SMALL PROLINE RICH PROTEIN-2 EXPRESSION AND REGULATION IN THE CACO-2 MODEL OF INTESTINAL EPITHELIAL DIFFERENTIATION ALONG THE CRYPT-VILLUS AXIS

by

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Abstract

Small proline-rich protein-2 (SPRR2) functions as a determinant of flexibility and permeability in the mature cornified envelope of the skin. SPRR2 is strongly upregulated by the commensal flora and may mediate signaling to differentiated epithelia of the small intestine and colon. Yet, SPRR2 function in the GI tract is largely unexplored. Using the Caco-2 model of intestinal epithelial differentiation along the crypt-villus axis, we hypothesized that SPRR2 would be preferentially expressed in post-confluent differentiated Caco-2 cells and examined SPRR2 regulation by the protein kinase A pathway (PKA) and short chain fatty acids (SCFAs).

Differentiation-dependent SPRR2 expression was examined in cytoskeletal-, membrane-, and nuclear-enriched fractions by immunoblotting and confocal immunofluorescence. We studied the effect of SCFAs, known inducers of differentiation, on SPRR2 expression in pre-confluent undifferentiated Caco-2 cells and explored potential mechanisms involved in this induction using MAP kinase inhibitors. SPRR2 expression was also compared between HIEC crypt cells and 16 to 20 week primary fetal villus cells as well as in different segments in mouse small intestine and colon. We determined if SPRR2 is increased by gram negative bacteria such as S. typhimurium.

SPRR2 expression increased in a differentiation-dependent manner in Caco-2 cells and was present in human fetal epithelial villus cells but absent in HIEC crypt cells. Differentiation-induced SPRR2 was down-regulated by 8-Br-
cAMP as well as by forskolin/IBMX co-treatment. SPRR2 was predominantly cytoplasmic and did not accumulate in Triton X-100-insoluble cytoskeletal fractions. SPRR2 was present in the membrane- and nuclear-enriched fractions and demonstrated co-localization with F-actin at the apical actin ring. No induction was seen with the specific HDAC inhibitor trichostatin A, while SCFAs and the HDAC inhibitor SBHA all induced SPRR2. SCFA responses were inhibited by MAP kinase inhibitors SB203580 and U0126, thus suggesting that the SCFA effect may be mediated by orphan G-protein receptors GPR41 and GPR43. *S. typhimurium* induced SPRR2 in undifferentiated cells.

We conclude that SPRR2 protein expression is associated with differentiated epithelia and is regulated by PKA signaling and by by-products of the bowel flora. This is the first report to establish an *in vitro* model to study the physiology and regulation of SPRR2.
Co-Authorship

Unless stated otherwise, all work presented in this thesis was performed by Patrick Hui. The experimental design and data interpretation were a joint effort between Patrick Hui and Dr. Mark Ropeleski. The establishment and harvesting of cells in the co-culture model of *Salmonella typhimurium* with Caco-2 cells was performed by Brandon Ritcey. However, Western blots for these experiments were performed by Patrick Hui.
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List of Abbreviations

8-Br-cAMP - 8-bromo-cyclic adenosine monophosphate
APC - adenomatosis polyposis coli
ATF-2 - activating transcription factor 2
BEC - biliary epithelial cells
BRE - butyrate response element
cAMP - cyclic adenosine monophosphate
CDX - caudal-related homeobox
DNA - deoxynucleic acid
E-cadherin - epithelial cadherin
EDC - epidermal differentiation complex
ERK - extracellular signal regulated kinase
Gab - Grb2-associated binder
GEF - guanine-nucleotide exchange factor
GPR - G-protein coupled receptor
Grb - growth factor receptor-bound protein
HDAC - histone deacetylase
HNF4 - hepatocyte nuclear factor 4
IBMX – isobutylmethylxanthine
IAP – intestinal alkaline phosphatase
IL-13 - interleukin 13
JNK1/2 - Jun n-terminal kinase
LRP - lipoprotein receptor-related proteins
MAP - mitogen-activated protein
MAPK - mitogen activated protein kinase
mRNA - messenger ribonucleic acid
NHE3 - sodium hydrogen exchanger-3
PI-3K – phosphatidylinositide 3-kinase
PKA - Protein Kinase A
RT-PCR - Reverse Transcription-Polymerase Chain Reaction
SB203580 – p38 MAP kinase inhibitor
SBHA - suberoyl bis-hydroxamic acid
SCFA - short chain fatty acids
SGLT1 - sodium-glucose co-transporter
SI – Sucrase isomaltase
Sos - son of sevenless
SPRR - small proline rich
STAT-6 - signal transducer and activator of transcription-6
TEF-1 – transcription enhancer factor-1
TCF- T-cell factor
TG2 – transglutaminase-2
TSA - trichostatin A
U0126 – MEK1/2 inhibitor
ZO-1 - zonula occludens 1
Chapter 1
Introduction

Small Proline Rich (SPRR) proteins have been best described as a characteristic of differentiated squamous epithelia. Biochemical evidence from studies in the epidermis has suggested that SPRRs are important scaffolding proteins involved in cross-linking several insoluble proteins to form the mature cornified envelope. Furthermore, SPRRs have been implicated in several physiological and pathophysiological settings including epithelial adaptation to bowel resection (1), remodeling of the uterus (2), helminth infection (3) and cell stress (4). Importantly, intestinal epithelial SPRR2a mRNA expression was found to increase 205-fold in mice mono-associated with the commensal bacterium, Bacteroides thetataomicron, when compared to germ-free mice. In addition, DNA microarrays revealed that SPRRs were preferentially expressed in the villus compartment as opposed to the crypts. Using the Caco-2 model, a human colon carcinoma cell line that exhibits spontaneous differentiation post-confluence and recapitulates events along the crypt-villus axis in vivo, we hypothesized that SPRR2 is preferentially expressed in the differentiated state of this epithelial model. Furthermore, we examined the effects of short-chain fatty acids, known inducers of differentiation, on SPRR2 expression and explored the mechanisms involved in this relationship. We also characterized the cellular localization of SPRR2 using immunocytochemical techniques and cellular fractionation. We report herein the first study on the regulation of SPRR2 in the intestinal epithelium in vitro.
Chapter 2
Literature Review

2.1 The Human Intestinal Epithelium

The human intestine is lined with a single layer of columnar epithelial cells that are continuously renewed through a process involving rapid proliferation of stem cells at the base of the crypts of Lieberkühn, followed by migration and differentiation into functional cells along the villus and eventual extrusion of terminally differentiated cells at the villus tip (5). Differentiated villus cells of the intestinal epithelium arise from four principal lineages: absorptive, goblet, enteroendocrine, and Paneth cells (6). The colonic epithelium, however, is comprised primarily of absorptive enterocytes and goblet cells. These lineages arise from anchored stem cells which possess the ability to self renew and generate more committed precursor cells, which also proliferate but ultimately assume a functional phenotype in the differentiated villus compartment. The crypt-villus axis along with a gradient of several functional markers corresponding to distinct cell populations is shown in Figure 1. In addition to expression of functional markers, there are several morphological and structural changes that have been well studied. However, the lack of normal human intestinal epithelial cell lines makes the study of these cells difficult. In fact, a majority of information gathered regarding intracellular events has been through cell culture using several cell lines derived from colon carcinomas. To date, only
Figure 1 - The crypt-villus axis: schematic of the functional unit of the intestinal epithelium. The stem cell, proliferating, differentiating, absorptive, and extrusion compartments are outlined along with the corresponding functional and structural expressed genes (7).
one carcinoma cell line (Caco-2) has been shown to exhibit spontaneous differentiation characteristics of mature absorptive enterocytes and this cell culture model will constitute the basis of this study.

2.2 Phenotype of Differentiated Absorptive Cells

Absorptive cells comprise a majority of differentiated cells in both the small bowel and colon. Morphologically, these cells display a brush border, a microvilli-covered surface containing several proteins required for nutrient absorption including sucrase isomaltase, the sodium-glucose co-transporter (SGLT1), and lactase (7, 8). Functionally, these cells also act as a selective semi-permeable physical barrier to macromolecules and the intestinal flora. This is mediated primarily by two junctional complexes: the tight junction and the adherens junction. Tight junctions are formed by a number of transmembrane proteins, including occludins, claudins, and junctional adhesion molecules. Tight junctions are intimately associated with the actin cytoskeleton via various junctional molecules such as ZO-1, occludin, and claudins (9). The tight junction is dynamically regulated and actin-myosin interactions mediated by myosin light-chain kinases constitute an important mechanism by which tight junction permeability is regulated in both health and disease states. Similarly, adherens junctions are comprised of E-cadherin molecules, which are connected to the actin cytoskeleton by proteins such as α and β-catenin. In addition, these junctions are calcium dependent and require the recruitment and activation of
p85/PI-3K for assembly (10). Together these apical junction complexes perform a number of vital functions including the maintenance of cell-cell contacts, prevention of the passage of molecules and ions through the paracellular space, and also block the movement of integral membrane proteins between the apical and basolateral compartments (fence function) of the cell thereby helping to maintain the epithelial polarity of these cells (11).

### 2.3 Intracellular Signaling Events

The capacity for self-renewal and the dynamic nature of the intestinal epithelium serves as an ideal system to study complex biological processes such as proliferation, differentiation, and cell-cycle mediated events. The intracellular mechanisms in epithelial cells must be strictly regulated to allow for the organized control of proliferation, migration, differentiation and ultimately shedding along the crypt-villus axis in a timely manner. To date, the molecular mechanisms underlying these concepts are incompletely understood. However, there is evidence to suggest that hormones, growth factors, cytokines, changes in extracellular matrix and cell-cell interactions all have significant involvement in these processes (12). In addition, a number of intracellular signaling pathways have also been identified which control intracellular signaling events during differentiation including the Wnt/β-catenin, p38 MAP kinase, protein kinase A, and MEK/ERK signaling pathways. A schematic of these signaling pathways is presented in Figure 2.
2.3.1 Wnt signaling in Intestinal Epithelial Cells

The canonical Wnt signaling pathway plays a central role in maintaining the precise balance between proliferation and differentiation in the intestinal epithelium. In the absence of Wnt signaling, intracellular levels of β-catenin are regulated by the ‘destruction complex’ which includes several kinases and scaffolding proteins that bind and phosphorylate β-catenin, thus targeting it for ubiquitination and proteasomal degradation (13). The Wnt family includes several secreted glycoprotein ligands. Binding to their receptors, Frizzled and low-density lipoprotein receptor-related proteins (LRP5/6), results in an inhibition of the destruction complex comprised of glycogen synthase kinase3β, APC, and axin, thus allowing cytosolic accumulation of stabilized β-catenin. Accumulation of β-catenin results in nuclear translocation where interactions with the TCF family transcription factors occur which ultimately sustains gene expression of targets required to maintain the proliferative state of crypt cells (6).

2.3.2 Regulation of cellular differentiation by Protein Kinase A

The cyclic nucleotide (cAMP) is a well described regulator of a number of cellular processes including proliferation, differentiation, and cell growth. The primary function of cAMP is to activate cAMP-dependent protein kinase (PKA). Activated PKA modulates cellular events either at the transcriptional level by
Figure 2 - Intracellular signaling pathways involved in the regulation of proliferation and differentiation in epithelial cells. Pathways include the Wnt signaling, cAMP/PKA, MEK/ERK, and p38 MAPK pathways (10, 13, 16, 23, 24)
phosphorylating transcription factors such as transcription enhancer factor-1 (TEF-1) or at the post-translational level through its ability to phosphorylate cellular substrates such as transglutaminase 2 (TG2) (14). PKA signaling in the intestinal epithelium has not been well defined. In the Caco-2 cell model, stimulation of the PKA pathway acutely inhibits the apical brush border Na⁺/H⁺ exchanger (NHE3) (15). Additionally, Boucher et al. have shown that activation of the PKA pathway can negatively modulate adherens junction integrity as well as differentiation in intestinal epithelial cells (10). Using the Caco-2 model, they demonstrated that treatment with the highly potent membrane permeable analogue of cAMP, 8-Br-cAMP, inhibited the expression of several markers of differentiation including villin and sucrase isomaltase. Furthermore, they showed that activation of the PKA pathway attenuates epithelial cell polarity, brush border formation, while reducing the amount of E-cadherin and catenins at the junctional complexes of neighboring cells. Taken together, these results suggest that PKA signaling favors the proliferative state while inhibiting differentiation of epithelial cells.

2.3.3 The p42/p44 Mitogen Activated Protein Kinase signaling cascade

The p44 and p42 MAP kinases (MAPK), also termed ERK1 and ERK2 for extracellular signal regulated kinases, have been implicated in mediating cell division and differentiation in intestinal epithelial cells. MAPK is activated by MAP kinase kinase (MEK), which in turn, is activated by a complex of adaptor
proteins including Raf, Ras and Grb2, which are bound to activated receptor tyrosine kinases(16). Upon activation, MAPK can translocate to the nucleus and phosphorylate a number of downstream targets including other kinases as well as transcription factors such as c-JUN, Elk-1, CHOP, and ATF-2(17, 18). In fibroblasts, activation of MAPKs have been suggested to be involved in targeting genes involved in maintaining a state of cell proliferation. Conversely, inhibition of MAPK activity was shown to block cell cycle progression (19). In the Caco-2 model, p42 and p44 MAPK activities apparently occupy a dual role as they are necessary for both cell cycle progression as well as differentiation. More specifically, elevated activities of MAPK corresponded to increased DNA synthesis and maintenance of proliferation whereas low sustained levels of MAPK correlated with cell cycle arrest and increased expression of markers of differentiation including sucrase isomaltase (20).

2.3.4 p38 Mitogen Activated Protein Kinases

Whereas p42 and p44 MAPKs are generally thought to regulate cell cycle dynamics and differentiation, JNK1/2 and p38 (α,β), two stress-activated MAPK families, have been shown to play important roles in responding to stress and the regulation of apoptosis (21, 22). However, in the intestinal epithelium, there is a growing literature suggesting that activation of p38 MAPK leads to the phosphorylation and activation of the homeobox transcription factor CDX2 (23). CDX2 is a pro-differentiation transcription factor that is expressed in
differentiating enterocytes and over-expression of this transcription factor leads to growth arrest and differentiation in the Caco-2 model (24). Moreover, in the rat intestinal crypt cell line IEC-6, over-expression of CDX1 induces phenotypic changes characteristic of differentiating enterocytes (25). Houde et al. showed that p38 MAPK activity correlated strongly with sucrase isomaltase, alkaline phosphatase, and villin expression. Furthermore, immunoprecipitation experiments revealed a direct interaction between p38 and CDX3. Interestingly, the same laboratory also showed that cell-cell contact dependent signals were mediated through phosphatidylinositol 3-kinase (PI-3K) and required for the activation of p38 MAPK and the subsequent expression of markers of differentiation (26).

2.4 Short Chain Fatty Acids

In the presence of normal anaerobic microbiota, most undigested dietary fibers which traverse the small intestine and enter the colon intact are fermented to short chain fatty acids (SCFAs). Three major SCFAs are produced by this process including butyrate, propionate, and acetate. SCFAs have been reported to serve a number of functions in the intestine. SCFAs are rapidly absorbed by the bowel, stimulating water and sodium absorption through direct effects on the NHE3 sodium hydrogen exchanger, and are thus a key contributor to water homeostasis (27). Additionally, butyrate serves as the primary energy source for colonocytes. Anaerobic fermentation produces 300 to 800 mmol of SCFAs per
day and the total SCFA concentration in excreted stool ranges from 100-240 mmol suggesting that the colon consumes or absorbs most of the butyrate in stool (28). Butyrate, the most potent and best studied of the three SCFAs, has been implicated as both a trophic and a differentiation factor for intestinal epithelial cells. Reports have implicated SCFAs as stimulators of proliferation through increased DNA synthesis and the mitotic index \textit{in vivo} (29, 30). Conversely, \textit{in vitro} models predominantly exploiting cancer-derived cell lines have shown that butyrate inhibits cell proliferation, stimulates differentiation, and leads to apoptosis (31). For example, in colorectal adenoma cells (PC/BH/C1), butyrate induces several markers of differentiation including alkaline phosphatase activity and E-cadherin protein expression (32). In the latter setting, the classical mechanism by which SCFAs have been suggested to alter gene expression is through their histone deacetylase inhibition properties. Upon butyrate treatment, the resulting hyperacetylation of histones allows for specific transcription factors to induce genes required for cell cycle arrest and differentiation (33).

### 2.4.1 GPR41 and GPR43 – Two previously identified orphan G-protein coupled receptors for short chain fatty acids

Mechanisms by which SCFAs exert their effects have been intensely debated over the years. In addition to putative SCFA/HCO$_3^-$ exchangers, SCFAs
including butyrate, propionate, isobutyrate and acetate have recently been identified as ligands for the orphan G-protein coupled receptors GPR41 and GPR43 in polymorphonuclear cells (34). In this setting, propionate was the most potent agonist for both receptors. Interestingly, activation of GPR43 was coupled to inositol 1,4,5-triphosphate formation, intracellular Ca\(^{2+}\) release, ERK1/2 activation, and inhibition of cAMP accumulation, suggesting a possible mode for induction of differentiation (35). Furthermore, in a human breast cancer cell line (MCF-7), SCFAs treatment selectively increased the phosphorylation of p38 MAPK which was abolished upon silencing of GPR43 (36). Recently, GPR43 was identified to be limited to the mucosa of the human colon with specific expression shown in colonocytes and enteroendocrine L cells (37). Taken together, this new arm of SCFA signaling could be a plausible pathway for the induction of differentiation and the regulation of proliferation in the intestinal epithelium.

2.5 SPRR Proteins

Human Small Proline-Rich (SPRR) proteins exist as a multi-gene family, containing at least 11 members, and are located on human chromosome 1q21 in a region called the epidermal differentiation complex (EDC) (38). Presently, there are four recognized classes in the SPRR family numerically named SPRR1, SPRR2, SPRR3, and SPRR4 (39, 40). Among these classes, SPRR1 and SPRR2 contain multiple isoforms with two and seven, respectively. SPRR3 and
SPRR4, however, have only been shown to exhibit one isoform. All SPRR proteins have highly homologous amino and carboxy terminal domains containing several glutamine and lysine residues, as well as a proline-rich central repetitive domain (41). Furthermore, SPRRs all display similar genomic organization: a short first exon followed by a single intron and a second exon containing the entire open reading frame (41). To date, SPRRs have been shown to function as a scaffolding protein and a precursor in the formation of the mature cornified envelope in the skin (42). In human epidermal keratinocytes, SPRR regulation occurs in concert with other members of the EDC such as loricrin and involucrin in response to differentiation factors such as calcium and phorbol ester treatment, as well as to stressors such as UV-irradiation (43). SPRRs also increase during the normal differentiation process of human keratinocytes both in vitro and in vivo (43, 44). To emphasize the strict correlation between SPRR expression and differentiation, cultured cell lines derived from squamous cell carcinomas were assessed for their ability to form a cornified envelope relative to SPRR2 mRNA expression. Levels of SPRR2 transcript were found to directly correlate with the ability to form a differentiated cornified envelope (45).

To date, SPRR expression has best been described as a characteristic feature of squamous epithelia. The four SPRR classes are differentially expressed in a variety of tissues such as skin, tongue, uterus, cervix, ovary, and esophagus (40, 46, 47). In skin, SPRRs are differentially expressed in response to external insults such as UV irradiation, aging, and disease states such as
psoriasis (43, 47). In addition, a number of studies have shown an upregulation of SPRRs in response to a variety of stressors including oxidative stress in the duodenal epithelium, exposure to smoke in bronchial epithelium, and even ischemic cardiovascular stress (4, 48-50). The regulation and function of SPRRs in non-squamous intestinal epithelia is not well understood. SPRRs have been shown to have a potential protective role as they are upregulated in response to a range of infections. Upon infection with the intraepithelial nematode *Trichinella spiralis*, jejunal epithelia from BALB/c mice display a 37-fold increase in SPRR2a transcript (3). Similarly, a murine model of *Helicobacter pylori* infection revealed an upregulation of SPRR2a which, in this setting, was suggested to play a role in launching a mucosal immune response (51). In mice with deficient gastric acidification, an increased bacterial load is observed in the stomach, which is paralleled by an up-regulation of epithelial protective genes in the small intestine including SPRR2a (52). In the lungs, specific isoforms of SPRR1 and SPRR2 are differentially regulated upon exposure to pro-inflammatory cytokines and pathogenic viruses (53, 54). In pulmonary epithelium, SPRR2a and 2b mRNAs are induced by allergen ovalbumin challenge, suggesting a potential role in the inflammatory response (55). Similarly, allergens which induce inflammation of the intestine also lead to SPRR2 mRNA upregulation which is mediated by elevated levels of IL-13 in a STAT-6 dependent manner.

Based on the high sequence homology between the 11 isoforms, identifying a functional role for SPRRs *in vivo* by knockdown would prove to be a
daunting task. Instead, a number of biochemical approaches have been performed to elucidate the role of SPRRs in the epidermis, where they have been studied most extensively. A characteristic feature of the epidermis is the specialized barrier of highly insoluble protein that is deposited on the intracellular surface of the squamous cell plasma membrane. Termed the cornified envelope, this barrier consists of several proteins including: involucrin, cystatin, filaggrin, loricrin, as well as SPRRs. These proteins have been shown to be cross-linked either through disulfide bonds or through the isodi peptide cross-links, formed by transglutaminases (56). Several approaches have been taken to elucidate the relative composition as well as order of binding of these proteins. Mathematical models have revealed that loricrin is the major component (70%), followed by filaggrin and elafin (8% and 6%, respectively), SPRRs and cystatin (5% each), and involucrin (2-5%) (57). In addition, time-dependent digestion with proteinase K followed by mathematical modeling revealed a three-stage model for the structure of the cornified envelope (57). As shown in Figure 3, the outer third consists mostly of loricrin, SPRRs, and filaggrin; the middle third consists mostly of elafin, loricrin, and SPRRs, while the inner third consists of involucrin and cystatin. Furthermore, sequence analysis of protease digested proteins shows both intra- and inter-chain cross-linking between loricrin and SPRR1 while SPRR2 was shown to cross-link between loricrin units (58). Further biochemical evidence suggests that these proteins contribute to the formation of the cornified envelope that provides structural integrity, flexibility and limits permeability of the
Figure 3 - A schematic outlining the present understanding of the mature cornified envelope. The outer third consists of loricin (L), filagrin (F), and SPRRs. The middle third consists of loricin (L), elafin (E), and SPRRs. The inner third consists of involucrin (I), and cystatin (C) (57).
skin (59). Moreover, it has been shown that the relative composition of different cross-bridging SPRR protein family members largely determines the rigidity and flexibility of the cornified envelope thereby suggesting that differential expression patterns reflect specific barrier requirements of different epithelia (42). To date, the concept of an insoluble functional barrier in the intestine has not been explored. As such, there is no clear data suggesting a functional role for SPRRs in the intestine. Interestingly, in biliary epithelial cells (BEC) that stably over-express SPRR2a, there is increased resistance to oxidative injury, and increased wound restitution as a function of enhancing migration of injured cells. It has been proposed that this reflects increased SPRR dependent epithelial to mesenchymal transition (60). Also, this study showed that SPRR2a can function as a SH3 domain ligand, suggesting that SPRRs may function as an important scaffolding protein for signaling pathways activated in response to injury (60). Outside of the intestine, a number of studies have also implicated SPRRs in repair and remodeling of epithelia ranging from peripheral neurons to the epithelial lining of the uterus. In neurons, SPRR1a is induced in response to axotomy as well as injury to the optic nerve (61, 62). At least ten-fold increases in SPRR2a transcript was observed in the uterus during the estrogen phase, at the site of implantation as well as estrogen-induced remodeling of the epithelial uterine in mice (63-65). After massive bowel resection in mice, a 4-fold increase in SPRR2a is observed (1). While transgenics and knockouts have yet to be exploited in the study of SPRR physiology, in vivo studies in mice over-
expressing claudin-6, a protein contributing to the formation of functional tight junctions, results in neonatal death two days after birth apparently due to the lack of an epidermal permeability barrier, increased water loss, and the penetration of X-gal through the skin. In this setting, overexpression of claudin-6 results in dysregulation of several proteins involved in maintaining an epidermal permeability barrier including many isoforms of the SPRR2 class (66).

An evolving literature exists on SPRR expression in the epithelium of the upper and lower gastrointestinal tract. Our interest in intestinal epithelial SPRR expression stems from the study by Hooper et al. where, using laser captured microdissected RNA from the differentiated villus compartments of the mouse small intestinal epithelium, SPRR2a mRNA was shown to exhibit a 2050-fold increase in expression in the jejunum of mice mono-associated with Bacteroides thetaiotaomicron when compared to germ-free mice (67). Furthermore, in another study using microarrays, which compared gene expression profiles of epithelial cells isolated from the villus and crypt indicated that SPRR2a mRNA was preferentially expressed in the villus as opposed to the crypt (68). SPRR expression coincided with numerous genes related to transport and metabolism, therefore making it tempting to speculate that SPRRs may play a role in epithelial differentiation or in the maintenance of epithelial polarity.

In light of the above, we formulated the hypothesis that SPRR2 expression would be preferentially expressed during differentiation in a well-described Caco-2 model of intestinal epithelial differentiation in vitro and that this model may set
the stage for future studies on the physiological regulation and compartmentalization of SPRR2 expression during intestinal epithelial differentiation. In parallel, we hypothesized that short-chain fatty acids, by-products of commensal bacterial fermentation of dietary fiber and known inducers of differentiation, would stimulate SPRR2 proteins in pre-confluent undifferentiated Caco-2 cells.
Chapter 3
Materials & Methods

3.1 Reagents

All cell culture reagents and fetal bovine serum were obtained from Sigma Aldrich (St. Louis, MO). All chemicals were of molecular biology grade and obtained from Sigma Aldrich (St. Louis, MO) unless otherwise stated. The MEK1/2 inhibitor U0126 was obtained from Calbiochem (LaJolla, CA). The p38 MAP kinase inhibitor (SB203580) was obtained from Sigma Aldrich.

3.2 Cell Culture

Caco-2 cells (ATCC #HTB-37, Manassas, VA) were seeded on 60mm dishes and cultured in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (4.5 g/L) and supplemented with 10% fetal bovine serum, 1% glutamine, 0.5% penicillin/streptomycin and 0.2% sodium pyruvate in an atmosphere of 5% CO₂ and 95% atmospheric air at 37 °C. The cells were passed every 3-4 days using 0.05% trypsin/0.53 mM EDTA at a ratio of 1:8. T84 cells (ATCC #CCL-248, Manassas, VA) were seeded in 75-cm² tissue culture flasks and grown in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium (1:1), supplemented with 5% fetal bovine serum, 1% glutamine, 0.5% penicillin/streptomycin, and 0.1% sodium pyruvate in an atmosphere of 5% CO₂ and 95% atmospheric air at 37 °C. Cells were passed every 3-4 days using 0.05% trypsin/0.53 mM EDTA at
a ratio of 1:6. Cell lines were cultured for three (two days pre-confluent, undifferentiated), seven (two days post-confluent), and fourteen days (ten days post-confluent, differentiated) after seeding in 60mm plates. Prior to harvesting, cells were washed twice with ice-cold PBS, scraped on ice, pelleted and stored at -70 °C until used. Cells were treated with the short-chain fatty acids butyrate, propionate, and isobutyrate (5mM) and harvested at 6, 12, 24, 48, 72, and 96 hours after treatment. Similarly, the well described histone deacetylase inhibitors trichostatin A (TSA, Sigma Aldrich, St. Louis, MO) and suberoyl bishydroxamic acid (SBHA, Biomol, Plymouth Meeting, PA) were used to treat two days pre-confluent Caco-2 cells at a concentration of 2 µM and 15 µM, respectively (69, 70). To measure dose responsiveness, cells were treated with butyrate, propionate, or isobutyrate for 24 hours at various concentrations (1-20 mM), TSA (0.2-10 µM) or SBHA (3-30 µM). Confluent cells were also treated with 8-Br-cAMP (1 mM), forskolin (10 µM), dideoxyforskolin (10 µM), 3-isobutyl-1-methyl xanthine (IBMX, 1 mM) (71, 72).

3.3 Immunoblotting

Frozen cell pellets were re-suspended in a hypotonic lysis buffer containing 10 mM Tris pH 7.3, 5 mM MgCl₂, 1X Complete protease inhibitor cocktail (Roche Biochemicals, Indianapolis, IN), DNase I 50 U/mL (GE Healthcare, Baie D’Urfé, Quebec) and RNase Cocktail 5 µL/mL (Ambion, Austin,
Aliquots were taken for protein concentration determination using the bicinchoninic acid reagent (Sigma Aldrich, St. Louis, MO). Lysates were added to 0.5 volumes of 3X SDS sample buffer containing 187.5 mM Tris·HCl (pH 6.8), 6% SDS, 30% glycerol, and 0.03% bromophenol blue and heated to 100°C for five minutes. Protein samples were stored at -70°C until further use. Lysates from Human Intestinal Epithelial Crypt (HIEC) cells as well as frozen human fetal villus cells obtained from weeks 16.5 to 20 in utero were generously provided by Dr. J.F. Beaulieu and Dr. N. Rivard, respectively (Université de Sherbrooke). Upon arrival, lysates were supplemented with 1X Complete protease inhibitor cocktail (Roche Biochemicals, Indianapolis, IN), aliquots were taken for protein concentration determination, and lysates were diluted in sample buffer as described above.

Fifty µg of protein were separated by SDS-PAGE using 16.5% polyacrylamide gels. The separated proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA) for two hours at 400 mAmps in 1X Towbin’s transfer buffer containing 25mM Tris, 192 mM Glycine, and 20% methanol. Membranes were blocked in 7.5% milk in Tris-buffered saline with Tween 20 (TBS-T) containing 10 mM Tris, 50 mM NaCl, and 0.1% Tween 20 for one hour to prevent non-specific binding and were incubated with rabbit polyclonal anti-SPRR2 antibody (Apotech Corporation, Geneva, Switzerland) at a concentration of 1:3000 in 7.5% milk overnight with agitation at 4°C. After incubation, membranes were washed five times for ten minutes in
TBS-T and then incubated with donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch, Westgrove, PA) at a dilution of 1:10000 for one hour. After repeated washes, signals were detected by chemiluminescence using the Pico West detection kit (Pierce, Rockford, IL) and Kodak Biomax Light film. To assess protein loading, blots were stripped using 200 mM glycine/0.05% Tween-20 (pH 2.5) for 1 hour at 60º C and reblotted with antibody detecting β-actin at a dilution of 1:20000 (Sigma Aldrich, St. Louis, MO).

3.4 Isolation of cytoskeleton-associated proteins

Cytoskeletal enriched fractions were isolated as described by Boucher et al. (10). Briefly, frozen cell pellets were lysed in cytoskeleton stabilization buffer (0.5% Triton X-100, 50 mM NaCl, 10 mM PIPES pH 6.8, 300 mM sucrose, 3 mM MgCl₂, 0.2 mM orthovanadate, 1X Complete protease inhibitor cocktail (Roche Biochemicals, Indianapolis, IN) and extracted by centrifugation at 13000 rpm at 4ºC for 20 minutes. The cytoskeleton-associated proteins (insoluble fraction) were solubilized in 1 M Tris pH 7.3 and an aliquot was taken for protein determination. An aliquot was also taken from the cytoplasmic proteins (soluble fraction) for protein concentration determination. Lysates from both fractions were added to 0.5 volumes of 3X SDS sample buffer heated to 100°C for five minutes. Protein samples were stored at -70ºC until further use.
3.5 Isolation of membrane- and nuclear-associated proteins

Prior to harvesting, cells were washed twice with ice-cold PBS, scraped on ice, and pelleted. Cell pellets were resuspended in lysis buffer containing 10 mM Tris pH 7.4, 5 mM EDTA, 1X Complete protease inhibitor cocktail (Roche Biochemicals, Indianapolis, IN), 1 mM NaF, 1 mM Na-Orthovanadate, 1 mM beta-glycerophosphate, and 2.5 mM Na-pyrophosphate. Lysates were dounce homogenized on ice for 50 passes and left at 4°C for 15 minutes. Nuclei were isolated by centrifugation (500xg for 5 minutes at 4°C). The supernatant was then centrifuged to pellet mitochondria (10,000xg for 10 minutes at 4°C). The final supernatant was centrifuged (49200xg for 60 minutes at 4°C) and the resulting membrane pellet was resuspended in 10 mM Tris pH 7.4, 5 mM EDTA, and 1X Complete protease inhibitor cocktail (Roche Biochemicals, Indianapolis, IN). An aliquot was taken for protein concentration determination as mentioned above. Lysates were added to 0.5 volumes of 3X SDS sample buffer and heated to 100°C for five minutes. Protein samples were stored at -70°C until further use.

Extraction of nuclear proteins was performed as directed by the Nuclear & Cytoplasmic Reagents kit (Pierce, Rockford, IL). An aliquot of nuclear extract was taken to determine the protein concentration. Nuclear extracts were added to 0.5 volumes of 3X SDS sample buffer and heated to 100°C for five minutes. Protein samples were stored at -70°C until further use.
3.6 RNA extraction and semi-quantitative RT-PCR

Cells were grown in conditions as mentioned above. On days three, seven, and fourteen, cells were harvested. Cells were rinsed twice with PBS, scraped on ice, pelleted, snap frozen and stored at -70°C until used. Frozen cell pellets were re-suspended in 1 mL Trizol (Invitrogen, Carlsbad, CA), separated with 0.2 mL of chloroform, extracted with additional phenol-chloroform (pH 4.3), precipitated in 0.5 mL of isopropyl alcohol and re-dissolved in RNAse-free water. Five µg of RNA was reverse transcribed in a 100 µL reaction mixture by AMV reverse transcriptase (Roche Biochemicals, Indianapolis, IN) using random primers (hexamers), 1x reaction buffer, 25 mM MgCl₂, 10 mM deoxynucleotides, RNase inhibitor, and sterile water. Two µL aliquots were used as template in a series of PCR reactions (20 µl) with Qiagen Taq, 25 mM MgCl₂, 1x Qiagen buffer, 10 mM deoxynucleotides, and specific sets of primers for SPRR2a-g and GPR41 and GPR43 (35, 40 see Table 1). Reaction conditions and cycle parameters for SPRR2 isoforms were: 5 minutes at 94°C; 40 cycles of: 1 minute at 94°C, 1 minute at optimal primer pair-specific annealing temperature (which varied per primer pair); 1 minute at 72°C; and 10 minutes at 72°C. Reaction conditions and cycle parameters for GPR41 were: 5 minutes at 94°C; 40 cycles of: 1 minute at
Table 1 - A list of primers used in RT-PCR for SPRR2a-g and GPR41/GPR43 in Caco-2 undifferentiated and differentiated cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Fragment Size</th>
<th>Annealing Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPRR2a</td>
<td>F – TGTTACCTGAGCACCCTGAGTCTG</td>
<td>318 bp</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>R – CCAGATATCTCTGACCTGAGTCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPRR2b</td>
<td>F – CATCAAGCTTTATCATGGGATCTCT</td>
<td>199 bp</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R – TTCCGAGCCGACACGCTGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPRR2c</td>
<td>F – TACTTGAGCACCCTGAGTCTGCTC</td>
<td>521 bp</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>R – GAGAGCTCTGAAAGGCGGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPRR2d</td>
<td>F – GAGCTAAGAAAAGGAAGTCTCTCA</td>
<td>179 bp</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R – TTATCAGGGAGTGAAGGATAAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPRR2e</td>
<td>F – AGCCTACCATGGATACACAGTT</td>
<td>227 bp</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>R – GACACAGAAAAACATCAACAGCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPRR2f</td>
<td>F – TCCAGGAGAGAGCTGCTCT</td>
<td>159 bp</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>R – TCAGTCGGATGAGCTGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPRR2g</td>
<td>F – GCCATGAGTTGTATGCTTTG</td>
<td>481 bp</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R – GCGAGATTAGCAATGATCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPR41</td>
<td>F – TACGTCATAGAATTCCTCAGG</td>
<td>508 bp</td>
<td>52</td>
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<tr>
<td></td>
<td>R – TGGTACTGAGCTTACTTCTTCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPR43</td>
<td>F – TTCTACAGCAGCACTACTG</td>
<td>439 bp</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>R – GAAGCACACAGGAAATTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-actin</td>
<td>F – TGACCGGGGTACCACCACCTGTCG</td>
<td>556 bp</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>R – CTAGAAGCATTGACGGGTAGAGGG</td>
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</tr>
</tbody>
</table>
94°C, 1.5 minutes at 52°C, 35 seconds at 72°C; and 5 minutes at 72°C. Reaction conditions and cycle parameters for GPR43 were: 5 minutes at 94°C; 40 cycles of: 1 minute at 94°C, 1.5 minutes at 53°C, 40 seconds at 72°C; and 5 minutes at 72°C. The results of the PCR amplification were analyzed using 2% ethidium bromide-stained TAE gel electrophoresis and a 100bp DNA ladder (Invitrogen, Carlsbad, CA). Photos were taken with the Alphalmager Gel Imaging System (Alpha Innotech, San Leandro, CA). Images were processed using the AlphaEase FC software (Alpha Innotech, San Leandro, CA).

### 3.7 Immunofluorescence & Confocal Microscopy

Caco-2 Cells were plated on glass coverslips in 6-well plates (Corning, Corning, NY) and grown for three, seven, and fourteen days. For analysis, cells were washed 3x for 5 minutes with ice-cold PBS and fixed for 10 minutes in ice-cold methanol:acetone (1:1). Cells were permeabilized in PBS supplemented with 0.2% Triton X-100 for 15 minutes with gentle rotation at room temperature. After several washes with PBS, a blocking solution containing 1% BSA and 1% goat serum in PBS was applied for 1 hour. Following 3x 5 minute washes in PBS, anti-SPRR2 antibody at a concentration of 1:1000 in antibody diluent solution (Dako, Glostrup, Denmark) was applied overnight at 4°C in a humidified chamber. Cells were subsequently washed 5x for 10 minutes with PBS and a goat anti-rabbit secondary antibody conjugated to an Alexa 555 dye was applied
at a concentration of 1:2000 for one hour. Additionally, an Alexa 488 conjugated phalloidin at a concentration of 1:1000 (Invitrogen Carlsbad, CA) was added at this time. Lastly, cells were washed 5x for 10 minutes and mounted using one drop of ProLong Gold antifade reagent (Invitrogen Carlsbad, CA). Slides were visualized using a Leica immunofluorescent microscope or Olympus confocal microscope (IX70, Center Valley, PA) at 488 and 534 nm. Images were analyzed using the Fluoview FV300 software.

3.8 Harvesting Mouse Tissue

Five week old male C57/Bl6 mice were purchased from Charles River (Wilmington, MA). Animals were allowed to acclimatize for one week and were subsequently sacrificed by halothane anesthesia and cervical dislocation. Segments of the gastrointestinal tract including the esophagus, stomach, duodenum, jejunum, mid ileum, terminal ileum, cecum, ascending, transverse, descending colon and rectum were excised and immediately snap frozen. In a separate subset of mice, superficial mucosal scrapings were obtained using glass slides and both the epithelial enriched fraction and remaining mucosal tissues were snap frozen. All tissues were subsequently finely ground using a mortar and pestle, re-suspended in hypotonic lysis buffer as described above and sonicated for ten seconds. Samples were centrifuged at 14000 rpm for 10 minutes to discard debris and supernatants were removed. An aliquot was taken for protein concentration determination as mentioned above. Lysates were
added to 0.5 volumes of 3X SDS sample buffer and heated to 100°C for five minutes. Protein samples were stored at -70°C until further use.

3.9 Co-culture with Salmonella typhimurium

One day prior to co-culture, a single colony of NLM2217- wild type Salmonella typhimurium was grown in 5 mL of LB broth overnight at 37°C with aeration. After 24 hours, the culture was centrifuged at 14000g for 2 minutes to pellet cells. The supernatant was removed and the resulting pellet was resuspended in 5 mL of sterile PBS. A 1:100 dose of S. typhimurium was added to the media of two days post-confluent Caco-2 cells and harvested at 30 minutes, one, two, four, and six hours after treatment and processed for immunoblotting as described above.

3.10 Densitometry and Statistical Data Analysis

Densitometry was carried out using Adobe Photoshop Elements 3.0 (Adobe, San Jose, CA). Bands were initially normalized to β-actin density. Controls were assigned a value of 1 with all other treatment groups expressed in relation to this value. Where indicated, a two-tailed student’s t-test was used to determine statistical significance between independent groups. Results are means ± SE of three or more experiments.
Chapter 4
Results

4.1 SPRR2 expression coincides with differentiation of Caco-2 cells

Caco-2 cells are a human intestinal epithelial cell line derived from a colon adenocarcinoma (73), which recapitulates many of the events in vivo during epithelial absorptive cell differentiation along the crypt-villus axis. These cells have distinct characteristics as they are undifferentiated in the pre-confluent state but spontaneously differentiate after reaching confluence. Given that Caco-2 cells act as a model of intestinal epithelial differentiation along the crypt-villus axis, we employed immunoblotting to measure SPRR2 expression during the transition from the undifferentiated state to a post-confluent differentiated state. As shown in Figure 4-A, using mouse esophagus and skin as a positive control, SPRR2 expression is observed in Caco-2 cells ten days post confluence, coinciding with the differentiated state of these cells. Importantly, SPRR2 was not expressed in T84 cell lines which display polarity and tight junction formation, but does not spontaneously differentiate into absorptive cells in vitro (Appendix A). To elucidate a more detailed profile of SPRR2 expression, Caco-2 cells were harvested at two day intervals and an immunoblot displaying SPRR2 expression is shown in Figure 4-B. The SPRR2 expression profile exhibits a steady increase concomitant with a
**Figure 4A:** A Western blot analysis of SPRR2 expression two days pre-confluent, two days post-confluent, and 10 days post confluent in two separate passages of Caco-2 cells (n=3). **4B:** A Western blot analyzing a twelve day time course of villin and SPRR2 expression (n=3). **4C:** A Western blot analysis of SPRR2 expression in human intestinal epithelial crypt cells versus fetal villus cells ranging from 16-20 weeks gestation. In all Western blots, β-actin expression was probed to control for protein loading and mouse esophagus (ESO) and/or skin were utilized as positive controls for SPRR2.
differentiation-dependent rise in villin expression. β-actin detection was used to control for protein loading.

As shown in Table 2, a detailed inspection of the epitope of the anti-SPRR2A/B antibody reveals that there is upwards of 73% homology with other members of the SPRR2 class. As a result, we performed semi-quantitative RT-PCR on all SPRR2 isoforms. In Appendix B, mRNA transcript is upregulated over a twelve day time course for SPRR2d and SPRR2e. Unfortunately, the high sequence homology exhibited by all isoforms did not allow us to accurately analyze these mRNA transcripts with consistency, so the thesis proceeded to focus on SPRR2 protein expression.

Human intestinal epithelial crypt cells (HIEC) express numerous crypt cell markers but no villus markers (73). Importantly, this cell line is unable to differentiate spontaneously thus making it a useful tool to study the intestinal crypt. Conversely, primary human fetal villus epithelial cells display a more differentiated state of the intestinal epithelium and express many of the markers associated with the differentiated epithelium. In combination, these two models provide an in vitro recapitulation of the crypt-villus axis similar to that of the Caco-2 cell line and more importantly, allow us to extrapolate our findings to the developing normal human intestine. As shown in Fig 4-C, SPRR2 protein expression was not found in HIEC cells, however, it was observed in the human fetal villus cells, recapitulating our findings in the Caco-2 cell model.
Table 2 – A sequential comparison of the SPRR2a/b epitope compared with seven isoforms of the SPRR2 class.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Protein Sequence</th>
<th>Percentage Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPRR2a/b Antibody Epitope</td>
<td>pcpepccccpckppcppccpp</td>
<td>100%</td>
</tr>
<tr>
<td>SPRR2a</td>
<td>msyqqqqckq pcqppvctp pkcpepcppp kpcpepcppp kpcpepcppp cpqcppcppqqc qkypvvtpsp ppcqkcppk sk</td>
<td>100%</td>
</tr>
<tr>
<td>SPRR2b</td>
<td>msyqqqqckq pcqppvctp pkcpepcppp kpcpepcppp kpcpepcppp cpqcppcppqqc qkypvvtpsp ppcqkcppk sk</td>
<td>100%</td>
</tr>
<tr>
<td>SPRR2c</td>
<td>Non expressed Pseudogene</td>
<td>0%</td>
</tr>
<tr>
<td>SPRR2d</td>
<td>msyqqqqckq pcqppvctp pkcpepcppp kpcpepcppp kpcpepcppp cpqcppcppqqc qkypvvtpsp ppcqkcppk sk</td>
<td>94%</td>
</tr>
<tr>
<td>SPRR2e</td>
<td>msyqqqqckq pcqppvctp pkcpepcppp kpcpepcppp kpcpepcppp cpqcppcppqqc qkypvvtpsp ppcqkcppk sk</td>
<td>100%</td>
</tr>
<tr>
<td>SPRR2f</td>
<td>msyqqqqckq pcqppvctp pkcpepcppp kpcpepcppp kpcpepcppp cpqcppcppqqc qkypvvtpsp ppcqkcppk sk</td>
<td>88%</td>
</tr>
<tr>
<td>SPRR2g</td>
<td>msyqqqqckq pcqppvctp pkcpepcppp kpcpepcppp kpcpepcppp cpqcppcppqqc qkypvvtpsp ppcqkcppk sk</td>
<td>73%</td>
</tr>
</tbody>
</table>
4.2 SPRR2 expression is localized to the apical actin ring and shows enrichment in membrane and cytoplasmic protein fractions

Little is known about the localization of SPRR2 other than its relative abundance in the cytoplasm as well as its tissue transglutaminase-dependent cross-linking and localization at the skin cell membrane to form the cornified envelope (42, 56, 58). We proceeded to delineate the compartmentalization of SPRR2 expression in our intestinal epithelial model using differential cellular fractionation and immunofluorescent confocal microscopy. The Triton X-100 soluble and insoluble, as well as membrane- and nuclear-enriched fractions were analyzed by immunoblotting over a twelve day time course. As shown in Fig 5-A, SPRR2 increases in both membrane- and cytoplasmic-enriched fractions with some lesser expression in the nuclear fraction. Interestingly, no expression is seen in the Triton x-100 insoluble fraction. To confirm these findings, two immunocytochemical approaches were taken. First, as shown in Figure 5-B, immunofluorescent microscopy was employed where negative controls using either no primary antibody or a mismatched secondary antibody were performed for both day -2 and day +10. An increase in SPRR2 expression was observed in day +10 compared to day -2, thus, confirming our western blot analysis. In addition, SPRR2 expression is observed in both membrane and nuclear compartments.
Figure 5A - A Western blot analysis of SPRR2 expression over twelve days in membrane-enriched, nuclear-enriched, and Triton X-100 soluble and insoluble fractions (n=3).
Figure 5B - B: Immunofluorescent analysis of SPRR2 expression in Caco-2 cells (n=3). Mismatched secondary (i,ii) and omitted primary antibody (iii,iv) were used as negative controls. Caco-2 cells two days pre-confluent (v) and ten days post-confluent (vi) were stained with anti-SPRR2 (1:1000 dilution). White arrows indicate positive staining in the cytoplasm (v) and membrane staining (vi).
Figure 5C: X-Y confocal microscopy (40x) of two days pre-confluent and ten days post-confluent Caco-2 cells (n=3). Cells were probed for F-actin and SPRR2 expression and composite images were subsequently merged. Z-plane (40x) analysis of ten days post-confluent Caco-2 cells stained for F-actin and SPRR2 expression (n=3). White arrows indicate positive staining localized to the apical actin ring.
Parallel time points were examined by confocal microscopy. As shown in Fig 5-B, SPRR2 shows enrichment in the cytoplasm as well as the cell membrane in both day -2 and day +10 time points. Using the apical actin ring as a reference, day +10 cells were stained for F-actin and SPRR2 and observed in the X-Y plane. The resulting merged image in Fig 5-C displays a clear co-localization of SPRR2 and F-actin. In addition, this association is further strengthened by analyzing the X-Z plane, where expression of SPRR2 and F-actin is observed to show concentration in the vicinity of the junctional complexes in the apical domain of differentiated Caco-2 cells.

4.3 8-Br-cAMP reduces SPRR2 induction in Caco-2 cells

Differentiation of the intestinal epithelium has been shown to be PKA dependent in Caco-2 cells (74)(10). Work by Boucher et al. has shown that stimulation of the PKA pathway by the addition of 8-Br-cAMP can inhibit markers of differentiation such as villin and sucrase isomaltase as well as negatively modulate adherens junction integrity by reducing the amount of E-cadherin and β-catenin (10). Therefore, we studied the effects of daily treatment of 1 mM 8-Br-cAMP on SPRR2 expression in the Caco-2 model. As shown in Fig 6-A, the induction of SPRR2 expression is reduced by 8-Br-cAMP treatment starting at day 0.
Figure 6A - A Western blot analysis of SPRR2 expression over a twelve day time course in (i) 0.1 N NaOH Vehicle Control versus 8-Br-cAMP; (ii) Control versus dideoxyforskolin; (iii) Dideoxyforskolin versus forskolin; (iv) IBMX versus forskolin/IBMX. In all Western blots, β-actin was probed to control for protein loading. The experimental concentrations of reagents used were: dideoxyforskolin (10 µM), forskolin (10 µM), and IBMX (1 mM).
**Figure 6B:** A western blot analysis of SPRR2 expression over a twelve day time course after treatment with control, dideoxyforskolin, forskolin, IBMX, and forskolin/IBMX (n=2). **6C:** An analysis of SPRR2 expression compared to day +10 control. Treatments that reached statistical significance (p < 0.05) are indicated (*).
To confirm this mechanism, we employed the well studied pharmacological activator of adenylyl cyclase, forskolin, and utilized its biologically inactive analogue, 1,9-dideoxyforskolin, as a negative control (15, 75). As shown in Figure 6-A, treatment with 10 µM 1,9-dideoxyforskolin and 10 µM forskolin every second day had minimal effect on SPRR2 expression over ten days. To increase the potency of the forskolin effect, isobutylmethylxanthine (IBMX), a non-specific inhibitor of phosphodiesterases, was administered with or without forskolin at a concentration of 1 mM (76). IBMX alone had no effect on differentiation-dependent SPRR2 expression but co-treatment with forskolin resulted in a significant decrease in SPRR2 expression.

4.4 SCFA induce SPRR2 expression in undifferentiated Caco-2 cells

Short-chain fatty acids such as butyrate, propionate and isobutyrate are produced by bacterial fermentation and are an important energy source for colonic epithelial cells. In addition, they have well described roles in regulating cell growth, proliferation and the ability to induce differentiation, with butyrate being the most potent and well studied (31, 77-79). Utilizing these pro-differentiation properties, day -2 undifferentiated Caco-2 cells were treated with varying concentrations of short-chain fatty acids. As shown in Fig 7-A, villin expression was measured over 96 hours as a marker of the differentiated state of
Caco-2 cells. Interestingly, in our model, only butyrate was potent enough to increase villin expression. Furthermore, as shown in Figure 7-B, there was no obvious difference in villin expression over 24 hours compared to control in any treatment group. However, SPRR2 expression is induced by 5 mM butyrate, propionate and isobutyrate within 24 hours (Figure 7-C). Additionally, all SCFAs show a concentration-dependent expression pattern of up-regulation of SPRR2 although at 20 mM, significant toxicity was seen with butyrate and propionate. The conventional mechanisms of action of short-chain fatty acids have been well described and include potent histone deacetylase inhibition properties. Two potent histone deacetylase inhibitors, trichostatin A and suberoyl bis-hydroxamic acid, were used to determine if histone deacetylase inhibition by itself was sufficient to induce SPRR2 expression. As shown in Figure 7-C/D, the induction of SPRR2 expression due to trichostatin A was less than that of short-chain fatty acids, thus suggesting that other mechanisms might be accountable for the SCFA effect.
Figure 7A: A Western blot analysis of villin expression over ninety six hours after treatment with control, butyrate, propionate, isobutyrate, TSA, and SBHA (n=3).

Figure 7B: A comparative analysis of villin expression over twenty four hours after treatment with control, butyrate, propionate, isobutyrate, and TSA (n=3).
**Figure 7C** - Twenty four hour time course and concentration range analysis of SPRR2 protein expression with or without treatment of butyrate, propionate, isobutyrate, TSA, and SBHA. Concentrations used for time course experiments (left) were: butyrate (5 mM), propionate (5 mM), isobutyrate (5 mM), TSA (2 µM), SBHA (15 µM). For all dose responses, cells were exposed to their respective drugs for twenty-four hours and compared to their controls at zero hours and twenty four hours (C0, C24, n=3).
**Figure 7** – D: SPRR2 expression after treatment with control at zero and twenty four hours (C0, C24), butyrate, propionate, isobutyrate, TSA and SBHA at twenty four hours (n=4). In all Western blots, β-actin was probed to control for protein loading. The experimental concentrations of reagents used were: butyrate (5 mM), propionate (5 mM), isobutyrate (5 mM), TSA (2 µM), and SBHA (15 µM). Treatment groups reaching statistical significance by a two-tailed student’s t-test (p < 0.05) are indicated (*).
4.5 SPRR2 induction by propionate is mediated through p38 MAPK and MEK/ERK pathways

Amongst all short chain fatty acids, butyrate is the most well studied and has been shown to be the most potent inhibitor of histone deacetylases and inducer of differentiation in a number of settings including vascular endothelial cells, the human colon carcinoma cell line HT-29, and the Caco-2 cell line (28, 31, 80, 81). In accordance with the literature, upregulation of villin expression was detected only by treatment with butyrate. However, treatment with propionate and isobutyrate exhibited a greater induction of SPRR2 expression when compared with butyrate suggesting a mechanism independent of their well-documented histone deacetylase inhibition properties. Recently, two orphan G protein-coupled receptors, GPR41 and GPR43, were identified as receptors for SCFAs in polymorphonuclear cells (35). Propionate was the most potent agonist for both receptors and activation of these receptors was coupled to intracellular Ca\textsuperscript{2+} release, ERK1/2 activation, and inhibition of cAMP accumulation. In addition, in the MCF-7 human breast cancer cell line, propionate has been shown to induce phosphorylation of p38 MAP kinase through the activation of GPR43. To confirm that these receptors were indeed present in our model and in the absence of commercially available antibodies, we performed qualitative RT-PCR for GPR41 and GPR43 based on published primer sequences at two days pre-confluence and ten days post-confluence. As shown in Figure 8-A, mRNA
transcript for both GPR41 and GPR43 are present at both time points. To delineate the signaling pathways involved in mediating SPRR2 induction by propionate, we pretreated cells for two hours with the p38 MAP kinase inhibitor SB203580 (10 µM) and the ERK1/2 inhibitor U0126 (10 µM). As shown in Figure 8-B, co-treatment with SB203580 and U0126 inhibited the induction of SPRR2 expression by propionate suggesting that the propionate effect depends on pathways associated with GPR41 and GPR43 activation.

4.6 SPRR2 is present in the upper and lower gastrointestinal tract of C57/Bl6 mice

We proceeded to isolate tissue from the upper and lower gastrointestinal tract from conventionally housed C57/Bl6 mice fed a normal diet. As shown in Fig 9-A, SPRR2 is expressed in both the upper and lower gastrointestinal tracts of C57/Bl6 mice (n=3). Although not reaching statistical significance, an increasing trend in SPRR2 expression can be seen along the longitudinal axis of the gastrointestinal tract. Furthermore, separation of intestinal mucosal scrapings from the remaining tissue showed that SPRR2 is not only localized to the intestinal epithelium, but that it is also expressed in the submucosa and deeper constituents of the wall of the intestine which includes the smooth muscle and surrounding connective tissues.
Figure 8 - A: Qualitative detection of GPR 41 and 43 mRNA transcripts at two days pre-confluence and ten days post-confluence (n=2). Forty PCR cycles were used. B: Western blot analysis of SPRR2 expression after twenty four hours of treatment with (i) propionate versus SB203580/propionate (n=2) and (ii) propionate versus U0126/propionate (n=2). β-actin expression was probed to control for equal protein loading.
**Figure 9** - A: Western blot analysis of SPRR2 expression throughout the upper and lower murine gastrointestinal tract (n=3). B: Western blot detection of SPRR2 expression in mucosal fraction (M) versus remainder (R) in the murine duodenum, terminal ileum, ascending colon, and descending colon (n=3). Abbreviations used for the gastrointestinal tract: esophagus (ESO), stomach (STO), duodenum (DUO), jejunum (JEJ), middle ileum (MI), terminal ileum (TI), cecum (CEC), ascending colon (AC), transverse colon (TC), descending colon (DC), and rectum (R). In all Western blots, β-actin expression was probed and utilized as a control for equal protein loading.
4.7 SPRR2 is induced by components of *Salmonella typhimurium*

It is well known that the gram negative bacterium such as *Bacteroides thetataomicron* induces a 205-fold increase in SPRR2a mRNA in villus epithelial cells of mono-associated germ-free mice. In addition, a number of studies have suggested a potential adaptive role for SPRR2 (40, 82, 83). With an aim to build on previous reports that gram negative colonization of the gut induces SPRR2 expression as well as with an aim to start delineating physiological roles of SPRR2 proteins in intestinal epithelia, we analyzed the effect of the pathogen *S. typhimurium* on SPRR2 expression in undifferentiated Caco-2 cells. As shown in Appendix C, SPRR2 expression is induced at 6 hours with a 1:100 dilution of *S. typhimurium* (n=2). Interestingly, co-culture with heat killed *S. typhimurium* also resulted in a similar induction of SPRR2 (n=1), suggesting that bacterial cell constituents rather than invasive viable bacteria themselves may account for the up-regulation of SPRR2 proteins in epithelia in the presence of bacteria.
Chapter 5
Discussion

It has been proposed that SPRRs function as scaffolding proteins during the multi-step process leading to the formation of the mature cornified envelope in differentiated keratinocytes (58). Based on the differential expression of various SPRR isoforms in a variety of tissues, a number of authors have suggested that the SPRR family of proteins may play a cell- and tissue-specific role in the provision of the integrity and flexibility required by epithelial barriers by acting as integral cross-bridging proteins (84, 85). In the intestine, however, there are few studies focusing on the function of SPRR family of proteins. The intestinal epithelium must be selectively permeable to allow for transport of ions, water, and nutrients yet, must also discriminate against potentially harmful dietary proteins and commensal bacteria. Using a model to study the relationship between *Bacteroides thetataomicron* and gut development, Hooper *et al.* found that this commensal bacterium can fortify the epithelial barrier by inducing the expression of decay-accelerating factor, which inhibits cytotoxic damage from secreted microbial components (86). Most importantly, this study provided evidence that mice mono-associated with *Bacteriodes thetataomicron* anaerobic bacteria cause a 205-fold increase in SPRR2 expression when compared to germ-free mice (67). These results suggest that SPRR2 may contribute to the fortification of the epithelial barrier in response to the colonization of bacteria.
Furthermore, the coordinate upregulation of SPRR2 along with numerous transporter genes such as sucrase isomaltase and sodium glucose co-transporter (SGLT-1) suggests that in addition to identifying a potentially important role for SPRR2 in normal barrier development and function, a key role in the regulation of nutrient transport can also be suggested. We have reported herein novel data which importantly, will help facilitate the elucidation of the role that SPRR proteins play in the intestinal epithelium. Polarized Caco-2 cell monolayers have been well-studied and function as an important model which has played a pivotal role in the advancement of our understanding of the process of differentiation of the small intestinal absorptive enterocyte, the regulation of nutrient transport and epithelial barrier function in health and disease (87). We have shown that SPRR2 expression coincides with the differentiated state of Caco-2 cells. In addition, we have found that the SPRR2 protein, while absent in HIECs, is expressed in human fetal villus epithelial cells which suggests that a role for SPRRs must exist in development of the small intestine even prior to birth. These results recapitulate our findings in the Caco-2 cell model, suggesting that SPRRs have functional relevance and are not merely an epiphenomenon characterisitc of this transformed cell line.

In transgenic mice over-expressing claudin-6 in skin driven by the involucrin promoter, the SPRR2 profile in the epidermal permeability barrier is altered. This modified expression of a variety of SPRR isoforms is accompanied by the altered expression of a number of claudin family members. Taken
together, these changes in gene expression are associated with altered skin permeability and suggest that the interaction and balance of both SPRR2 and claudins may be important in maintaining normal barrier function. Unfortunately, it is difficult to extrapolate further from this skin-specific model as claudin-6 is amongst those claudins which do not appear to be expressed in the mouse intestine.

The primary role of claudins is to regulate paracellular transport across epithelia (88). In the intestine, several claudin family members are expressed and show unique profiles along the length of the lower gastrointestinal tract which suggests that the composition of specific claudin family members contribute to the functional needs of these intestinal barriers (88). Although the interaction between SPRR2 and claudins has yet to be characterized, claudin-1 and claudin-5 have been detected in the cytoskeletal Triton X-100 insoluble fraction in Caco-2 cells (89). Moreover, confocal analysis has shown claudin-1 and claudin-5 to colocalize with the apical actin ring. Therefore, we hypothesized that SPRR2 might localize to the apical actin ring much like other well-documented tight junction proteins such as zonula occludens-1, occludin, and claudin-1/5 (89). Through confocal microscopy, SPRR2 was found to colocalize with the apical actin ring and was also found in the cytoplasm. Interestingly, confirmation of these findings by differential cell fractionation of post-confluent differentiated Caco-2 cells clearly indicated that SPRR2 was not associating with the Triton X-100 insoluble cytoskeletal fraction. Instead, we observed that SPRR2 was
expressed in the membrane- and cytoplasmic-enriched fractions, suggesting that while SPRR2 is localized to the apical actin ring, it may for example, function as an actin binding protein displaying loose interactions with the cytoskeleton. From a functional perspective, these results can be explained in a number of settings. While claudins themselves do not possess SH3 domains, zonula occludens-1 (ZO-1), a tight junction protein that interacts closely with claudins, does possess a SH3 domain. In addition, considerable attention has been dedicated to finding the ligands that bind to the SH3 domain on ZO-1 and ultimately, that are responsible for recruiting ZO-1 to the tight junctions. To date, α-catenin and the serine-threonine kinase ZAK have been shown to bind to this SH3 domain (90, 91). In our hands, SPRR2, a recently identified SH3 domain ligand (60), was found to associate with the actin cytoskeleton by confocal microscopy as well as show expression in the cytoplasm and it is tempting to speculate that they may have a role in recruitment of junctional proteins such as ZO-1. In further support of this is the apparent concentration of the SPRR2 signal on the Z-plane reconstruction at the level of the tight junction.

Further speculation for a role for SPRR2 in the intestine stems from the report that SPRR2 act as a SH3 domain ligand (60). Many studies have shown that interactions between cell surface receptors and SH3 domain-containing adaptor proteins are required for intact function downstream intracellular signaling pathways. For example, Grb2-associated binder (Gab) family of proteins act as scaffolding adaptors through their SH2/SH3 domains and signal
to downstream effector proteins such as Ras/MAPK pathway-specific guanine nucleotide exchange factors (GEF) and son of sevenless (Sos) (92). Whereas in vivo mouse knockouts of Gab-2 and 3 result in viable and generally healthy mice, knockout of Gab-1 results in death between embryonic days 12.5 to 18.5 apparently due to defects in the heart, placenta, and the liver (93). Furthermore, Li et al. showed that the SH2/SH3 adapter Nck/Dock’s biological function was to signal to the actin cytoskeleton. They suggested that the binding of Nck (SH3 domain) and Pak1 (SH3 ligand) led to an enhancement in Pak1 kinase activity and resulted in actin polymerization after treatment with PDGF in fibroblasts (94, 95). In the cornified envelope of the skin, it has been proposed that SPRR2 functions as a scaffolding protein. Therefore, it is tempting to speculate that its role in the intestine may be to function as a scaffold which facilitates signaling to the actin cytoskeleton in various physiological settings.

An important aim of this thesis was to begin to understand how the increase in differentiation-dependent SPRR2 expression is regulated. When we examined putative transcription factor binding sites in the SPRR2e promoter for example, we found many GATA-1 and HNF4 sites but no significant CDX2 binding sites. Another study showed that stimulation of the PKA pathway inhibits numerous markers of differentiation including villin, sucrase isomaltase as well as epithelial polarity in Caco-2 cells. This led us to test the effect of 8-Br-cAMP on SPRR2 expression. We found that differentiation-dependent SPRR2 was inhibited and confirmed by co-treatment with forskolin and IBMX, albeit not as
potent as 8-Br-cAMP. In parallel, we also uncovered that SPRR2 expression was induced in response to SCFA treatment. Butyrate, propionate, and isobutyrate have been shown to alter gene expression by reducing histone deacetylase (HDAC) activity, shifting histones towards a hyperacetylated state, and subsequently inducing changes in gene transcription. In addition, a butyrate response element (BRE) has also been identified that may be involved in the regulation of a number of genes (96, 97). As a result, SCFAs have been used extensively to induce differentiation in different cell lines. At 5-10 mM, butyrate, propionate, and isobutyrate were able to induce SPRR2 expression without undue toxicity. However, at 20 mM, expression of SPRR2 is abolished in parallel with significant cell death. This is likely explained by previous work showing that high concentrations of SCFAs cause apoptosis in cancer cells (77, 98). Interestingly, the more potent and specific HDAC inhibitor, trichostatin A did not induce SPRR2 expression in a similar manner to SCFA. Furthermore, the poorly metabolized isomer of butyrate, isobutyrate as well as an equimolar concentration of propionate displayed an equipotent induction of SPRR2 to that of butyrate. Yet, only propionate achieved statistical significance. Butyrate, however, was the only SCFA to induce villin expression over 96 hours. Taken together, these results suggest a non-classical mechanism by which SPRR2 is induced by SCFAs. SCFA induction of SPRR2 may be a result of cellular acidification as pH, although not measured, was observed to decrease in media as a result of SCFA and SBHA treatment. Indeed, there are few studies showing
that SCFA treatment results in the formation of regulated extracellular microdomains, however, there has yet to be any reports linking acidification with targeted changes in gene expression (99). Furthermore, we allowed time for the culture media to buffer the pH shift induced by SCFAs, prior to applying to the Caco-2 cells. A more probable explanation arises from an evolving literature suggesting that short chain fatty acids such as propionate and isobutyrate can signal through the previously described orphan G-protein coupled receptors 41 and 43, which function independently of intracellular pH. Specifically, interaction with GPR43 increases the intracellular concentration of Ca$^{2+}$ while reducing the intracellular concentration of cAMP, leading to the activation of pro-differentiation genes. Moreover, activation of GPR43 by propionate was also coupled to an acute phosphorylation of p38 MAPK and ERK1/2. In accordance, we showed that pre-treatment with the inhibitors SB203580 and U0126 inhibited the propionate induced SPRR2 expression. Although not examined in this study, it is within reason to speculate that the increased induction shown by SCFAs could, in part, be due to an interaction with GPR43. However, further studies including differential knockdown of GPR41 and GPR43 by siRNA will be required to fully characterize the significance of this pathway.
In conclusion, we have presented novel discoveries that SPRR2 coincides with differentiation in the Caco-2 model. Using immunocytochemical approaches, we have shown that SPRR2 co-localizes with the apical actin ring, yet does not associate with the cytoskeleton by traditional Triton X-100 based approaches, suggesting a loose interaction between SPRR2 and the apical actin ring. Moreover, we have shown that SPRR2 can be inhibited by activation of the PKA pathway and induced by SCFAs ascribing a putative role to GPR41 and GPR43 mediated signaling. We have also taken the first steps to delineate the mechanisms behind this induction. Lastly, we have found that SPRR2 is upregulated in response to certain components of Salmonella typhimurium, suggesting a possible role for SPRR2 in adaptive barrier function. This work sets the stage for a closer examination of the physiological roles of SPRR2 in the regulation of epithelial barrier function, nutrient absorption, and host microbe interactions both in vitro and in vivo.
References


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Appendix A

Appendix A – Western blot analyzing SPRR2 expression in T84 cells two days pre-confluent, two days post-confluent, and ten days post-confluent. Mouse skin was used as a positive control for SPRR2 expression. β-actin expression was probed to control for equal protein loading.
Appendix B: Semi-quantitative RT-PCR analysis of SPRR2d (179 bp), 2e (227 bp) mRNA expression over a twelve day time course. β-actin was used to control for loading.
Appendix C

A

WT - S. typh (1:100)

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SPRR2

β-ACTIN

B

WT - S. typh (heat killed)

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SPRR2

β-ACTIN

Appendix C: A: Western blot analysis of SPRR2 expression after co-culture with wild-type *S. typhimurium* (1:100, n=2). B: Western blot analysis of SPRR2 expression after co-culture with heat killed *S. typhimurium* (n=1). In all blots, β-actin was probed to control for protein loading.