the other cell lines (KH29, KH33, KH36) had decreased expression of DNMT1, HDAC2, and HDAC5 (Fig. 15C). This suggests that DNA
methylation and histone hypoacetylation might account for the decreased expression of SMA and SM22 in some normal human cells, but does not completely explain why repeated passage decreases the contractile phenotype in these cells.
Figure 15. Protracted growth of normal ISMC isolated from normal ileum of tissue resected for colon cancer decreases contractile phenotype and induces epigenetic changes. A. Representative western blot of low and high passage ISMC from normal non-CD humans. B. Quantification of the band density of SMA and SM22 protein in ISMC isolated from non-Crohn’s disease humans. Compared to low passage ISMC, high passage cells had decreased SMA expression (n=4 ± sem, p<0.05), and appeared to have decreased expression of SM22 (n=4 ± sem, p=0.09). *p<0.05 for paired student’s t-test compared with low passage ISMC. C. Quantitative PCR analysis of DNMT1, HDAC2, and HDAC5 mRNA expression in low and high passage ISMC isolated from non-Crohn’s disease humans. Changes in mRNA expression were normalized to low passage cells, and separated across each human cell line. High passage ISMC from only one cell line (KH25) had increased DNMT1, HDAC2, and HDAC5 expression compared to low passage cells.
Chapter 4

Discussion

Prolonged proliferation decreases the contractile phenotype of ISMC, increases their proliferative capacity, and decreases their neurotrophic function (Han et al., 2015; Nair et al., 2011). The mechanisms for how prolonged proliferation induces this altered phenotype are unknown. Elsewhere, recent work has identified a role for epigenetic changes, namely DNA methylation and histone acetylation, in modulating smooth muscle phenotype (reviewed in Alexander and Owens, 2012; Wright et al., 2013). Therefore, we tested whether the same epigenetic mechanisms might account for the altered phenotype of high passage ISMC, and found evidence supporting acquired epigenetic changes in these cells. Principally, we found that inhibition of DNMT and HDAC through AZA and TSA treatment, respectively, restored the altered phenotype of high passage cells. Since high passage ISMC mimic the phenotype from strictures in a rat model of intestinal inflammation (Marlow & Blennerhassett, 2006; Nair et al., 2011), we wanted to test if the same changes in contractile phenotype were present in ISMC from human strictures, and if similar epigenetic mechanisms account for this altered expression. Our results showed that ISMC from human strictures have a decreased contractile phenotype, which was also associated with increased expression of DNMT and HDAC.

4.1 Epigenetic changes underlie the altered phenotype of high passage ISMC

4.1.1 Inhibition of DNMT and HDAC activity suppresses serum-induced proliferation of ISMC

Previous work by our lab showed that proliferation of ISMC is associated with decreased expression of contractile markers (Nair et al., 2011). We showed that the onset of proliferation was inversely associated with expression of the contractile marker SM22 in low passage cells. However, it is unknown whether proliferation of high passage cells is also associated with decreased
expression of contractile markers. High passage cells expressed low levels of SM22 independent of serum exposure. Furthermore, high passage cells had similar levels of SMA and SM22 protein when grown with or without serum. This suggests that high passage cells have a permanently altered phenotype, that is irreversible through growth manipulation.

We found PCNA-positive high passage ISMC in serum-free cultures, suggesting that these cells still grow in serum-deprived conditions. Unpublished work from our lab showed that high passage ISMC express higher levels of PDGF-BB, the principal mitogen for ISMC (Stanzel et al., 2010), than low passage cells, and that blocking the PDGF-BB receptor, PDGF-Rβ, decreases their growth response. These findings suggest that high passage cells might grow in the absence of serum due to auto-stimulation with PDGF-BB. Therefore, we hypothesize that TSA and AZA decrease the expression of PDGF-BB or PDGF-Rβ in high passage cells.

It is also possible that TSA and AZA upregulate expression of genes which function to inhibit PDGF-BB-mediated growth. Recent work has identified a number of different factors that inhibit PDGF-BB-induced proliferation of smooth muscle cells, including peroxisome proliferator-activated receptor-δ activation, tunicamycin, and Slit2 (Liu et al., 2013; Ning et al., 2011; Yi et al., 2012). Other work also identified downstream elements involved in PDGF-BB-mediated proliferation, including ERK, p38, Kruppel-like factor 4, and MAPK (reviewed in Alexander and Owens, 2012). Therefore, future work should determine if TSA and AZA increase expression of genes that inhibit PDGF-BB expression or signaling, to gain a complete understanding of how these compounds reverse the growth response in high passage ISMC.

There are a wide variety of factors suggested to influence smooth muscle proliferation, in addition to growth factors like PDGF-BB (reviewed in Owens, 1995). Overexpression of the contractile marker SM22 induced cell-cycle arrest in VSMC exposed to PDGF-BB (Dong et al., 2010). Furthermore, SM22 expression prevented VSMC proliferation in vivo, inhibiting subsequent neointimal thickening in a model of atherosclerosis (Dong et al., 2010). Likewise, SM22 knockout
mice had increased plaque growth attributable to VSMC (Feil et al., 2004). These results suggest that this contractile marker also plays a role in regulating the phenotype of smooth muscle cells. Our results showed that TSA and AZA treatment increased SM22 expression in high passage cells. Therefore, the ability of TSA and AZA treatment to reduce the growth response in high passage ISMC conditions might be attributable to increased SM22 expression and SM22-mediated suppression of proliferation. Further work should attempt to block the actions of SM22 using siRNA in TSA- and AZA-treated high passage cells, or transfect non-treated cells with an SM22-expressing plasmid to assess if SM22 directly regulates ISMC growth. Understanding the mechanisms that control proliferation of high passage ISMC might provide insights into specific molecular therapeutic targets for restoring proper smooth muscle function in strictures.

4.1.2 Epigenetic changes cause decreased contractile phenotype in high passage ISMC

The altered phenotype of high passage ISMC includes decreased expression of contractile markers (Nair et al., 2011). Elsewhere, TSA and AZA treatment restored expression of contractile markers in smooth muscle cells (Jin et al., 2011; Ning et al., 2013; Okamoto et al., 2006; Zhuang et al., 2016). Our results showed that TSA- and AZA-treated cells had increased SMA and SM22 mRNA and protein expression. This suggests that promoter hypermethylation and hypoacetylation of histones associated with SMA and SM22 genes account for their decreased expression in high passage cells.

Importantly, we used three independent techniques to assess changes in SMA and SM22 expression in high passage ISMC: qPCR, western blotting, and immunocytochemistry. The use of cell lines derived from independent animals further validates our results, and suggests that increased expression of contractile markers through TSA and AZA treatment is a highly significant and reproducible finding.

However, our results did not directly show that DNA hypermethylation occurs at the promoters of SMA and SM22, nor that histones associated with these genes are hypoacetylated.
Future experiments should measure the level of DNA methylation using quantitative methylation specific PCR, and histone acetylation using chromatin immunoprecipitation with antibodies against acetylated histones to confirm that TSA and AZA directly increase expression of SMA and SM22.

Elsewhere, the transcription factor serum response factor and its coactivator myocardin are important elements in regulating expression of smooth muscle markers (reviewed in Alexander & Owens, 2012; Owens et al., 2004). It is possible that serum response factor or myocardin are epigenetically downregulated in high passage cells, and that TSA and AZA treatment restore their expression and subsequent expression of contractile markers. Further work should determine if expression of serum response factor and myocardin is decreased in high passage ISMC, and if TSA and AZA treatment can increase their expression.

Since TSA and AZA are pan-HDAC and DNMT inhibitors, respectively, one unintended consequence of treating high passage cells with these chemicals is non-specific increased expression of unwanted genes. Recent work has identified specific inhibitors for DNMT1 (SW155246), HDAC2 (ST088357), and HDAC5 (miR-2861) (Fischer et al., 2015; Kilgore et al., 2013; Wang et al., 2015). To avoid inhibiting all DNMT and HDAC activity, future experiments should use these specific inhibitors. The use of specific DNMT and HDAC inhibitors would also confirm that DNMT1, HDAC2, and HDAC5 are mechanistically involved in altering gene expression in high passage cells. However, there were no changes in proteins β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in TSA- and AZA-treated cells, compared to untreated controls (data not shown). There were also no differences in pAKT or AKT protein levels between untreated and TSA- or AZA-treated ISMC. This supports our hypothesis that TSA and AZA only increase expression of contractile marker proteins and other epigenetically downregulated genes in high passage ISMC. To confirm this, further experiments should assess changes in expression of genes known to increase with TSA and AZA treatment, including p21, α-tubulin, and thymidylate synthase (Glaser et al., 2003).
Our findings showed that high passage ISMC expressed less MLCK2 and M₃ than low passage cells, which was restored by TSA and AZA treatment. These results agree with work done in other smooth muscle types, where AZA treatment restored PDGF-BB-suppressed expression of MLCK2 in cultured airway and intestinal SMC (Ning et al., 2013; Shi & Sarna, 2013). Restored expression of MLCK2 in ASMC and ISMC was associated with increased contractility of these cells. Therefore, we predict that restored expression of MLCK2 and M₃ in high passage cells might improve their contractile function.

Although we found that TSA and AZA induced similar changes to gene expression in most of our experiments, AZA but not TSA induced a significant increase in MLCK2 and M₃ mRNA expression in high passage cells. Elsewhere, TSA and AZA upregulated overlapping and distinct genes in different multiple myeloma cell lines (Heller et al., 2008) and human retinal endothelial cells (Xie et al., 2014). Likewise, expression of the progesterone receptor-B increased only in response to AZA treatment and not TSA in two different human astrocytoma cell lines (Hansberg-Pastor et al., 2013). This suggests that the epigenetic mechanisms which control transcription can vary for different genes. Therefore, it is possible that the promoters of MLCK2 and M₃ are hypermethylated in high passage ISMC, but that histones associated with these genes are not hypoacetylated. However, since select cell lines exhibited an increase in MLCK2 and M₃ expression following TSA treatment, different high passage cell lines might acquire dissimilar epigenetic changes: some cell lines might develop hypoacetylation of histones associated with these genes, while other cell lines might not. Variation in TSA-induced changes to MLCK2 and M₃ expression might correlate with the variation of stricture formation in CD patients, as discussed later.

Our results showed that TSA and AZA treated ISMC had an increased ratio of pAKT:AKT in response to carbachol stimulation. Importantly, there were no significant changes in the ratio of pAKT:AKT before carbachol addition between treated and untreated cells. This suggests that TSA
and AZA upregulates the intracellular response to carbachol, and not pAKT expression directly. Previously, it was shown that carbachol-induced phosphorylation of AKT and $M_3$ expression was reduced in proliferating ISMC isolated from rats (Nair et al., 2011). We predict that restored expression of MLCK2 and $M_3$, and increased cholinergic responsiveness through TSA and AZA treatment will increase the contractility of high passage ISMC. Since the rat high passage model mimics the altered phenotype of rat and human strictured ISMC, we predict that dysmotility \textit{in vivo} might stem from altered expression of these contractile elements. Future work should assess if ISMC from strictures have decreased MLCK2 and $M_3$ expression, and a decreased response to cholinergic stimuli, and if TSA and AZA can restore their expression. Interestingly, the ratio of pAKT:AKT increased in all cell lines treated with TSA, suggesting that the variation in $M_3$ and MLCK2 expression does not translate to cholinergic responsiveness in these cells. It is possible that changes in mRNA expression of $M_3$ and MLCK2 do not reflect changes in protein levels, or that TSA upregulates other elements in the excitation-contraction cascade of smooth muscle cells. Thus, further experiments should evaluate the effects of TSA and AZA treatment on expression of other contractile elements, including the 20-kDa regulatory light chain of myosin II, the protein phosphatase 1 regulatory subunit, and serotonin receptors, and assess the protein levels of $M_3$ and MLCK2 with western blotting.

In other obstructive diseases associated with smooth muscle hyperplasia, including atherosclerosis, increased strain on SMC can induce changes in gene expression that affect their phenotype (reviewed in Chistiakov et al., 2015; Owens et al., 2004). In healthy arteries, normal laminar blood flow downregulates expression of factors which induce VSMC proliferation, including PDGF-BB (Goldman et al., 2007). However, disturbed blood flow upregulates PDGF-BB expression and autocrine action on VSMC (Wilson et al., 1993), leading to increased VSMC proliferation (Fan & Karino, 2010). Recent work showed that a similar mechanism affects gene expression of ISMC. Obstruction of the intestine due to dysmotility, as observed in intestinal
strictures, increases luminal content, subsequently increasing luminal pressure and distention proximal to the stricture (Shi et al., 2011). Strain induced expression of cyclooxygenase-2 in cultured rat ISMC, and in animal models of intestinal obstruction (Shi et al., 2011, Li et al., 2012). Increased cyclooxygenase-2 was associated with increased prostaglandin E2, which has been shown to inhibit smooth muscle contractility (Westergaard et al., 1997). Therefore, it is possible that protracted growth of ISMC increases cyclooxygenase-2 and prostaglandin E2 production, leading to an altered contractile phenotype. Further, increased strain on ISMC at the site of or proximal to intestinal strictures might induce changes in gene expression. Since we showed that TSA and AZA restored expression of elements involved in carrying out contraction, including MLCK2, M3, and cholinergic signaling, it is possible that these compounds can restore contractility of ISMC at strictures. Consequently, we hypothesize that restored contractility of ISMC may improve motility at the site of the stricture, thus decreasing strain-induced changes to gene expression of the smooth muscle layers.

4.1.3 Epigenetic changes cause decreased neurotrophic function of high passage ISMC

Recent work from our lab showed that high passage cells had decreased GDNF expression (Han et al., 2015), compared to low passage cells. Our results showed that TSA and AZA treatment increased GDNF mRNA and protein expression in high passage ISMC. Importantly, we showed increased GDNF expression using 3 independent techniques: qPCR, western blotting, and an established co-culture bioassay, reinforcing the legitimacy of our results. Interestingly, GDNF products at different molecular weights were detected in cell pellets and from conditioned media of TSA- and AZA-treated high passage cells. To understand why TSA and AZA might induce expression of different GDNF forms, it is important to discuss how GDNF is produced and processed by smooth muscle.

GDNF protein is synthesized as a zymogen that gets cleaved to produce the mature and biologically active 15 kDa form (Sun et al., 2014). However, there is variation in the detected
molecular weights of precursor and intermediate forms of GDNF. A 34 kDa GDNF precursor was found in PC12 cells (Geng et al., 2011), while Chinese hamster ovary cells produced a 25 kDa precursor (Lonka-Nevalaita et al., 2010). A 39 kDa form of GDNF was isolated from the smooth muscle layer of rats in vivo (David Mario Rodrigues, thesis), while a 50 kDa dimerized product was detected in the substantia nigra of rats (Gonzalez-Barrios et al., 2006). The dissimilarity in the molecular weights of precursor GDNF is further complicated by variations in the protein processing machinery across different cell types, and the fact that different growth conditions can alter processing of GDNF (Piccinini et al., 2013). Our lab found that intracellular MMP-9 expression by ISMC is responsible for the cleavage of full length GDNF into its mature 15 kDa form (Cirella, thesis). Although there were no consistent changes in 15 kDa GDNF protein expression, there was an increase in a GDNF product detected at 40-50 kDa in conditioned media from TSA- and AZA-treated cells. This suggests that expression of intracellular MMP-9 or precursor forms of GDNF is variable across high passage cell lines. Future work should assess the effects of repeated passage on MMP-9 expression in ISMC, and if TSA or AZA alters MMP-9 expression, compared to untreated high passage cells.

Elsewhere, TSA and AZA treatment have been shown to act synergistically by increasing gene expression in different cancer cell lines (Heller et al., 2008; Nie et al., 2010). Since TSA and AZA upregulated all genes of interest, except for M3 and MLCK2, it is possible that combining these compounds will further enhance expression of these genes. Preliminary results showed that high passage cells had substantial cytotoxicity in the presence of combined 0.1 µM TSA and 2 µM AZA. Future work should attempt to find a non-cytotoxic concentration of either compound when combined, and determine if TSA and AZA exert synergistic effects on reversing the altered phenotype of high passage ISMC.
4.2 ISMC from human strictures have a decreased contractile phenotype

Hyperplasia of smooth muscle cells is the most common structural change in strictures of CD patients (Chen et al., 2017; Koukoulis et al., 2001), and some evidence shows phenotypic switching of ISMC in human strictures (Suekane et al., 2010). However, the specific phenotypic changes of ISMC in human strictures are unknown. Cultured ISMC from strictures had decreased SMA and SM22 protein expression compared to ISMC from normal margin tissue. These results corroborate with our high passage rat model, where strictured cells have a decreased contractile phenotype compared to normal margin cells. Interestingly, no obvious changes were seen in sections of strictured and normal margin tissue labelled for SMA and SM22. This might be due to decreased sensitivity and ability for protein changes to be observed in immunohistochemistry, compared to a semi-quantifiable technique like immunoblotting. SMA staining appeared more intense in the perimeter of tissue in normal margin sections than strictured sections. The apparent lack of SMA staining in all cells from the strictured section might suggest an inability of the antibody to properly penetrate and coat the tissue, the use of an inappropriate fixation reagent, or improper fixation time.

Furthermore, it is well-known that placing smooth muscle cells in culture decreases expression of contractile markers (reviewed in Owens, 1995). Indeed, we showed that ISMC cultured for 4 days have decreased SMA and SM22 expression compared to freshly isolated cells (Nair et al., 2011). Therefore, it is possible that the difference between our western blot and immunohistochemistry findings results from changes in contractile marker expression when placing human ISMC in culture. However, since both strictured and normal margin ISMC were treated the same in culture, and prepared equally for western blotting, it is reasonable to compare protein expression between these cells. Future work should compare contractile marker expression in freshly isolated ISMC from strictures and normal margin ISMC, to confirm they agree with our cell culture measurements.
4.3 DNMT and HDAC activity induce epigenetic changes that underlie the phenotypic changes in ISMC from rats and humans

4.3.1 DNMT1 and HDAC2 activity induce epigenetic changes in high passage rat ISMC

Our results showed that high passage ISMC have increased DNMT1 and HDAC2 expression, compared to low passage cells. These results confirm the hypothesis that repeated passage induces changes to the expression of enzymes which modify epigenetic control of transcription. Our results also agree with other work which showed repeated passage of various cell types increased DNA methylation (Bork et al., 2010), and expression of DNMT1 and HDAC2 (Li et al., 2014).

Exposure to PDGF-BB increased expression of DNMT1 in cultured ASMC (Ning et al., 2013; Zhuang et al., 2016), and increased activity of HDAC2, HDAC4, and HDAC5 in cultured VSMC (Usui et al., 2014; Yoshida et al., 2007). Similarly, we showed that low passage cells exposed to serum for 24 hr. had increased DNMT1 and HDAC2 expression, compared to serum-deprived cells. Since PDGF-BB is the growth factor in serum that induces ISMC growth (Nair et al., 2014), we predict that PDGF-BB increases expression of DNMT1 and HDAC2 in low passage ISMC. High passage cells undergo numerous rounds of proliferation and growth in culture, and have therefore had increased exposure to PDGF-BB than low passage cells. Furthermore, unpublished data from our lab shows that high passage ISMC have increased expression of PDGF-BB, compared to low passage cells. Since we found that high passage cells have increased DNMT1 and HDAC2 than low passage ISMC, this reinforces our hypothesis, and suggests that increased PDGF-BB production might further increase the expression of these enzymes.

We also found that removal of serum from high passage ISMC did not alter the expression of DNMT1 and HDAC2 in high passage cells. These results support our earlier findings, showing that the altered phenotype of high passage is not reversible through serum deprivation. However, since PDGF-BB increases expression of DNMT and HDAC in other SMC (Ning et al., 2013; Usui,
2014; Yoshida et al., 2007; Zhuang et al., 2016), it might be expected that exposure to serum would further increase expression of these enzymes in high passage cells. We suggest two possible explanations for why the expression of these enzymes did not change in high passage cells exposed to serum: 1) high passage ISMC produce PDGF-BB regardless of environmental conditions, including serum exposure or serum-deprivation in culture or 2) DNMT1 and HDAC2 expression peaks after passaging ISMC 10 or more times, so that exposure to serum for 24 hr. cannot cause any further changes in expression of these epigenetic enzymes.

### 4.3.2 Strictures from ISMC display two distinct epigenetic modifications

Importantly, we found that ISMC from human strictures have increased expression of DNMT1 and HDAC5, compared to normal margin cells. This suggests that the altered phenotype of SX-ISMС is also due to epigenetic changes, namely DNA hypermethylation and histone hypoacetylation. Further work should determine if TSA and AZA treatment on SX-ISMС can restore expression of contractile markers.

Increased DNMT1 and HDAC5 expression was only observed in 3 cell lines, while the other 2 cell lines showed increased HDAC2 expression. This suggests that there are two independent mechanisms of acquired epigenetic changes in human ISMC from strictures. We hypothesize that the variation in these epigenetic mechanisms are likely to account from variations in the stricture environment or nature of the disease across each patient. Interestingly, there were no obvious correlations for the disease course and patient history in the two groups of human SX-ISMС. Although the two cell lines that only showed increased HDAC2 expression (KH37 and KH38) were isolated from female patients aged 37 and 38, another cell line (KH42) was also derived from a female patient, while a different cell line (KH39) was isolated from a male patient aged 47. This suggests that sex or age cannot completely explain the two distinct mechanisms of acquired epigenetic change in cultured SX-ISMС. There were also no clear differences in the past and current medications, time of diagnosis, previous resection surgeries, or lifestyle factors across...
all patients. However, we had a very small samples size, and it is possible that including more CD patients will provide clear associations between patient information and acquired epigenetic changes.

Since ISMC undergo hyperplasia in strictures from human and animal models (Marlow & Blennerhassett, 2006; Suekane et al., 2010), it is possible that these cells are exposed to increased levels of growth factors, including PDGF-BB. In other diseases of smooth muscle hyperplasia, including atherosclerosis and asthma, increased PDGF-BB levels account for enhanced smooth muscle growth (Hirota et al., 2011; Ross et al., 1990). Similarly, chronic inflammation of the intestine is associated with increased PDGF-BB production by circulating platelets, suggesting increased PDGF-BB at the site of strictures (reviewed in Chang et al., 2015). TNBS-induced colitis in rats showed increased expression of PDGF-Rβ in ISMC, which was associated with smooth muscle hyperplasia (Stanzel et al., 2010). Therefore, it is possible that increased PDGF-BB levels and PDGF-Rβ signaling might also account for increased growth of ISMC in human strictures. This suggests an in vivo mechanism for the development of the altered phenotype of SX-ISMC: Increased TNF-α and IL-1β during chronic inflammation increases expression of PDGF-Rβ by ISMC, which respond to increased circulating PDGF-BB produced by platelets, leading to increased DNMT1 and HDAC5 expression, and subsequent silencing of markers of the contractile phenotype. Further work should assess if SX-ISMC have increased expression of PDGF-Rβ, and determine if serum or PDGF-BB exposure or deprivation can alter expression of DNMT1 and HDAC5 in human ISMC.

Interestingly, the expression profile of epigenetic enzymes in ISMC from human strictures does not perfectly corroborate with that of high passage rat ISMC: DNMT1 expression is increased in both human and rat, while HDAC2 expression is only increased in rat and some human cell lines, and HDAC5 expression is only increased in some human cell lines. This variation might be attributable to differences in the mechanisms which control transcription of these enzymes in either
animal, or differences in how these cells respond under cell culture conditions. Future experiments should assess the effects of ST088357 and miR-2861, inhibitors of HDAC2 and HDAC5, respectively, to confirm a specific role of either enzyme in affecting gene expression in rat or human ISMC. However, the correlation of epigenetic changes in rat and human ISMC suggests that TSA and AZA might be potential treatment options for restoring proper smooth muscle function in vivo. The effect of TSA and AZA on ISMC growth and phenotype in vivo should be assessed in the TNBS-model of colitis in chronically inflamed and strictured regions.

Although we found changes in DNMT1, HDAC2, and HDAC5 expression in rat and human ISMC, we did not consider other isoforms of DNMT or HDAC. Increased DNMT3a expression induced DNA methylation that altered gene expression in human bladder SMC in vitro (Jiang et al., 2013), supporting a potential role for DNMT3 in regulating gene expression in ISMC. In contrast, DNMT1 but not DNMT3a was shown to affect VSMC proliferation and phenotypic alteration in cultured ASMC (Zhuang et al., 2016). Similarly, AZA-treatment mimicked the effects of DNMT1 knockdown on gene expression in human cancer cell lines (Ghoshal et al., 2004). Although these results suggest an important role for DNMT1 and not DNMT3 in affecting SMC gene expression, future work should assess DNMT3 expression in high passage cells, and compare outcomes of DNMT1 or DNMT3 specific inhibition.

We assessed changes in HDAC2 and HDAC5 as they represent two families of HDAC, and were found were to be expressed in colonic tissue in an analysis of the human transcriptome (Caron et al., 2001). However, other potential candidates that have shown promise include HDAC8 and HDAC4 (Waltregny et al., 2004; Wedel et al., 2006; Usui et al., 2014; Yoshida et al., 2007). Likewise, inhibition of class I but not class II HDAC suppressed proliferation and migration of cultured pulmonary arterial SMC (Galletti et al., 2014). Therefore, future work should assess if changes in expression all isoforms of HDAC are associated with changes in the contractile phenotype of ISMC from human strictures and the high passage rat model.
There is little work done showing a beneficial role of AZA treatment in diseases of SMC
dysfunction, although one study showed that AZA treatment reduced the atherosclerotic lesion area
in a mouse model of atherosclerosis (Zhuang et al., 2016). Another study showed that DNMT
inhibition reduced atherosclerotic lesions \textit{in vivo} by restoring appropriate mechanotranscription on
endothelial cells, suggestive of improved VSMC contractility (Dunn et al., 2014). Atherosclerotic
plaques from a murine model of atherosclerosis also showed decreased expression of TET
methylcytosine dioxygenase 2, an enzyme which demethylates DNA, suggesting a beneficial role
of AZA treatment in reversing epigenetic changes \textit{in vivo} (Liu et al., 2014).

The role of HDAC inhibitors have shown therapeutic promise in various diseases of SMC
hyperplasia, including asthma, pulmonary arterial hypertension, and atherosclerosis. HDAC
inhibition reduced SMC-mediated airway remodeling in a model of asthma (Ren et al., 2016), and
suppressed pulmonary arterial hypertension \textit{in vivo}. HDAC inhibition also reduced neointimal
thickening and formation in a rat and murine model of vascular injury (Findeisen et al., 2011; Jin
et al., 2011).

The beneficial effects of TSA and AZA as cancer therapeutics, and their ability to attenuate
disease progression in animal models of diseases of SMC hyperplasia suggest that these compounds
might be future treatment options for restoring ISMC phenotype and function in strictures. To prove
a potential role of these compounds as IBD treatment options, it will be important test the effects
of TSA and AZA on human ISMC \textit{in vitro} and in animal models of CD.

4.4 \textbf{Protracted growth of human ISMC mimics the rat high passage model}

Elsewhere, repeated passage of cancer cell lines induced epigenetic changes, including
increased methylation at CpG sites (Bork et al., 2010), and increased expression of DNMT and
HDAC (Li et al., 2014). We found that repeated passage of rat ISMC also increased expression of
DNMT1 and HDAC2. Since we found that SX-ISMC have an altered phenotype and increased
DNMT1 and HDAC5 expression compared to NM-ISMC, we wanted to confirm that the changes in rat high passage ISMC modeled human cells. High passage human ISMC were generated from two different models: 1) Repeated passage SX-ISMC and NM-ISMC and 2) repeated passage ISMC isolated from the ileum of specimens resected for colorectal cancer.

Our results also showed that epigenetic changes are likely responsible for the altered contractile phenotype in human ISMC at high passage. However, DNMT1, HDAC2, and HDAC5 expression in these cells were highly variable: DNMT1 expression was increased in high passage NM-ISMC in one cell line, while the other cell line showed increased DNMT1, HDAC2, and HDAC5 in SX-ISMC and NM-ISMC at high passage. Similarly, only one cell line of ISMC isolated from normal tissue in cancer patients showed increased DNMT1, HDAC2, and HDAC5 expression at high passage. This variation might be attributable to the fact that human ISMC did not grow well in culture through repeated passaging, and often required increased serum exposure and premature passaging to maintain cell viability. It is possible that these culture manipulations might have influenced expression of epigenetic enzymes in high passage human ISMC. Consequently, only two strictured and normal margin cell lines were testable for DNMT and HDAC expression at high passage. Repeating these experiments with a larger sample size might show more consistent changes in these epigenetic enzymes.

Interestingly, variation in gene expression was observed in rat and human high passage ISMC in most experiments. In human CD, the prevalence of strictures is 28% at the time of diagnosis (Henriksen et al., 2007), which increases to approximately 50% 5 years after diagnosis (Louis et al., 2001). We found that 25% (1 of 4) of high passage ISMC cell lines from ileal tissue resected for colon cancer tissue had increased DNMT1, HDAC2, and HDAC5 expression. We also found that 50% (1 of 2) of cell lines from human strictures and normal margin regions had increased expression of these epigenetic enzymes at high passage. Therefore, it is possible that the variation observed in our in vitro models reflects the variation of the disease prevalence.. We predict that the
events which cause stricture formation in CD patients might occur at a similar frequency in cultured high passage ISMC. To confirm this hypothesis, a larger sample size of human ISMC at high passage should be generated. It is important to note the impact of using different cell lines on the statistical significance of our experiments. Since each cell line were generated from independent rats and humans, variation across individual animals was accounted for, suggesting that the observed significant changes in gene expression are convincing and believable results.

4.5 Summary

Using a model of inflammation-induced protracted growth in vitro, this thesis showed that the altered phenotype of high passage rat ISMC was reversible through inhibition of HDAC and DNMT via TSA and AZA treatment, respectively. Our findings also showed that the phenotype of ISMC from human strictures have an altered phenotype, associated with changes in DNMT and HDAC expression. Our repeated passage rat model was verified using high passage human ISMC, which developed an altered phenotype, and showed variable changes in DNMT and HDAC expression. Overall, this thesis shows that the phenotype of ISMC in vitro is associated with epigenetic changes, and suggests that TSA and AZA treatment in vivo might restore proper contractility of smooth muscle.
References


Cirella, K. R. (2016). *Inflammation promotes the processing of glial cell-line derived neurotrophic factor by matrix metalloproteinase-9 in intestinal smooth muscle cells.*


muscle cell proliferation and neointima hyperplasia. *Art, 361*, 683–691.


Gougeon, P.-Y., Lourenssen, S., Han, T. Y., Nair, D. G., Ropeleski, M. J., & Blennerhassett, M. G. (2013). The pro-inflammatory cytokines IL-1 and TNF are neurotrophic for enteric neurons. *Journal of Neuroscience, 33*(8), 3339–3351.


