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Mechanisms of Induction and Maintenance of Constitutive Ptgs2 Expression in Colonic

Mesenchymal Stem Cells

by

Monica Rae Drylewicz

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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Saint Louis, Missouri

ABSTRACT OF THE DISSERTATION

Mechanisms of induction and maintenance of Ptgs2 in colonic mesenchymal stem cells

by

Monica Rae Drylewicz

Doctor of Philosophy in Biology and Biomedical Sciences
Immunology

Washington University in St. Louis, 2012

Professor Thaddeus Stappenbeck, Chairperson

Colonic injury and inflammation exhibit high morbidity and mortality in western society and are increasingly diagnosed in human patients. In many models of such gastrointestinal insults, prostaglandin endoperoxide synthetase 2 (Ptgs2; cyclooxygenase 2) and its downstream synthetic products, most notably prostaglandin E2, support proliferation and modulate the inflammatory immune response, two functions involved in proper injury response. We identified a primary source of this mediator as a population of stromal cells constitutively expressing Ptgs2 that appear to be required in two different model injury systems: administration of dextran sodium sulfate (DSS) and colonic biopsy.

The central role of Ptgs2-expressing cells in injury demonstrates the importance of understanding Ptgs2 regulation. We therefore created a robust *in vitro* system for studying the pathways and molecules involved by isolation of Ptgs2-expressing colonic stromal cells and identified the isolated lines as colonic mesenchymal stem cells (cMSCs). We then demonstrated the dominance of post-transcriptional regulation of Ptgs2 expression in cMSCs. Subsequent global gene array analysis comparing these cMSCs to

a similar cell isolated from the bone marrow revealed a number of candidate genes with potential roles in post-transcriptional regulation of Ptgs2 expression.

We determined that Fgf9 can regulate Ptgs2 through Fgf receptor expressed preferentially in cMSCs. Downstream of Fgf9, phosphorylation of Erk and increased abundance of mRNA binding protein CUGbp2 augments Ptgs2 expression. Furthermore, Fgf9 expression in the adult colonic epithelium ideally positions this growth factor to affect Ptgs2 expression *in vivo*. We also found that loss of insulin growth factor 2 binding protein 1 (Igf2bp1 or Imp1) results in a decrease in Ptgs2 and confirmed the ability of Imp1 protein and Ptgs2 mRNA to interact in the cellular environment. Additionally, we discovered increased Imp1 expression in the colonic biopsy wound bed compared to normal mucosa and co-localization of Imp1 in wound bed Ptgs2-expressing cells.

Overall, this work has uncovered multiple mechanisms involved in the complex post-transcriptional regulation of Ptgs2 in isolated stromal cells and demonstrated the role of these pathways in response to multiple forms of colonic injury.

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TABLE OF CONTENTS

Abstract of the Dissertation ii

Acknowledgements..... iv

Table of Contents v

List of Figures and Tables..... vi

List of Abbreviations viii

Chapter 1 1

Introduction: Ptgs2 Expression and Regulation in Colonic Injury

Chapter 2 20

Fgf9 Regulation of Ptgs2 Expression in Colonic Mesenchymal Stem Cells

Abstract 21

Introduction 22

Materials and Methods 24

Results 30

Discussion 55

Chapter 3 61

Imp1 is a novel trans-acting factor involved in Ptgs2 regulation

Abstract 62

Introduction 63

Materials and Methods 65

Results 69

Discussion 79

Chapter 4 82

Summary and Future Directions

References..... 96

LIST OF FIGURES AND TABLES

CHAPTER 1

Figure 1.1 Depiction of Imp1 conserved protein domains14

CHAPTER 2

Figure 2.1 *Ptgs2*-expressing stromal cells co-localize with markers of mesenchymal stem cells *in vivo*31

Figure 2.2 Isolated colonic stromal cells are mesenchymal stem cells and maintain *Ptgs2* expression34

Figure 2.3 High constitutive *Ptgs2* expression is unique to cMSCs37

Figure 2.4 High constitutive expression of *Ptgs2* is not due to TLR activation40

Figure 2.5 Comparison of cMSCs to bmMSCs reveals possible mechanistic pathways involved in *Ptgs2* expression44

Figure 2.6 Fgf9 is sufficient to stabilize *Ptgs2* mRNA48

Figure 2.7 Fgf9 stabilizes *Ptgs2* partially through Erk activation and increased CUGbp2 protein52

Figure 2.8 Model of Fgf9 regulation of *Ptgs2* expression in cMSCs54

CHAPTER 3

Figure 3.1 *Ptgs2* is required for and present during proper wound healing70

Table 1 Effect of decreased mRNA binding protein candidates on *Ptgs2* expression73

Figure 3.2 Imp1 interacts physiologically with *Ptgs2* mRNA75

Figure 3.3 Imp1 is expressed in *Ptgs2*-expressing wound bed stromal cells77

CHAPTER 4

Figure 4.1 Imp1 phosphorylation and Imp1-Ptgs2 interactions in 0% serum88

Figure 4.2 Imp1 regulation in cMSCs is not affected by Wnt, TNF α , or IFN γ 90

LIST OF ABBREVIATIONS

ARE	AU-rich element
bmMSC	bone marrow mesenchymal stem cell
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CCL5	chemokine ligand 5
cDNA	complementary deoxyribonucleic acid
CIP	calf intestinal phosphatase
cMSC	colonic mesenchymal stem cell
CMV	cytomegalovirus
CNS	central nervous system
ColEP	colonic epithelial progenitor
CONV	conventionally raised
Cox	cyclooxygenase
CRD-BP	coding region determinant binding protein
Csf-1	colony stimulating factor 1
CUGbp2	CUG binding protein 2
DMEM	Dulbecco's modified eagle's media
DSS	dextran sodium sulfate
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EP4	prostaglandin E receptor 4
FACS	flow cytometry
FBS	fetal bovine serum
Fgf	fibroblast growth factor
Fgfr	fibroblast growth factor receptor
GF	germ free
GFP	green fluorescent protein
GI	gastrointestinal
GO	gene ontology
HBSS	Hank's buffered salt saline
HCV	hepatitis C virus
HEK293T	human embryonic kidney 293T cells
HES-1	hairy and enhancer of split 1
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HuR	Hu antigen R
I-A ^b	MHC Class II
IFN γ	interferon γ
Igf	insulin-like growth factor
Igf2bp1	Insulin-like growth factor 2 binding protein 1
IL	interleukin
Imp1	Insulin-like growth factor 2 binding protein 1
IP	immunoprecipitation

IRAK-M	interleukin-1 receptor-associated kinase 3
IRES	internal ribosome entry site
KH	hnRNP K homology
lacZ	β -galactosidase gene
LPS	lipopolysaccharide
MAP	mitogen activate protein
MD-2	lymphocyte antigen 96
MEK	mitogen-activated protein kinase kinase 1
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
NCBI	National Center for Biotechnology Information
NIH3T3	NIH Swiss mouse embryonic fibroblasts
NK	natural killer
n.s.	not significant
NSAID	non-steroidal anti-inflammatory drug
OCT	optimal cutting temperature
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PFA	paraformaldehyde
PGE2	prostaglandin E2
PI3K	phosphoinositol 3 kinase
PKC	protein kinase C
PLB	polysome lysis buffer
PSC	Ptgs2-expressing stromal cell
Ptgs2	prostaglandin endoperoxide synthetase 2
q	quantitative
Rbm3	RNA binding motif protein 3
RIPA	radio-immunoprecipitation assay buffer
RRM	RNA recognition motif
RT-PCR	reverse transcriptase polymerase chain reaction
SDF-1	chemokine (C-X-C motif) ligand 12
SDS	sodium dodecyl sulfate
shRNA	short hairpin RNA
SMA	smooth muscle actin
SNP	single nucleotide polymorphism
TBS(T)	tris-buffered saline (tween 20)
TGF β	transforming growth factor β
Th1/2	T helper cell
Tlr	toll like receptor
TX	thromboxane
TNBS	2,4,6-trinitrobenzene sulfonic acid
UTR	untranslated region
UV	ultra-violet
VCAM	vascular cell adhesion molecule
Vgl	vegetal 1

WAE	wound associated epithelium
WT	wild-type
ZBP-1	zipcode binding protein 1

CHAPTER 1: INTRODUCTION

Ptgs2 Expression and Regulation in Colonic Injury

Chapter 1: Introduction

A variety of injurious insults to the colon are seen in human patients from cancer lesions to chronic inflammation to parasitic infections with many detrimental sequelae. The cost in mortality and in morbidity is high. Understanding the mechanisms resulting in various injuries and the mechanisms involved in resolving these injuries could create better diagnosis and prognosis methodologies and more effective, targeted treatment for specific situations. We and others have recognized the important role of prostaglandin endoperoxide synthetase 2 (Ptgs2) and its downstream synthetic products, prostaglandins, in mediating responses to injury via regulation of proliferation and modulation of the immune system. A precise knowledge of the regulation of this gene is therefore necessary to fully understand, develop, and evaluate interventions in colonic injury in human medicine.

Biology and histology of the colon

The colon of the mouse, a model of the human organ, is a tubular organ consisting of various layers of cellular tissues. The inner most layer, abutting an inner lumen containing an abundant microbial population, is simple columnar epithelium and the surrounding mesenchyme that together make up the mucosa. Beyond this layer, located radially are the muscularis mucosa, submucosa, muscularis propria made up of circular and longitudinal layers, and outer serosa. The enteric nervous system runs the length of the colon with the muscularis. The colon can also be divided from proximal to distal: ascending colon, transverse colon, descending colon, and finally the rectum.

The inner epithelium of the colon serves an indispensable barrier function separating the host organism and the microbial contents of the colonic lumen. This barrier allows

the luminal bacteria and host to exist symbiotically rather than antagonistically. The colonic epithelium is made up of many invaginations along the length of the organ called the crypts of Lieberkuhn. These crypts contain the epithelial stem cell putatively at the base, colonic epithelial progenitors (ColEPs), and the three mature epithelial cell types: absorptive enterocytes, secretory goblet cells, and enteroendocrine cells. The ColEPs are also referred to as transit amplifying cells which rapidly proliferate and provide the epithelium with a constant supply of maturing cells.

Surrounding these crypts are many cells which collectively make up the colonic mesenchyme and support the epithelium. The area most closely associated with the proliferative/stem cell compartment is often referred to as the stem cell niche as it is at least partially responsible for the support, maintenance, and regulation of the stem cell¹. The most abundant cell types in the mesenchyme are myofibroblasts, endothelial cells, macrophages, and dendritic cells. Other populations are also represented including colonic stromal cells expressing Ptgs2, B cells, T cells, plasma cells, and dendritic processes of the enteric nervous system. Many different signaling pathways operate in the regulation of epithelial organization and differentiation^{2,3}. For example, the Wnt pathway is highly active in the lower crypt where the ColEPs are constantly undergoing division to supply the rest of the epithelium. The role of the Wnt pathway has been well illustrated by a number of studies. Neonatally, deficiency of Tcf4, the transcription factor activated by canonical Wnt stimulation, results in an endoderm made up purely of differentiated epithelial cells and devoid of a proliferative compartment⁴. Likewise, in the adult, targeted expression of the Wnt inhibitor dickkopf-1 leads to a vast decrease in epithelial proliferation and apparent loss of crypts⁵. Suggestive of a role in inhibiting

proliferation and/or inducing differentiation, TGF- β and the type II TGF- β receptor are both localized in the upper epithelial compartment.⁶ The activity of bone morphogenetic proteins seems to be similar to that of its family member TGF- β as demonstrated by an association between its loss-of-function and dysregulated proliferation shown by high rates of polyposis³. The Notch and Hedgehog signaling pathways seem to function primarily in cell fate decisions. Animals deficient in Notch signaling (via loss of HES-1) lose absorptive cells while expanding the secretory compartment containing goblet cells and enteroendocrine cells⁷. Further downstream of Notch, Neurogenin 3 is suggested to be responsible specifically for the enteroendocrine lineage⁸. Sonic hedgehog is expressed only at very low mRNA levels in the base of the crypts. It seems to be more involved in patterning the radial aspects of the intestine⁹. Indian hedgehog is expressed in differentiated colonic enterocytes and thus hints at a possible role in differentiation of these cells. Indeed, blockade of the hedgehog pathway in general by cyclopamine-mediated inhibition of Smoothened leads to increased proliferation of the epithelium¹⁰.

These various signaling pathways control homeostasis in the colon which can be simplistically viewed as directing proliferation toward the base of the crypt and differentiation toward the cuff. Various injuries, discussed below, place different stresses on the epithelium and often alter this delicate programming. Combining knowledge of the basic biology of the normal colon along with responses during injury will increase the general ability of medicine to treat and heal these insults.

Models of colonic injury

A variety of injury models have been developed and studied with the goal of understanding the mechanisms involved in the development and healing of inflammation,

injury, and cancer. The primary interest of our lab has been focused on inflammatory and localized insults with the goal of forming a complete understanding of the mechanisms involved in the development and healing of colonic diseases in human patients. Such knowledge may provide for improved diagnostic techniques and targeted treatments for many common colonic insults.

Models of chronic inflammation

A variety of colonic injury models for chronic inflammatory bowel disease have been developed over the past four decades¹¹. Many have been based on gene knockouts and transgenics of various immune genes such as IL-10. Some models have been found by spontaneous development during breeding such as the C3H/HeJBir. Adoptive transfer of T cells has also resulted in colitis. Many inducible models of colitis have been discovered as well through administration of various compounds/insults including dextran sodium sulfate (DSS), TNBS (2,4,6-trinitrobenzene sulfonic acid), indomethacin, iodoacetamide, acetic acid, oxazolone, peptidoglycan-polysaccharide, and radiation. All of these models result in varying degrees of inflammation and ulceration of the epithelium, often accompanied by either or both a decrease in epithelial proliferation or an increase in epithelial apoptosis.

One of the first reports rigorously documenting the injury induced by DSS was published in 1990¹². Depending on the percentage of DSS given, the severity of colitis can be controlled. Furthermore, the specific effects of the chemical on the colon are location dependent; for example, the transverse colon is more severely affected than the descending colon. Our lab recognized the importance of another variable in this model: diet. Mice fed a special diet intended for germ free animals during DSS administration

are less susceptible to the effects of the chemical compound¹³. As mentioned, due to proximal-to-distal variations, the location of injury analysis must be strictly distinguished. In our model, administration of DSS does not apparently affect the ascending colon or the majority of the descending colon in WT mice. The transverse colon is afflicted with a number of smaller ulcerations surrounded by hyperproliferative crypts while the distal rectum simply shows ulceration. Our lab has also discovered that DSS-treated Myd88^{-/-} and Ptgs2^{-/-} mice lose epithelial proliferation in rectal crypts that can be rescued by administration of prostaglandin E2 (PGE2) during DSS administration¹⁴. By analyzing this model with the goal of identifying the cellular source of PGE2, we discovered a population of cells in the mesenchyme that constitutively express prostaglandin endoperoxide synthase 2 (*Ptgs2* or *cox-2*). During DSS injury, these cells redistribute to the epithelial stem cell niche, the mesenchyme apposed to the base of the colonic crypts, seemingly to maintain proliferation of the stem cell compartment. As shown herein, isolation of colonic stromal cells was accomplished, and these cell lines allowed rigorous examination of the mechanisms involved in Ptgs2 regulation. We show that Ptgs2 regulation can be mediated by Fgf9 signaling through Erk activation and CUGbp2 stabilization.

Model of physical injury

Our laboratory has pioneered the development and study of a new model of localized physical colonic injury that allows localization and precise timing of injury to be specifically studied. This model is the colonic biopsy model¹⁵. In this model, a portion of the mucosa of the colon is removed by biopsy and the injury is followed over time by endoscope until sacrifice of the mice followed by gene expression and histological

studies. It has been demonstrated that the healing of these wounds is predictable and occurs over approximately 6-8 days depending on the size of the wound¹⁵ (unpublished, H. Miyoshi and N. Manieri). Histology and cytokine expression were also found to be predictably reproducible. The wound bed, consisting of granulation tissue, is rapidly covered by a single cell epithelial layer. It was found that proper expression of IL-13 and IL-4 was required for timely and organized wound healing.

An understanding of the mechanisms underlying wound healing will lead to a better overall picture of how the colon reacts in injury and will identify the molecules most important in healing. It is demonstrated herein that one of these molecules is Ptgs2 and that its regulation in this case is likely partially mediated by a novel trans-acting factor, insulin growth factor 2 binding protein 1 (Igf2bp1 or Imp1).

Biology of Ptgs2

Ptgs2 is one of the two rate-limiting catalysts in the synthesis pathway of prostaglandins (PG) and thromboxane (TX). These enzymes catalyze the oxygenation of arachidonic acid to prostaglandin H₂ which is further converted by specific PG and TX synthases to PGI₂, PGE₂, PGF₂, PGD₂, and TXA₂¹⁶. In general, Ptgs1 is considered to have housekeeping and homeostatic functions while Ptgs2 is considered to be the inducible form upregulated primarily in inflammation.

The Ptgs2 knockout mouse was generated in 1995 and analyzed¹⁷⁻¹⁹. The primary phenotype of an unperturbed Ptgs2 KO mouse is renal dysplasia with some cardiac fibrosis, and females are infertile. In the absence of an injurious insult, the colon is normal by all measurable aspects in a Ptgs2 KO mouse. However, upon injury, Ptgs2 is required for proper response both in radiation injury²⁰ and DSS-mediated injury^{14,21}.

These studies also demonstrated the ability of PGE2 to rescue the observed phenotypes, strongly suggesting that *Ptgs2* was necessary due to its synthetic function upstream of the secretion of PGE2.

PGE2 signals through four different G-coupled protein receptors termed EP1-4. These receptors are expressed in various cells throughout the gastrointestinal (GI) tract²² and seem to have a role in a variety of basic GI functions including gastric acid, sodium bicarbonate, and mucus secretion, motility, and cytoprotection. The most important of the four receptors in cytoprotection against DSS-induced colitis is EP4 as demonstrated by the severity of injury in mice lacking EP4 or treated with an EP4 antagonist²³. The expression of the EP4 receptor by crypt epithelial cells further suggests the importance of this receptor in responding to DSS injury. Intriguingly, a genome wide association study recently identified a SNP located in the EP4 gene as a risk factor in the inflammatory condition Crohn's disease²⁴. As mentioned, our lab has described the role of *Ptgs2* and subsequent PGE2 in maintaining epithelial proliferation in DSS injury¹⁴. It appears that EP4 is the receptor important in this pathway.

Though normally considered to be expressed upon induction, *Ptgs2* is expressed constitutively in certain cells, a fact that has been recognized in the small²⁵ and large¹⁴ intestine. Taking together the developmental problems and injury response deficiencies of the *Ptgs2*^{-/-} mouse and the constitutive expression of *Ptgs2* in some, albeit rare, cells, it seems that a better understanding of the regulation of *Ptgs2* is necessary to a complete understanding of homeostasis and healing.

The regulation of *Ptgs2* is complex and involves many transcriptional and post-transcriptional mechanisms^{26,27}. Regulation at the transcriptional level has been highly

studied, especially in terms of upregulation during inflammation. Less well-studied is the post-transcriptional regulation of Ptgs2 as well as recognition of the constitutive expression of this gene in adult tissues. Creation of a lacZ Ptgs2 reporter mouse clearly demonstrated the expression of Ptgs2 in adult tissues in the mammal in the absence of inflammatory insult²⁸ with the greatest expression in the colon and the testis. Many growth factors normally present in various tissues of an adult animal have been implicated in at least partial control of Ptgs2 expression level including EGF²⁹, PDGF³⁰, basic Fgf³¹, and TGF β ³². In these investigations, these factors were demonstrated to act through Src and various MAP kinase pathways.

We and others have noted that Ptgs2 is very strongly regulated at the post-transcriptional level. The area of focus of many investigations is the 3' untranslated region (UTR) of the Ptgs2 mRNA transcript. This region contains multiple AUUUA sequences also known as AREs or AU-rich element^{33,34}. This sequence is known to be targeted by multiple mRNA binding proteins that, while stabilizing the mRNA, have varying and sometimes unclear effects on translation of the transcript. One well-recognized binder of AREs and regulator of Ptgs2 is CUGbp2 (CUG triplet binding protein³⁵) which is reported to stabilize the transcript and decrease translation³⁶. Interestingly, binding to the ARE is degenerate and the identity of the binding proteins seems dependent on the physiologic conditions. For example, CUGbp2 and HuR can both bind Ptgs2 but CUGbp2 decreases while HuR increases translation. Following radiation injury, the CUGbp2 abundance increases and may be able to out-compete HuR binding, thus decreasing Ptgs2 translation^{37,38}. It has also been shown that the binding of trans-acting factors can be affected by more than just expression level. Phosphorylation

of CUGbp2 by Src is capable of increasing binding affinity of the protein for Ptgs2 mRNA³⁰. Thus the post-transcriptional regulation of AREs is complex and dynamic; however, the degenerate nature of this binding sequence that is found in a great abundance of mRNA transcripts suggests that in order to specifically regulate Ptgs2, there must be further mechanisms involved. In order to study these mechanisms in more detail within this thesis, we employed a cell culture line of colonic stromal cells that constitutively express Ptgs2.

Mesenchymal Stem Cells

This work uses isolated colonic stromal cells that constitutively express high levels of Ptgs2 as a primary tool to investigate the regulation of Ptgs2. Herein we identify these cells as mesenchymal stem cells (MSCs, also known as multipotent stromal cells), a cell type found in virtually all tissues³⁹ and capable of differentiating into multiple mesenchymal lineages. MSCs were originally recognized and isolated in 1974 by Friedenstein from the bone marrow⁴⁰. These cells were isolated simply by plastic adherence and showed a fibroblastic morphology. In fact, the cells were originally termed “precursors to fibroblasts” due to their morphological features and simultaneously of their relatively undifferentiated state. The terminology of “mesenchymal stem cell” surfaced later as it was recognized that these cells could undergo differentiation into a variety of different mesenchymal cell types including chondrocytes, osteoblasts, adipocytes^{41,42}, and myocytes⁴³. This multipotency *in vitro* remains the gold standard for the definition of MSC though more criteria have been proposed including the expression of certain surface markers⁴⁴.

In addition to their stem cell-like abilities, MSCs retain a number of other properties that can be generally categorized as ‘signaling functions’. They have been shown to modulate the immune system *in vitro* in a number of different assays, generally inducing a more tolerant or anti-inflammatory phenotype in different immune effector cells. Aggarwal and Pittenger demonstrated the ability of MSCs to decrease TNF α and increase IL-10 secretion by dendritic cells, inhibit Th1 and NK cell IFN γ secretion, and increase Th2 IL-4 secretion. They also showed MSC ability to increase the number of T regulatory cells⁴⁵. Modulation of the immune system seems to be a necessary function for the beneficial effects of MSCs⁴⁶. The precise mechanism for these various effects has not been fully elucidated and probably cannot simply be attributed to one molecule or pathway within MSCs. The mechanism(s) likely varies from one cellular interaction to the next. Many soluble secreted factors have been implicated including PGE2⁴⁷, hepatocyte growth factor, TGF- β , indoleamine 2,3-dioxygenase, nitric oxide, IL-10⁴⁸, heme oxygenase 1⁴⁹, and IL-6⁵⁰. Most of these were first determined by *in vitro* studies. More recently, other molecules have been identified including an IL1R antagonist⁵¹ *in vivo*. Indeed, even contact-dependent mechanisms have been proposed, adding to the very complex mechanisms involved in the various functions of MSCs^{52,53}. While the effects of MSCs on the immune system *in vitro* are well-accepted, there remains some debate on the relevance and strength of similar interactions *in vivo*^{54,55}. One study even suggests that the effect of MSCs on T cells remains limited to their proliferation and do not affect effector function as measured by upregulation of activation markers and cytotoxic assay⁵⁶.

Stimulation or recognition of signaling molecules and cytokines have been proposed to affect MSC immune modulation properties. $\text{TNF}\alpha$ and $\text{IFN}\gamma$ are able to increase secretion of various immune modulators by MSCs^{57,58}. Expression of TLRs has been evaluated in MSCs as well and proposed to affect function^{59,60}. Also acting in their capacity as signaling cells, MSCs have been implicated in promoting cancerous conditions, especially metastasis. Recently it has been demonstrated that coculture with MSCs increases the metastatic capability of breast cancer cells dependent on the secretion of CCL5 by MSCs⁶¹. However, opposing viewpoints have also been published such as a model of hepatocarcinoma that is suppressed by interaction with MSCs⁶². The precise role of MSCs in cancer may vary on a case by case basis⁶³.

MSCs have become a very hot topic in recent years as a result of their recognized immune modulation and tissue regenerating abilities⁶⁴. The natural source of MSCs during development is currently under investigation with evidence that they originate as perivascular cells⁶⁵. However, MSCs derived from bone marrow have been employed therapeutically in cardiac ischemia models⁶⁶, kidney ischemia and drug-induced injury models⁶⁷, colitis models⁶⁸, gastric ulcer models⁶⁹, nervous system injury models⁷⁰, and tendon regeneration models⁷¹. MSCs are even currently being used in human clinical trials. Osiris Therapeutics Inc is enrolling patients for Phase III trials for treatment of Graft-vs.-Host Disease and Crohn's Disease and for Phase I trials for acute myocardial infarction with the drug Prochymal, an intravenous infusion of ex vivo-expanded bone marrow-derived MSCs⁷². Naturally, trafficking of such cells through the endothelium has come under intense investigation in order to better understand migration and homing

of these cells in the body⁷³. Some of the currently proposed players include VCAM, β 1 integrins, MMP-2, various cytokines⁷³, and SDF-1^{66,70}.

The vast majority of investigations involving MSCs are centered on those derived from bone marrow. However, MSCs, as mentioned above, can be isolated from virtually every organ in the body³⁹ and demonstrate small differences in their various properties⁷⁴. Each organ-specific MSC is unique though all hold to the same definition of multipotency. The field continues to suffer from a lack of a precise marker for this cell type. The most consistent two markers appear to be CD29 and CD44³⁹ but these are highly non-specific. Different investigations have profiled MSCs in various manners but have yet to identify a precise marker. Microarray analysis of expanding and differentiating bmMSCs demonstrated that expansion *in vitro* does not significantly alter expression profiles⁷⁵. Others have attempted to uncover a specific marker⁷⁶ but such studies have not proven beneficial to future applications. Cytokine and stem cell molecular profiles comparing the most commonly employed MSCs, those derived from adipose and bone marrow, concluded that differences between these two types were minimal⁷⁷. Identification of a specific marker could greatly aid future studies of MSCs.

Insulin growth factor 2 binding protein 1 (Igf2bp1, Imp1)

This work provides evidence of regulation of Ptgs2 post-transcriptionally by Igf2bp1 or Imp1, an interaction that has never before been recognized. A widely-known mRNA binding protein, Imp1 is a highly conserved 577 amino acid protein made up of two RRM (RNA recognition motif) domains at the N terminus and four KH (hnRNP K homology) domains at the C terminus (Fig 1.1). In fact, the primary protein sequence in humans and in mice differs by only 4 amino acids. Imp1 has been reported to bind up to 3% of the

transcriptome in one cell line⁷⁸ primarily in predictably sized ribonucleoprotein complexes. The binding sequence of Imp1 targets is not fully characterized and appears to be degenerate and at least partially dependent on 3D structure of the mRNA molecule as well as on primary sequence⁷⁹. Due to its wide range of targets^{78,79}, Imp1 has been studied in many different contexts and has been cloned and identified through multiple lines of investigation. Its various functions are the focus of many continuing studies.

Figure 1.1

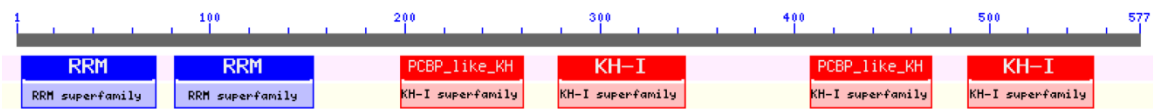


Figure 1.1 Depiction of Imp1 conserved protein domains.

Conserved domains were found using the conserved domains search at NCBI. RRM is RNA recognition motif and KH is hnRNP K homology, both of which can interact with nucleotide sequences. All binding activity has been reported to reside in the KH domains⁸⁰. Blue boxes, RRM; red boxes, KH.

Identification of Imp1

The gene termed Imp1 in humans was virtually simultaneously identified by multiple groups in multiple organisms in the mid 1990s^{81,82}. The laboratories of Singer⁸³ and Yisraeli⁸⁴ each identified the protein ZBP-1 or Vg1 RBP respectively as the factor responsible for localization of β -actin mRNA to the leading edge of fibroblasts in culture and specific for the so-called zip code binding sequence in its sequence. Similarly, Vera was cloned as a binding protein for the vegetal localization element in *Xenopus oocytes*⁸⁵. Yet another group interested in c-myc RNA stabilization identified CRD-BP (coding

region determinant binding protein) as a protein that stabilizes c-myc mRNA by binding within the coding region⁸⁶. The protein is also known as insulin growth factor 2 binding protein 1 (Igf2bp1) for its discovery by its ability to bind and regulate insulin growth factor II RNA⁸⁷.

Expression of Imp1

Imp1 is broadly referred to as an oncofetal protein in reference to its general expression pattern: it is found in the embryo, generally not in adult tissues, and again upregulated in various cancers. In the mouse, Imp1 is expressed beginning at day 11.5, peaking at day 12.5, and dropping off rapidly thereafter in the whole embryo⁸⁷. These authors report expression most prominently at day 12.5 in the basal layer of the epidermis, in the skeletal muscle, and in trophoblasts of the placenta. Another group finds expression by RT-PCR analysis in the distal intestine and strongly in the testis of adult animals. They also find expression within the epithelium of villi by in situ hybridization⁸⁸.

Overexpression of Imp1 has been reported in a variety of cancers. One report estimated that mesenchymally-derived tumors of all kinds showed increased expression of Imp1 in about 65% of cases⁸⁹. Later, the same group investigated tumors of the CNS (specifically brain) and non-small-cell lung carcinomas and noted 55% and 27% respective tumors that showed Imp1 misexpression⁹⁰. Most intriguing from the perspective of our lab, Ross et al⁹¹ found that up to 81% of colorectal carcinomas express Imp1 as measured by RT-PCR. Also, a subject of recent focus, Imp1 has been found to be overexpressed in 60% of breast carcinomas⁹² and in malignant melanoma⁹³.

The precise functions of Imp1 in development and in cancer progression are in various stages of understanding and remain the subject of investigation. A complete knowledge of the various roles and functions of Imp1 may potentially show some promise in cancer diagnosis, prognosis, and treatment.

Functions and regulation of Imp1

As mentioned, Imp1 has been studied in many contexts from embryo polarization to translational repression. Some of the most important are discussed here.

A knockout mouse deficient in Imp1 has been produced by gene trap inserted between exons 2 and 3 and examined⁹⁴ though, despite its being published 6 years ago, this initial report remains the only published study of this animal. The Nielsen group documented increased perinatal mortality with 50% death within 3 days while those animals that survived were 40% smaller than litter mates throughout life. The authors noted poor development of various organs with histological changes in the kidney and intestine. They found small, hypoplastic glomeruli in the kidney and greatly decreased size of villi in the small intestine. Furthermore, decreased cartilage was demonstrated by examination of the skeletons of mice. These data indicate a necessary role for Imp1 in proper development. Whether the need for Imp1 is purely embryonic could not be addressed by this experimental system and remains an important question: what is the role of Imp1 in the adult animal?

As mentioned above, Imp1 is also known as CRD-BP, named for its affinity for the coding region of c-myc mRNA and its stabilization of this message in various *in vitro* systems. The mechanism of stabilization of c-myc mRNA has been elucidated recently and may potentially prove similar in the case of Ptg2 regulation. An endonuclease

capable of cleaving c-myc mRNA within the coding region determinant was purified and shown to be inhibited from this degradative function in the presence of CRD-BP⁹⁵. Subsequent studies identified a mechanism of activity of CRD-BP that may eventually prove to have broad applicability to the method of trans-acting factor stabilization of mRNA. Specifically, Elcheva et al⁹⁶ demonstrated that the destabilizing region mRNA of another target of CRD-BP (β TrCP1) is subject to degradation downstream of a specific micro RNA. When CRD-BP is present, access to this coding region by the micro RNA is blocked which inhibits its ability to cause degradation of the mRNA transcript. This suggests that the mechanism of stabilization is essentially steric hindrance by the presence of CRD-BP which blocks the function of proponents of degradation pathways.

Another mechanism of Imp1 is alluded to by another name for the gene: zipcode binding protein 1. It acts to localize RNA to the leading edge of fibroblasts, specifically β -actin mRNA. There is strong evidence that an unphosphorylated version of ZBP-1 binds to β -actin mRNA and this binding is lost upon Src-mediated phosphorylation of a specific tyrosine residue of ZBP-1. Though stability of β -actin mRNA is increased by binding of ZBP-1, translation is decreased. Upon release of the transcript purportedly by phosphorylation of the protein, the mRNA can then be translated into protein. The authors speculate that this phosphorylation control allows for the buildup and localization of β -actin protein to the leading edge of cells⁹⁷, but this remains to be rigorously demonstrated.

Imp1 is also able to interact with multiple RNA transcripts unique to viruses. One of these interactions involves the HIV protein Gag, a relationship that seems to decrease the infectivity of the viral particles⁹⁸. Here too, the binding interaction is mediated by the C-

terminal KH domains of the protein, specifically the two most C-terminal iterations.

Imp1 also interacts with the 5'UTR and 3'UTR of the hepatitis C virus, but in this case, the presence of Imp1 seems to enhance translation of the RNA⁹⁹.

The regulation of Imp1 itself has not been highly studied but has been touched on in an effort to understand the function of Imp1 overexpression in various cancers. Noubissi et al¹⁰⁰ found that Imp1 expression could be upregulated by activation of β -catenin/Tcf signaling. Subsequently, a specific β -catenin element within the promoter of Imp1 was identified, and Imp1 protein was shown to interact with β -catenin mRNA and increase its stability, suggesting a coordinate regulation of the two genes¹⁰¹. The same group has subsequently proposed evidence for prognostic ability of Imp1 in breast cancer metastasis¹⁰² though this remains in its infancy and will require specifically designed studies in order to fully address this hypothesis. Essentially, the authors recognize that metastatic lines of breast cancer express lower levels of Imp1 and present evidence that within patient populations, in situ cancer cells express greater levels of Imp1 than metastatic cells; the expression of Imp1 in these cases can be at least partially mediated by β -catenin. This observation raises the intriguing possibility of prognostic and interventional techniques based on employing Imp1 as a target.

Summary

The work described herein primarily focuses on increasing our understanding of the mechanisms of regulation of Ptgs2, an enzyme that is important in a variety of models for the proper response to and healing of colonic injury. It seems that Ptgs2 likely acts on both proliferation of tissue as well as on modulation of the immune system and controlling the inflammatory response. It is demonstrated that Ptgs2 is highly regulated

at the post-transcriptional level by various trans-acting factors: (1) CUGbp2 downstream of Fgf9 and Erk activation and (2) Imp1 through an as-yet uncharacterized pathway.

Moving forward, clarification of the complex regulation of Ptgs2 may provide the ability to precisely manipulate this system in order to maximize healing and minimize morbidity in various human colonic injuries.

CHAPTER 2

FGF9 Regulation of Ptgs2 Expression in Colonic Mesenchymal Stem Cells

Chapter 2: Abstract

We previously found that a population of colonic stromal cells that constitutively express high levels of prostaglandin-endoperoxide synthase 2 (Ptgs2, also known as Cox-2) altered their location in the lamina propria in response to injury in a Myd88-dependent manner.¹⁴ At the time of this study, the identity of these cells and the mechanism by which they expressed high levels of Ptgs2 were unknown. Here we found that these colonic stromal cells were mesenchymal stem cells (MSCs). These colonic MSCs expressed high Ptgs2 levels not through interaction with bacterial products, but instead as a consequence of mRNA stabilization downstream of fibroblast growth factor 9 (Fgf9), a growth factor that is constitutively expressed by the intestinal epithelium. This stabilization was mediated partially through a mechanism involving endogenous CUG binding protein 2 (CUGbp2). These studies suggest that Fgf9 is an important factor in the regulation of Ptgs2 in colonic MSCs and may be a factor involved in its constitutive expression *in vivo*.

Chapter 2: Introduction

Homeostasis in the mammalian colon relies on proper function of the epithelium and underlying mucosal immune system. Both of these cellular components are challenged by a local environment of a robust and diverse array of microbes that normally reside in the lumen of the colon¹⁰³⁻¹⁰⁵. An important feature of the epithelial barrier is that it undergoes constant renewal during the lifetime of the organism. This process can be enhanced in response to either infection or injury^{15,106}. Proliferative colonic epithelial progenitors (ColEPs) located in the basal one-third of the crypts of Lieberkühn are central players in the maintenance of the barrier epithelium in health and during injury response¹⁰⁷. ColEPs serve as a continuous source of maturing epithelial cells that migrate up crypts and terminally differentiate into one of three lineages: absorptive enterocytes, mucous-secreting goblet cells, and enteroendocrine cells.

The source of the signals responsible for regulating ColEP proliferation and differentiation of their daughter cells both during homeostasis and in response to injury is the local microenvironment surrounding the crypt base termed the progenitor cell niche. This niche is partially composed of the surrounding mesenchyme¹ that includes myofibroblasts, endothelial cells, neural cell extensions from the autonomic enteric nervous system, and mobile stromal cells capable of trafficking into and within the mesenchyme, such as immune cells^{13,108}. Signals released from these many varied cell types likely interact with each other and the epithelium to regulate ColEPs and their progeny in homeostasis and in response to injury.

One additional cell type that we found appears to be an important component of the ColEP niche is Ptgs2-expressing stromal cells¹⁴. The key signaling molecule secreted by

these cells is one of Ptgs2's downstream synthetic products: prostaglandin E2 (PGE2)^{14,16}. The immune modulatory properties of this and other downstream products of this gene family (Ptgs1 and Ptgs2), have been well-established *in vitro*⁴⁵. In the gut, PGs, most notably PGE2, have been shown to improve inflammation, ulceration, and other common measures of disease in model injury systems^{13,109-112}.

Our goal in the current studies was to define and identify these Ptgs2-expressing colonic stromal cells and determine the mechanism by which their expression of Ptgs2 is mediated. We identified Ptgs2-expressing stromal cells as consistent with tissue resident mesenchymal stem cells (MSC). We isolated colonic MSCs using previously established protocols^{39,40} and found that their properties further supported the hypothesis that the Ptgs2-expressing stromal cells were colonic MSCs. We found that the high level of Ptgs2 expression in the colonic MSCs was not dependent upon exposure to bacterial products but rather to local growth factors, specifically Fgf9. We found that Fgf9 signaling was sufficient to maintain the high levels of Ptgs2 expression in cMSCs and that it appears to do so partially by Erk-mediated increase in mRNA binding protein CUGbp2 and subsequent stabilization of Ptgs2 mRNA. These studies suggest further the recognized role of various growth factors in stabilizing Ptgs2 mRNA and suggest part of the mechanism in play is constitutive, as opposed to induced, expression of this important enzyme.

Chapter 2: Materials and Methods

Mice- All animal experiments were performed in accordance with approved protocols from the Washington University School of Medicine Animal Studies Committee. Mice involved in this study were housed in microisolator cages, in a specified pathogen-free barrier facility following a 12 hour light cycle and fed a standard irradiated chow diet (PicoLab Rodent Chow 20, Purina Mills) and water ad libitum. *Ptgs2*^{-/-} mice¹⁹ were generated previously on a C57Bl/6 background. C57Bl/6 mice were obtained from the National Cancer Institute and C57Bl/6 germ free mice were obtained from Jeffrey Gordon (Washington University).

Isolation of organ-specific MSCs- Lung, stomach, colon and bone marrow (flushed from isolated tibias and femurs) were removed from the mouse and were rinsed thoroughly with 1:1 HBSS:Dulbecco's modified eagle's medium (DMEM). Each tissue was separately minced with scissors. The tissue fragments were placed in 20mls of 10mM HEPES-DMEM and 5µg/ml Type I collagenase (Gibco) and were incubated in an oscillating shaker at 225rpm at 37°C for 35min. Dithiothreitol (Sigma) was then added to a final concentration of 20mM and incubation was continued for an additional 20min. The resulting cell suspension was filtered through a 70µm filter, pelleted at ~400g for 5 min, washed once in complete media (low glucose DMEM with 10mM HEPES and 10% fetal bovine serum with penicillin/streptomycin), and plated on standard tissue culture plates (VWR) in complete media. Cells were cultured in a humidified chamber at 37°C and 5% CO₂. After 1 hour, non-adherent cells were removed and adherent cells were maintained in culture, feeding every 3-4 days in complete media and were passaged (1:3) when they reached 90-100% confluence.

Isolation of bone marrow-derived macrophages- Bone marrow-derived macrophages were cultured by flushing femurs and tibias of mice and culturing the cell suspension in standard media supplemented with L-cell supernatant containing Csf-1¹¹³ for 7 days before replating and experimentation.

Splenocyte proliferation assay- This assay was performed by isolation and activation of 5×10^4 splenocytes in a 96-well dish by incubation with anti-CD3 ϵ and anti-CD28 antibodies¹¹⁴ in the presence or absence of cMSCs at a ratio of 5:1 – 50:1 splenocytes:cMSC or in the presence of cMSC culture supernatant. Cells were incubated for 72hrs and pulsed with H³ thymidine. Plates were harvested and H³ thymidine measured. All conditions were performed in triplicate for each experiment.

Manipulation of cell lines To differentiate MSCs into adipocytes, isolated MSCs were treated with 10^{-8} dexamethasone and 5 μ g/ml insulin for 21 days⁴¹. Verification of lipid stores was done by staining with Oil Red O. To differentiate MSCs into osteocytes, MSCs were treated with 10^{-8} dexamethasone, 5 μ g/ml ascorbic acid 2-phosphate, 10mM β -glycerophosphate for 21 days⁴¹. Verification of Ca²⁺ deposits was performed by Alizarin Red S staining.

LPS (Sigma) treatment of cells was carried out on cells plated at 10^5 cells/6-well dish well at a concentration of 1ng/ml to 100ng/ml.

Serum starvation experiments were performed by slightly different methods based on specific application. Experiments were carried out on cells plated in 24-well or 6-well dishes using 5×10^4 cells/well or 1×10^5 cells/well respectively. For Fgf9 treatment, cells were starved for 2-3 hours in low glucose DMEM with 10mM HEPES before treatment with Fgf9 (Peprotech, 1 – 500ng/ml) for one hour before cells were lysed for RNA and

protein isolation. For transcriptional inhibition coupled with starvation, treatment with actinomycin D (4µg/ml, Sigma) was begun simultaneously with or without 250ng/ml Fgf9, and RNA was analyzed at 3hrs. For MEK inhibition, treatments with PD98059 (100µM-300µM, Enzo Life Sciences) was performed in the same method as actinomycin D, and RNA and protein were collected at 3 hours.

Prostaglandin E2 assay- Cells were cultured with or without LPS (1-100ng/ml; Sigma-Aldrich) in 6-well plates at a density of 10^5 per well. Following 21-22 hour incubation, supernatants were collected for analysis using a PGE2 Elisa assay kit (Cayman Chemical) according to the manufacturer's protocol. Cells were also counted and RNA isolated for qRT-PCR analysis.

Cell transfection for shRNA treatment- HEK293T cells were transfected with Mission shRNA constructs specific for CUGbp2 and non-targeting control (CUGbp2 clones NM_010160.1-131s1c1, NM_010160.1-305s1c1; Sigma) and lentiviral packaging plasmids using FuGene HD reagent (Roche). Following one day transfection, cell media was refreshed and allowed to accumulate virus 24 hours. Viral-containing supernatant was used undiluted directly on plated cMSCs for 24 hours before adding puromycin (Sigma)-containing media for selection of positively infected cells.

Quantitative RT-PCR analysis- Cells were treated as noted in various experiments before RNA isolation. RNA was isolated and purified using QiaShredders and a Qiagen RNEasy mini kit according to manufacturer's protocol. cDNAs were synthesized using a Superscript III reverse transcriptase (Invitrogen), primed using random primers. Quantitative RT-PCRs were performed in triplicate for each sample using SYBR-green master mix (Clontech) and analyzed by either a Stratagene Mx3000P or Eppendorf

realplex Mastercycler. The following primers were used: 18S
(AACCCGTTGAACCCATT, CCATCCAATCGGTAGTAGCG); Ptgs2
(TGCCTGGTCTGATGATGTATG, GCCCTTCACGTTATTGCAGATG); Human Ptgs2
(ATATGTTCTCCTGCCTACTGGAA, GCCCTTCACGTTATTGCAGATG); Primer 1
(Fig. 2.6D, AGCGAGGACCTGGGTTTAC); Primer 2 (Fig. 2.6D,
TGGGTGTGATTTGTTTGGCATGG); Primer 3 (Fig. 2.6D,
GGATACACCTCTCCACCAATGAC); Fgfr1b (GGGAATTAATAGCTCGGATG,
CCACAGGTCTGGTGACAGTG); Fgfr1c (CCAGATCCTGAAGACTGCTG,
GAGTCCGATAGAGTTACCCG); Fgfr2b (CTCGGGGATAAATAGCTCCA,
GGAAGCCGTGATCTCCTTCT); Fgfr2c (GGGAATCGCTAGAGTTGCAG,
TGTCGTCCTCATCATCTCCA); Fgfr3b (CAAGTTTGGCAGCATCCGGCAGAC,
TCTCAGCCACGCCTATGAAATTGGTG); Fgfr3c
(CAAGTTTGGCAGCATCCGGCAGAC, CACCACCAGCCACGCAGAGTGATG);
Fgfr4 (GCCCTTCACGTTATTGCAGATG, CCTCTCCAACCCCGTACTC); Ptgs1
(AGTGCGGTCCAACCTTATCC, GCAGAATGCGAGTATAGTAGCTC); CD14
(CTCTGTCCTTAAAGCGGCTTAC, GTTGCGGAGGTTCAAGATGTT); Tlr4
(GCCTTTCAGGGAATTAAGCTCC, AGATCAACCGATGGACGTGTAA); Myd88
(AGGACAAACGCCGGAACTTTT, GCCGATAGTCTGTCTGTTCTAGT); MD-2
(CGCTGCTTTCTCCCATATTG, GTCTTATGCAGGGTTCAGAAC); IRAK-M
(CTGGCTGGATGTTTCGTCATATT, GGAGAACCTCTAAAAGGTCGC); TNF- α
(CCCTCACACTCAGATCATCTTCT, GCTACGACGTGGGCTACAG); I-A^b
(CCCTCATCAGGGAGAGGTCTA, CCGATGCCGCTCAACATCT); CUGbp2
(GCTGCTTCAACCCCAATTC, CGCCATACCTGCTAGTGCAT).

Immunoblotting- Colonic MSCs were treated as noted. Following treatment, cells were lysed in RIPA buffer (Sigma) containing Protease and Phosphatase inhibitor cocktails (Sigma) and frozen at -80°C. Protein content was reduced by heating to 95°C for 5min in a ratio of 1:1 with 2X Laemmli's buffer. Protein was loaded onto 10% or 4-12% Bis-Tris gels (Invitrogen) and electrophoresed with MOPS buffer (Invitrogen). Samples were transferred to a 0.45µm nitrocellulose membrane (BD) and blocked in 5% milk in 0.05% Tween 20 TBS (TBST) overnight at 4°C. Blots were incubated for 2hrs at room temperature in primary antibody, washed three times in TBST, incubated for 1hr in HRP conjugated anti-rabbit secondary (1:10,000, Bio-Rad) before development using SuperSignal West Dura chemiluminescent kit (Pierce). Primary antibodies were specific for phospho-Erk (diluted 1:500; Cell Signaling Technologies), CUGbp2, and actin (each diluted 1:500; Sigma). Quantitation was performed using ImageJ software.

FACS analysis- For analysis of extracellular molecules, cultured cells were treated with trypsin/EDTA, pelleted, and resuspended in 100µl FACS buffer (1% BSA in PBS) with the appropriate primary antibody for one hour, washed three times in FACS buffer, incubated in 100µl FACS buffer with the appropriate secondary antibody, washed again, and analyzed on a FACSCalibur flow cytometer. Analysis was performed using FlowJo software. For analysis of intracellular enzymes, cells were fixed after suspension in 50% methanol in PBS for 10min at -20°C followed by 90% methanol overnight at -20°C. Cells were then stained and analyzed by the same methodology for extracellular molecules. Antibodies employed at 1:100 dilution were CD3ε (BD Pharmingen), CD11b (BD Pharmingen), CD11c (BD Pharmingen), CD29 (BD Pharmingen), CD31 (BD Pharmingen), CD34 (eBioscience), CD44 (BD Pharmingen), CD45 (BD Pharmingen),

CD54 (eBioscience), CD90 (Caltag Laboratories), CD105 (eBioscience), CD106 (eBioscience), Sca-1 (BioLegend), B220 (BD Pharmingen), F4/80 (Caltag Laboratories), NK1.1 (BD Pharmingen), Gr-1 (BD Pharmingen), I-A^b (BioLegend), and Ptgs2/Cox-2 (BD Pharmingen).

Global Gene Array analysis- RNA from two cultured cell lines each of cMSCs and bmMSCs was isolated using QiaShredders and Qiagen RNEasy mini kit according to instructions. RNA was measured on a nanodrop machine and amplified in one-step using RiboAmp Plus from MDS Analytical Technologies according to provided protocol. Amplified RNA was labeled with Turbo Labeling Biotin (MDS) and fragmented before hybridization onto MOE430A microarray chips from Affymetrix. Analysis of data was performed using dCHIP software¹¹⁵ and gene ontology comparisons¹¹⁶. Significance of differential expression was set at 1.3 fold difference with $p < 0.05$.

Immunohistochemistry- Mice were sacrificed and colons removed, flushed with room temperature PBS, opened, and pinned in 2% paraformaldehyde for 30 minutes. Tissue was then rinsed in 5% sucrose PBS and blocked in OCT. Seven micron sections were cut and stained by blocking in 2% dry milk PBS, incubating for 1 hour in primary antibody, washing, incubating for 45 minutes in secondary antibody, staining with Hoechst nuclear dye, and finally coverslipping with 1:1 glycerol:PBS solution. Sections were viewed with a Zeiss Axiovert 200 with AxioCam MRM camera. Antibodies used in staining are found in the FACS analysis section.

Chapter 2: Results

Ptgs2-expressing stromal cells co-localized with markers of mesenchymal stem cells (MSCs). Previously we found that stromal cells in the colonic mesenchyme expressed high levels of Ptgs2 in stained tissue sections co-labeled with CD44 but not with definitive hematopoietic, myofibroblast, or endothelial cell markers¹⁴. We noted that other investigators found that CD44 was common in all mesenchymal stem cells (MSCs) isolated from a wide array of mouse tissues³⁹. Therefore, we tested the hypothesis that Ptgs2-expressing stromal cells were tissue resident MSCs by screening a variety of MSC markers. CD29 (integrin β 1) and CD44 (hyaluronate binding protein) were selected as the best current defining markers for MSCs of all tissues³⁹ while CD54 (Icam1), CD105 (endoglin), and CD106 (Vcam1) are commonly described in numerous MSC lines (e.g.⁴⁵). We confirmed our previous work that demonstrated Ptgs2-expressing cells co-expressed CD44¹⁴ (Fig. 2.1A). Using double label immunofluorescence on colonic sections from WT mice, we also found Ptgs2-expressing cells co-stained with the remaining MSC markers (Fig. 2.1B-E), supporting our hypothesis. We also confirmed our previous analysis that the Ptgs2-expressing stromal cells lacked expression for all hematopoietic markers tested including F4/80 (macrophages, Fig 2.1F), CD68 (macrophages), CD3 ϵ (T cells), CD45, B220 (B cells), Gr-1 (granulocytes), and NK1.1 (NK cells) as well as markers of endothelial cells (CD31/Pecam) and prolyl-4-hydroxylase and fibroblast specific protein¹⁴. Taken together, these data generate a hypothesis that the Ptgs2-expressing stromal cells are MSCs.

Figure 2.1

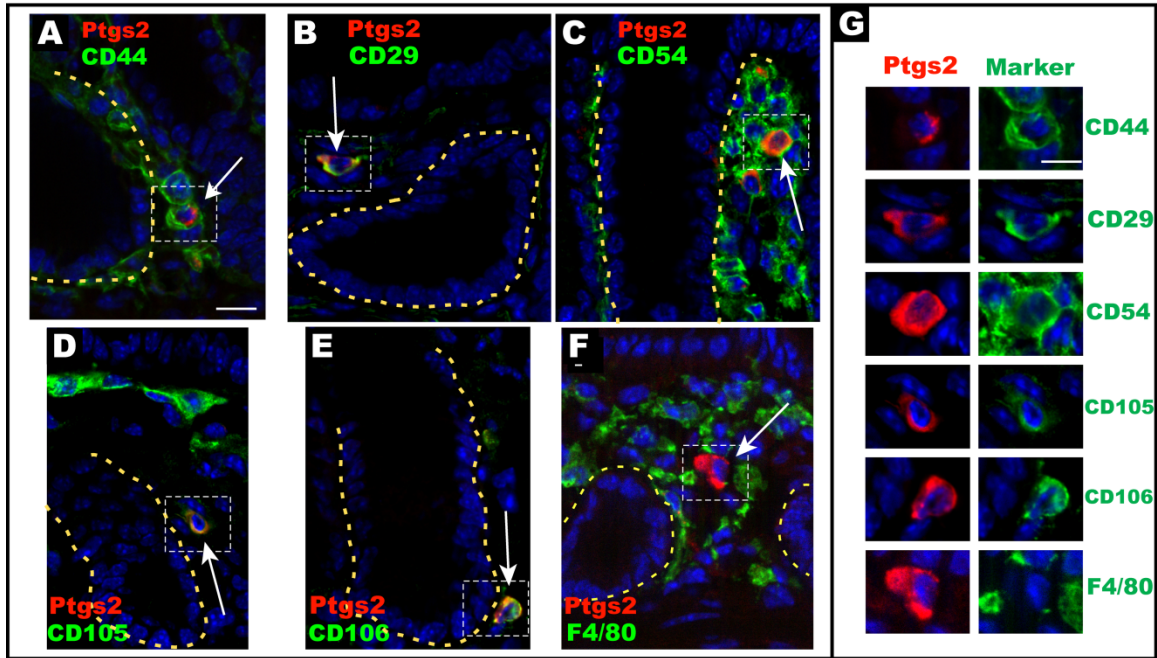


Figure 2.1. *Ptgs2*-expressing stromal cells co-localize with markers of mesenchymal stem cells *in vivo*.

Sections of the rectum from WT C57Bl/6 mice co-stained with Alexa fluor 594 Zenon labeled-anti-mouse *Ptgs2* IgG1 (red) and various MSC and hematopoietic markers. All MSC markers (A-E) were visualized with an Alexa Fluor 488-conjugated anti-rat Ig secondary used to detect (A) rat anti-mouse CD44, (B) rat anti-mouse CD29, (C) rat anti-mouse CD54, (D) rat anti-mouse CD105, and (E) rat anti-mouse CD106. The hematopoietic co-staining marker was (F) FITC-conjugated F4/80. Yellow dashed lines denote the basolateral membrane of the epithelium. (G) Magnified insets of the dashed boxed portions of A-F. *Ptgs2* co-labels with all MSC markers but not F4/80. Bars = (A-F) 20 μm (G) 15 μm .

Isolated colonic stromal cells were identified as MSCs and exhibited marker expression similar to Ptgs2 expressing stromal cells in vivo. MSCs are multi-potent progenitors for a number of mesenchymal cell types that have been isolated from many mouse and human tissues^{39,41}. One of their functions is to mediate tissue repair either through the secretion of various factors or by differentiation into specific cell types (e.g.¹¹⁷). To test our hypothesis that Ptgs2-expressing stromal cells are MSCs, we isolated MSCs from the adult mouse colon by the technique originally developed by Freidenstein and colleagues⁴⁰ and modified for a wide array of mouse organs³⁹. Briefly, collagenase-dissociated colonic cells were plated for one hour, and the adherent cells were expanded and analyzed. We have maintained cell lines produced in this manner for at least 40 passages in culture as has been described for MSCs isolated from other organs.

We characterized the putative MSC cell lines isolated from the mouse colon in order to determine whether these cells were representative of the Ptgs2-expressing cells that we identified *in vivo*. Cells cultured in low-glucose DMEM with 10% serum were used to analyze expression of pertinent surface proteins identified *in vivo*. Single channel flow cytometric analysis of the colonic stromal cells showed expression of CD29, CD44, CD54 and CD106 in all lines isolated from WT colons (n=5) at early passages (P3-5; Fig 2.2A). Expression of these markers was stably maintained up to P40 (data not shown). Interestingly, we could detect expression of CD105 in cells after initial isolation and expansion, but expression of this marker was undetectable by P3 (Fig 2.2A). These putative MSCs also lacked expression of hematopoietic markers (CD11b, CD11c, B220, CD3ε, F4/80, CD45, NK1.1, Gr-1, I-A^b) and endothelial markers (CD31/Pecam). Most significantly, these cells co-expressed Ptgs2 with CD44 and CD106 as demonstrated by

dual label flow cytometry (Fig. 2.2B). Taken together, these data suggested that the isolated and expanded stromal cells were representative of the stromal cells identified by strong Ptgs2 immunofluorescence *in vivo*¹⁴.

We then performed expression analysis of CD90, Sca-1 and CD34. These proteins are additional markers of various, organ-specific MSCs (isolated from spleen, muscle, blood vessels, kidney, lung, liver, brain, and thymus) that are known to show variation in expression³⁹. The isolated colonic stromal cells expressed all three of these markers (Fig. 2.2A). This finding recapitulated analysis of marker proteins from bone marrow (bm) MSCs with the exception of CD34, which is commonly reported to be absent on bmMSCs^{44,118}.

We then determined if the isolated colonic stromal cell lines had properties of *bona fide* MSCs. Like all other MSCs, the colonic stromal cells were able to differentiate into osteocytes and adipocytes when cultured under appropriate conditions as demonstrated by staining by Alizarin Red S and Oil Red O respectively^{41,42} (Fig. 2.2C). This capacity confirmed the multipotency of these cells. Furthermore, the isolated cells were capable of modulating immune function as has been previously shown for bmMSCs^{45,53}. Co-culture of the colonic stromal cells with anti-CD3/CD28-activated splenocytes resulted in significantly decreased splenocyte proliferation (to a ratio of splenocytes:cMSCs of 20:1) in a dose-dependent manner as measured by incorporation of H³ thymidine (Fig 2.2D). As in similar experiments with bmMSCs,⁴⁵ culture media from the colonic stromal cells also significantly decreased splenocyte proliferation (Fig. 2.2D). Taken together, these data confirm that our isolated colonic stromal lines have the properties of MSCs and were therefore termed cMSCs.

Figure 2.2

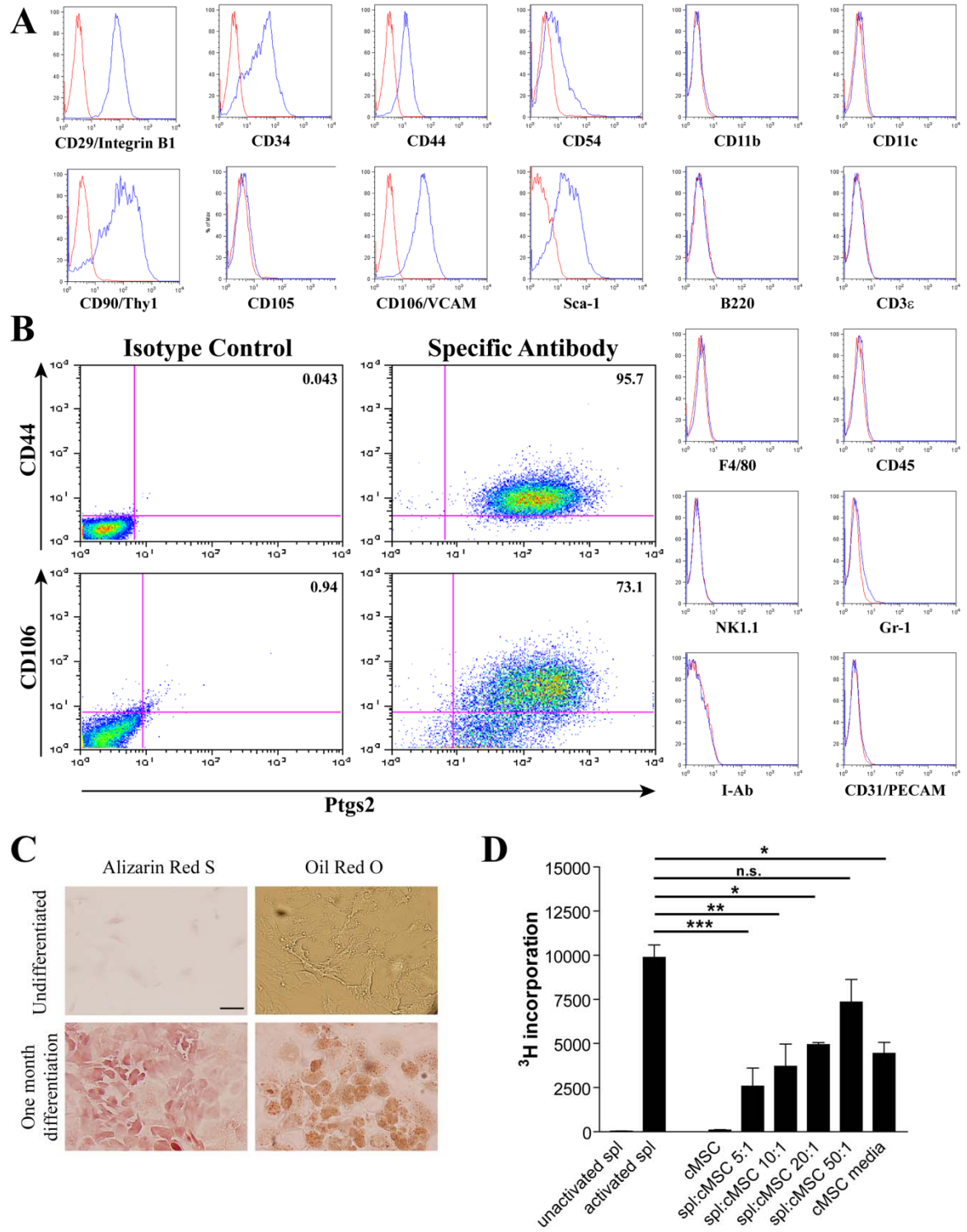


Figure 2.2. Isolated colonic stromal cells are mesenchymal stem cells and maintain *Ptgs2* expression

(A) Representative histograms of flow cytometric analysis of cultured colonic stromal cells (n=5 lines) at passage 3-5 stained for the markers shown (blue lines). Antibodies to CD11b, CD11c, CD90, Sca-1, B220, CD3 ϵ , F4/80, CD45, NK1.1, Gr-1, and I-A^b were pre-conjugated FITC- or Alexa-fluor 488-labeled primary mouse antibodies. Cells labeled with CD29, CD34, CD44, CD54, CD105, CD106, and CD31 were detected with an Alexa-fluor 488 conjugated anti-rat secondary. Controls were antibodies of the same isotype (red lines). (B) Representative double label flow cytometric analysis of colonic stromal cells (n=3 lines) co-stained with either CD44 or CD106 as described above and *Ptgs2* detected by Alexa-fluor 647 conjugated anti-mouse antibody. (C) Representative histological images of colonic stromal cells (n=3 lines) which were plated on coverslips and treated for 21 days in appropriate media conditions (see Materials and Methods). Following incubation, cells were fixed and stained for calcium deposition by Alizarin Red S or for lipid deposition by Oil Red O. Undifferentiated cells plated two days before staining were likewise visualized. Bar=50 μ m. (D) Graph of H³ thymidine incorporation by splenocytes (spl). Splenocytes were plated with or without activation by antibodies to CD3 ϵ and CD28 in triplicate in 96 well plates. Colonic stromal cells were similarly plated either alone or at varying ratios with splenocytes. Error bars represent standard deviation. Statistical analysis by ANOVA and Bonferroni post-test *(p<0.05), **(p<0.01), ***(p<0.001), n.s.(not significant).

High constitutive *Ptgs2* expression was unique to cMSCs. Based on previous experiments in colonic injury repair^{13,16,109,110}, our hypothesis was that one key defining characteristic of cMSCs is their expression of high levels of *Ptgs2* and consequent production of high levels of PGE2. We first directly compared the expression of *Ptgs2* in and secretion of PGE2 from cMSCs isolated from WT versus *Ptgs2*^{-/-} mice. This analysis showed that *Ptgs2* activity accounted for > 99.9% of secreted PGE2 (Fig. 2.3A, B). We also compared our cMSC lines to other major colonic cell types capable of expressing high *Ptgs2* in certain circumstances: macrophages¹¹⁹ and epithelial tumor cells¹²⁰. We found that cMSCs contained 74-fold higher levels of *Ptgs2* mRNA when compared to bone marrow-derived macrophages and >1000-fold higher levels of secreted PGE2. In comparison of gene expression in cMSCs and a model tumor line (Caco-2 cells), we saw that the cMSCs contained 4-fold higher levels of *Ptgs2* and produced >4000-fold higher levels of PGE2.

We then assessed whether the high levels of *Ptgs2* in cMSCs were a general property of MSCs, as bmMSCs have also been reported to produce PGE2⁴⁵. Therefore we isolated MSCs from a variety of adult mouse organs including the bone marrow, lung, and stomach and maintained these MSCs in culture as we did for cMSCs. Interestingly, the cMSCs contained the highest levels of *Ptgs2* mRNA (range of 5-10 fold greater message) and produced the highest levels of PGE2 (range of 10-25 fold) in comparison with all of these other MSC lines (Fig. 2.3A, B).

Figure 2.3

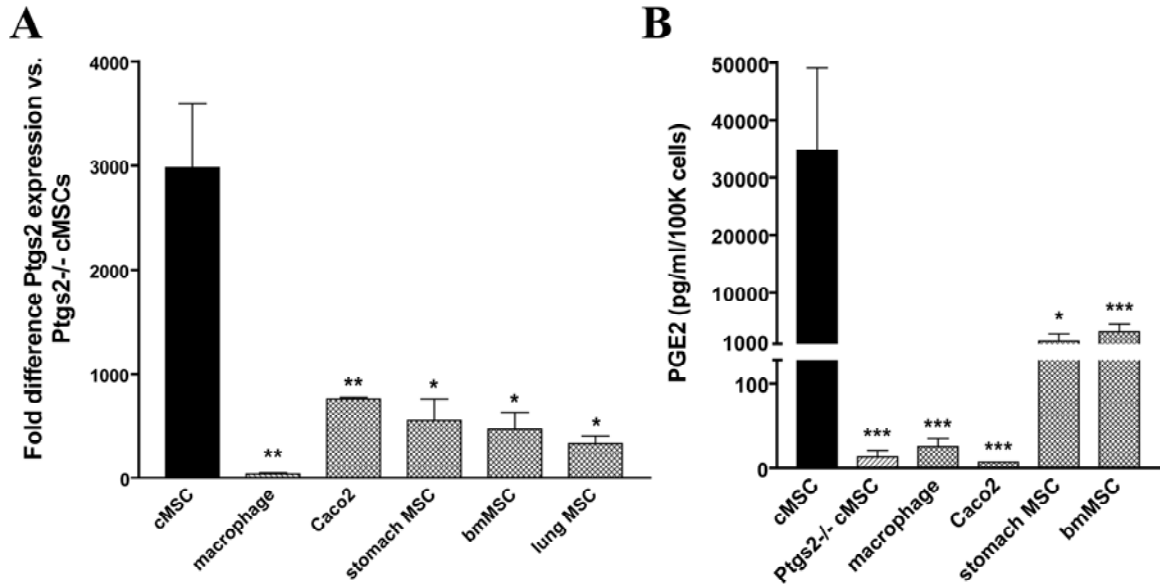


Figure 2.3. High constitutive *Ptgs2* expression is unique to cMSCs.

(A) Graph comparing *Ptgs2* expression by quantitative RT-PCR in various cell lines (baseline=cMSCs isolated from *Ptgs2*^{-/-} mice). MSC lines were isolated from the colon (n=7 lines), stomach (n=4 lines), bone marrow (n=4 lines), and lung (n=2 lines) of WT C57Bl/6 mice and colon of *Ptgs2*^{-/-} mice (n=2 lines). Macrophages (n=2 lines) were derived from bone marrow of C57Bl/6 mice with differentiation in L-cell supernatant. Caco2 cell data represents n=2 different passages. All cDNAs used in qRT-PCR were synthesized using random hexamer primers. Statistical markings (asterisks) refer to comparison to WT cMSC *Ptgs2* expression. (B) Graph of PGE2 secretion measured by ELISA assay of various cell lines described above. Statistical markings refer to comparison to WT cMSC PGE2 secretion. Error bars represent standard deviation. Statistical analysis by Student's *t* test: *(p<0.05), **(p<0.01), ***(p<0.001), n.s.(not significant).

High constitutive *Ptgs2* expression was not due to TLR stimulation. We considered two possible explanations for the observed high constitutive expression of *Ptgs2* in cMSCs: 1) that it is cell autonomous or 2) that it requires endogenous signal(s) from the local environment of the colon that educate the cells to express abundant *Ptgs2*. These endogenous signals could be derived from the host and/or from the indigenous microbes.

Because cMSCs are located in the colon and would thus potentially be exposed to microbes and/or microbial products, we first tested the hypothesis that the high levels of *Ptgs2* expression may be induced/maintained via toll-like receptor (Tlr) signaling. To test this idea, we isolated cMSCs from adult C57Bl/6 germ free (GF) mice that lacked all microbes. Interestingly, GF cMSCs contained similar levels of *Ptgs2* expression as conventionally raised (CONV) mice that contained the normal gut microbiota (Fig. 2.4A), indicating that *in vivo* TLR stimulation was not necessary during cMSC development to produce high levels of *Ptgs2*.

The striking result obtained using GF cMSCs led us to determine if cMSCs could respond to TLR stimulation. In the normal colon, one dominant TLR ligand is lipopolysaccharide (LPS). Therefore, we tested the response of cMSCs isolated from CONV mice to LPS stimulation. We considered the possibility that a lack of response in the CONV mice could be the result of attenuation of TLR signaling due to microbial stimulation of these cells while in the mouse gut¹²¹, so we also evaluated the response of GF cMSCs to account for this possibility. We found that stimulation of either CONV or GF cMSCs with LPS (at concentration ranges from 1 – 100 ng/ml) showed no significant induction of *Ptgs2* as compared to untreated CONV and GF cMSCs, respectively. As a positive control, we similarly treated bone marrow-derived macrophages (a cell type

known to respond to LPS) isolated from adult C57Bl/6 mice and found that these cells demonstrated >150 fold increase in *Ptgs2* expression upon incubation with LPS (Fig. 2.4B) that corresponded with an increase of >200 fold in prostaglandin E2 (PGE2) secretion (Fig. 2.4C). These results suggested that the high constitutive level of *Ptgs2* expression in cMSCs was not the result of an *in vivo* microbe-dependent maturation.

We next identified a potential mechanism for the lack of cMSC response to LPS by comparing expression of various members of the Tlr4 pathway in cMSCs versus bone marrow-derived macrophages. By qRT-PCR analysis, expression levels of many genes important in relaying the LPS signal were statistically significantly lower in untreated cMSCs than untreated macrophages, including Tlr4 (fold difference = -30) and CD14 (fold difference = -50; Fig. 2.4D). The near lack of CD14 expression in cMSCs is likely important as this molecule is a necessary component for recognition of LPS by Tlr4¹²². Lastly, macrophages predictably responded to LPS stimulation by alteration of the expression of TLR-pathway members including *IRAK-M*, MHC Class II (*I-A^b*), and *Ptgs2* (Fig. 2.4E). Analysis of similarly treated cMSCs did not show significant changes in the expression of any of these genes (Fig. 2.4E). This expression analysis confirms that the high constitutive expression of *Ptgs2* in cMSCs was likely not the result of TLR-stimulated maturation.

Figure 2.4

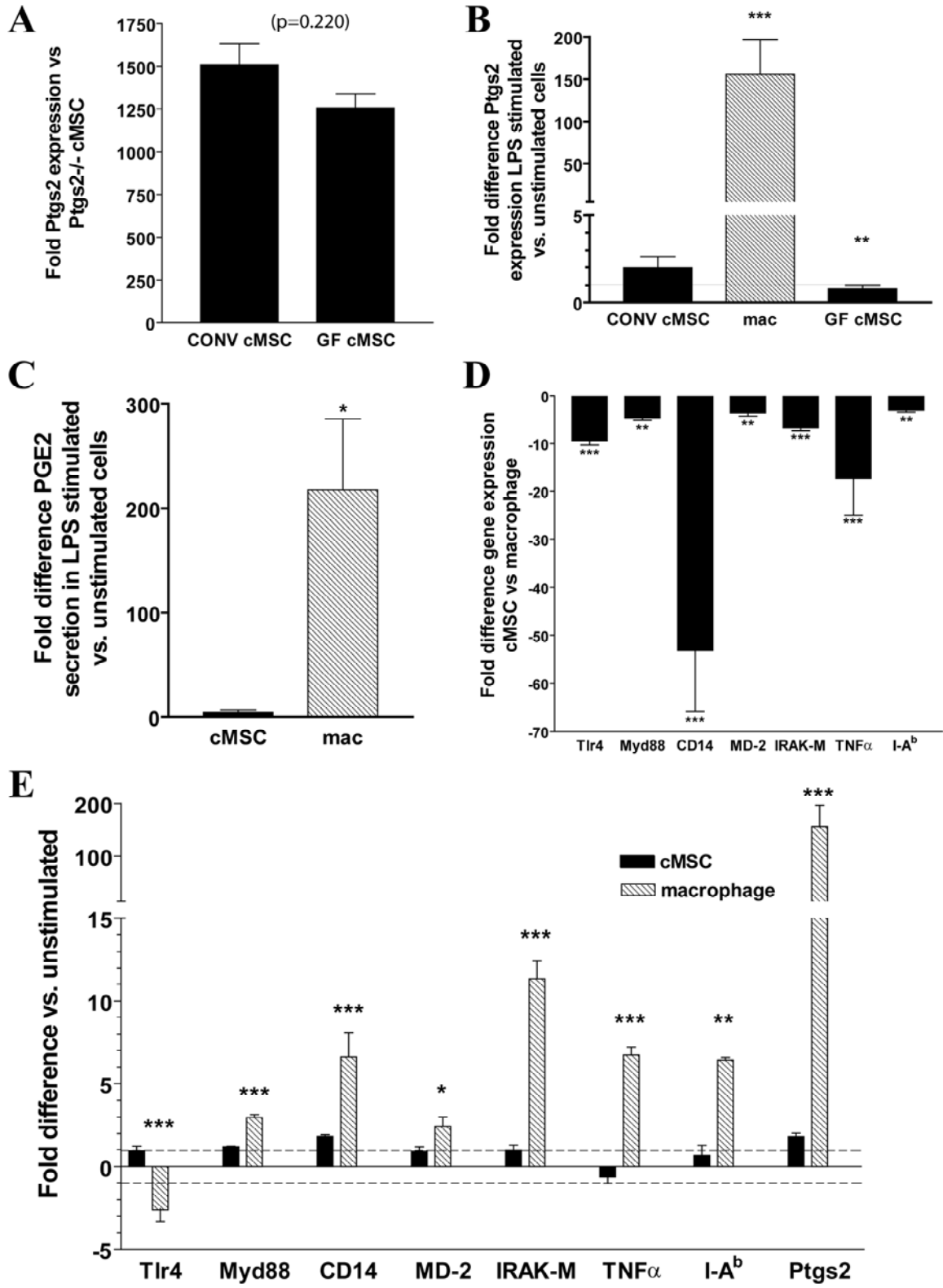


Figure 2.4. High constitutive expression of Ptgs2 is not due to TLR activation.

Colonic MSCs from CONV and GF mice and bone marrow-derived macrophages were isolated and analyzed. (A) Graph of Ptgs2 expression difference in cMSCs isolated from CONV (n=3 lines) and GF mice (n=2 lines) measured by qRT-PCR (baseline: cMSCs from CONV Ptgs2^{-/-} mice). p=0.220, Student's t test. (B) Graph of Ptgs2 expression difference in MSCs and macrophages plated and stimulated by LPS (1-100ng/ml) for 21 hours (baseline: unstimulated cells). (C) Graph of PGE2 secretion fold difference (baseline: secretion from unstimulated cells) from cells stimulated by LPS measured by ELISA assay of supernatants from cMSC (n=3 lines) and macrophages (n=3) (baseline=secretion from unstimulated cells). (D) Graph of TLR4 pathway member gene expression fold difference in cMSCs (n=3 lines) baselined to macrophages (n=3 lines) for each gene measured by qRT-PCR. (E) Graph of fold expression difference in members of the TLR4 pathway and target genes measured by qRT-PCR in 21-hour LPS-stimulated cells (baseline: unstimulated cells). Statistical markings represent significance in the fold difference of a given gene in cMSCs compared to the fold difference of that gene in macrophages. Error bars represent standard deviation. Statistical analysis by ANOVA and Bonferroni post-test (B) or Student's t test (A, E): *(p<0.05), **(p<0.01), ***(p<0.001).

Comparison of global gene expression between organ-specific MSCs revealed candidate pathways that could enrich for Ptgs2 expression in cMSCs. As microbially-derived signals did not appear to participate in the high endogenous levels of Ptgs2

expression in cMSCs, we evaluated signals from other host cells. A proven method for identifying upstream candidates in a target cell type is global gene expression analysis. To identify cMSC-specific Ptgs2 regulatory pathways, we compared these cells to bmMSCs because: 1) they expressed less Ptgs2/PGE2 than cMSCs and 2) they shared important properties with cMSCs (i.e. multipotency and immune modulation).

We probed Affymetrix MOE430A microarrays with cRNA amplified from total RNA procured from two independently isolated lines of cMSCs and bmMSCs. We analyzed the data using dCHIP software¹¹⁵ and generated a list of 575 probe sets (corresponding to 427 unique genes) that were elevated in the cMSCs as compared to bmMSCs (using a 1.3 expression fold difference cut-off and $p < 0.05$ ¹³; this data is deposited on the GEO database: <http://www.ncbi.nlm.nih.gov/geo/>). Importantly, this list included Ptgs2 (11-fold difference) and CD34 (200-fold difference) which correlated with the observed differences in protein expression between these two cell lines (data not shown).

We then functionally categorized the genes that were enriched in cMSCs and bmMSCs using gene ontology (GO) terms¹¹⁶. The three most highly represented categories of genes preferentially expressed in cMSCs were ‘integral to membrane’ (fractional representation: 0.284), ‘protein binding’ (fractional representation: 0.275), and ‘membrane’ (fractional representation: 0.275). Conversely, the three most highly represented GO terms in bmMSCs were ‘protein binding’ (fractional representation: 0.354), ‘membrane’ (fractional representation: 0.320), and ‘nucleus’ (fractional representation: 0.296). The most striking feature of this analysis was that the ‘integral to membrane’ GO term was the most highly represented in the cMSC list. This is quite unusual for a stem cell¹²³, and we therefore focused closely on this subset of genes.

In order to narrow the list of candidates involved in the regulation of high levels of Ptgs2 in cMSCs from the 94 genes in the ‘integral to membrane’ category, we reasoned that genes of interest would also be capable of receiving and transducing an extracellular signal. Therefore, we required that genes of interest also contain the GO terms ‘integral to plasma membrane’ (genes = 25) and ‘receptor activity’ (genes = 35). The intersection of ‘integral to membrane’ genes that contained these two additional GO terms generated a list of 13 genes (Fig. 2.5A) that were enriched in cMSCs as compared to bmMSCs (Fig. 2.5B). Interestingly, these 13 genes could be loosely categorized into the receipt of three general types of signals: growth, immune, and neural (Fig. 2.5B).

We further evaluated the possible role of Fgfr1 because previous studies in our laboratory demonstrated that communication between Fgf9 and MSCs in the intestinal tract exists during development¹²⁴, thus demonstrating the ability of at least one type of MSC to respond physiologically to Fgf signaling. PCR analysis of cDNA isolated from whole WT adult colon showed that all four Fgf receptors were expressed, including all ‘b’ and ‘c’ splice forms. The ‘b’ forms are expressed predominately in epithelial cells, and the ‘c’ forms are expressed predominately in mesenchymal cells^{125,126} (Fig. 2.5C). We then performed qRT-PCR for Fgfr1, 2, 3, and 4 using total RNA isolated from cMSCs and found that cMSCs contained detectable expression of only the ‘c’ form of Fgfr1 and Fgfr2 (Fig. 2.5D). This expression analysis confirmed that signaling by Fgf ligands to cMSCs was possible. We then focused on the role of Fgf9 as it is expressed in the epithelium of the colon¹²⁷ and appears to signal to MSCs during development¹²⁴.

Figure 2.5

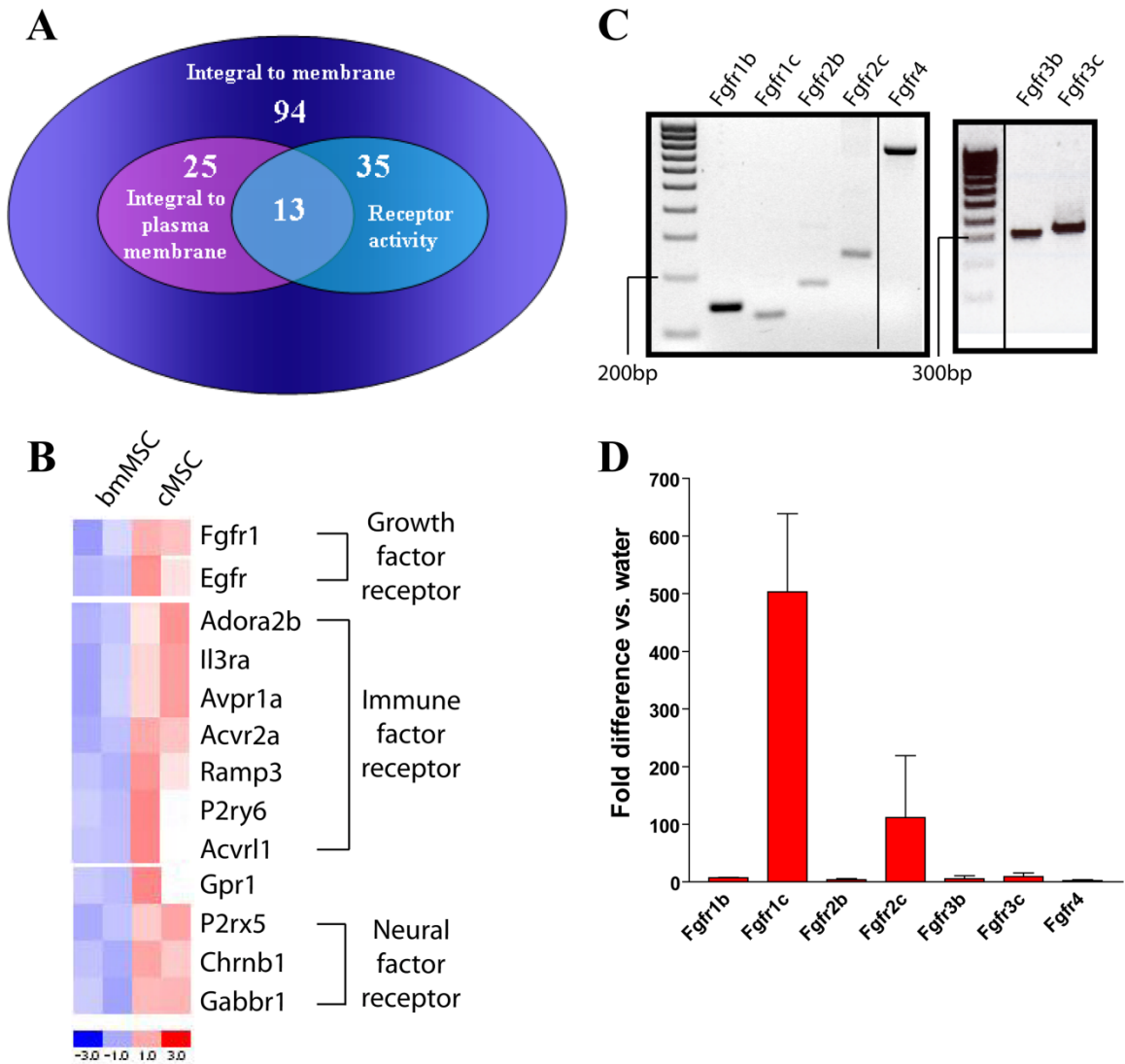


Figure 2.5. Comparison of cMSCs to bmMSCs reveals possible mechanistic pathways involved in *Ptgs2* expression.

(A) Venn diagram illustrating the intersection of the gene ontology (GO) terms ‘integral to plasma membrane’ and ‘receptor activity’ obtained through GO analysis of genes preferentially expressed in cMSCs (compared to bmMSCs). Data for analysis was obtained by isolation of total RNA from cMSCs (n=2 lines) and bmMSCs (n=2 lines), amplification and cRNA labeling, hybridization to

Affymetrix MOE430A microarrays, and dCHIP analysis of genes preferentially expressed in one cell type or the other at a 1.3 fold level, $p < 0.05$. (B) Heat map generated by dCHIP software of genes fitting into the logical intersection of 'integral to plasma membrane' and 'receptor activity' showing relative expression levels in each cell line. (C) Electrophoresis gel of PCR reaction amplifying cDNA obtained from whole WT C57Bl/6 mouse intestine for the Fgf receptor splice forms: Fgfr1b, Fgfr1c, Fgfr2b, Fgfr2c, and Fgfr4 (annealing temperature = 60°C) and Fgfr3b and Fgfr3c (annealing temperature = 65°C). (D) Graph of qRT-PCR measurements of the various splice forms of the Fgf receptors expressed in cMSCs (n=3 lines) compared to water control. Error bars represent standard deviation.

Fgf9 was sufficient to maintain high levels of Ptgs2 expression in cMSCs. We hypothesized that Fgf9 stimulation may be responsible for induction and/or maintenance of *Ptgs2* expression in cMSCs. Therefore, we studied its effects on expression in cMSCs *in vitro*. Colonic MSCs were serum starved to remove the growth factor signaling that was present during standard tissue culture. As little as 3 hours of starvation resulted in an approximately 25-fold decrease of *Ptgs2* expression while expression of other genes, notably *Ptgs1*, was unaffected within this time frame (Fig. 2.6A). This loss of *Ptgs2* mRNA was rescued in a dose dependent manner by the addition of Fgf9 (range of 1 – 500ng/ml) to the culture media for one hour (Fig. 2.6B). This treatment had no effect on expression of *Ptgs1*. Addition of Fgf9 was sufficient to rescue *Ptgs2* expression to a similar level as the addition of 10% serum. The effects of exogenous Fgf9 on *Ptgs2*

mRNA levels could be blocked by addition of a neutralizing antibody directed against Fgf9 (Fig. 2.6C).

In order to determine whether *Ptgs2* mRNA was increased due to transcriptional activation or post-transcriptional regulation/stabilization, we designed a transcript-specific qRT-PCR assay. Primers were designed to amplify a product specifically from the unspliced (unprocessed) form of *Ptgs2* RNA (primers 1 and 2) or to amplify a product specifically from the spliced (mature) form of *Ptgs2* RNA (primers 1 and 3; Fig. 2.6D). Both the unspliced and spliced forms of *Ptgs2* RNA expressed by cMSCs showed decreased levels after serum starvation for 3 hours but the effect on spliced mRNA was much more pronounced. Treatment with 100ng/ml of Fgf9 after starvation significantly rescued both the spliced and unspliced forms of *Ptgs2* RNA (Fig. 2.6E). These data suggest that Fgf9 may regulate *Ptgs2* RNA expression at either the transcriptional or post-transcriptional level.

In order to better delineate between the two levels of regulation, we devised an experiment to specifically test the effect of Fgf9 on the post-transcriptional regulation of *Ptgs2*. To do this, we removed the possibility of Fgf9 affecting *Ptgs2* expression at the transcriptional level by blocking transcription in cMSCs with actinomycin D treatment (4µg/ml). Blockade of transcription in cMSCs cultured with 10% serum in the media had no significant effect on the mature *Ptgs2* mRNA levels in 3 hours (Fig. 2.6F). This finding suggested that a growth factor present in the serum was able to maintain the mature form of *Ptgs2* mRNA synthesized before transcriptional blockade. As in serum starved conditions alone, a significant decrease in *Ptgs2* mature mRNA was seen when serum starvation was compounded by transcriptional repression by actinomycin D (Fig.

2.6F). We suspected that Fgf9 would be sufficient to mediate the stabilization of mature Ptgs2 mRNA seen in transcriptional blockade in the presence of 10% serum and tested this hypothesis. We found that serum starved cMSCs treated with actinomycin D in the presence of Fgf9 (250ng/ml) showed no loss of mature Ptgs2 mRNA, indicating that Fgf9 is capable of maintaining Ptgs2 expression independent of transcriptional activation (Fig. 2.6F). Also, as expression of unspliced Ptgs2 should depend completely upon transcription, we showed that Fgf9 was unable to rescue the total loss of unspliced Ptgs2 expression upon blockade of transcription by actinomycin D treatment (Fig. 2.6G). These series of experiments confirmed that the serum starvation-induced decrease of mature Ptgs2 mRNA expression could be rescued by Fgf9 independent of transcription. Thus, Fgf9 was sufficient to maintain mature Ptgs2 mRNA expression and affected this expression at a post-transcriptional level.

Figure 2.6

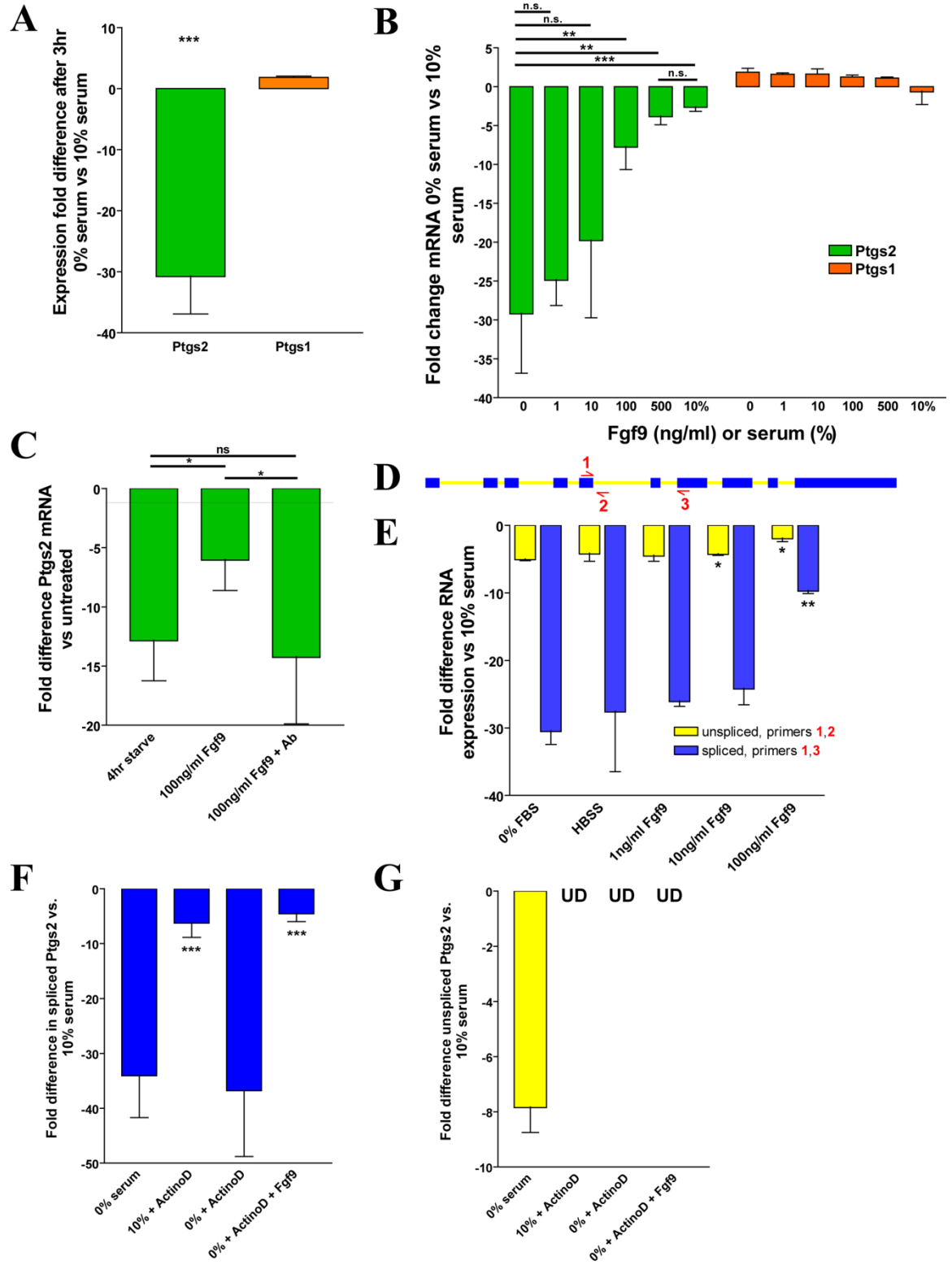


Figure 2.6. Fgf9 is sufficient to stabilize Ptgs2 mRNA.

(A-D, G-I) qRT-PCR and (E) immunoblot analysis of cMSCs. (A) Graph of the fold difference for Ptgs2 and Ptgs1 expression of serum starved cMSCs versus cMSCs grown in 10% serum. (B) Graph of the fold difference for Ptgs2 and Ptgs1 expression in cMSCs after 1hr Fgf9 treatment (range=1 – 500ng/ml) following 3hr serum starvation. (C) Graph of the fold difference for Ptgs2 expression after treatment with Fgf9 or Fgf9 plus anti-Fgf9 neutralizing antibody following 3hr serum starvation. (D) Schematic illustrating the location of primers 1, 2 and 3 on the Ptgs2 gene. (E) Graph of the fold difference for the spliced and unspliced forms of Ptgs2 isolated from 3hr serum starved cMSCs additionally treated for 1hr with Fgf9. (F) Graph showing the fold difference for Ptgs2 spliced RNA after treatment with or without serum starvation, 4µg/ml actinomycin D, and 250ng/ml Fgf9. Asterisks compare data to fold difference in cells treated with 0% serum and actinomycin D. (G) Graph showing the fold difference for Ptgs2 unspliced RNA with or without serum starvation, 4µg/ml actinomycin D, and 250ng/ml Fgf9 (UD = undetectable). All data is representative of three independent experiments. Error bars represent standard deviation. Statistical analysis by Student's t test: *(p<0.05), **(p<0.01), ***(p<0.001), n.s.(not significant).

Fgf9 stabilizes Ptgs2 mRNA in part through CUGbp2. To investigate the mechanism of Fgf9-mediated Ptgs2 mRNA stabilization, we first tested the role of Erk, a component of the MAP kinase cascade capable of transducing Fgf signaling in many cells¹²⁸. Erk activation was inhibited by use of the MEK inhibitor PD98059. We found that an increasing dosage of PD98059 in cMSCs decreased phosphorylation of Erk and that this

treatment also corresponded to a decrease in *Ptgs2* mRNA expression (Fig. 2.7A,B). As expected Erk phosphorylation is also decreased in the absence of serum. These results support the role for Erk activation in *Ptgs2* stabilization.

We then undertook a candidate molecule approach to identify possible proteins which could be downstream targets of Fgf9/Erk activation. Our microarray data (Fig. 2.5) identified multiple mRNA binding proteins that were preferentially expressed in cMSCs compared to bmMSCs. One of these was CUGbp2, a protein known to stabilize *Ptgs2* mRNA^{36,38} and whose activity can be regulated by phosphorylation downstream of growth factors³⁰. We evaluated this gene at both the mRNA and protein level in the context of Erk inhibition and found that although CUGbp2 mRNA was not significantly altered by either serum starvation or by Erk inhibition (Fig. 2.7A), the level of endogenous CUGbp2 protein was decreased both in the absence of serum and upon Erk inhibition (Fig. 2.7B, C).

This result suggested that CUGbp2 protein was required to stabilize *Ptgs2* mRNA in cMSCs. We tested this idea by specifically inhibiting CUGbp2 protein translation through shRNA-mediated knockdown of CUGbp2 expression. Lentiviral transfection of cMSCs with two different CUGbp2-specific produced significant knockdown of CUGbp2 mRNA as measured by qRT-PCR (Fig. 2.7D). Importantly, transfection with control shRNA did not affect CUGbp2 expression. The shRNA-mediated decrease of CUGbp2 mRNA corresponded to a decrease in CUGbp2 protein expression as demonstrated by immunoblot (Fig. 2.7E). We then assessed the effect of CUGbp2 loss on *Ptgs2* expression and observed that in cMSCs. We found a significant decrease in *Ptgs2* message level in agreement with other studies in other cell lines^{30,36} (Fig. 2.7F).

Based on these results, we hypothesized that Fgf9 may act through Erk to increase CUGbp2 protein. To test this hypothesis, we repeated our previous experiments and assessed phosphorylation of Erk and quantity of CUGbp2 protein in cells treated in normal serum, in 0% serum, and in 0% serum supplemented only with Fgf9 protein. We observed an increase in both Erk phosphorylation in Fgf9-treated cells as well as increased levels of endogenous CUGbp2 protein (Fig. 2.7G, H). Taken together, these observations suggest that the mechanism of Ptgs2 mRNA stabilization by Fgf9 signaling is partially mediated through phosphorylation of Erk, leading to increased CUGbp2 protein, resulting in CUGbp2-mediated stabilization of Ptgs2 mRNA (Fig. 2.8).

Figure 2.7

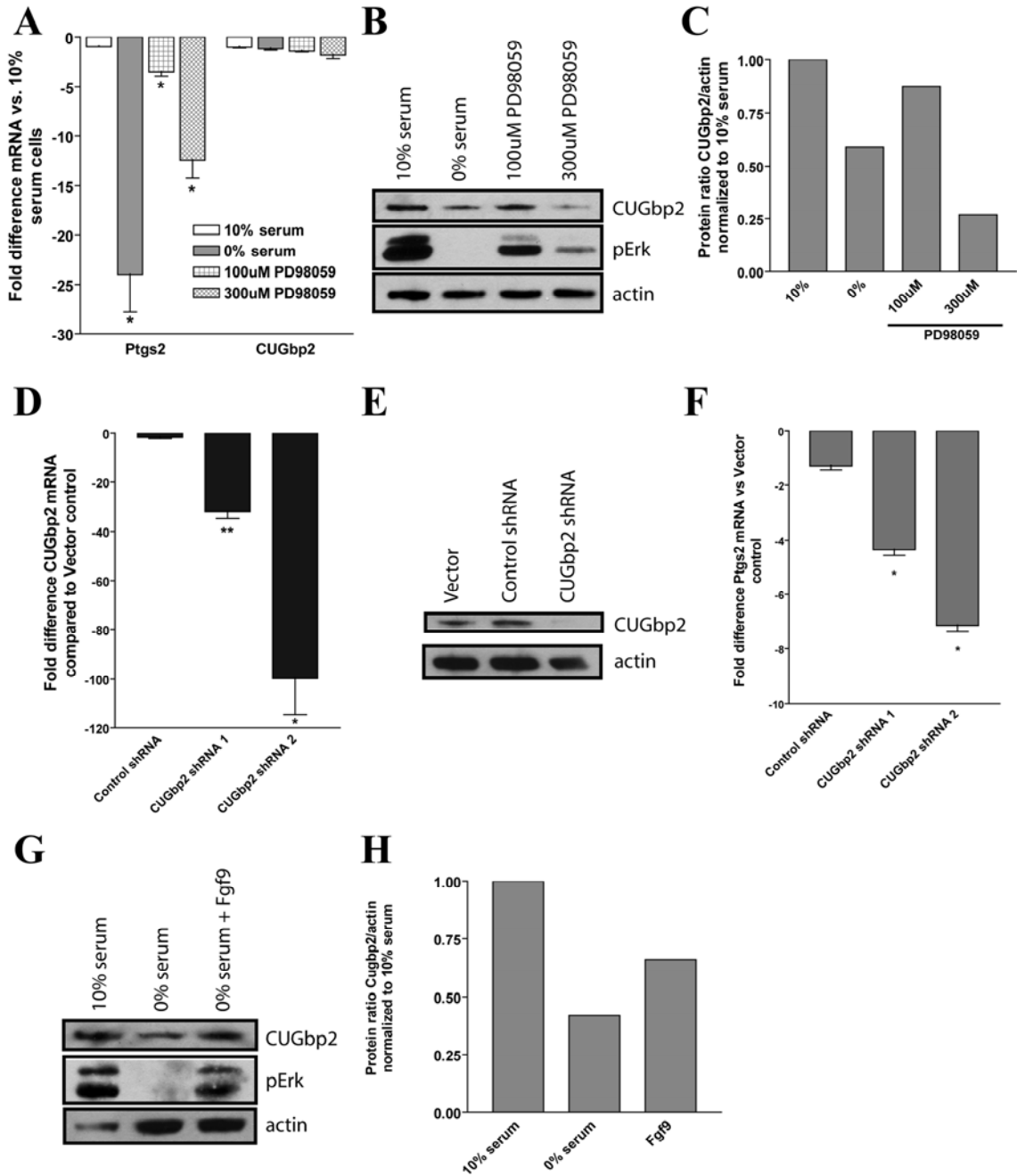


Figure 2.7. Fgf9 stabilizes Ptg2 partially through Erk activation and increased CUGbp2 protein.

(A) Graph of the fold difference for Ptg2 and CUGbp2 mRNA following a 3h treatment of cMSCs with an Erk kinase inhibitor, PD98059 (100μM or 300μM), or

0% serum. (B) Reducing immunoblot of PD98059-treated cMSCs probed for actin (loading control), phosphorylated-Erk, and CUGbp2. (C) Graph of the quantification of (B) showing the ratio of CUGbp2/actin protein quantity in cMSCs treated by serum starvation or with PD98059. Baseline (1.00) = ratio of cMSCs grown in 10% serum. (D) Graph of the fold difference in CUGbp2 mRNA in cMSCs lentivirally transfected with control shRNA or CUGbp2 shRNAs compared to vector control. (E) Reducing immunoblot of cMSCs transfected with vector control, control shRNA, or CUGbp2 shRNA showing specific knockdown of CUGbp2 protein. (F) Graph of the fold difference for Ptgs2 mRNA in cMSCs transfected with control shRNA and CUGbp2 shRNA versus vector control. (G) Reducing immunoblot of cMSCs treated with 10% serum, 0% serum, or 0% serum plus Fgf9 (250ng/ml) and quantification (H) of the ratio of CUGbp2/actin in these cells. Baseline (1.00) = ratio of cMSCs grown in 10% serum. Immunoblots and quantifications were representative of three independent experiments. (A,D,F) Asterisks compare data to fold difference in cells grown in 10% serum. Statistical analysis was performed by Student's t test: *($p < 0.05$), **($p < 0.01$).

Figure 2.8

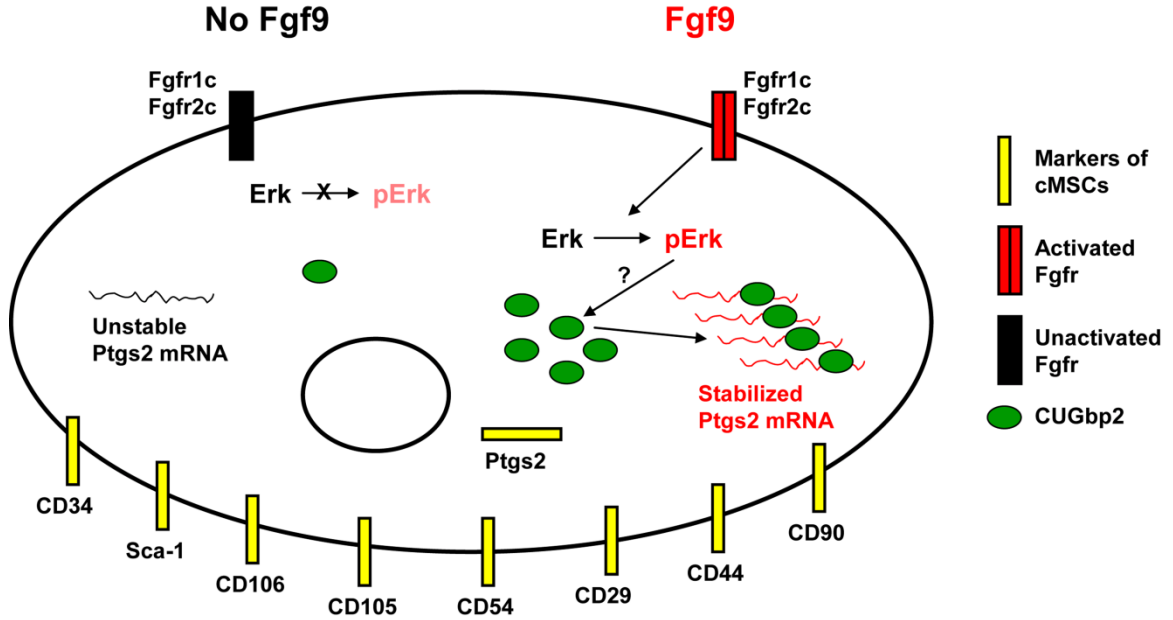


Figure 2.8. Model of Fgf9 regulation of Ptg2 expression in cMSCs.

Colonic MSCs express CD29, CD34, CD44, CD54, CD90, CD105, CD106, Sca-1, and Ptg2. When Fgf9 is present, it signals through Fgfr1c and/or Fgfr2c on the cell surface. Transduction of this signal via Erk phosphorylation and activation results in an increase in CUGbp2 protein. This protein in turn binds to Ptg2 mRNA and stabilizes the message. In the absence of Fgf9, Erk phosphorylation is decreased, resulting in decreased CUGbp2 protein quantity and loss of Ptg2 mRNA stabilization.

Chapter 2: Discussion

Here we define a previously unidentified Ptgs2-expressing cell population within the adult mammalian intestine as mesenchymal stem cells. We demonstrated a novel mechanism by which the observed constitutive expression of Ptgs2 is maintained within these cells downstream of Fgf9, a growth factor expressed in the adult mammalian intestine. Our investigations suggest that the stabilization of Ptgs2 mRNA downstream of Fgf9 is mediated via phosphorylation of the MAP kinase signaling cascade member Erk followed by increased abundance of CUGbp2 protein (Fig. 2.8).

Fgfs: role in development and homeostasis. There are at present 22 recognized Fgf ligands that can be grouped into seven subfamilies based on phylogenetic analysis. The Fgf of interest in these studies, Fgf9, is the namesake for one of these subfamilies that also includes Fgf16 and Fgf20. Fgf ligands signal specifically through certain Fgf receptors. There are four total receptors with Fgfr1, 2, and 3 expressed as either a 'b' or 'c' splice form creating essentially 7 different Fgf receptors^{129,126}. Fgfs are known to play important roles in the morphogenesis and expansion of developing structures including the lung^{130,131}, limb¹³², intestine^{133,124,127}, epidermis¹³⁴, and nervous system¹³⁵.

We have demonstrated an interaction between Fgf9 and Ptgs2 in a mesenchymal cell type unique to the colon, the colonic mesenchymal stem cell. Our findings potentially expand the growing recognition of the role of Fgfs in homeostasis and normal physiology within the adult organism. In particular, Fgfs have recently been shown to aid regulation of nutrient and mineral homeostasis. For example, Fgf15 can be induced in the small intestinal epithelium and subsequently signal to the liver where it regulates enzymes of the bile acid synthesis pathway¹³⁶. Thus Fgf15 has an important role in enterohepatic

homeostatic regulation of bile acid production. In other studies, Fgf23, produced by the bone, plays a role in regulating calcium and phosphate homeostasis that requires precise control of the kidney, parathyroid, intestine and bone. Fgf23 appears to have multiple cellular targets in this regulation^{137,138}. Also, nutrient availability and composition can impact expression of specific Fgfs including keratinocyte growth factor¹³⁹ (KGF also known as Fgf7). Our studies raise the intriguing possibility that another Fgf may be acting in a homeostatic role in the adult organism, specifically that Fgf9 may be important in maintaining the constitutive expression of Ptgs2 in this specific subset of mesenchymal cells which have already been implicated in injury response¹⁴.

Growth factors affecting Ptgs2 expression. Our studies have implicated Fgf9 as a factor sufficient to mediate Ptgs2 mRNA stability. However, a variety of other growth factors are also likely to be capable of regulating Ptgs2 expression in this cell type. These factors include Fgfs other than Fgf9¹⁴⁰, Egf^{29,141}, and Igf^{142,143}.

These various growth factors may act via a number of signaling networks that have been implicated in the regulation of Ptgs2, many of which are involved in the induction of gene expression during the inflammatory response rather than in constitutive expression²⁶. Our *in vitro* studies strongly indicate that, rather than acting primarily via transcriptional upregulation, Fgf9 acts at the post-transcriptional level and suggest that the mechanism of this interaction partially involves CUGbp2 protein expression. This protein and others including HuR and Apobec-1 have been implicated in the regulation of Ptgs2 expression in various tumor cell lines^{36,38} and in an *in vivo* model of irradiation injury¹⁴⁴. Comparing the ability of Fgf9 to increase CUGbp2 protein with its ability to increase Ptgs2 mRNA stabilization suggests that increased CUGbp2 is only a portion of

the overall mechanism downstream of Fgf9 involved in Ptgs2 expression. It is very likely that this growth factor is also signaling through other kinase cascade pathways including MAPK through Jnk and p38, PKC, and PI3K¹⁴⁵. Intriguingly, the Src family of kinases has recently also been implicated in the stabilization of Ptgs2 mRNA downstream of signaling by platelet derived growth factor³⁰. The targets of kinase activity must also be greater than one. There are a variety of further mRNA binding proteins that are likely involved in this cascade, some well-researched such as HuR³⁸ and others unknown.

The role of CUGbp2 in stabilizing Ptgs2 expression. Previous studies have shown that CUGbp2 is capable of stabilizing Ptgs2 mRNA via a mechanism requiring binding of the protein to the AU rich region of the 3'UTR of the Ptgs2 message^{30,36,38}. Our findings contribute to this field by recognition of a mechanism of the regulation of Ptgs2 mRNA stability by endogenously expressed CUGbp2. We demonstrated that Ptgs2 stabilization is in part dependent on the quantity of endogenous CUGbp2 protein and appears to be independent of altered transcription of CUGbp2. The question now is the mechanism by which endogenous CUGbp2 protein is increased consequent of Erk activation. The fact that CUGbp2 protein is increased downstream of Erk activation suggests a mechanism involving protein phosphorylation. Erk may directly phosphorylate CUGbp2 itself resulting in stabilization. Alternatively, it may act indirectly through other proteins that then stabilize CUGbp2, disrupt CUGbp2 degradation, or increase CUGbp2 translation from available mRNA. Further studies are needed to understand the regulation of CUGbp2 itself.

Constitutive expression of Ptgs2. The presence of constitutive Ptgs2 expression in the adult mammal has been well demonstrated^{14,28,146,147}. However, Ptgs2 remains primarily

recognized as the inducible cyclooxygenase largely due to its upregulation in inflammation and in various cancers¹⁴⁸. However, Ptgs2 knockout mice, though largely healthy, do exhibit multiple problems that arise from loss of constitutive expression specifically of Ptgs2 that are not found in Ptgs1 knockout mice. These include defects in kidney development and renal function, in reproduction, and in proper bone maintenance¹⁸. These data highlight the necessity of understanding the regulation of constitutive Ptgs2 expression in addition to understanding the mechanisms involved in the upregulation of Ptgs2 in pathological situations. Our colonic MSCs can serve as a prime vessel for investigating the various mechanisms involved in regulating constitutive Ptgs2 expression as they are a primary cell type that naturally maintains extremely high levels of Ptgs2 mRNA. The mechanistic findings can then be evaluated in the whole organism in order to better understand the basic biology underlying Ptgs2 expression.

Colonic MSC: monitors of the homeostatic environment? Our findings raise the highly intriguing question of the precise role of colon resident MSCs in homeostasis. We have demonstrated a probable role as an immune modulator through the production of PGE2. Interestingly, cMSCs do not express MHC class II, even after LPS stimulation, indicating that they likely are not acting as non-professional antigen presenting cells. We followed this line of inquiry from our analysis of receptors whose expression was enriched specifically in cMSCs as compared to bmMSCs. Twelve other receptors were uncovered in this analysis, each of which raises possibilities for future experimental pathways and inquiries into the role of cMSCs.

We propose that cMSCs, due to their location in the complex environment of the mature colonic mesenchyme made up of a great variety of host cell types, are well-

positioned to act as monitors of the colonic environment. These cells specifically express receptors that are capable of receiving signals from a variety of immune and nervous cells, rendering them ideal for integrating signals indicative of the state of the organism as a whole and of the level of immune activation locally. The cells would thus be poised to combine and interpret neural and immune signals, respond to slight changes in their activity, and mediate responses directly or indirectly to these fluctuations before serious consequences such as full inflammation result.

Receptors on the surface of the cMSC capable of receiving neurally-derived signals include a cholinergic receptor (Chrn1), a gamma-aminobutyric acid receptor (Gabbr1), and a purinergic receptor (P2rx5). These are intriguing in colonic immune homeostasis as there is an emerging literature on the cross-talk between the autonomic enteric nervous systems and cellular constituents of the immune system^{149,150}. Other receptors on the surface of the cMSC identified by our chip analysis are capable of receiving immune signals either directly derived from immune effector cells or indirectly as a consequence of immune effector cell action. Two of these are activin receptors (Acvr11 and Acvr2a) that recognize this member of the TGF- β superfamily, a factor that may be secreted by Th2 cells¹⁵¹ and by dendritic cells¹⁵². The expressed adenosine receptor, Adora2b, can receive signals important to NK cell activity and regulation¹⁵³. A receptor capable of sensing extracellular nucleotides released in inflammation is also present (P2ry6¹⁵⁴). Other receptors include interleukin-3 receptor (Il3ra), a vasopressin receptor (Avpr1a), and a calcitonin receptor (Ramp3). Indeed, many more receptors capable of sensing other neural, immune, and growth factor signals are also likely to be present; we have only highlighted those that appear to be enriched in cMSCs compared to bmMSCs. The

simultaneous expression of these many and varied receptors on cMSCs and the localization of these cells in the complex environment of the colon place them in a prime position to act as monitors of the colonic homeostatic environment. This proposed role suggests a wealth of future investigative pathways that could aid the gastrointestinal field in gaining a greater understanding of the cellular players and mechanisms involved in the complex regulation of colonic homeostasis.

CHAPTER 3

Imp1 is a novel trans-acting factor involved in Ptgs2 regulation

Chapter 3: Abstract

Prostaglandins have been shown to play important roles in different injury models, acting both on proliferation of injured tissue and in modulation of the immune response. These lipid mediators are synthesized downstream of the two prostaglandin endoperoxide synthetase genes (Ptgs or cyclooxygenase/cox). Specifically, Ptgs2 has been shown to be both inducible in inflammatory cells and constitutively expressed in rare stromal cells of various organs. The expression of this gene is necessary for proper injury response in a variety of colonic injury models from chronic inflammation to radiation exposure. Herein we show that Ptgs2 is also necessary in the response of the colon to a localized biopsy injury. We demonstrate that insulin growth factor 2 binding protein 1 (Igf2bp1 or Imp1) is necessary for full expression of Ptgs2 in cell lines and can interact with Ptgs2 transcript in the cellular environment. Furthermore, we show that Imp1 is upregulated within the wound bed of colonic biopsy injury and is expressed in the same cell type as Ptgs2, suggesting that Imp1 plays an important role in Ptgs2 regulation in response to injury.

Chapter 3: Introduction

Injury to the colonic mucosa can occur in a variety of settings including foreign body insult and helminth infection. Mechanical injury during various scope procedures that are now common place for screening and intervention of colonic inflammation and cancers may occur, though rare¹⁵⁵. Furthermore, acute inflammatory states may also develop in infection and diverticulitis¹⁵⁶. The natural sequence of healing and the biological signals and cellular interactions required in such injuries are not fully understood.

In order to address this issue, we pioneered a model injury system that induces acute, localized trauma, subsequently followed by confined inflammation and healing¹⁵. Mice are injured by colonic biopsy, removing a portion of the colonic mucosa. Injuries then heal and can be followed endoscopically over time until sacrifice and histological examination. We have found that healing occurs with a predictable progression over 6 – 8 days. Initially we observe increased macrophage and neutrophil infiltration of the wound while a single cell epithelial layer, referred to as wound associated epithelium (WAE), covers the injured area. Granulation tissue rapidly forms with a high degree of vascularization. Knowledge of the biological signals involved in healing is necessary in order to understand the mechanisms directing healing. Previously, we identified the Th2 cytokines IL-4 and IL-13 as cytokines that are necessary for proper healing, likely through induction of an M2 macrophage phenotype¹⁵. We have become interested in other local candidate signaling molecules that may be important in healing.

We hypothesized that prostaglandin endoperoxide synthetase 2 (Ptgs2, more commonly known as cyclooxygenase-2/cox-2) may play a role in healing as it is known

to mediate response to other types of injury. Ptgs2 is one of the two rate limiting enzymes responsible for the conversion of arachidonic acid to prostaglandin H₂ which is the precursor for the various prostaglandins, prostacyclin, and thromboxane that are then secreted to mediate various biological functions. Specifically, prostaglandin E₂ has been demonstrated to play a role in maintaining proliferation¹⁴ and in modulation of the immune system in various models of colonic injury including colitis¹⁵⁷ and radiation¹¹¹. Additionally, it has been proposed that mesenchymal stem cells which are currently in a phase III trial for treatment of Crohn's disease⁷² may mediate at least some of their anti-inflammatory effects via secretion of PGE₂⁴⁷. Finally, evidence for the importance of Ptgs2 in colonic injury comes from various case-control studies and case reports demonstrating correlation between non-steroidal anti-inflammatory drugs (NSAIDs), which function by inhibiting the Ptgs genes, and induction of colitis or exacerbation of established disease¹⁵⁸.

We therefore asked whether Ptgs2 may be similarly required for the proper response to a more localized traumatic injury by investigating wound healing in Ptgs2 deficient mice. We found that Ptgs2 is necessary for proper healing response and is expressed in a population of stromal cells in the wound bed. To uncover novel regulators of Ptgs2, we employed a candidate approach and demonstrate for the first time that Imp1 protein interacts with Ptgs2 mRNA in the cellular environment, is upregulated in colonic biopsy, and is co-localized with Ptgs2 in the wound bed. Understanding the various mechanisms and pathways involved in the response to biopsy injury could aid medicine in decreasing the morbidity of similar conditions and in accelerating treatments.

Chapter 3: Materials and Methods

Immunoprecipitation and RNA isolation. The open reading frame of Igf2bp1 was cloned from cDNA isolated from colonic mesenchymal stem cells with specific primers, cccgaattcatgaacaagctttacatcggc (forward) and cccggatcccttctccgagcctg (reverse), and directionally ligated into p3XFLAG-CMV-14 expression vector (Sigma-Aldrich) using restriction sites EcoR1 (5') and BamH1 (3') in order to create a FLAG-tagged protein construct. Igf2bp1 deletion mutants RRM (primers: Igf2bp1 F and cccggatccctgctgccacgggcg) and KH (primers: cccgaattcatgatccctctccggctcc and Igf2bp1 R) were similarly cloned and tagged. NIH3T3 cells were plated in 10cm dishes and transfected using FuGene HD reagent (Roche). After 18-24 hours, dishes were rinsed in cold PBS and exposed to 300mJ 254nm UV light on ice. Cells were scraped, pelleted, and lysed by passage through a 26.5 gauge needle using polysome lysis buffer¹⁵⁹ (PLB: 100mM KCl, 5mM MgCl₂, 10mM HEPES) supplemented with 0.05% NP-40, 2mM vanadyl ribonucleoside complex, 100U RNase inhibitor, 1:100 phosphatase inhibitor cocktails 2 and 3 (Sigma), and 1:40 protease inhibitor cocktail (Sigma). Cellular debris was removed by centrifugation before preclearing the lysate for one hour tumbling at 4°C with mouse IgG-conjugated Protein A beads, and samples were saved for total protein and RNA fractions. Lysates were then incubated overnight at 4°C with M2 antibody-conjugated agarose beads (Sigma). Beads were washed 3 times with PLB and 3 times with PLB-500mM NaCl. In some cases, bound FLAG-tagged proteins were eluted using 100µg/ml 3X FLAG peptide (Sigma) for 3 hours. Beads, total protein, and eluate fractions were then either boiled in Laemmli's buffer for protein isolation or incubated at 55°C with 30µg Proteinase K and 0.1% SDS in 100µl TBS for 30min, phenol-chloroform

extracted, and ethanol precipitated to isolate RNA. RNA quantity was measured on a nanodrop (ND-1000 Spectrophotometer).

Mice. All animal experiments were performed in accordance with approved protocols from the Washington University School of Medicine Animal Studies Committee. Mice involved in this study were housed in microisolator cages, in a specified pathogen-free barrier facility following a 12 hour light cycle and fed a standard irradiated chow diet (PicoLab Rodent Chow 20, Purina Mills) and water ad libitum. *Ptgs2*^{-/-} mice¹⁹ were generated previously on a C57Bl/6 background. C57Bl/6 mice were obtained from the National Cancer Institute.

Colonic biopsy. We used a high-resolution miniaturized colonoscope system^{160,161} in order to visualize the luminal side of the colon, create discrete injury of the mucosal layer, and monitor regeneration of the mucosa. This system consisted of a miniature rigid endoscope (1.9-mm outer diameter), a xenon light source, a triple chip high resolution CCD camera, and an operating sheath with 3 French instrument channel and water injection bulb used to control inflation of the mouse colon (all from Karl Storz). In order to perform the procedure, we monitored the protocol with high resolution (1024 × 768 pixels) live video and collected images on a color monitor.

At the beginning of the procedure, mice were anesthetized by ketamine and xylazine. The endoscope with outer operating sheath was inserted to the mid-descending colon and any feces pellets were removed. We then inserted 3 French flexible biopsy forceps, and removed full thickness areas of the entire mucosa and submucosa. We were careful to avoid much removal or full penetration of the muscularis propria. Each mouse was biopsy injured at 3–5 sites along the dorsal side of the colon.

Immunofluorescence. Wounded C57Bl/6 or Ptgs2^{-/-} mice were sacrificed and perfused with 4% PFA. After dissection, the colon was flushed with PBS, inflated/flushed with 4% PFA for 10 seconds, opened longitudinally, and pinned flat in PFA overnight. Colons were changed with 20% sucrose-PBS before wounds were dissected and frozen in OCT compound. Samples were cut into 5 μ m sections and stored at -80°C. For staining, slides underwent antigen retrieval in citrate buffer for 10min, rinsed in PBS, blocked with 3% BSA, and incubated with primary antibody for one hour. Slides were then rinsed, incubated with secondary antibody followed by Hoechst dye and mounting with 1:1 PBS:glycerin. Sections were viewed with a Zeiss Axiovert 200 with AxioCam MRM camera. Antibodies used were rabbit anti-Ptgs2, goat anti-Imp1 (Santa Cruz), rat anti-CD31, rat anti-CD45, rat anti-CD44, rat anti-CD29, and rat anti- β -catenin.

Cell isolation and culture. Performed as in Chapter 2. NIH3T3 cells were similarly cultured.

shRNA Transfection. HEK293T cells were transfected with Mission shRNA constructs specific for Igf2bp1 and non-targeting control (Igf2bp1 clones NM_009951.2-1611s1c1, NM_009951.2-1874s1c1; Sigma) and lentiviral packaging plasmids using FuGene HD reagent (Roche). Following one day transfection, cell media was refreshed and allowed to accumulate virus 24 hours. Viral-containing supernatant was used undiluted directly on plated cMSCs for 24 hours before adding puromycin (Sigma)-containing media for selection of positively infected cells.

qRT-PCR. Cells were treated as noted in various experiments before RNA isolation. RNA was isolated and purified using a Nucleospin kit (Clontech) according to

manufacturer's protocol or as noted for IP experiments. cDNAs were synthesized using a Superscript III reverse transcriptase (Invitrogen), primed using random primers.

Quantitative RT-PCRs were performed in triplicate for each sample using SYBR-green master mix (Clontech) and analyzed by an Eppendorf realplex Mastercycler. The

following primers were used: 18S (AACCCGTTGAACCCATT, CCATCCAATCGGTAGTAGCG); Ptgs2 (TGCCTGGTCTGATGATGTATG, GCCCTTCACGTTATTGCAGATG); Igf2bp1 (CGGCAACCTCAACGAGAGT, GTAGCCGGATTTGACCAAGAA); CUGbp2 (GCTGCTTCAACCCCAATTC, CGCCATACCTGCTAGTGCAT).

Immunoblotting. Colonic MSCs were treated as noted. Following treatment, cells were lysed in RIPA buffer (Sigma) containing Protease and Phosphatase inhibitor cocktails (Sigma) and frozen at -80°C. Protein content was reduced by heating to 100°C for 5min in a ratio of 1:1 with 2X Laemmli's buffer. Protein was loaded onto 10% or 12% Tris-Gly gels (BioRad) and electrophoresed with Tris-Glycine-SDS buffer (BioRad). Samples were transferred to a 0.45µm nitrocellulose membrane (BD) and blocked in 5% milk in 0.05% Tween 20 TBS (TBST) overnight at 4°C. Blots were incubated one hour with HRP-conjugated M2 antibody (anti-FLAG, Sigma) before development using SuperSignal West Dura chemiluminescent kit (Pierce).

Chapter 3: Results

Ptgs2^{-/-} mice show impaired healing of colonic biopsies.

In order to determine whether Ptgs2 is required for the proper healing of localized injury generated by colonic biopsy, we injured Ptgs2^{-/-} mice and allowed them to heal. Histologically, by day 6, wild-type (WT) mice exhibit a contained area of granulation tissue bordered by the WAE cells covering the wound bed and by intact muscularis propria. These discrete cellular tissues can be visualized by smooth muscle actin, marking the muscularis propria, as well as by CD31, marking endothelial cells (Fig 3.1A). CD31 staining demonstrates the vascularization of the granulation tissue at this point. Notably, CD31 is primarily absent in the muscularis propria. By contrast, Ptgs2^{-/-} mice (n=6) exhibit a transmural granulation tissue phenotype with degradation of the muscularis propria at day 6 demonstrating impaired healing of the wound. CD31 positive cells throughout the wall of the colon suggest that the entirety of the organ in this location is made up of granulation tissue (Fig 3.1B). In support of this interpretation, these deficient mice also lack a healthy muscularis propria as shown with SMA (Fig 3.1B). These results suggest that Ptgs2 is indeed important in healing in this discrete injury model.

Figure 3.1

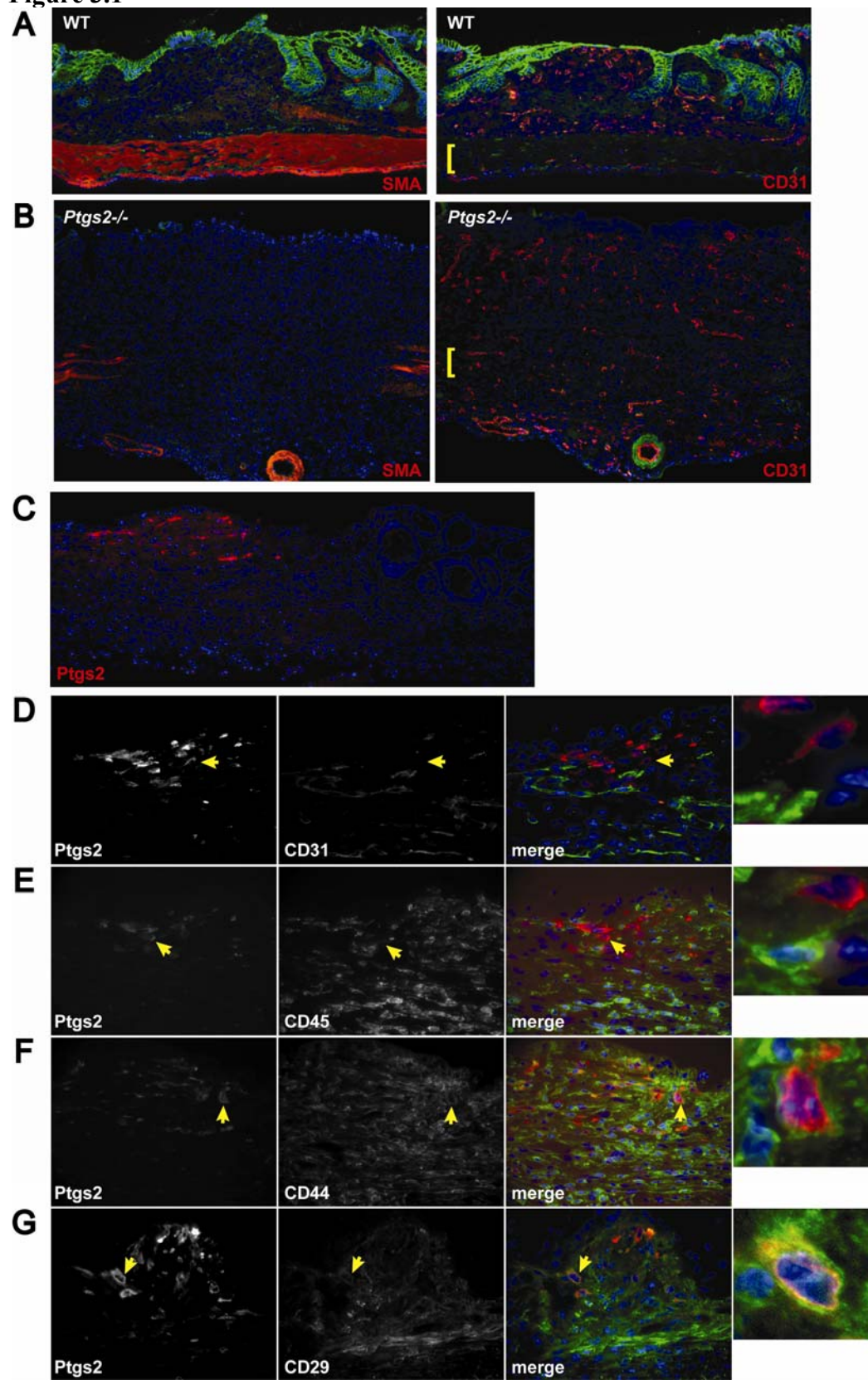


Figure 3.1 Ptgs2 is required for and present during proper wound healing.

WT C57Bl/6 or Ptgs2 ^{-/-} mice were injured by colonic biopsy and allowed to heal for six (A,B) or four (C-G) days before sacrifice and fixation with 4% paraformaldehyde. Seven micron sections were cut from frozen blocks and stained by indirect immunofluorescence and Hoechst dye. Primary antibodies used were anti-SMA (A,B, red), anti-CD31 (A,B, red; D, green), anti- β -catenin (A,B, green), anti-Ptgs2 (C-G, red), anti-CD45 (E, green), anti-CD44 (F, green), or anti-CD29 (G, green). Parenthesis (A,B) denotes area of muscularis propria in CD31-sections. Parenthesis marks muscularis propria. Arrowheads denote Ptgs2-positive cells that are enlarged to the right.

Ptgs2-positive cells in the wound bed are probable MSCs

As Ptgs2 seemed to be important in proper wound healing, we performed immunofluorescence using a specific Ptgs2 antibody in order to identify cells that may be the source of this gene within the wounded area. A large, discrete population of cells expressing abundant Ptgs2 protein in the upper wound bed, directly apposed to the WAE cells, was easily detected (Fig. 3.1C). In an attempt to identify the cell type(s) expressing Ptgs2, we co-stained sections with various lineage and surface markers. The lack of co-staining by CD31 or CD45 respectively demonstrated that these cells were neither of the endothelial nor hematopoietic lineage (Fig. 3.1D,E). We did, however, observe co-localization of Ptgs2 with both CD44 and CD29 (Fig. 3.1F,G). This pattern of surface markers, in conjunction with high Ptgs2 expression, has been recognized previously in our lab and marks a population of cells known as mesenchymal stem cells. We have

previously isolated MSCs from the colons of WT mice and used them to study the regulation of Ptgs2¹⁶². In these earlier investigations, we showed that the regulation of Ptgs2 has a strong post-transcriptional component and thus hypothesized that a trans-acting protein may be present in these cells capable of interacting with and regulating Ptgs2. Performing a gene ontology analysis as previously reported¹⁶², we identified a number of candidate mRNA binding proteins, specifically HuR, CUGbp1, CUGbp2, Rbms3, Rbpms, Rbm3, and Imp1, as exhibiting preferential expression in colonic MSCs and screened these genes to identify a novel trans-acting factor that may be playing a role in the regulation of Ptgs2 and consequently in the healing of colonic biopsy injury.

We screened these mRNA binding candidates by shRNA-mediated knockdown of the gene in cMSCs *in vitro* and observation of the effect of gene loss on Ptgs2 expression (Table 1). We focused further investigations on insulin-like growth factor 2 binding protein 1 (Igf2bp1 or Imp1) because, while it has been highly studied as an mRNA binder⁹⁵⁻⁹⁷, interaction with Ptgs2 has never been reported. Imp1 exhibits oncofetal expression, i.e. it is high during development, especially around day 12.5 in mice⁸⁷, and is also upregulated in various tissue specific cancers including colonic carcinoma, breast carcinoma, and melanoma⁸¹. However, at least one investigation reported detection of Imp1 in the distal intestine and testis of adult mice⁸⁸. Despite intense efforts^{78,79}, the target mRNA binding sequence of Imp1 has never been precisely defined and appears to be degenerate and affected by 3D structure of the transcript⁷⁹. Furthermore, while some targets are known, many remain to be elucidated. In fact, in one study estimated that Imp1 may bind up to 3% of the transcriptome⁷⁸. Taken together, these published data

along with our knockdown screen strongly suggested that Imp1 would be a worthwhile gene on which to focus for a potential novel function in regulating Ptgs2.

Table 1

Gene Knocked Down	Knockdown achieved	Ptgs2 fold change
HuR	-20	-3
CUGbp2	-30	-8
CUGbp1	-11	2.5
Rbms3	-35	-3.5
Rbm3	-9	-2
Rbpms	-12	-1.5
Igf2bp1	-7	-5

Table 1 Effect of decreased mRNA binding protein candidates on Ptgs2 expression.

Annotated mRNA binding protein candidates were identified by a cutoff of 2 fold preferential expression in cMSCs vs. bmMSCs on global gene array analysis. shRNAs specific for the candidates or control shRNA were transfected into HEK293T cells along with lentiviral packaging vectors to produce lentivirus that was then used to infect cMSCs in culture. After 1-2 passages, expression of the targeted gene and Ptgs2 were assessed with qRT-PCR. Values reflect the strongest effect on Ptgs2 expression. Control shRNA produced no more than a -1.5 fold change in Ptgs2 mRNA.

Imp1 protein interacts with Ptgs2 mRNA in the cellular environment.

In order to observe whether Imp1 and Ptgs2 interact within the physiological cell environment, a (3X)FLAG-tagged Imp1 construct was created and driven by a CMV-promoter in NIH3T3 cells. The FLAG-tagged construct was immunoprecipitated using a specific antibody for FLAG, and the protein was eluted from the pellet specifically by competition with 3X FLAG peptide. Total protein, eluate, and pellet fractions were

analyzed for protein content by immunoblot (Fig 3.2A) and associated RNA by quantitative RT-PCR (Fig 3.2B). We hypothesized that if Imp1 does indeed interact with Ptgs2 mRNA, then this transcript should be specifically pulled down by Imp1 in the eluate and pellet fractions of the immunoprecipitation procedure. As a control, we observed an 18S RNA abundance ratio in transfected vs. untransfected cells of near 1.0 in all fractions. However, while the Ptgs2 abundance ratio was approximately 1.0 in the total protein fraction, this ratio was significantly increased in both the eluate and pellet fraction (Fig 3.2B). These data demonstrated that Imp1-FLAG must specifically interact with Ptgs2 thereby enriching Ptgs2 in these fractions. Similarly, two FLAG-tagged deletion mutants of Imp1 containing either the four C-terminal KH domains or the two N-terminal RRM domains were also tested (Fig 3.2C). We observed that KH-FLAG similarly enriched Ptgs2 while RRM-FLAG did not (Fig 3.2D). The specific pull-down of Ptgs2 was thus neither an artifact of the transfection protocol nor the FLAG tag. The mutant data suggested that the functional interaction between Imp1 and Ptgs2 RNA resides in the KH domains though the strength or frequency may be decreased in the absence of the full-length protein. We concluded that Imp1 is indeed capable of interacting with Ptgs2 RNA in the cellular environment.

Figure 3.2

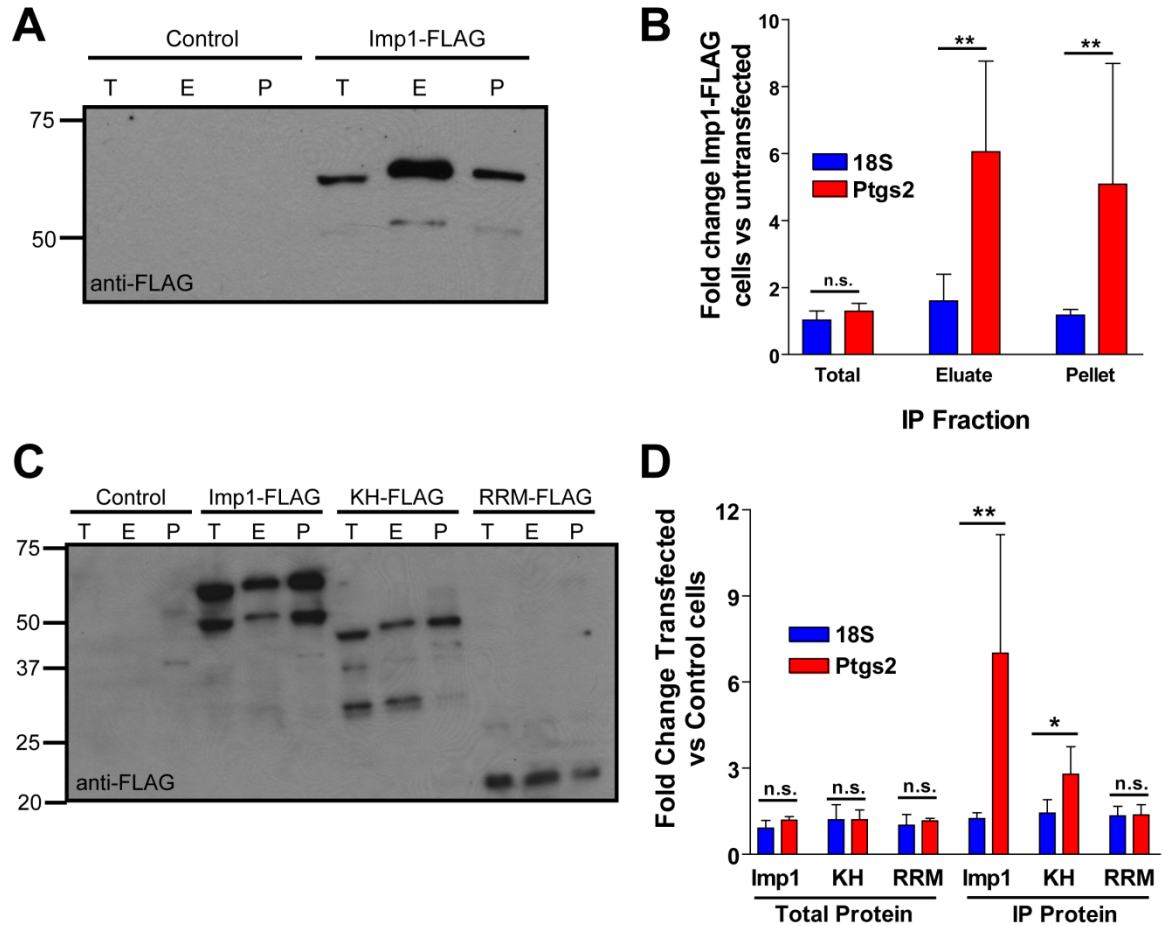


Figure 3.2 Imp1 interacts physiologically with Ptg2 mRNA.

NIH3T3 cells were transiently transfected with Imp1-FLAG, KH-FLAG, or RRM-FLAG, crosslinked with 300mJ 254nm UV, and lysed. Cell lysates were immunoprecipitated using M2 anti-FLAG conjugated agarose beads, and protein and RNA were isolated from different fractions. (A) Immunoblot with anti-FLAG-HRP showing expression of Imp1-FLAG and successful pulldown and elution of the protein. (B) Quantitative RT-PCR graph of 18S and Ptg2 RNA measured in the various fractions during immunoprecipitation baselined to untransfected control. (C) Immunoblot with anti-FLAG-HRP as in (A) including transient transfection and

immunoprecipitation of KH-FLAG and RRM-FLAG. (D) Quantitative RT-PCR graph of 18S and Ptgs2 RNA measured in the total and pellet fractions during immunoprecipitation baselined to untransfected control. Immunoblots are representative of at least 3 individual experiments and qRT-PCR graphs include data from 3-4 individual experiments. Abbreviations: T, total protein; E, eluate; P, pellet. Statistics: unpaired student's t test; n.s. not significant, * $p < 0.05$, ** $p < 0.01$.

Imp1 expression is increased in the wound bed and co-localizes with Ptgs2 expression.

After demonstrating Imp1 and Ptgs2 interaction, we investigated whether Imp1 plays a role *in vivo* in the colonic biopsy injury model. Measurement of Ptgs2 and Imp1 expression in the wound bed compared to adjacent normal mucosa by qRT-PCR revealed strong upregulation of both genes (Fig. 3.3A), demonstrating that expression of Imp1 coincided in place and time with Ptgs2 upregulation. Notably, another well-known binder of Ptgs2 mRNA, CUGbp2, did not show similar upregulation..

We next assessed whether Imp1 was found in the Ptgs2-positive wound bed stromal cells. Co-immunofluorescence was performed using antibodies for Imp1 and Ptgs2. In agreement with our expectations following the *in vitro* pull-down experiments and *in vivo* RNA expression data, we found that Imp1 and Ptgs2 proteins were indeed located in the same cells in the upper portion of the wound bed (Fig 3.3B). Quantitatively, we have observed that approximately 30% of Ptgs2-positive cells are also Imp1-positive while all Imp1-positive cells are also Ptgs2-positive (Fig 3.3C). These data suggest that the presence of Imp1 may actually mark a specific type of Ptgs2-positive cell. Alternatively,

in some cells, Imp1 may have been down-regulated below detectable levels while Ptgs2 remains more strongly expressed within the time frame observed.

Figure 3.3

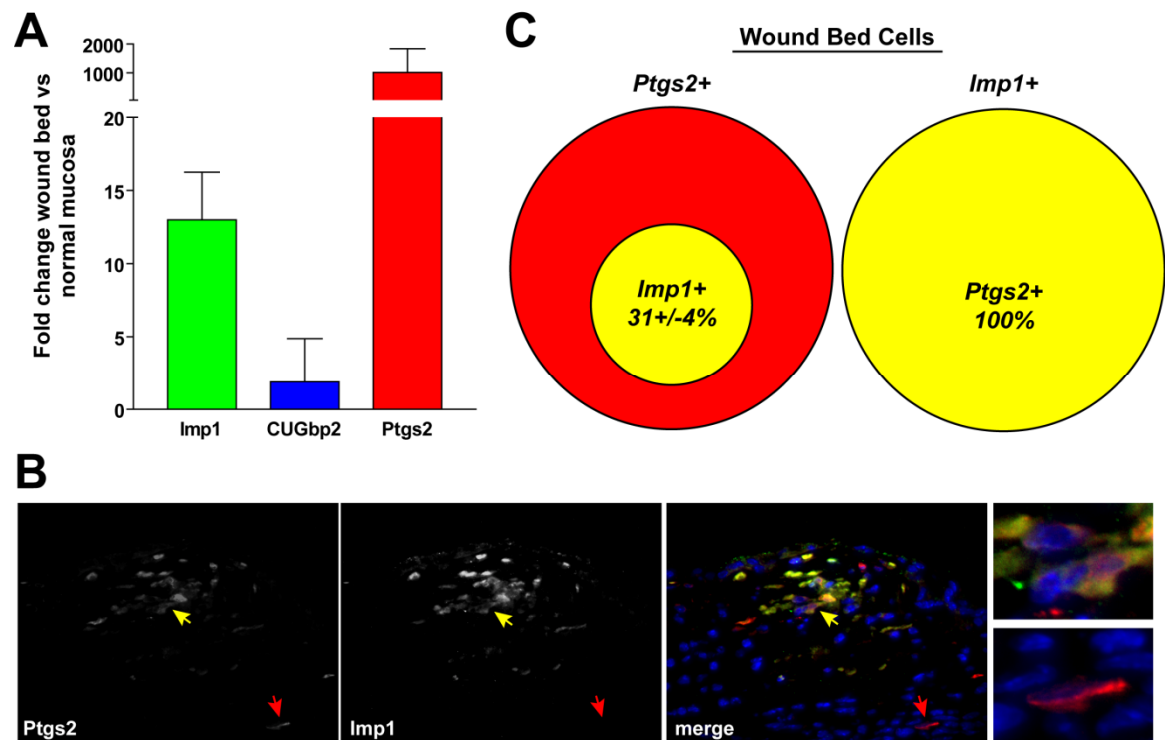


Figure 3.3 Imp1 is expressed in Ptgs2-expressing wound bed stromal cells.

(A) Quantitative RT-PCR graph showing relative RNA abundance of Imp1, CUGbp2, and Ptgs2 in biopsy injured mucosa compared to uninjured mucosa. (B) Indirect co-label immunofluorescence of the wound bed using rabbit anti-Ptgs2 and goat anti-Imp1. Mice were sacrificed two days following colonic biopsy wounding, transfused with 4% paraformaldehyde and tissues processed for immunofluorescence. Yellow arrowhead depicts representative co-labeled cells in upper right enlargement. Red arrowhead depicts single-label Ptgs2-positive cell in lower right enlargement. (C) Venn diagram

depicting the percentage of Ptgs2-positive cells in the wound bed that is also Imp1-positive and the percentage of Imp1-positive cells that is also Ptgs2-positive.

Chapter 3: Discussion

Mechanisms of Ptgs2^{-/-} colonic biopsy phenotype

We observed transmural granulation tissue at the site of colonic biopsy wounding by CD31 staining rather than intact healthy tissue layers such as the muscularis propria normally present and visualizable by SMA. It is likely that this degradation of tissue and replacement by granulation tissue occurs due to the overactivity of the immune response in the absence of modulatory signaling from Ptgs2 mediators. Furthermore, we have noted that the overlying WAE cells do not seem to be fully formed in Ptgs2^{-/-}. If the integrity of this layer is compromised, bacteria and other antigens may infiltrate the wound and further enhance the inflammatory response. The specific effects of Ptgs2 expression on the healing of colonic biopsy injury remain to be precisely elucidated. Prostaglandins, as the downstream products of Ptgs2 expression and activity, are likely to be playing at least a partial role in colonic biopsy healing. We are currently investigating the timing of secretion of these mediators and whether the exogenous addition of specific prostaglandins, such as PGE₂ or PGI₂, may aid in healing.

Imp1 as a marker

We have described a population of stromal cells within the wound bed of colonic biopsy injured mice that express both Ptgs2 and Imp1. The recognition of Imp1 expression in this discrete subset of stromal cells during healing in adult tissues demonstrates the potential usefulness of Imp1 as a novel cell marker. Fibroblasts are a poorly defined cell type so-called primarily due to their morphology alone, though the idea that different subsets exist is widely-accepted¹⁶³. However, the lack of defined markers specific to their identity and/or function hampers the classification of specific

populations of fibroblasts and their unique functions. In our injury model, we have found Imp1 expression confined to a unique population of stromal cells in both time and space. Imp1 may thus serve as a marker of this subset. Targeting this cell population genetically by Imp1 driven genetic tools could prove valuable in the definition and functional description of a unique subset of fibroblasts.

Imp1 expression and function in the adult

The observations herein also raise interesting questions as to the role of Imp1 in the adult animal. Considering the common overexpression of Imp1 and Ptgs2 in various cancers, it would be worthwhile to investigate whether these two genes are correlated both in presence and in level of expression in the same oncologic samples. Such descriptive studies could help precisely define different cancer subtypes from common tissues of origin as well as develop directed targets that may have fewer side effects than more generic inhibitors of Ptgs2.

Furthermore, recent data detailing Imp1 interactions with viral RNAs^{98,99} demonstrates the need for understanding the relevance of Imp1 expression *in vivo*. The ability of Imp1 to increase replication of HCV as well as the presence of Imp1 in the HIV ribonucleoprotein complex in cells raises interesting questions. Is Imp1 expressed at sufficient levels in the adult mammal in certain cell types susceptible to these viruses? Would the loss of this mRNA binding protein decrease the infectivity of the virus in whole organisms? Most expression studies of Imp1 have been whole organism- or organ-oriented and thus small populations of cells expressing the gene could easily have been overlooked or diluted out in samples. The answers to the questions posed will

require detailed expression studies within adult mammals, especially of cells known to be targeted by HIV or HCV virus.

No studies to date report upregulation or visualization of Imp1 in wounded or healing tissue within the context of the organism. The reported role of Ptgs2 in other models of injury in the lung and other tissues^{164,165} and the newly demonstrated interaction between Imp1 protein and Ptgs2 mRNA provide the tantalizing proposition that Imp1 expression may be found in many other injury models. Might Imp1 expression in injury and healing be a generalizable phenomenon? Histochemical and expression analysis of stromal tissues involved in injury and repair of other organs holds the potential of uncovering discrete Imp1 expression. These studies may reveal a new role of Imp1 in the adult mammal in addition to the well-recognized correlation in cancer.

Summary

Ptgs2 expression and function are required for the proper response to colonic biopsy injury, a localized physical insult resulting in inflammation and subsequent tissue regeneration. Its expression correlates with the expression of the mRNA binding protein Imp1 that can interact with Ptgs2 mRNA within the cellular environment. This novel interaction of Imp1 and Ptgs2 as well as their common localization in wounded tissue highlights the need for further exploration of this novel relationship and may reveal a more generalizable role of Imp1 in adult tissue.

CHAPTER 4

Summary and Future Directions

Chapter 4: Abstract

The role of Ptgs2 and its downstream synthetic products in colonic injury has been well-documented. The studies detailed in this thesis sought to uncover and define the mechanisms involved in the regulation of this important enzyme and focused on the post-transcriptional regulation of this gene. While the transcriptional regulation of Ptgs2 has been a focus of many investigations due to its recognized upregulation downstream of macrophage activation in inflammation, the post-transcriptional regulation may be more relevant in homeostasis in cells that constitutively express the gene. We have shown that Ptgs2 post-transcriptional regulation can be mediated by Fgf9 signaling through Erk activation and CUGbp2 stabilization. In addition, the mRNA binding protein Imp1 can also interact with Ptgs2, and loss of expression of this gene results in decreased Ptgs2. Notably, these various mechanisms of Ptgs2 post-transcriptional regulation may be important in homeostasis, response to injury, and healing.

Chapter 4: Summary of the Thesis and Future Directions

Interactive roles of multiple mRNA binders on Ptgs2

It has become more widely recognized that mRNA transcripts are generally found not free-floating in the cytoplasm but bound and organized in larger structures called RNPs or ribonucleoproteins¹⁶⁶. Ptgs2 can be bound by a number of proteins¹⁶⁷, and this thesis demonstrates that Imp1 is a new addition to the list of mRNA interactors. The identities and interactions among these various proteins must be fully understood in order to create an accurate illustration of the overall regulation of Ptgs2 at the post-transcriptional level.

The most highly-studied sequence within the Ptgs2 transcript that is a target for mRNA binders is the ARE (AUUUA) signature. This sequence is degenerate in that it is present in a number of different mRNA transcripts. Indeed, the presence of AREs in many inflammatory and anti-inflammatory transcripts upregulated by TNF α stimulation help control the timing of the expression of these genes¹⁶⁸. How then is regulation of the transcript accomplished differentially from other inflammatory genes? It is conceivable that cells may employ a number of different proteins targeted to different RNA sequences that would allow for more precise control of stability, translation, and even localization of Ptgs2 independent of other ARE-containing transcripts. Our data, as well as that of others¹⁶⁷, demonstrates that expression of Ptgs2 can be affected by a number of different mRNA binding proteins. A more complete understanding of the interaction of transcripts and binding proteins will elucidate the mechanisms underlying control of one specific transcript post-transcriptionally.

A goal yet to be attained is an accurate knowledge of the interactions of the various proteins and their transcripts, including but certainly not limited to Ptgs2. What is the

stoichiometry and in which circumstances are certain binders required? Some insight has been provided into these sorts of questions in the case of Ptgs2 with proof of interaction between various mRNA binding proteins by co-immunoprecipitation^{78,169}. Interaction of other binding proteins of Ptgs2, specifically HuR and Rbm3, has also been demonstrated using a yeast two hybrid system¹⁷⁰. Descriptive and functional studies investigating these protein-protein and likely protein-protein-RNA interactions will facilitate our ability to predict and understand the intricacies of post-transcriptional regulation.

This work also raises the very simply stated question: Can Imp1 and CUGbp2 interact with Ptgs2 simultaneously? While CUGbp2 and HuR are both able to bind to Ptgs2, it seems that different circumstances allow for more productive or greater affinity binding of one or the other to their common ARE target sequence in the 3' UTR of Ptgs2. What is the role of Imp1 in these various circumstances? Is Imp1 capable of interacting with Ptgs2 simply whenever the protein is present or is this interaction mediated by certain conditions and the signaling state within the cell? To begin these investigations, co-immunoprecipitation experiments of the protein and of a biotinylated Ptgs2 RNA probe can be performed. These questions must be answered to address the hypothesis that Imp1 and CUGbp2 cooperate in the precise regulation specifically of Ptgs2.

One last line of inquiry stemming from the findings of this thesis is how exactly Ptgs2 is destabilized. As briefly mentioned in Chapter 3, CUGbp1 knockdown results in an *increase* in Ptgs2 expression; however, CUGbp1 has never been reported to affect or bind Ptgs2 mRNA. Considering the high degree of similarity of CUGbp2 and CUGbp1, it is likely that they may compete for binding of the same sequence of Ptgs2 mRNA. Could it be that in starvation conditions, the affinity of CUGbp2 for the ARE is diminished thus

allowing access of the transcript to CUGbp1? Binding of CUGbp1 mediates enhanced degradation of the targeted mRNA¹⁷¹ by deadenylation. Experiments designed to investigate the relative affinities and effects of CUGbp1 and CUGbp2 would test the hypothesis that CUGbp2 out-competes CUGbp1 during normal conditions while under alternate conditions, such as starvation, CUGbp1 binding relative to CUGbp2 increases, resulting in decreased Ptgs2 due to an increased rate of RNA degradation.

Thus, many further intriguing lines of investigation into the various roles and interactions of Ptgs2 trans-acting factors are suggested by the experimental findings herein.

Roles of mRNA binding protein phosphorylation.

It has been demonstrated that, in at least one interaction of Imp1 with mRNA, tyrosine phosphorylation controls post-translational Imp1 activity⁹⁷. Considering the rapidity with which Ptgs2 is lost in starvation, the possibility that phosphorylation is important in the pathways regulating Ptgs2 is high as this post-translational change can have rapid effects on function. This work demonstrates that activation of Erk by phosphorylation downstream of Fgf9 seems to act in the stability of Ptgs2. It has already been demonstrated that CUGbp2 activity can be affected by tyrosine phosphorylation³⁰, and our results suggest that there is likely a serine/threonine phosphorylation that may also play a role downstream of Erk. This remains to be investigated.

Whether phosphorylation of newly-identified Ptgs2 regulator Imp1 is important its control of Ptgs2 must also be investigated in order to understand the mechanism of activity. Phosphorylation of Imp1 can affect its functional activity in binding to β -actin mRNA⁹⁷. We also have found evidence that suggests that phosphorylation may play a

role in Imp1 activity. Visualization of Imp1 on immunoblot using a commercially available goat polyclonal antibody demonstrates a shift in the band upon starvation suggesting that some post-translational modification such as phosphorylation has been altered in these conditions. Detection of the protein by another antibody, a mouse monoclonal, is complicated by its nonspecific detection of a band in fetal bovine serum of a similar size to Imp1. However, starvation allows removal of this band. Phosphatase treatment for 15min using calf intestinal phosphatase (CIP) of cell lysates was performed to begin to investigate these observations. Treatment was monitored using Gapdh and pErk to confirm phosphatase activity. We found that CIP and not buffer alone results in loss of detection of Imp1 by this mouse monoclonal antibody while a band is still detected by the goat polyclonal (Fig 4.1A). A possible explanation for this could be that the mouse monoclonal shows specificity for a phosphorylated form of Imp1. This remains to be further investigated. We have also asked whether starvation of cells results in differential interaction between Imp1 protein and Ptgs2 mRNA using our Imp1-FLAG pull-down protocol. Pull-down fractions of cells either starved 25min or normally treated showed no significant difference in enrichment of Ptgs2 (Fig 4.1B). This suggests that the Ptgs2 transcripts that are still present after this starvation protocol remain associated with Imp1. It could be that Ptgs2 can be degraded while still associated with Imp1 or that Ptgs2 is immediately degraded upon dissociation from Imp1. These questions must be tested in order to understand the mechanism of Ptgs2 degradation and of Imp1 interaction with Ptgs2.

Figure 4.1

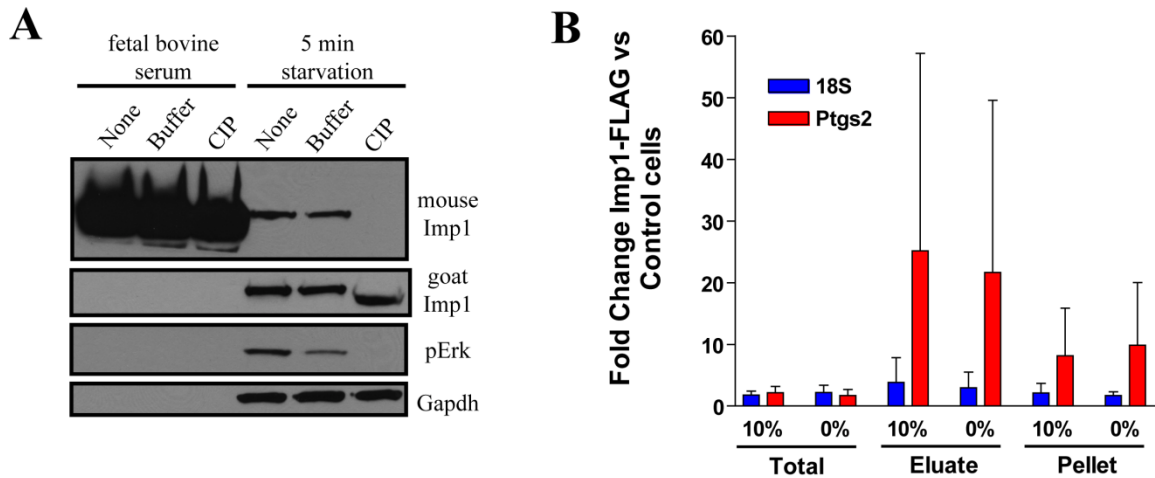


Figure 4.1 Imp1 phosphorylation and Ptgs2 interactions in 0% serum.

(A) Immunoblots of CIP-treated cell lysates demonstrating that treatment affects the detection of Imp1 by a mouse monoclonal antibody. Fetal bovine serum alone or 5min serum starved cells were lysed in RIPA with protease inhibitors only, and aliquots were treated either with buffer alone or with CIP for 15min at 37°C. Antibodies used were mouse monoclonal anti-Imp1, goat polyclonal anti-Imp1, anti-pErk, and anti-Gapdh and were indirectly visualized by secondary HRP-conjugated antibodies. (B) qRT-PCR graph of 18S and Ptgs2 RNA measured in the total, eluate, and pellet fractions of the immunoprecipitation protocol baselined to untransfected control. Control and transfected cells were either starved (0%) or not (10%) for 25min prior to IP protocol.

Control of Imp1 regulation

The regulation involved in Imp1 expression in colonic injury has not been determined. Identification of the extracellular signal(s) capable of upregulating Imp1 during injury

within the wound bed would add to the overall understanding of the biology of both Imp1 and of wound healing. The signals involved could be singular or multiple. One or multiple soluble factors could be required such as the many growth factors found within the wound bed or a precise cytokine signature from the local immune cells. Membrane-bound signals may also play a role. Indeed, the discrete area of expression of Imp1 in the wound bed suggests that such factors may be important either from neighboring cells or from the extracellular matrix of the wound bed. It does appear to be likely that multiple factors are involved based on data acquired thus far.

We have shown multiple soluble factors that, singularly acting on either cMSCs or NIH3T3 cells, have no discernable effect on Imp1 expression. These include Wnt3a, BMP4 and BMP8A, TNF α , IFN γ , LPS, and EGF (Fig 4.2 and data not shown). It is interesting to note that, while others have shown that β -catenin activation can increase Imp1 expression in different circumstances¹⁰⁰, activation of the same pathway by canonical Wnt signal has no effect on cMSC or NIH3T3 cell expression of Imp1 after 2-3 hours.

Figure 4.2

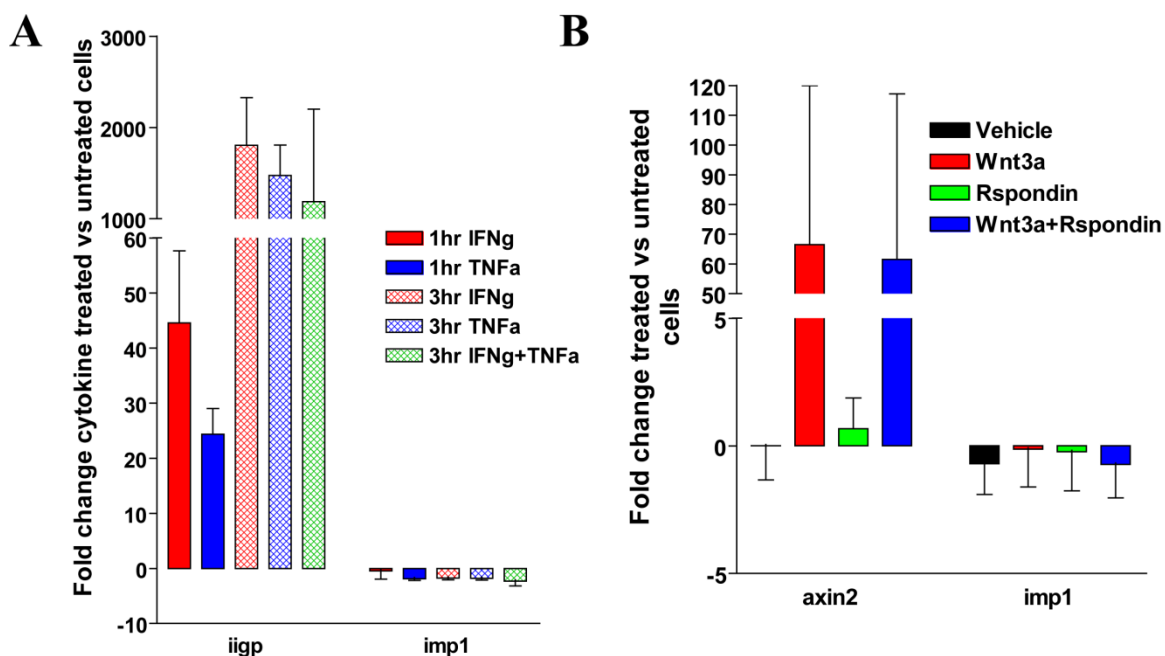


Figure 4.2 Imp1 regulation in cMSCs is not affected by Wnt, TNF α , or IFN γ .

Graphs depicting fold change of genes measured by qRT-PCR. (A) Fold change of *iigp* and *Imp1* in cells treated with IFN γ , TNF α , or both. (B) Fold change of *axin2* and *Imp1* in cells treated with Wnt3a, Rspondin, or both. Error bars represent standard deviation.

One potential source of this signal is the epithelium surrounding the wound bed, with the overlying WAE cells as the strongest candidates due to the close localization of *Imp1* positive cells. We have isolated RNA from the WAE cells, adjacent epithelium, and normal epithelium by laser capture microscopy and analyzed this material by microarray (unpublished, C. Luo). This data may provide candidates for further studies into the regulation of *Imp1* of soluble and/or membrane-bound basis.

Imp-1 is not simply an oncofetal protein

The findings described herein show that Imp1 cannot simply be regarded as an oncofetal protein. We have shown that it is upregulated in at least one injurious circumstance, and others have shown that Imp1 is expressed under homeostatic conditions in the mouse⁸⁸. It is logical to hypothesize that, with the demonstrated ability of Imp1 to interact with Ptgs2 mRNA and the documented necessity of Ptgs2 within various colonic injury models^{14,20,23}, that the upregulation of Imp1 partially mediates increased Ptgs2 expression. We also witness preferential expression of Imp1 in cMSCs versus bmMSCs suggesting that Imp1 is expressed in the normal colonic Ptgs2-expressing stromal cell. It may indeed be that the expression of Imp1 by qRT-PCR reported by others in the adult colon is found primarily within the Ptgs2-expressing stromal cell type. Imp1 presents itself thus as a strong candidate for lineage specification of this relatively undefined cell type. Up to this point, Ptgs2 expression has been the strongest and most specific marker of this mesenchymal stem cell stromal cell type in the colonic mesenchyme. However, Ptgs2 as a marker provides considerable complications as it can also be upregulated in myeloid-derived hematopoietic cells in inflammation. Imp1 may be a new, more specific marker of this lineage. Creation of a mouse in which lacZ or GFP is expressed by the Imp1 promoter may provide new insight into the origin, lineage, and movement of these cells.

Furthermore, the role of Imp1 within the adult animal requires further definition. While a global knockout of Imp1 has been created⁹⁴, the fact that Imp1 is highly expressed during embryogenesis complicates the interpretation of possible Imp1

functions in the adult. The ideal tool to investigate the potential roles of Imp1 during post-natal life is a mouse expressing a floxed Imp1 gene. This mouse could then be bred to various strains that express Cre under different promoters, allowing for the investigation of the role of Imp1 in specific cell types or tissues. Alternatively, and potentially more powerfully, this mouse could be bred to an inducible Cre in order to remove Imp1 at specific stages in the adult followed by investigation of the effect of such deletion. For example, Imp1 could be deleted in the mouse just prior to colonic biopsy which would allow for specific investigation of the role of Imp1 in this injury model. Further defining the role of Imp1 is essential not only to uncover the potential biological roles of Imp1 but also to understand the precise function of this gene in injury.

The role of Ptgs2 in acute injury

The mechanistic progression underlying the observed phenotype of transmural granulation tissue in colonic endoscopic biopsy in Ptgs2^{-/-} mice remains to be elucidated and may be multi-faceted. One strong possibility is that the inflammatory response occurs unchecked by the normal upregulation of Ptgs2 and consequent secretion of prostaglandins. A careful count of the ratio of neutrophils and macrophages at the early stages could help to address this issue as well as measurement of local and serum inflammatory cytokines. It has been shown that M2 phenotype of macrophages and Th2 cytokines are important in healing¹⁵; thus, determination of the phenotype of macrophages present in the wound of Ptgs2^{-/-} mice could demonstrate increased inflammatory state. Assessment of the level of microbial infiltration in the wound bed is also warranted. It appears that the integrity of the epithelial layer in Ptgs2^{-/-} may be compromised. Luminal bacterial populations may therefore invade the wound bed,

which could also contribute to a differential or greater immune response compared to WT mice.

Another interesting line of investigation involves testing whether the observed transmural granulation tissue phenotype can be rescued by exogenous addition of specific mediators. Two of the best possibilities are a long-lasting PGE2 (dmPGE2)¹³ or hematopoietic cells from WT mice that express Ptgs2.

Finally, the required cellular source of Ptgs2 remains to be rigorously demonstrated. It is highly likely that Ptgs2 must be expressed by the observed stromal cell population in the wound bed. A few new genetic tools must be created in order to reliably test this hypothesis. While a floxed Ptgs2 mouse has been made¹⁷² and used for similar cell-specific studies¹⁷³, a stop flox Ptgs2 driven by a ubiquitous promoter would allow targeting of Ptgs2 expression to specific cell types. The cellular source of Ptgs2 can then be tested both by lineage-specific knockout as well as by lineage-specific expression of Ptgs2. One potential experiment involves specific Ptgs2 knockout of the mesenchyme, along with expression of Ptgs2 in hematopoietic cells either genetically-driven or by bone marrow chimera. Alternatively, creation of a knock-in Imp1-driven Cre-IRES-GFP may be valuable as it seems to be capable of knocking out Ptgs2 in a very specific subset of cells. Intriguingly, this last model would allow for Ptgs2 expression in all other cells except those expressing Imp1 while the GFP would allow for visualization of the cells expressing Imp1 and therefore lacking Ptgs2.

Imp1 as a marker for MSC/fibroblast

Fibroblasts are a highly heterogeneous population of cells found in every organ. However, referring to all morphologically fibroblastic cells as fibroblast is akin to

referring to all T cells, B cells, NK cells, macrophages, etc as immune cells. It lacks precision. This fact has become more recognized as fibroblasts have begun to be sorted into somewhat smaller subsets such as ‘tumor stromal cell’ or, as is of interest here, ‘MSC’¹⁶³. A complete understanding of the various types of fibroblasts and the potentially differing activities and functions is hindered by a deficiency in precise markers such as those that have been recognized in other cellular types. The finding that Imp1 is expressed in injury in a specific subset of stromal cells suggests a new marker of an as-yet-undefined fibroblast population. Creation of an Imp1 reporter mouse (or, as mentioned above, Cre-IRES-GFP for greater flexibility of use) may allow lineage tracing of these cells, definition of a fibroblast subset, and contribute to the general field of fibroblast biology.

Final Summary

The work contained in this thesis provides a deeper understanding of post-transcriptional Ptgs2 regulation and places these observations within the context of multiple forms of colonic injury. The applicability of these findings to Ptgs2 in other injury systems or in other cell types may prove to be useful as well, especially in understanding the constitutive expression of this gene. Further investigations into the mechanisms driven by Ptgs2 involved in mediating colonic injury will be aided by this additional knowledge of the activity of various mRNA binding proteins. Most notably, this work uncovers the novel interaction of Imp1 and Ptgs2 and provides intriguing observations on the likely role this may play in physical colonic injury. Future studies will benefit from building upon these findings in order to create a complete mechanistic

picture of Ptgs2 regulation and its function in colonic injury, and hopefully, will aid the medical field in creation of targeted diagnostic tools and treatment protocols.

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