HOMEOSTATIC ROLES FOR ADULT INTESTINAL HEDGEHOG
SIGNALING: INSIGHT INTO SMOOTH MUSCLE MAINTENANCE
AND INFLAMMATORY CONTROL

by

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Chapter 1

Introduction

Cell to cell communication is essential for embryonic development; soluble

signals control multiple aspects cell migration, cell proliferation, tissue patterning and

specification of cell fate. A surprisingly small number of conserved signaling pathways,

including the Hedgehog (Hh), Wnt, BMP, TGF-β, Notch, and receptor tyrosine kinase

signaling pathways, are used to regulate a myriad of patterning steps in the context of

multiple organs (1). In fact, many birth defects in humans and other animals have been

directly tied to genetic alterations in these developmental signaling pathways. These

pathways have been the object of intense investigation and while their general roles -

including control of fate specification, proliferation, and migration - are clearly outlined,

much less is known about the precise molecular pathways, cellular targets, and regulatory

gene networks that are modified by these signaling pathways during development.

In addition, a growing body of evidence supports an important role for these same

developmental signaling pathways in the function of adult organs. Moreover, pathway

activity is often dysregulated in pathological conditions such as carcinogenesis. The

importance of these developmental pathways during homeostasis of adult organs is

difficult to study, however, given that key genetic knockout and transgenic mouse models

with modifications in signaling often show severe phenotypes before or immediately after

birth, precluding adult study. Also, it is not always possible to extrapolate developmental findings to adult life, since, due to redundancy and context-dependent function, molecules that are apparently dispensable in fetal life can be critical regulators of adult biology. Thus, studies designed to probe the role of developmental signaling pathways during adult life may have important implications for understanding adult biology and the development of pathological conditions in animals and humans. The aim of the work described in this thesis is to uncover details underlying the activity of one of these signaling pathways, Hedgehog, in the homeostatic control of the adult intestine.

The Hedgehog Signaling Pathway

The Hedgehog signaling pathway is an important mediator of cellular communication that is utilized in multiple contexts in multicellular organisms. The pathway was initially identified by genetic screening for molecules that affected larval segmental patterning in the fruit fly *Drosophila Melanogaster* (2-4), and named for the distinctive bristled appearance of embryos mutant in the *hh* gene. Other major members of the Hh pathway in the fly include two transmembrane molecules, patched (ptc) (5) and smoothened (smo) (6), and an intracellular transcriptional mediator of Hh pathway activation, cubitus interruptus (ci) (7; 8). In the absence of hh ligand, ptc inhibits the activity of smo (9). As a result of smo inhibition, an intracellular complex which includes costal2 (cos2), fused (fu), and suppressor of fused (sufu) is activated; this complex proteolytically cleaves ci, generating a repressor form which translocates to the nucleus and inhibits the expression of pathway target genes. In the presence of hh ligand,

direct interaction of hh with ptc prevents ptc from inhibiting smo, smo inhibits the cos2

complex, ci is not degraded, and full length ci translocates to the nucleus where it

activates hh target genes (10-12).

The Hh pathway also is present in vertebrate lineages, and overall the pathway is

very conserved with that of *Drosophila*. In mammals, three distinct hh orthologues are

expressed – Sonic (Shh), Indian (Ihh), and Desert (Dhh) hedgehog (13). The difference

in the pathway activation achieved by these ligands is not clear, and seems to be related

primarily to expression domain and level. Shh is the most broadly expressed, with

prominent roles during development in left-right asymmetry (14), as well as patterning of

the neural tube (15), the GI tract (16; 17), the lung (18), the limb (19), the urological

system (20), the skin (21; 22), and other organs. Ihh is required in the GI tract (16), bone

(23), and during lineage differentiation in the hematopoietic system (24; 25). Dhh is

involved in the development of the sheaths of peripheral neurons (26) as well as the

testes (27; 28). In several organs, including the gonad (29), prostate (30), and GI tract

(16; 17), multiple Hh ligands are co-expressed and presumably cooperate to drive Hh

pathway activation. All of these ligands interact with Ptch1 and/or Ptch2 (mammalian

orthologues of *Drosophila* ptc), and this interaction prevents Ptch from inhibiting the

activity of Smo (orthologous to smo). Recent studies have indicated a critical role for

localization of Ptch1 and Smo to cilia in the vertebrate Hh signaling pathway, and

disruption of primary cilia is capable of recapitulating many aspects of loss of Hh

signaling (31; 32). Also present in the mammalian pathway is a complex including Cos2

(cos2) and Sufu (sufu) that regulates the activity of the Gli family of ci-related

transcription factors both in the nucleus and the cytoplasm (33).

There are 3 distinct ci orthologues in vertebrates, Gli1, Gli2, and Gli3. Gli2 and Gli3 are closest in function to Drosophila ci: they are expressed in cells which are not actively responding to Hh signaling, and are processed to repressor forms in the absence of Smo activation (34). Interaction of one of the Hh ligands with Ptch1 leads to activation of Smo, inhibition of the Cos2 complex, and translocation of activator forms of Gli2 and Gli3 to the nucleus. Gli3 has been shown to act primarily as an inhibitor of Hh signaling in the limb (35; 36) and neural tube (37). The situation is complex, however, as Gli3 can act as an activator in other contexts, sometimes even during different stages of the same development processes (38). The propensity of Gli3 to act as a repressor may be related to the fact that it is more easily converted to a repressor form than Gli2 (34). In contrast to Gli2 and Gli3, Gli1 appears to act exclusively as a transcriptional activator, and is expressed only in cells that are actively responding to Hh signals (39); knockout of Gli1, unlike Gli2, Gli3, or Ptch, results in no obvious developmental abnormalities (40). Gli1 is a direct, early target gene regulated by Gli2, and is expressed at high levels in cells undergoing a robust Hh response (39). Gli1 is therefore not usually regulated by the Cos2 complex, and the activity of Gli1 is instead regulated through control of expression (39), interaction with inhibitors including Sufu (33), and by degradation; indeed, control of protein stability was recently demonstrated to be a key modulator of Gli1 activity (41).

The Hh pathway includes a number of other molecules known to regulate the activity of the pathway; these are primarily modulators of Hh signaling that are expressed

on the surface of Hh-responsive cells. This class of molecules includes ihog in *Drosophila*, the ihog orthologues Cdo and Boc in mammals, and the mammalian-specific factors Hhip and Gas1. Mammalian Gas1 binds to Hh ligands and promotes Hh signaling, particularly at a distance from the source of ligand (42; 43). In *Drosophila* and mammals, ihog, Cdo, and Boc act in a similar but not conserved manner (44), binding to Hh ligand and promoting Hh binding to ptc/Ptch1 (43; 45; 46). In contrast, Hhip is a cell surface molecule that binds to Hh ligand with high affinity and sequesters Hh ligand away from Ptch1 (47); a form of Hhip that does not include the transmembrane domain has been used by our laboratory as a secreted inhibitor of Hh signaling (48).

Several of the Hh pathway members are also regulated, directly or indirectly, by Hh pathway activation. Gli1 is a direct early target of Hh signaling, and is expressed only in cells which are actively responding to Hh signals, making it an ideal marker of active Hh response (39); mRNA expression of Gli1 and LacZ expression in Gli1$^{+/LacZ}$ animals are both sensitive readouts for Hh pathway activation. Ptch1 is also a direct Hh target. It is upregulated in cells responding to Hh signals and acts as a feedback inhibitor limiting the extent of intracellular pathway activation in the absence of continued ligand presence (49). Hhip is also upregulated in Hh expressing cells, acting as a feedback inhibitor of Hh signals (47). In addition, ihog, Cdo, Boc, and Gas1 are negatively regulated by Hh signaling (43; 45; 46), and are expressed in cells that are not currently responding to Hh signals, where they act to improve the response of this population to Hh signals. Regulation of these factors allows fine-tuning and feedback control in the Hh pathway; given that Hh is known to act as a morphogen (50), these factors may help

provide specificity of responses to a gradient of Hh ligand.

Functions of the Hh Signaling Pathway in Vertebrate Development and Disease

During vertebrate development, Hh signals have been implicated in control of cellular proliferation, cell fate decision-making, and tissue patterning in multiple contexts. Often these roles happen simultaneously during organogenesis, but certain systems have allowed careful examination of specific roles for Hh in development. Together these examples provide information about the effects of Hh signaling on target tissues during development, and insight into how dysregulated Hh signaling can lead to pathological conditions.

Hh signals have been implicated in control of proliferation in several systems, including the neural tube, limb, and small intestine. Careful studies have been done interrogating this role in the skin, where loss of Hh signaling prevents the progression of hair follicle morphogenesis beyond the follicular stage (21; 51; 22). In particular, studies from both mice and humans demonstrate that increased activation of the Hh signaling pathway leads to an increased proliferative response and the subsequent development of skin cancers (52; 53). Notably, even relatively subtle modification of the pathway in the context of expression of a form of Gli1 with reduced degradation is sufficient to accelerate the development of skin tumors in mice (41). Indeed, recent Stage One clinical trials utilizing a pharmacological inhibitor of Hh signaling to treat patients with Basal Cell Carcinoma have shown promising results (52), further emphasizing the

importance of the Hh pathway in aberrant proliferation. These studies demonstrate the important role that Hh signaling plays in controlling proliferation in the skin.

The Hh pathway is also critical in the control of cell fate decisions. Several studies in the vertebrate neural tube have demonstrated an important role for Hh as a critical ventralizing signal. Hh expression from the floor plate and notochord is required for inducing ventral neuron fates, and modulation of Hh signaling through knockout of Gli factors or Shh causes loss and mispatterning of ventral neuron populations (54; 38). Strikingly, knockout of both Shh and Gli3 partially restores ventral populations (37), indicating that both activator and inhibitor functions of Gli proteins are required for proper pattering of the neural tube. Recent studies have shown that Hh-dependent patterning in the neural tube also requires pathway modulators including Gas1 and Cdo (42; 43). Together these studies have demonstrated how careful modulation of Hh signaling level can help refine tissue patterning, and emphasized the importance of a controlled temporal and spatial Hh response in the establishment of developmental fate.

Another common effect of Hh signaling is polarization of a tissue during patterning. For example, during limb development, Hh signals produced in the zone of polarizing activity (ZPA) are instrumental in the anterior/posterior patterning of the limb (55), and act in part by inducing the expression of other signaling modulators (e.g. Fgf4) in the population that responds to Hh signaling (56). Later in limb development, proper digit specification requires Gli3; knockout of Gli3 leads to marked polydactyly (35; 36). Recent studies have suggested that while a gradient of Gli3 activity is present during limb

patterning (35), Shh$^{-/-}$ and Gli3$^{-/-}$ double knockout animals demonstrate proper limb outgrowth and skeletal development but clear polydactyly (57), suggesting that in the limb, as in the neural tube, Hh signals play multiple roles during limb development. Polarity determination is also important in the GI tract, where reduction in Hh concentration disrupts the polarity of the crypt/villus axis (48).

Studies in multiple organs have described the important role of Hh signaling in control of proliferation, cell fate, and tissue patterning during development. In accordance with these roles, dysregulation of Hh signaling during development or adult life has clear consequences. Hh signals have been implicated in many birth defects, most notably those which fall into the spectrum of VACTERL, a constellation of birth defects which include vertebral, anal, cardiac, tracheo-espophogeal, renal, and limb defects (58). Many animal models with defects in Hh signaling develop phenotypes similar to those seen in VACTERL patients (59; 60). In addition, Hh signaling has been implicated in many cancers (53; 61-65). The clear association between Hh signaling and pathological conditions in humans emphasizes two important conclusions: 1) proper Hh signaling is critical in normal development and homeostasis, and 2) studying the roles of the Hh signaling pathway in animals may yield important data applicable to the understanding or therapy of clinically important disease.

Hh Signaling in the Developing Vertebrate Intestine

One powerful system for the study of both developmental and adult roles of

signaling pathways is the gastrointestinal (GI) tract. In vertebrates, the GI tract initially

forms shortly after gastrulation as the endoderm and associated mesoderm are molded

into a tube that becomes progressively patterned along the anterior/posterior axis,

eventually giving rise to the esophagus, stomach, small intestine, and colon.

Epithelial/mesenchymal crosstalk plays a critical role in establishing each of these

distinct organs, as well as placement and specification of endodermal buds that will

become the lung, liver, and pancreas (66; 67). The luminal epithelial surfaces of the

individual organs are remarkably similar until embryonic day 14.5 (E14.5), but shortly

thereafter each organ develops specific morphology and function. For example, between

E14.5 and E16.5, the small intestine undergoes remarkable remodeling to form the

absorptive villi, fingerlike projections of the epithelium that are the functional absorptive

unit of the intestine. Prior to villus remodeling, nearly all of the multilayered, stratified

epithelial cells are proliferative; after villi are formed, proliferation is confined to the

epithelium of the newly specified intervillus zone. The mesenchymal cells of the villus

cores and lamina propria subsequently develop into supportive structures including blood

vessels, lymphatic vessels, villus smooth muscle, and intestinal subepithelial

myofibroblasts (ISEMFs). The intestinal crypts, flask-like structures that contain the

epithelial stem cells, arise in the first week after birth. ISEMFs surrounding the crypts

are believed to provide at least some of the structural and signaling components of the

stem cell niche (68). Throughout these developmental and morphogenic processes, the

Hh signaling pathway is involved in epithelial to mesenchymal communication; active

Hh signaling has been documented in the intestine from E10.5 through adulthood (17).

Early in gut tube development, Hh ligands are expressed throughout the endoderm, with the exception of the pancreatic primordia, which requires exclusion of Hh signals for further development (66; 69-71). Little is known about the mechanisms that activate Hh ligand expression in the intestinal endoderm, though FoxA family members have been implicated in control of Hh expression in the endoderm-derived epithelium of the lung (72). Foxa1 null animals exhibit no clear developmental phenotype in the intestine, however, suggesting that other mechanisms (or compensation by other FoxA factors) contribute to intestinal Hh expression (73). Late in gestation, Hh levels in the small intestine have been reported to be dependent on proper epithelial integrin expression (74). After early regional specification of the mid and hindgut, Hh signals from the endoderm contribute to patterning of the mesoderm, activating expression of Ptch1 close to the source of Hh signaling, and activating mesenchymal expression of Bmp4 (69; 71). Differential effects of Hh signaling are observed in different anterior to posterior regions of the gut tube, suggesting that the response of mesoderm to Hh signaling is modulated to some extent by the anterior-posterior patterning of the mesoderm (69). Also during this period, Hh signaling is important in radial patterning of the gut tube, as it drives the positioning of the mesenchymal zone that will become the muscularis externa of the mature gut. Grafting studies in the chicken embryo suggest that removal of the endoderm from the luminal surface and placement of this endodermal layer, the source of Hh signals, on the outside of the gut tube caused the smooth muscle of the muscularis externa to develop on the former luminal side of the tube (71). The authors of this study hypothesized that high levels of Hh signaling inhibit smooth muscle differentiation in cells next to the epithelium while lower levels of Hh ligand promote

smooth muscle formation in cells at a distance from the source of Hh signaling.

In the mouse, the pattern of Hh responsive cells are similar to those observed in chick (17), and transgenic animals expressing a high level of a secreted form of the pan-Hh inhibitor Hhip from an intestine-specific Villin promoter (12.4KVillin-Hhip) show reduced smooth muscle in the muscularis externa (48). In addition, both Shh and Ihh null embryos exhibit a reduced muscularis externa (16); together these results are consistent with a role for Hh signaling in radial patterning in mammals. Following the formation of the muscularis externa, Hh signals are important in the process of villus development between E14.5 and E16.5. Hhip-expressing embryos exhibit branched villi and poorly polarized crypt/villus axes, indicating that Hh signaling is important in epithelial to mesenchymal crosstalk during the process of epithelial remodeling required to form a villus (48).

After villus formation, Hh ligand expression becomes restricted to intervillus epithelial cells, and eventually the cells of the crypt (17). Shh or Ihh null embryos examined at E18.5 showed reduced smooth muscle, expanded pancreas, and abnormal gut rotation (16). Notably, several contrasting phenotypes were observed in Shh and Ihh null intestines. Shh null embryos displayed overgrowth of the duodenal epithelium and reduction in neuronal populations, while Ihh embryos exhibited lack of epithelial growth and expanded, mispatterned enteric neurons (16). Different phenotypes were observed after moderate inhibition of both Shh and Ihh ligand in 12.4KVillin-Hhip animals; at E18.5, moderate inhibition of Hh signaling leads to defects in the development of villus

core smooth muscle (48), mislocalization of ISEMF populations, and ectopic proliferation in the intestinal epithelium (48). Because Hh signals in the intestine are strictly paracrine (17), it was clear that the ectopic epithelial proliferation observed in 12.4KVillin-Hhip animals must be indirect, a result of altered signaling from mesenchymal cells that received reduced Hh signals. The likely source of these altered mesenchymal signals was the ISEMF population, known to be a rich source of Wnt ligands (75); Wnt signals are critical in establishment and maintenance of the proliferative compartment of the epithelium (76-78). Indeed, in the presence of lowered Hh signals, ISEMFs were seen high in the villi, beneath patches of proliferative epithelium with the appearance of ectopic pre-crypt pockets, leading to oddly branched villus structures in these animals (48). Branched villus formation was also seen in mice treated with Hh-blocking antibodies, emphasizing the role of Hh signaling in proper organization of the crypt-villus axis (79). Together, these data underscore the importance of functional Hh signaling, and demonstrate how modulation of one signal (in this case, Hh) can affect others (e.g. Wnt), emphasizing the connections present during developmental signaling in the intestine.

Hh signaling in the adult intestine

While the large body of literature discussed above suggests a role for Hh signaling during intestinal development, roles for Hh signaling in adult intestine and colon remain much less clear. A few studies have suggested that Hh signaling may impact epithelial proliferation or differentiation responses during adulthood (80-82). One study suggested

that Hh signals directly impacted upon crypt epithelial cells in the colon, inhibiting Wnt

signals and promoting epithelial differentiation (81). Postnatal Hh signaling has also

been implicated in development of Paneth cell lineages in the intestinal crypt (80). A

third study evaluated Hh expression during intestinal inflammation in late post-resection

human IBD tissue and suggested that Hh signaling was increased in the epithelium during

chronic inflammation (83).   Interestingly, each of these studies proposed an autocrine

signaling mechanism for Hh signaling during adulthood; that is, they suggest that

epithelial cells responded directly to Hh signals. This is in contrast to the apparent role of

Hh signaling during development, which had been reported to be strictly paracrine (48;

67; 69; 71); However, since modulation of Hh signaling affected both the mesenchymal

and epithelial compartments of the intestine (16; 48; 79), the possibility that Hh signals

could directly impact epithelium could not be excluded at the beginning of the work

described in this thesis.

During the course of this thesis work, a careful analysis of the expression pattern

of Hh ligand and response from early development until adulthood was carried in our

laboratory. This study demonstrated that all Hh signaling in the intestine and colon is

paracrine during development and homeostasis (17); left open was the question of

whether Hh signaling could be paracrine under pathological stress. These data suggested

that epithelial phenotypes resulting from changes in Hh signaling were indirect, a finding

which emphasized the importance of directly addressing the role of Hh signals in the

adult intestine and colon.

The goal of the work described in this thesis was to directly investigate the role of

Hh signaling during the homeostasis of the small intestine and colon, and to identify the

cellular and molecular targets of Hh signaling during adulthood. We hypothesized that

Hh signals were important during homeostasis of the adult intestine, and that alternation

in Hh signals would causes changes in mesenchymal cells which were Hh-responsive,

leading to tissue alteration. These studies were designed to interrogate the mechanism by

which Hh signals modify specific cellular and molecular targets that have implications

for proper function of the small intestine or are implicated in the development of

pathological states. Our investigations focused on two major mesenchymal populations

that respond to Hh signaling during adulthood: smooth muscle cells and myeloid immune

populations. Therefore, the following sections will contain a review of the role of these

populations in the small intestine and colon.

Development of GI Smooth Muscle

Properly patterned, functional smooth muscle is required for many important

intestinal functions, most notably peristalsis and movement of luminal contents. Smooth

muscle in the GI tract is found primarily in three populations: the muscularis externa,

muscularis mucosa, and villus smooth muscle. The muscularis mucosa is a thin layer of

smooth muscle found in the lamina propria close the epithelium which has been proposed

to function in the structure and function of the small intestinal mucosa (84). The role of

the muscularis mucosa is essentially unknown, though studies have indicated that this

population contracts and is potentially involved in epithelial secretion (85; 86). In

contrast, villus smooth muscle populations are found within the core of the intestinal villi, where they have been proposed to interact with the muscularis mucosa to function in the mechanical stability of villi (87; 88).

Finally, the most well studied population is muscularis externa smooth muscle. The muscularis externa is found at a distance from the epithelium, close to the serosal surface of the intestine, and is made up of two layers of smooth muscle, longitudinal and circular. These populations are critical in the peristaltic transit of luminal contents through the length of the GI tract. The peristaltic contractions in smooth muscle which are responsible for gut motility are regulated by a network of interstitial cells of Cajal (ICCs), which are pacemaker cell populations located within the muscularis externa responsible for controlling peristaltic contraction (89). The prevalence of Irritable Bowel Syndrome, a disorder thought to arise from dysregulation of intestinal and colonic motility, has recently focused extensive attention on smooth muscle populations in the intestine. Hormonal (90) as well as inflammatory (91) signaling may have important implications in the function of these smooth muscle populations; it is reasonable to hypothesize that functional defects may also arise from altered development of GI smooth muscle.

Descriptive studies of early development in chicken embryos have helped elucidate the temporal ordering of events in GI smooth muscle development. A careful examination of the protein expression pattern of alpha-smooth muscle actin (αSMA) during chick ileal development demonstrated that the circular smooth muscle layer of the

muscularis externa develops first, followed by branching of smooth muscle cells which then give rise to the outer longitudinal layer (92). The muscularis mucosa develops several days later, also from cells arising from the circular smooth muscle layer, and the muscularis mucosa subsequently gives rise to the earliest villus smooth muscle cells (92). A similar relative order of smooth muscle development appears to occur in the mouse (17) and human (93). In the mouse, the muscularis externa arises first, between E10.5 and E14.5, and the muscularis mucosa and villus smooth muscle populations appear after villus development; development of these populations appears complete at P10 (17). In the chick, confocal microscopy allowed the visualization of numerous connections between these apparently disparate smooth muscle populations both during development and at later stages, in particular between the villus smooth muscle and the muscularis mucosa (92); connections between these populations have also been recently detected in perinatal mice (17).

Hh Signaling Drives Smooth Muscle Differentiation

The molecular analysis of GI smooth muscle development has to date focused primarily on the development of the muscularis externa, and studies have shown that Hh signaling has a prominent role in the specification of this muscle population. Initial formation of the muscularis externa occurs due to Hh signals that emanate from the epithelium (71). Both Shh and Ihh null animals exhibit significant reduction in muscularis externa smooth muscle at E18.5 (16) and mice expressing a soluble version of the pan-Hh inhibitor, Hhip (12.4KVillin-Hhip transgenic animals) also demonstrated

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reduced muscularis externa (48).   In addition, moderate Hhip transgene expression leads

to reduction in smooth muscle of the villus cores; this is the only study to report a clear

phenotype in this cell population (48). Together, these studies suggest a critical role for

Hh signaling upstream of smooth muscle differentiation in the GI tract.

Hh signaling is also involved in the development of smooth muscle in other

organs. Hh signals are crucial in the development of smooth muscle in the urinary

system, where Hh has been described as an important inducer of smooth muscle in the

ureter (20) and the bladder (94; 95). Hh signals are also required for proper airway

smooth muscle development in the lung, and Shh null lungs demonstrate clear reduction

of both bronchial and vascular smooth muscle (96). Recent data has also shown that Hh

signals are important in the maintenance of a smooth muscle progenitor population found

in the vascular adventitia, indicating that Hh signals may have a role in the development

of vascular smooth muscle populations. Notably, Hh is required for development of

vasculature in the lung (97) as well as both development (98) and maintenance (99) of

vasculature in the heart. Finally, vascular smooth muscle in the intestine and colon

respond to Hh signals and express both Ptch1 and Gli1 (17). Thus, Hh signals appear to

be important in the development of smooth muscle in multiple contexts.

In contrast to the detailed information regarding the role of Hh upstream of

smooth muscle patterning, however, little is understood about the molecular pathway

downstream of Hh during smooth muscle induction. While studies in models of Hh

reduction have shown that loss of Hh signaling leads to loss of smooth muscle

populations (16; 20; 48), several studies have postulated that high level Hh signals can be inhibitory to the development of smooth muscle by promoting proliferation rather than differentiation in cells near the source of Hh ligand (20; 71). In addition, several studies have highlighted the importance of the direct Hh target gene *Bmp-4* as a potential downstream mediator of Hh signaling in smooth muscle development (100; 71). However, just as with Hh, BMP signals have also been shown in other circumstances to inhibit smooth muscle development (69). Finally, Forkhead family transcription factors, some of which are also Hh targets (101), have been proposed as potential regulators of smooth muscle in the lung (102) and intestine (103); reduction of smooth muscle in the intestinal muscularis externa has been noted in Foxf2 null and Foxf1/Foxf2 double heterozygous animals (103) though these animals paradoxically appear to also display increased villus smooth muscle populations. A microarray study in Shh null lungs also identified several smooth muscle-expressed factors, including *Srfcp* (also known as Myocardin, an important regulator of smooth muscle differentiation (104-106)) as potentially downstream of Hh in smooth muscle development (96), but these targets were not investigated further.

Together, these studies highlight the need for experiments designed to probe the pathways downstream of Hh signaling in the specification of smooth muscle. We hypothesized that Hh signals would be important in the regulation of smooth muscle populations in the adult intestine, and set out to address several specific questions related to the differentiation of smooth muscle downstream of Hh:

1) Does Hh signaling directly impact smooth muscle populations?

2) Does induction of smooth muscle differentiation by Hh require BMP?

3) What intracellular drivers of smooth muscle fate (e.g. transcription factors) are important in the pathway downstream of Hh signaling in the induction of smooth muscle?

4) In the intestine, are the signals that drive development of muscularis mucosa and villus smooth muscle populations similar to those which drive the development of the muscularis externa?

Studies designed to directly address these questions in the small intestine are described in Chapter 2 of this thesis.

Function and Dysfunction of Adult Intestinal Immunity

The homeostasis of the adult small intestine and colon depends critically on the proper function of the intestinal immune system. The intestinal immune system consists of a robust epithelial barrier, an extensive population of innate immune cells, and specialized structures (such as Peyer's patches (107)) of the adaptive immune system. All of these populations must work in concert to provide protection from pathogens and prevent infection, diarrhea, chronic pathogen-induced ulceration, and other clinically significant digestive disorders. Therefore, the intestinal immune system must be capable of strong, fast response to antigens that break the epithelial barrier and enter the lamina

propria. However, this immune response must also be precisely balanced; intestinal immunity must be activated only in response to appropriate stimuli, and the response must be limited to only what is required to remove the pathogen. Indeed, precise immune control must also be in effect to prevent immune reaction to the billions of commensal bacteria present in the normal intestine and colon. Failure of these controls leads to intestinal inflammatory disorders. In the human population, several significant inflammatory disorders with high prevalence cause significant morbidity, emphasizing the importance of immune control in the small intestine. These disorders have been intensely studied, with extensive literature describing animal modeling, *in vitro* examination of both animal and human inflammatory cells, and human genetic studies focused on the identification of genetic variants associated with increased risk for disease. These studies have generated substantial information about both the normal functioning of the intestinal immune system and the causes for aberrant inflammation in the intestine; some of this information will be reviewed below.

There are three major types of intestinal inflammatory disease in humans: Crohn's Disease (CD), Ulcerative Colitis (UC), and Celiac Disease. Celiac disease is a distinct disorder associated with intolerance to dietary gluten, which is found in many common grains including wheat and oats (108). Patients with Celiac disease develop significant villus blunting, crypt expansion, and inflammation in the proximal small intestine. These morphological changes reduce the intestinal absorptive surface, and lead to malabsorption. Celiac disease manifests in children as weight loss and failure to thrive and in adults as diarrhea or weight loss (108). Extra-intestinal manifestations are also

common, especially dermatitis; dermatitis herpetiformis, a characteristic skin lesion identified by IgA deposition in the skin, is the most common extra-intestinal side effect of Celiac disease (109). Celiac disease results from aberrant immune response to a complex formed between gluten and an enzyme called tissue transglutaminase (TTG) (110), which is expressed in cells of the lamina propria; anti-TTG antibodies are a major diagnostic criterion for Celiac disease (108; 111). The treatment for Celiac disease involves dietary modification to avoid gluten (108). Several genetic factors are associated with the development of Celiac disease, primarily the presence of specific HLA-DQ2 and DQ8 isoforms (112). Genome wide association studies have identified other genetic variants associated with Celiac disease risk (113-115); some of these genes are also associated with risk for IBD or other auto-inflammatory disorders (116; 108).

CD and UC share several features, and are collectively categorized as subtypes of Inflammatory Bowel Disease, or IBD. These disorders present in patients with abdominal pain, diarrhea, and bloody stool, and cause significant morbidity and tissue damage (117). CD causes more extensive tissue damage and is found in both the distal small intestine and colon (117), while UC is found in the colon only, and is associated with significantly increased risk of colonic carcinoma (117). Treatment modalities for IBD have focused on minimizing symptoms and tissue damage through the use of anti-inflammatory medications, probiotics, and surgical resection of the most severely affected tissue (118). Genes and pathways involved in the pathogenesis of IBD have been identified primarily using two methods. First, genome-wide association studies have identified several genes associated with increased susceptibility to IBD in human

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populations (119-122); genes identified as important in the susceptibility to inflammatory

bowel disease included IL23R and other mediators of immune function (119; 121; 123).

In addition, combination of these genetic studies with animal modeling have identified

target genes that are associated with increased susceptibility to inflammation in multiple

species, including the IL23 axis (119; 121; 124-126) and NOD (127-131), the first

specific gene identified as an important susceptibility locus in IBD, and to date still one

of the most important loci in attributing risk. Together these approaches have identified

genes and pathways critical in the protection from intestinal inflammation.

Inflammatory Disorders in the Intestine Result From Innate Immune Dysfunction

While robust adaptive immune responses are noted in chronic inflammatory

disease in humans, intestinal inflammatory disorders are increasingly recognized to arise

from dysfunctions in innate immune function. The genetic and animal studies described

above have shown that risk for inflammation largely arises from 1) overly robust

activation of mucosal immune response in the presence of a normal antigen load (132), or

2) dysfunction of the epithelial barrier, allowing more antigens into the lamina propria.

Aberrant activation of several mucosal innate immune pathways and cell populations has

been implicated as important modulator of inflammation in the gut. First, modification of

innate immune populations has critical implications for inflammation in the GI tract. The

lamina propria contains a large number of myeloid cells, including Cd11b-positive

macrophages and dendritic cells and Cd11c-positive dendritic cells (133; 134; 107).

These populations act in the control of inflammation through presentation of antigens and

subtype specification in lymphocytes (133; 107; 135), but many subtypes of myeloid

cells exist and the mechanisms underlying their action are complex; for example, studies

have suggested both pro- and anti-inflammatory functions for dendritic cells during acute

inflammation in mice after dextran sodium sulfate (DSS) administration (136; 137).

Second, studies beginning with the identification of Nod2 have shown the critical

importance of bacterial recognition by a variety of cell types in the small intestine,

including epithelial cells and myeloid lineages (138; 139). Recognition of bacterial motifs

by the intracellular Nod family (138) or by extra-cellular Toll-like Receptor (TLR) family

receptors (140; 141) leads to activation of downstream inflammatory pathways mediated

by NF-κB. These responses are critical in proper clearance of pathogens and

maintenance of enteric bacterial populations (138; 142), and dysregulation of these

responses can predispose to inflammation in humans (138; 142). While TLRs are

expressed in both the epithelium and myeloid cells (142), epithelial TLR signaling is

critical in protection from inflammation even in the context of normal mucosal TLR

function (143; 144), as inhibition of the NF-κB pathway specifically in the epithelium

results in spontaneous inflammation.

Finally, recent genome-wide association studies have implicated the IL23

pathway as a critical regulator of inflammation in intestine of both mouse and human

(116; 119; 121; 123; 125; 126). IL-23 is a strongly pro-inflammatory cytokine produced

by antigen presenting cells, primarily activated myeloid cells (145). IL-23 drives the

development of T-helper 17 lineage T-cells, which produce IL-17, IL-6, TNF, and other

pro-inflammatory molecules (146). IL-23 and IL-17 are robustly expressed during

inflammation in the intestine, and inhibition of IL-23 prevents normal immune response

to pathogens (125). In IL-10 knockout animals, which normally develop spontaneous

inflammation (147), IL-23 is required for development of inflammatory disease (126).

Notably, the importance of the IL-23 pathways has been confirmed in humans, where

several genome wide studies have shown members of the IL-23 pathway to be important

susceptibility loci for development of human IBD (119; 121).

Epithelial Signals Regulate Intestinal Inflammatory Status

Disruption or dysfunction of the intestinal epithelial barrier is also an important

potential instigator of intestinal inflammation (148; 143). In the case of epithelial

disruption, not only does the mucosa experience a higher load of antigens that can

stimulate an intense immune response, but it is also deprived of the tolerogenic signals

provided by a normal epithelial layer. These signals play a critical role in controlling the

immune response under conditions of normal epithelial function, and lack of these signals

provides a signal to the underlying mucosal populations that the epithelium has been

damaged (149). Indeed, reduction in these signals can also predispose to immune

activation even in the presence of an otherwise normal epithelial barrier (149). The

identification of these epithelial signals therefore is critically important both for the

understanding of the mechanisms of inflammation in the intestine and for the potential

therapeutic value pharmacological use of these factors may hold for treatment of IBD.

Therefore, much work has focused on understanding how the epithelium provides these signals to the underlying cells. Recent studies have focused primarily on a molecule called thymic stromal lymphopoeitin (TSLP), which is critically involved in modulation of the inflammatory response in the intestine (150-152). TSLP is produced by intestinal epithelial cells in response to NF-κB pathway activation and signals to lamina propria myeloid lineages to induce these cells to an anti-inflammatory phenotype (135; 151). In addition, knockout of the TSLP receptor increases susceptibility to acute inflammation mediated by DSS (150). Together these studies indicate that TSLP is a critical anti-inflammatory epithelial signal. Importantly, however, TSLP alone is not capable of recapitulating the effect of colonic epithelial cells in the induction of anti-inflammatory dendritic cells (135), a clear indication that other epithelial-produced signals are important in the anti-inflammatory effect of the intestinal epithelium.

Development of and Developmental Signaling to Intestinal Immune Lineages

Despite the presence of an enormous body of literature investigating the function, dysfunction, and inflammatory role of intestinal innate immune lineages, next to nothing is known about the development of the innate immune compartment of the small intestine and colon. In fact, the intestinal immune system is large and specialized, containing a multitude of specific lineages with characteristic anatomical distribution in the gut (107). However, the signals that regulate the differentiation of these lineages are unknown. In addition, at the outset of the studies described in this thesis, very little was known about the importance or lack thereof of functional developmental signaling pathways in the

immune system or in inflammatory disease in the small intestine. In the case of Hh

signaling, myeloid (153) and lymphoid (154; 155) lineages were known to express Ptch

and respond to Hh signals outside of the intestine, but no studies had evaluated their

response in the gut. In addition, one study had investigated the expression of Hh pathway

members in intestine and colon samples taken from human IBD patients (83); the authors

of this study stated that the expression of Shh and Ihh ligand as well as both Ptch1 and

Gli1 appeared to be increased in the context of inflammation, but the tissue used in the

study was from late, severe lesions and no mechanistic studies had been performed to

evaluate the role of Hh signaling in the GI tract.

There were therefore hints that Hh signals may be involved in regulation of immune

cells or inflammation in the GI tract, and an abundance of evidence that signals

originating in the epithelium were critical in immune homeostasis in the gut. Thus, it was

important to ask the following specific questions:

1. Is Hh signaling involved in the regulation of immune cells or pathways during
   development or homeostasis of the small intestine and colon?
2. Would inhibition or overexpression of Hh signaling have implications for immune
   response in the intestine?

Studies described in Chapters 3 and 4 of this thesis directly investigate these questions

and establish that Hh signals indeed play an important role in immune control in the

intestinal mucosa.

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Chapter 2

Hedgehog signaling directs gastrointestinal villus smooth muscle differentiation via direct activation of Myocardin

Summary

Hedgehog signaling plays multiple patterning roles during development of the mammalian gastrointestinal tract, but its role in adult gut function has not been carefully examined. Here we show that Hh signaling is required for maintenance of villus smooth muscle populations in the adult small intestine. Inhibition of the combined paracrine Indian (Ihh) and Sonic (Shh) Hedgehog signal leads to loss of smooth muscle in the villus core and muscularis mucosa, mislocalization of intestinal subepithelial myofibroblasts, and crypt hyperplasia. In contrast, overexpression of epithelial Ihh in the adult intestine leads to dramatic expansion of villus smooth muscle and Hh treatment induces SM differentiation in primary mesenchyme cultures. Examination of the pathway downstream of Hh revealed that the smooth muscle master regulator Myocardin is a direct target of Hh signaling. In 10T1/2 cells, Hh signal activation promotes differentiation of these cells to SM; simultaneously, Myocardin is activated in a cell-autonomous manner. Finally, we identify a conserved Gli-responsive enhancer within the first intron of the Myocardin

gene, and perform luciferase experiments suggesting that this element is capable of driving Myocardin expression. Together, these data demonstrate that Hh signaling is a direct activator of Myocardin, and provide evidence that Hh is critical regulator of intestinal homeostasis.

Introduction

Pathological changes in visceral smooth muscle (SM) are responsible for such diverse conditions as asthma, bladder dysfunction, hydroureter, and gastrointestinal motility disorders. Thus, unraveling the molecular mechanisms underlying smooth muscle development and maintenance is an important clinical and therapeutic goal. Among signaling pathways that promote cell fate decisions, Hedgehog (Hh) signaling has been implicated in SM differentiation in the lung (1), ureter (2), bladder (3) and gastrointestinal tract (4-6). However, the molecular pathways by which Hh directs SM differentiation in these various organs have not been clearly defined.

In the small intestine and colon, paracrine Hedgehog signaling, driven by the interaction of epithelial Shh and Ihh ligand with the mesenchymally expressed Ptch receptor (7), plays an important role in multiple developmental patterning events, including radial patterning (4), villus formation, specification of the early pre-crypt/villus axis (6), gut rotation, migration and/or function of enteric neurons, development of the anus (5), and specification of smooth muscle. Hh signaling has also been reported to impact epithelial function, in particular lipid handling by enterocytes (8) and the

specification of Paneth cells (9). However, these epithelial effects are likely to be indirect as Hh signals are exclusively paracrine in the intestine from E10.5 in development through adulthood (7). The phenotypes observed in these animal models are similar to the presentation of humans with VACTERL (vertebral, anal, cardiac, tracheal, esophageal, renal, lung) syndrome, a collection of abnormalities thought to be related to reduced hedgehog signaling (10; 11).

Studies of SM differentiation in the intestine have primarily been focused on the muscularis externa (ME). However, multiple geographically distinct populations of intestinal SM exist in the intestine and develop in a characteristic temporal sequence (7; 12; 13). The muscularis externa is bi-layered and its inner circular layer is the first to develop at embryonic day 10 (E10). Formation of the outer longitudinal layer follows quickly, so that by E12, the external muscle layers of the intestine are in place. In contrast, the SM populations of the muscularis mucosa and villus cores are not fully established until postnatal day 10, after the mucosal surface has undergone striking morphological remodeling to form the villi.

Although the pathways impacting SM differentiation in the muscularis mucosa and villus cores are unknown, initial formation of the ME is believed to involve radial patterning that is mediated by Hh signals that emanate from the endodermally-derived epithelium (4). This model posits that in cells near the epithelium, high concentrations of Hh inhibit muscle formation while low levels of Hh signaling promote the differentiation of smooth muscle cells of the early ME at a distance from the endoderm (4). A similar

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mechanism has been proposed for the development of the visceral SM of the mouse

ureter (2). Loss of either Shh or Ihh, as well as over-expression of the pan-Hh inhibitor,

Hhip, results in significant reduction of the ME smooth muscle at E18.5 (5; 6), providing

further evidence for the role of Hh signaling in the development of the ME. Taken

together, these studies suggest a critical role for Hh signaling upstream of smooth muscle

differentiation in the muscularis externa, but the molecular pathway underlying the action

of Hh is poorly understood.

Recently, we demonstrated that throughout development and in adult life, smooth

muscle precursor cells and differentiated smooth muscle of the antral stomach, intestine,

and colon express Gli1, indicating that they are actively responding to Hh signals (7).

Taken with previous findings these data suggest that Hh might be directly activating an

intracellular smooth muscle differentiation pathway. One regulator critical in smooth

muscle differentiation is Myocardin, a SAP-family transcription factor that is considered

a master regulator of smooth muscle differentiation (14-18). Myocardin is expressed in

cardiac and smooth muscle populations in the heart (17), vasculature (16; 18), and

gastrointestinal tract (19). Myocardin knockout mice die at E9.5 due to defective

development of aortic smooth muscle. Within cardiac and smooth muscle cells,

Myocardin cooperates with SRF to bind and activate CaRG elements found in many

smooth muscle target genes (17-21). While the pathway downstream of Myocardin has

been well characterized, the factors that activate Myocardin and drive smooth muscle

differentiation have been described only in the cardiovascular system (22-24). Recent

microarray analysis of Shh$^{-/-}$ lungs, however, revealed down-regulation of a number of

SM genes, including Srfcp (an alternative gene symbol for Myocardin) (1). Though the results of the array were not independently verified and the putative regulation of Myocardin by Hh was not shown to be direct, these data suggested a potential link between Hh signaling and Myocardin.

We investigated the potential link between Hh signaling, smooth muscle, and Myocardin in more detail using two mouse models developed in our laboratory: 12.4KVFHhip, in which Shh and Ihh signals are inhibited by overexpression of Hhip, and 12.4KVil-Ihh, in which full length Ihh is overexpressed. Utilizing these models, we find that Hh signaling is necessary for the maintenance of smooth muscle populations of the villus core and muscularis mucosa and sufficient to induce smooth muscle differentiation in vivo and in isolated intestinal mesenchyme. In addition, we find that Hh can drive cell-autonomous smooth muscle differentiation in vitro, and identify the smooth muscle transcription factor Myocardin as a direct target of Hh signaling. Further, a Gli-responsive enhancer within the first intron of Myocardin is responsive to Hh signals and is conserved form mouse to fly, suggesting an evolutionarily ancient regulation of Myocardin by Hh signaling in the context of SM differentiation.

Materials and Methods

Development and Husbandry of Transgenic Animals

For all constructs, the vector backbone was removed, and transgenic animals were

created by injection into C57Bl/6 x SJL/J F2 oocytes. Injections were performed by the University of Michigan Transgenic Animal Core. Founders were bred and maintained on the C57Bl/6 background. Analysis in this manuscript includes animals in generations F1 to F4 for 12.4KVil-Ihh and crosses of generations F1 to F4 of Villin-flox-LacZ-flox-Hhip to Villin-Cre (25). Sequence and maps for all constructs used in the creation of these animals are available at Addgene (www.addgene.org).

Villin-flox-LacZ-flox-HhipΔTM: The 12.4kbVillin-flox-LacZ-flox-HhipΔTM construct was created by the addition of the following components (from 5' to 3') into the pUC18 vector: the 12.4kb Villin promoter (25), a floxed LacZ cassette constructed from the pFlox vector (Dr. Jamey Marth) and the pnlacZf (Dr. Richard Palmiter), bovine growth hormone polyA, HhipΔTM cDNA (25), and the SV40 late polyA/intron (subcloned from the pGL2-basic vector, Promega). The construct demonstrates LacZ expression in the predicted intestinal epithelial expression pattern driven by the 12.4kb Villin promoter (Figure 2.1), as HhipΔTM cDNA is held out of frame by the floxed-LacZ cassette until excision by Cre (25). Founders were genotyped using primers spanning the promoter/insert border. 5 founders were utilized for the creation of transgenic lines, and the two lines with the best LacZ expression without Cre-mediated recombination were selected for further analysis (see Figure 2.1).

12.4KVil-Ihh: The full length Ihh cDNA was amplified from P0 newborn whole jejunal cDNA, and cloned into the 12.4kb VillinΔATG vector (Addgene #19358).   Founders were genotyped using primers spanning the promoter/insert border (Supplemental Table

1). 20 founders and 2 lines were analyzed for phenotypes, Ihh expression, and Hh

pathway modulation. Founder and line data were very similar, and data in this

manuscript are from founders and F1 to F3 animals from each of the transgenic lines.

Tissue Preparation, Histology, and Immunofluorescence

Adult whole small intestine and colonic tissue were dissected in ice-cold PBS

supplemented with penicillin/streptomycin, linearized to expose the lumen, washed, and

fixed. For paraffin sectioning, intestines were fixed overnight in 4% paraformaldehyde

(PFA) at 4°C, dehydrated, infiltrated with paraffin, and sectioned at 5μm. For frozen

sectioning, intestines were fixed for 30 minutes in 4% PFA at 4°C, washed in PBS,

soaked overnight in 30% sucrose in PBS overnight, embedded in OCT, and sectioned at

6-8μm. H&E staining was performed using standard methods. Alkaline phosphatase

staining was performed using the Alkaline Phosphate Substrate Kit (Vector) and

PAS/Alcian Blue staining was performed using the PAS Stain Kit (Newcomer Supply)

following the manufacturer's protocol. Immunofluorescence was performed as described

(7) using the following antibodies and concentrations: mouse anti-α smooth muscle actin

(Sigma, 1:500), rabbit anti-desmin (Abcam 1:500), rabbit anti-activated caspase 3 (R&D

Systems, 1:500), rabbit anti-lysozyme (Zymed, 1:100), and anti-chromogranin A (26).

All secondary antibodies used were Alexa Fluor (Invitrogen, 1:500).

RNA Preparation and Quantitative RT-PCR

RNA was prepared from whole adult jejunal tissue, cultured intestinal mesenchyme, or 10T1/2 cells using Trizol (Invitrogen) and following the manufacturer's protocol. cDNA was prepared using the iScript cDNA Synthesis kit (Biorad), and quantitative PCR was performed using a Biorad iCycler. Data analysis was performed using the $\Delta\Delta$Ct method, and fold changes were normalized to WT or vehicle treatment, as applicable. p values were calculated using the Student's t-test.

Small Intestinal Mesenchyme Culture

Whole small intestine was harvested from E18.5 C57Bl/6 animals in PBS supplemented with penicillin/streptomycin, cleaned carefully of connective and pancreatic tissue, and opened lengthwise. Intestines were treated for 6-8 hours with Cell Recovery Solution (BD Biosciences) at 4°C to disrupt connections between epithelium and mesenchyme, followed by vigorous shaking on ice. Separated mesenchyme was washed in sterile PBS, minced, and pooled. Six-well plates coated with rat tail collagen (BD Biosciences) were seeded with mesenchyme and incubated in DMEM supplemented with 10% FBS, 1mM Hepes, and Penicillin/Streptomycin at 37°C in 5% CO_2. Media was changed at 24 hours, and after 48 hours adherent mesenchyme was treated with recombinant Shh or Ihh (R&D Systems, 2.5μg/ml), with or without Noggin (R&D Systems, 1μg/ml) for 24 hours, for a total culture of time 72 hours. For RNA analysis, cells were lysed in 1mL of Trizol, and total RNA was prepared following the recommended protocol. For

immunofluorescence, mesenchyme was cultured as above on collagen-coated coverslips, fixed for 30 minutes in 4% PFA in PBS at 4°C and staining was performed utilizing the antibodies at the concentrations described above. For quantification of IF results, 10 fields per coverslip were randomly selected, imaged, randomized, counted by three blinded observers. Statistics were calculated using the Student's t-test in GraphPad Prism.

Smooth muscle induction in C3H/10T1/2 cells

C3H/10T1/2 cells (clone 8) were purchased from ATCC (www.atcc.org) and cultured in DMEM supplemented with 10% FBS at 37° in 5% CO_2. Cells were passaged at low density of 30-50% confluency to prevent differentiation. For smooth muscle development, 6 well plates or coverslips were seeded with $2x10^5$ cells per well. Cells were cultured for 24 hours in maintenance media, transitioned to induction medium (DMEM with 0.5% FBS) for 12 hours, followed by addition of recombinant factors (all from R&D Systems) Tgfβ1 (10ng/ml final concentration), Shh (2.5μg/ml), or Ihh (2.5μg/ml) with or without Noggin (1μg/ml) in induction media for 24 hours.   RNA preparation, QPCR, and immunofluorescence were performed as described above.

Luciferase Assays

293T cells cultured in DMEM supplemented with 10% FBS at 37° in 5% CO_2 were plated in 12 well plates, transfected with 0.7μg/well Gli2ΔN, 0.4μg/well Myocd-pGL3 reporter plasmid, and 2ng/well pRL-TK Renilla (Promega) and analyzed for luciferase

expression 36 hours after transfection using the Dual-Luciferase Reporter Kit (Promega) following the manufacturer's protocol. Firefly luciferase expression was normalized by well to Renilla, and fold changes were calculated by comparing to Myocd-pGL3 transfected alone. Statistical analysis was performed using the Student's t-test.

Identification of Predicted Gli Binding Sites

Genomic sequences surrounding known or predicted Myocd orthologues was obtained from genome.ucsc.edu. Sequences were searched using Matinspector (genomatix.de) utilizing the Gli2 matrix. Poisson probabilities were calculated by comparison of the number and distribution of Gli binding sites in the mouse genomic sequence to the number of Gli binding sites in randomly generated sequence of the same size and overall GC content.

Results

A mouse model of reduced Hh signaling in the adult intestine

To evaluate the role of Hh signaling during postnatal intestinal development and homeostasis, we developed two new transgenic models with complementary modifications in Hh signaling. First, we created a bi-transgenic model of Hh inhibition utilizing a modified version of the previously described 12.4KVillin-HhipΔTM transgene (6). This modified transgene, called 12.4KVillin-flox-LacZ-flox-HhipΔTM (or 12.4KVFHhip), uses the intestinal-specific 12.4KVillin promoter (27) to drive expression of LacZ in the absence of Cre. Combination with the intestine-specific 12.4KVillin-Cre allele (27) causes excision of the LacZ cassette and expression of HhipΔTM, a modified form of Hhip that lacks the transmembrane domain and therefore acts as a secreted inhibitor of Hh signaling (6) (Figure 2.1). We derived two independent transgenic lines with strong expression of LacZ in the small intestine, and bred each to 12.4KVilCre. We confirmed that Cre caused recombination of the 12.4KVillin-flox-LacZ-flox-HhipΔTM locus, led to ablation of LacZ expression in the small intestine, and caused mRNA expression of the recombined transgene product (Figure 2.1) by 1 month of age. We examined activation of the Hh pathway by QPCR, and found that expression of Gli1, Ptch1, and Bmp4, all direct target genes of Hh signaling in the small intestine, are significantly reduced in response to Hhip expression at both 1 (data not shown) and 3 months of age (Figure 2.2A). These data confirmed that our strategy leads to effective postnatal inhibition of Hh signaling, and we therefore examined the phenotypes of bitransgenic animals starting at birth and through early postnatal life.
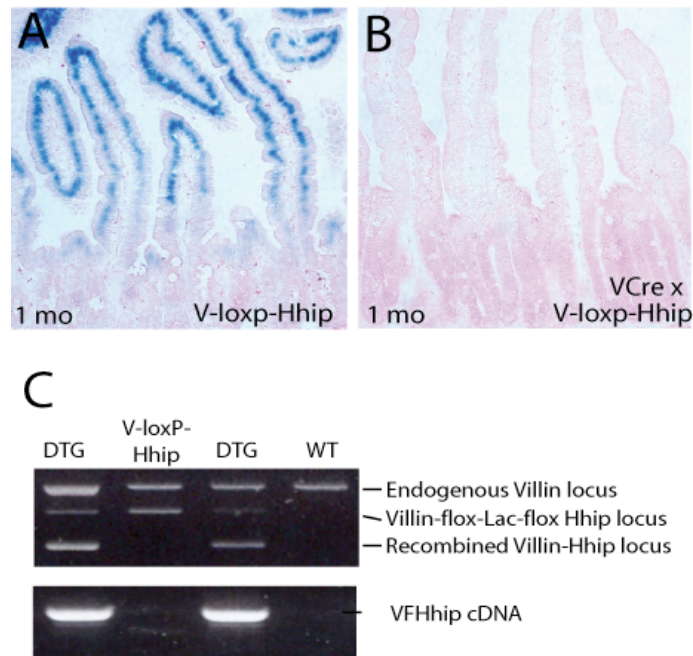
Figure 2.1: Validation of the VFHhip model for Bi-transgenic inhibition of Hh signaling in the small intestine. A) 12.4KVil-flox-LacZ-flox-Hhip animals display clear β-galactosidase enzyme activity in intestinal epithelial cells in the absence of Cre. B) Combination of the 12.4KVil-flox-LacZ-flox-Hhip and 12.4KVil-Cre alleles leads to recombination and loss of β-galactosidase activity in intestinal epithelial cells. C) Bitransgenic (DTG) VFHhip animals demonstrate recombination at the 12.4KVil-flox-LacZ-flox-Hhip locus and expression of Hhip RNA by RT PCR.
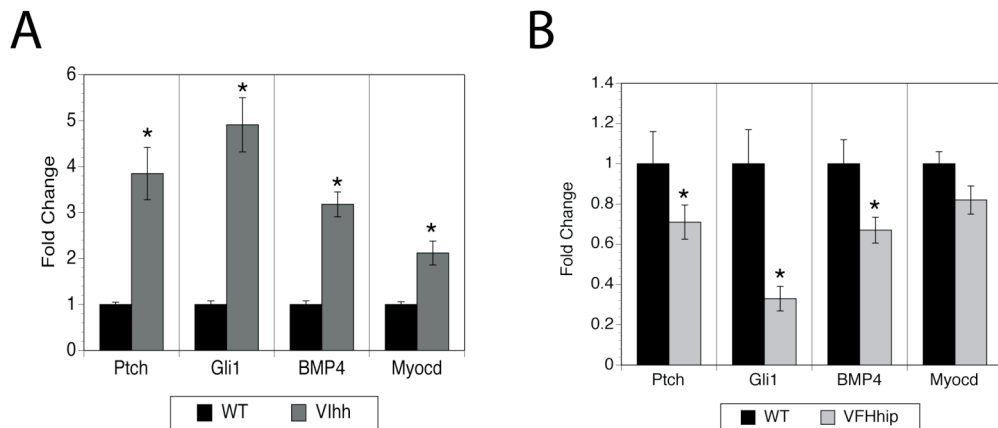
Figure 2.2: Analysis of Hh pathway response in 12.4KVil-Ihh and 12.4KVFHhip animals at 3 months of age using QPCR. A) Increased expression of Ihh in 12.4KVil-Ihh animals is associated with increased expression of the Hh target genes Gli1, Ptch, and Bmp4, as well as the putative target Myocd. B) Inhibition of Hh signaling in VFHhip animals is associated with decreased expression of Gli1, Ptch, and Bmp4. Despite loss of villus smooth muscle, Myocd is downregulated in a nonsignificant manner, likely due to the presence of a normal muscularis externa in VFHhip animals. $* = p < 0.05$ compared to WT.

At birth, 12.4KVFHhip animals appear normal. Over the first 3 months of post-natal life, however, 12.4KVFHhip animals progressively develop a phenotype reminiscent of that seen in the original Villin-Hhip model (6), with pathological changes in both the epithelial and mesenchymal compartments of the small intestine (Figures 2.3 and 2.4). In WT mice, intestinal sub-epithelial myofibroblasts (ISEMFs) are normally found in tight association with proliferative epithelial cells of the crypt. In 12.4KVFHhip animals, ISEMFs are associated with epithelium further up the crypt/villus axis (Figure 2.3D). As in our earlier study, we note that ectopic ISEMFs are associated with ectopically proliferative epithelial cells. Due to this ectopic proliferation, crypt depth in 12.4KVFHhip mice is nearly twice that of WT animals (Figure 2.3).

Examination of epithelial differentiation in 12.4KVFHhip animals reveals a reduced epithelial absorptive surface compared to WT animals, as shown by alkaline phosphatase staining (Figure 2.4A-B). Lineage allocation, however, is apparently not affected, as quantitation reveals that the number of enteroendocrine, goblet, and Paneth cells per villus is not significantly different in 12.4KVFHhip animals compared to WT animals (Figure 2.3). Examination of apoptosis with activated Caspase 3 staining did not reveal a change in cell death in either the epithelium or mesenchyme in the context of reduced Hh signaling (data not shown). Together, these data indicate that functional Hh signaling is required during intestinal homeostasis for correct localization of subepithelial myofibroblasts and control of crypt proliferation. Thus, while the levels of Hh signals are lower in adults than during development (7), the residual adult Hh signal is functional and plays a similar role with regard to myofibroblast localization as it does in the embryo.
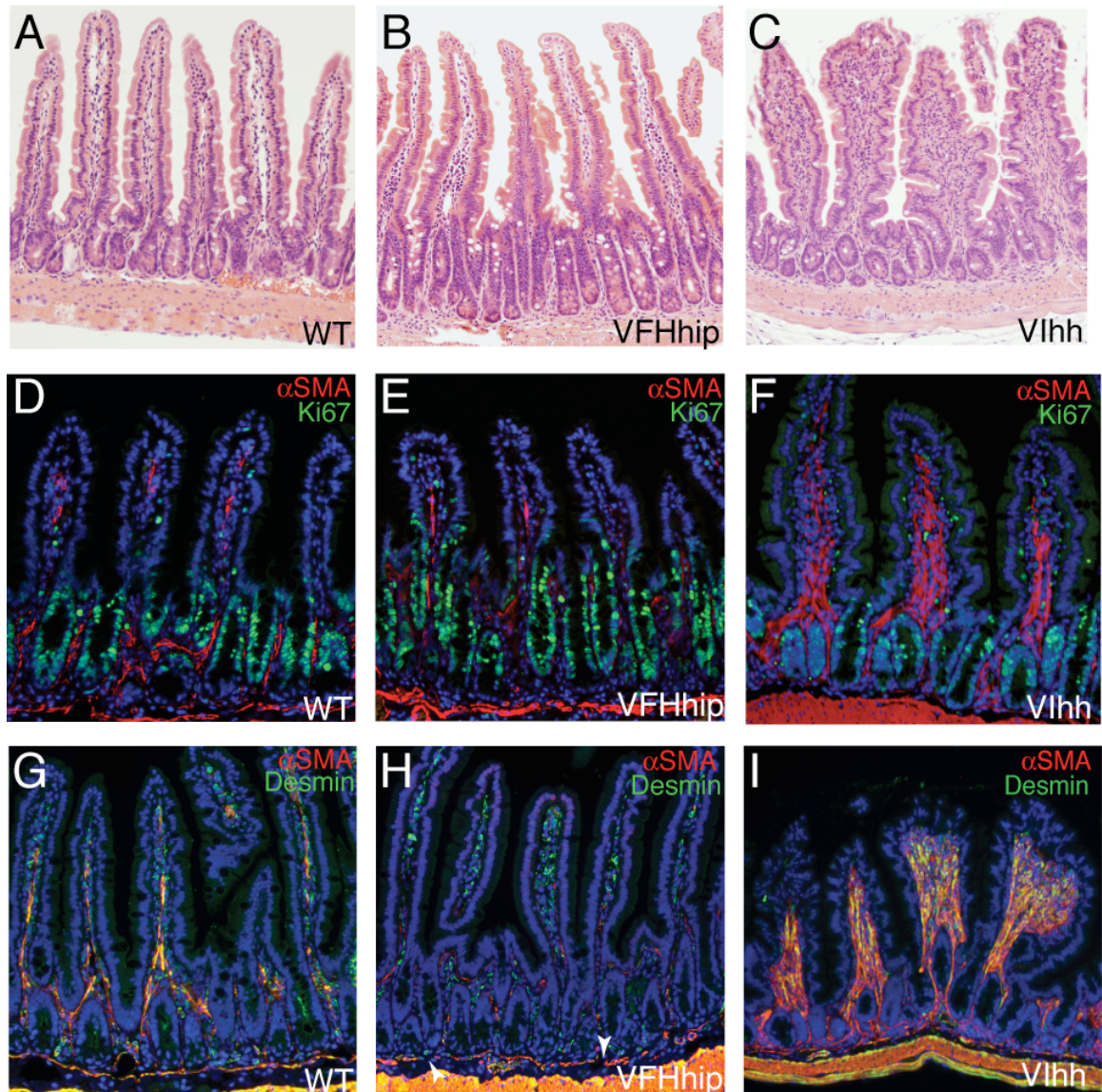
Figure 2.3: Transgenic modulation of Hh signaling leads to alterations in mesenchymal populations.
A-C) Histological examination of 12.4KVFHhip and 12.4KVil-Ihh phenotypes. D-F) Examination of αSMA (red) and Ki67 (green) staining for characterization of ISEMFs and proliferation. ISEMF populations are mislocalized further up the crypt/villus axis in response to Hh inhibition in 12.4KVFHhip animals (E), leading to crypt expansion. ISEMFs and epithelial proliferation are not changed in 12.4KVil-Ihh animals (F). (G-I) Examination of αSMA (red) and Desmin (green) expression for examination of smooth muscle populations. Smooth muscle precursors are Desmin positive (green), ISEMFs are αSMA positive (red), and differentiated smooth muscle cells are express both αSMA and Desmin (yellow). Inhibition of Hh signaling leads to loss of differentiated smooth muscle in the villi of 12.4KVFHhip animals (H), and smooth muscle loss also leads to clear gaps in the muscularis mucosa (arrows in H). Transgenic overexpression of Ihh leads to a dramatic increase in villus smooth muscle (I); note also that very few Desmin positive precursors are visible in the villi of 12.4KVil-Ihh animals. All images were taken at a magnification of 200x.

<u>Inhibition of Hh signaling causes loss of villus smooth muscle, disruption of the muscularis mucosa, and expansion of smooth muscle precursor cells</u>

In addition to myofibroblasts, SM precursors and differentiated SM cells are direct cellular targets of Hh signaling (6; 7). We therefore investigated whether inhibition of Hh signaling during adulthood impacts these adult smooth muscle populations. In 12.4KVFHhip animals, the muscularis externa, muscularis mucosa, and villus core smooth muscle appear normal at one month of age. However, by three months, SM is reduced in the cores of the villi and the muscularis mucosa (Figure 2.3G-H), although the muscularis externa is unaffected. In villus cores, αSMA- and desmin-positive differentiated SM is lost, with a corresponding increase in the number of desmin-positive SM precursor cells (Figure 2.3G-H). In addition, individual SM cells in the lamina propria are lost, causing the appearance of breaks and gaps in the muscularis mucosa (see arrows in Figure 2.3H). These findings suggest that Hh signals are required for maintenance of villus and muscularis mucosa SM populations during intestinal homeostasis.

<u>Increased expression of Ihh drives smooth muscle differentiation</u>

Since decreased Hh signaling compromises SM development, we tested whether increased Hh ligands promotes SM formation in vivo. We recently described 12.4KVil-Ihh transgenic founders, generated by linking the 12.4kb Villin promoter to a full length Ihh cDNA (7). Here, we derived 2 transgenic lines carrying this transgene, and examined

them over time for phenotypes, expression of trangenic Ihh mRNA, and regulation of the Hh pathway.

At birth, Villin-Ihh mice appear developmentally normal, and do not show clear modulation of the Hh signaling pathway as measured by Gli1 and Ptch1 levels, but demonstrate clear expression of transgenic Ihh mRNA (data not shown). Ihh expression remains robust during postnatal life, and by 3 months of age (a time when 12.4KVFHhip animals have lost all smooth muscle), villus cores in Villin-Ihh animals are distended with a population of cells that is mostly differentiated smooth muscle (Figure 2.3I). Very few desmin-positive precursors are visible in the villi at this stage, suggesting that this population has largely been converted to smooth muscle (Figure 2.3I). QPCR analysis demonstrates that the Hh pathway is significantly upregulated in 3 month old 12.4KVil-Ihh animals, with increased expression of *Gli1*, *Ptch1*, and *Bmp4* (Figure 2.2B). Despite this clear increase in the Hh pathway, we do not detect any alteration in the myofibroblast number or localization (Figure 2.3F). Additionally, no significant effects on epithelial lineage allocation is detectable in 12.4KVil-Ihh animals (Figure 2.4). Taken with previous findings that smooth muscle precursors and differentiated smooth muscle respond directly to Hh signals (7), these data indicated that Hh signaling is sufficient to drive smooth muscle differentiation in adult small intestine. Indeed, the adult lamina propria appears to be quite sensitive to the level of Hh ligand.
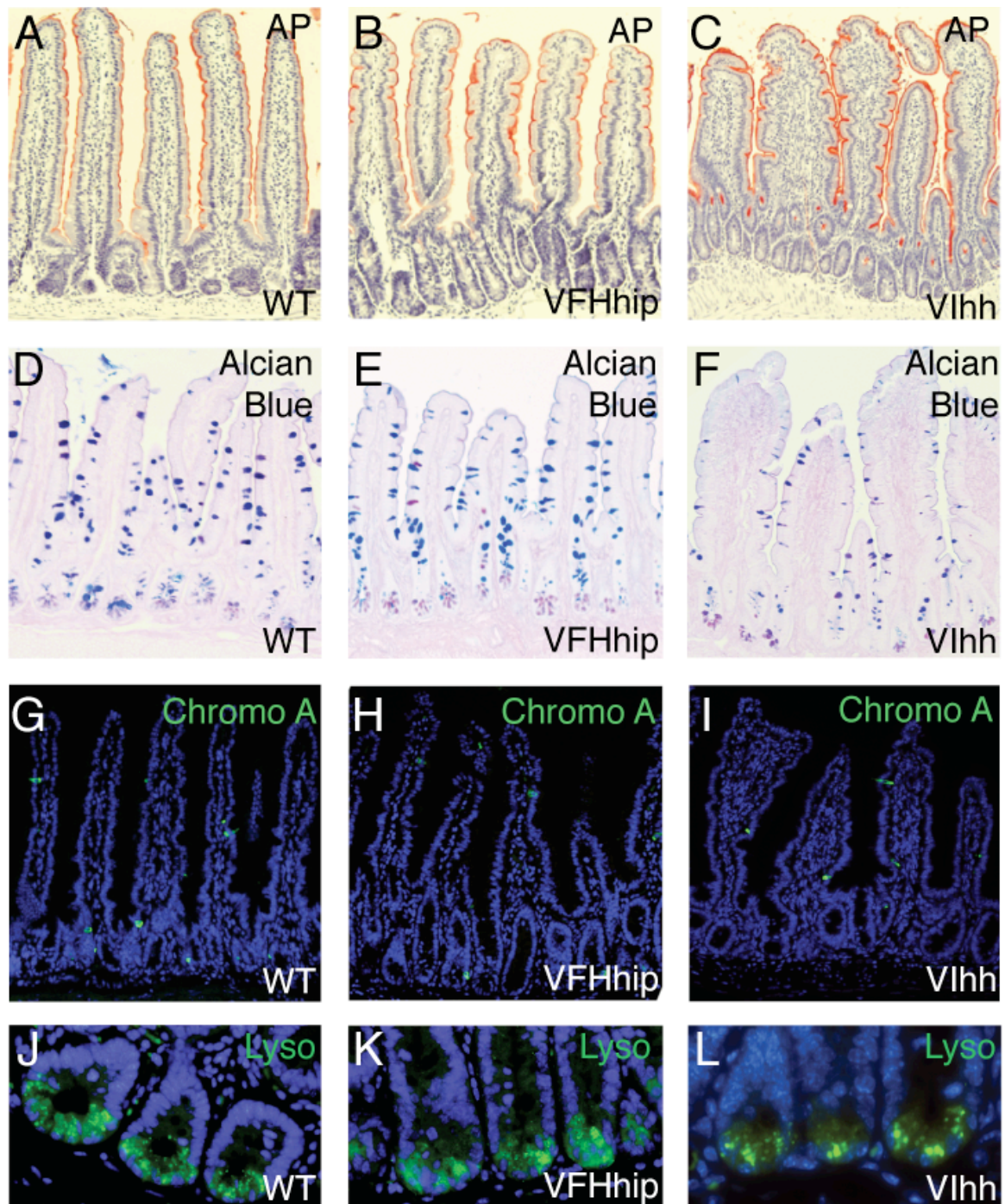
Figure 2.4: Transgenic modulation of Hh signaling does not alter epithelial lineage allocation. A-C) Alkaline Phosphatase (AP) staining to visualize the absorptive surface of enterocytes. The absorptive surface is reduced by expanded crypts in VFHhip animals (B) but is otherwise normal. VIhh animals (C) have a similar absorptive surface as WT (A). D-F) PAS/Alcian Blue stain to visualize goblet cells. Both VFHhip (E) and VIhh (F) animals have a similar number of goblet cells compared to WT (D). G-H) Chromogranin A (Chromo A) stain for endocrine cells, with DAPI nuclear counterstain. Neither inhibition of Hh (H) or overexpression of Hh (I) alters enteroendocrine cell number or position. J-L) Lysozyme (Lyso) staining for detection of Paneth cells, showing high magnification of crypts. No change in Paneth cell number or location is seen with Hh modulation. A-I shown at a magnification of 200x, J-K at 600x.

Hh signaling drives smooth muscle differentiation in E18.5 intestinal mesenchyme

To confirm the role of Hh in SM differentiation and to interrogate the pathway required downstream of Hh, we utilized cultures of isolated intestinal mesenchyme from E18.5 WT embryos. Before crypt formation, it is possible to cleanly separate intestinal epithelium and mesenchyme, and to culture the mesenchyme for several days on a collagen matrix (see Materials and Methods). Because the epithelium is the dominant source of Hh ligand in the small intestine (7), we hypothesized that Hh signal transduction in isolated mesenchymal cells would be reduced or inactive after 48 hours in culture, but that addition of recombinant Hh ligand would provoke a robust *Gli1* response. To test this, we isolated mesenchyme from Gli1$^{+/LacZ}$ animals, cultured the mesenchyme for 48 hours, treated with vehicle or Hh ligand for 24 hours, and performed LacZ staining to identify Hh response. Mesenchyme from Gli1$^{+/LacZ}$ animals treated with vehicle showed no apparent expression of LacZ after 48 or 72 hours of culture (Figure 2.5A). However, addition of either Shh or Ihh ligand induced strong LacZ expression, indicating robust Hh pathway activation in these cells (Figure 2.5B-C).

Using immunofluorescence for αSMA and Desmin, we confirmed that smooth muscle precursor cells, ISEMFs, and smooth muscle cells were all present in vehicle treated culture after 72 hours (Figure 2.5G), indicating that this system would be appropriate to examine the process of SM differentiation in response to Hh pathway activation ex vivo. We therefore treated isolated mesenchyme with vehicle, Shh, or Ihh (N=4 wells per treatment) and quantified SM precursors, ISEMFs, and differentiated SMC populations after each treatment. Vehicle treated mesenchyme contains a large

population of desmin positive, αSMA negative SM precursor cells (Figure 2.5). After treatment with Shh or Ihh, this smooth muscle precursor population is reduced by 50%, and the number of ISEMFs and SMCs increase (Figure 2.5H-I). These data suggest that Hh signaling promotes SMC differentiation in isolated mesenchyme, mirroring the findings seen in 12.4KVilIhh animals.

<u>Myocardin, a myogenic transcription factor, is downstream of Hh signaling</u>

The fact that SM precursors and differentiated SM cells express Gli1 (7) suggests that the effect of Hh on these cells might be direct. To understand the molecular pathway downstream of Hh signaling, we surveyed known intracellular inducers of SMC differentiation, focusing especially on transcriptional regulators of SMC fate. Myocardin is a crucial regulator of smooth muscle differentiation in several systems. Therefore, we tested the effect of Hh signaling on Myocardin expression in intestinal mesenchyme.

First, we determined that Myocardin is expressed in isolated intestinal mesenchyme, and that treatment with either Shh or Ihh ligand caused clear upregulation of Myocardin expression in mesenchyme (Figure 2.5D). Next, we investigated whether Myocardin was modulated in response to alteration of *in vivo* Hh signaling in our transgenic models. In 12.4KVil-Ihh animals, Myocardin expression is significantly increased; 12.4KVFHhip animals show a moderate reduction (Figure 2.2A-B). Taken together, these data are consistent with the idea that Hh signaling acts upstream of Myocardin both *in vivo* and *in vitro*.
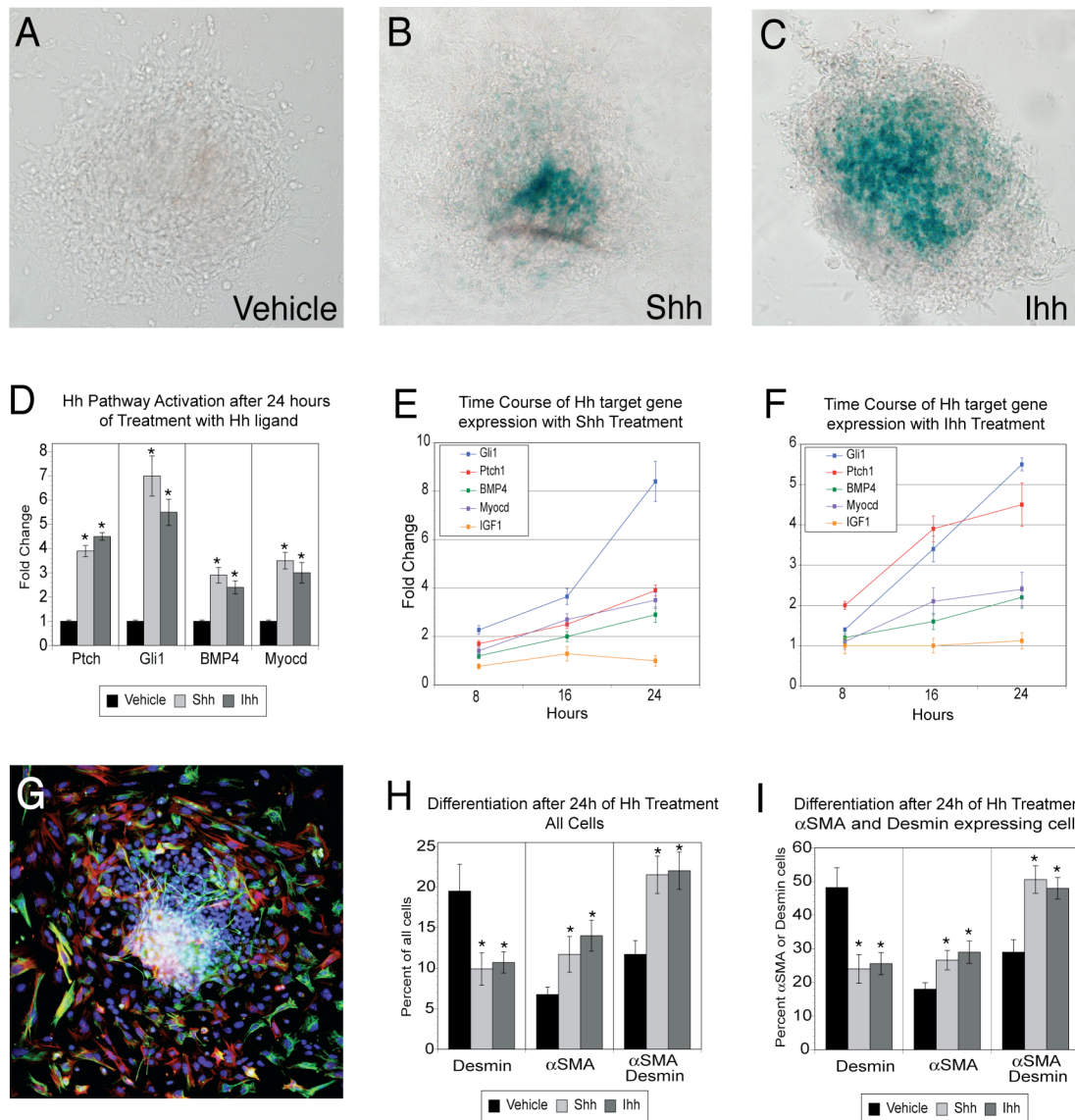
Figure 2.5: Hh treatment of E18.5 mesenchyme causes Hh pathway activation and smooth muscle differentiation. A-C) Activation of Hh signaling in E18.5 Gli1$^{+/lacZ}$ mesenchyme. Removal of the epithelium renders Hh signaling inactive in untreated isolated mesenchyme after 48 hours of culture (data not shown) and after 24 hours of vehicle treatment (A). Treatment with either Shh (B) or Ihh (C) ligand for 24 hours leads to robust expression of β-galactosidase, indicating robust Hh pathway activity. D) QPCR examination of Hh pathway activation in mesenchyme. After 24 hours of Shh or Ihh treatment, *Gli1*, *Ptch1*, *BMP4*, and *Myocd* are all robustly activated. E-F) Time course of Hh pathway expression after Hh treatment. Examination of the 8 and 16 hour time points reveals that the kinetics of Myocardin activation parallel those of the direct Hh targets *Gli1, Ptch1*, and *Bmp*4, but not *Igf1*, after treatment with either Shh (E) or Ihh (F) ligand. G) αSMA (red) and desmin (green) immunofluorescence demonstrating that ISEMFs (red), smooth muscle precursor cells (green) and differentiated smooth muscle cells (yellow) are present in untreated isolated mesenchyme. H-I) Quantification of ISEMF and smooth muscle populations after Hh treatment in isolated mesenchyme. Shown are the precursor (Desmin), ISEMF (αSMA), and smooth muscle cell (αSMA/Desmin) populations as a percentage of all cells (H) or of only the αSMA and/or desmin positive population (I). Treatment with either Shh or Ihh ligand causes a significant decrease in the precursor population and a significant increase in ISEMF and smooth muscle cells. Images were taken at 600x. * = p < 0.05 compared to vehicle treatment.

Next, we investigated the Hh-dependent upregulation in *Myocd* by comparing the

kinetics of *Myocd* expression to that of the known Hh direct targets *Gli1, Ptch1*, and

Bmp4. We included another smooth muscle expressed factor that was upregulated with

long term Hh treatment in mesenchyme cultures, *Igf1*. We found that *Myocd*, but not *Igf1*

was expressed with kinetics similar to those seen for known the direct targets of Hh

signaling, closely mirroring the induction of *Bmp4* and *Ptch1* (Figure 2.5E,F).

Hh signaling is sufficient to activate smooth muscle differentiation of C3H10T1/2 cells

Because the mesenchymal culture system is complex and contains many cells that

do not respond to Hh signaling, we sought to examine the process of Hh-mediated SMC

differentiation in a more homogeneous in vitro system. We therefore utilized the

C3H10T1/2 cell line, which has been shown to express Ptch1 and respond to Hh (28) and

to differentiate into recognizable smooth muscle cells in response to treatment with

Tgfβ1 in the presence of low serum (29). We confirmed that in our hands 10T1/2 cells

expressed *Ptch* and *Gli1* RNA, and Desmin but not αSMA protein (data not shown),

suggesting that they may be similar in phenotype to the smooth muscle precursor

population of the mouse intestine. To induce SMC differentiation, we treated low

passage, non-confluent 10T1/2 cells with Shh, Ihh, Tgfβ1, or vehicle in low serum media

and examined SMC differentiation. As previously reported, Tgfβ1 treatment drives

robust smooth muscle differentiation, causing morphological changes and robust αSMA

expression in the majority of cells (~70%, N=10 wells), whereas serum withdrawal alone

causes very little SMC differentiation (Figure 2.6A,D). Treatment with either Shh or Ihh

also causes robust SMC differentiation (N=10 wells) (Figure 2.6B,E), confirming the

results seen in our mesenchymal cultures. Quantification of smooth muscle

differentiation using QPCR for *Myocd, SM22α, and αSMA* confirms that Tgfβ1, Shh, and

Ihh drive robust smooth muscle differentiation (Figure 2.6G). Notably, treatment with

Shh or Ihh causes more significant upregulation of Myocd than treatment with Tgfβ1,

though the overall degree of SM differentiation in each treatment is similar.
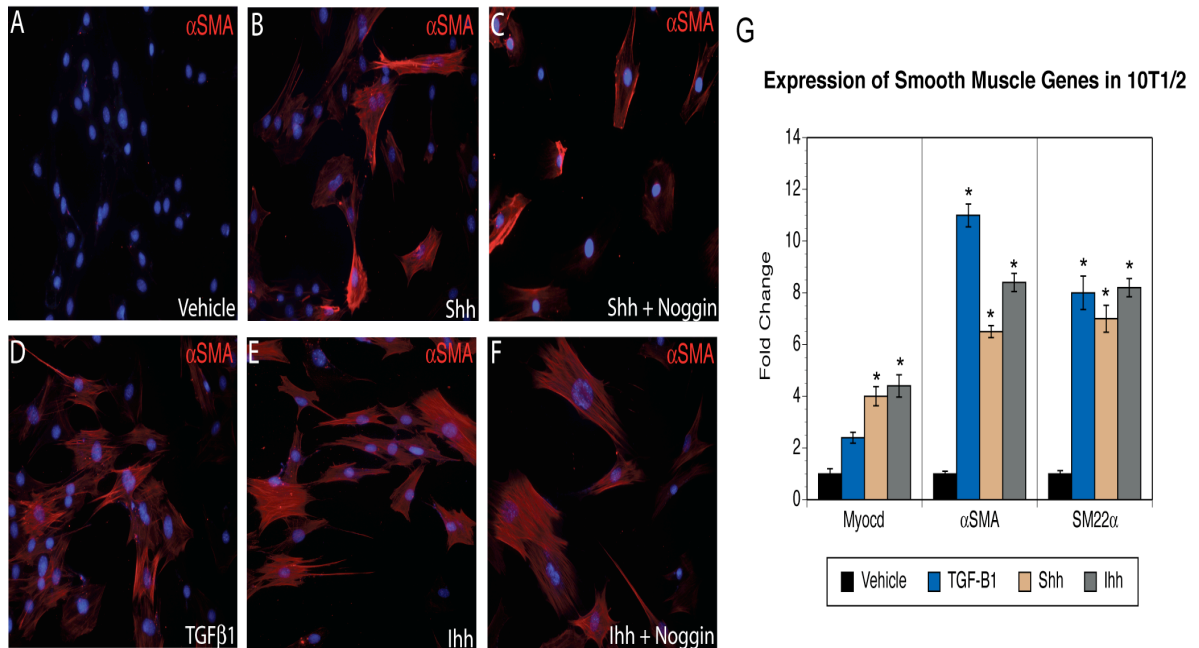
Figure 2.6: Hh promotes BMP-independent smooth muscle differentiation in 10T1/2 cells.
A) 10T1/2 cells do not express αSMA when treated with vehicle in the presence of low serum. B-F) Shh (B) or Ihh (E) treatment drives smooth muscle differentiation and αSMA expression in 10T1/2 cells in a similar manner to the known smooth muscle induction factor Tgfβ1 (D). This effect does not require BMP, as BMP ligand expression is not modulated by Hh treatment (citation 27 and data not shown) and co-treatment of Shh (C) or Ihh (F) with the BMP-inhibitor Noggin has no effect on αSMA expression in 10T1/2 cells. G) QPCR examination of smooth muscle differentiation with Tgfβ1 or Hh treatment. Note that while Tgfβ1 drives smooth muscle differentiation, the upregulation of Myocd is only significant with Hh treatment. $* = p < 0.05$

While these data provided further evidence that Hh signaling was sufficient to drive SMC development, they did not address whether this effect was direct or the result of secondary signaling. Indeed, Bmp4 is a direct Hh target that has been implicated independently in smooth muscle differentiation in the intestine. We therefore investigated whether Bmp4 was a major component of SMC differentiation downstream of Hh by investigating the BMP pathway in 10T1/2 cells treated with Hh. Previous studies have indicated that 10T1/2 cells express Bmp2 and Bmp4 but not Bmp5,6, or 7, but that the expression of these BMPs is not altered in response to treatment with Hh ligand (28). We confirmed that *Bmp4* RNA was not significantly upregulated in 10T1/2 cells in response to Hh treatment in this system (data not shown). However, to directly assess whether BMP signals were required for Hh to drive smooth muscle differentiation in 10T1/2 cells, we examined smooth muscle differentiation in 10T1/2 cells treated with both Hh and the BMP inhibitor Noggin; these cultures showed no change in the development of SMCs, indicating that Hh signaling is capable of driving SMC differentiation directly, and does not require BMP signaling (Figure 2.6C,F).

Transfection of activated Gli2ΔN induces cell autonomous smooth muscle differentiation

To confirm that Hh-directed SM differentiation is a result of cell autonomous reception of the incoming Hh signal (i.e., that Gli factors, activated directly in SM precursor cells, drives SM differentiation) we utilized Gli2ΔN, a version of Gli2 lacking the N-terminal repressive domain and a strong activator of Hh target genes (30). We transfected 10T1/2 cells with Gli2ΔN, then withdrew serum to induce smooth muscle
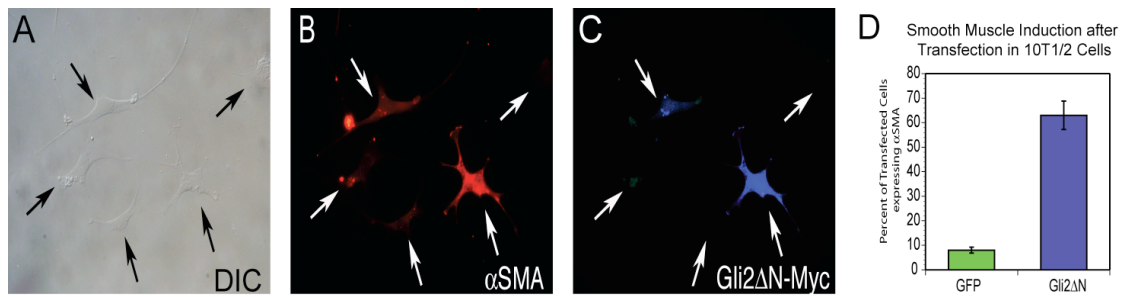
Figure 2.7: Intracellular activation of the Hh signaling pathway leads to cell autonomous smooth muscle differentiation of 10T1/2 cells. A-C) Representative images of Gli2ΔN transfected 10T1/2 cells exposed to differentiation media (see Materials and Methods). Cells that are positive for Gli2ΔN express αSMA and take on an SMC-like morphology, whereas adjacent untransfected cells do not express αSMA. D) Quantification of smooth muscle differentiation after transfection with GFP or Gli2ΔN. Quantification was performed on transfected cells positive for GFP or Gli2ΔN in 10 fields from N=4 wells from two separate transfection experiments. p<0.01 by Student's t-test.

differentiation, and assessed the extent of differentiation and the correlation of SMCs with cells positive for Gli2ΔN. We found that 63% of cells transfected with Gli2ΔN expressed αSMA and took on the morphology of SMCs, compared to 8% of cells transfected with GFP alone (N=4 wells, Figure 2.7). In addition, non-expressing cells immediately adjacent to Gli2ΔN-expressing cells did not develop into SMCs (Figure 2.7), further supporting the idea that activation of Hh signaling drives SMC differentiation in a cell-specific manner, rather than through induction of a second signal, such as BMP.

Myocardin is a direct target of Hh signaling

To assess whether Myocardin is a direct target of Hh signaling, we sought to identify a likely Hh-responsive enhancer near the Myocardin gene. Approximately 100kb of genomic sequence surrounding the mouse Myocd locus on chromosome 11 was searched for potential Gli binding sites. The searched region included 60kb upstream of the Myocd start site and all of the large intron 1 common to the 5' end of both described

Myocardin transcripts (22). We noted that a genomic element previously described to regulate myocardin in vascular smooth muscle (22) does not contain any predicted Gli binding sites; this element was therefore not examined further. In all, we located 5 Gli sites in 50kb upstream of the transcriptional start site and 10 within the 40kb intron 1. Among these 15 sites, there were two clusters of sites that stood out as potential regulatory elements: a group of 4 sites within 8kb found between 40 and 48kb upstream, and a group of 5 sites within 4.7kb found immediately 3' to exon 1 within the first intron. Next, we examined the conservation of these clustered binding sites. Neither cluster was well conserved on a sequence level between primates and rodents (data not shown). However, a cluster of Gli binding sites detected in the 5' region of intron 1 was identified in every mammalian lineage examined, as well as chicken and *Xenopus* myocardin. Moreover, a similar cluster of Ci sites was found within intron 1 of the *Drosophila* Myocardin ortholog MRTF (Figure 2.8A).

The potential functional conservation suggested by these observations led us to test whether the mouse intronic region is regulated by Gli factors downstream of Hh. We therefore cloned the core sequence of this putative intronic enhancer element, which contained 4 Gli binding sites in 2.8kb, and inserted this element into the pGL3 Promoter luciferase vector (Figure 2.8B). Co-transfection of Gli2ΔN and Myocd-pGL3 promoted a 2.5 fold induction of luciferase activity compared to Myocd-pGL3 transfected alone, indicating that Gli factors can bind to this intronic element and promote transcription. Taken with the kinetics of Myocd induction (Figure 2.5E,F), these results suggest that Hh signals may directly regulate Myocd through a functionally conserved intronic enhancer.
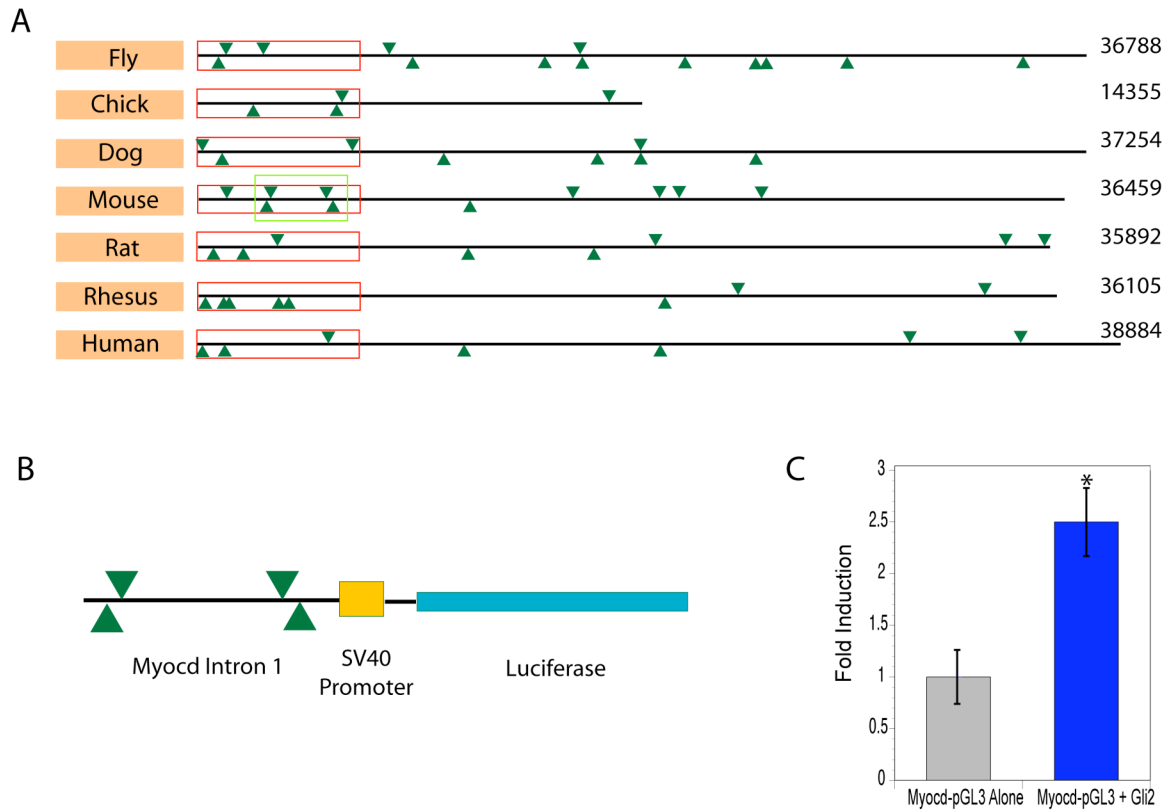
Figure 2.8: Identification of a novel Hh-responsive element in the 5' end of Intron 1 of the Myocd locus. A) Location of Gli binding sites in intron 1 of animal Myocd orthologues (MRTF in *Drosophila*). The full length of the intron 1 is shown. Green circles indicate predicted Gli binding sites; sites were identified using the MatInspector tool from Genomatix using the Gli2 binding matrix. In every animal examined, there was an unexpectedly high number of Gli binding sites based on the relatively low (42-44%) GC content seen in this region. In particular, the 5' 5kb of intron 1 in every Myocd orthologue examined contains an element of significant enrichment of Gli binding sites above what would be expected by random distribution; this region is highlighted with a red box. The Poisson probability of finding the element observed in mouse by random chance is approximately 1 to 10000 (based on examination of 1000 random mouse genomic sequences). B) Design of Myocd-pGL3 construct. The most clustered region of the mouse intron (shown as a green box in A) was cloned into the pGL3 promoter vector, and tested for activation of luciferase in combination with Gli2ΔN. C) Co-transfection of Myocd-pGL3 and Gli2ΔN causes significantly increased Luciferase activity compared to Myocd-pGL3 transfected alone. * = p<0.05 compared to Myocd-pGL3 alone.

Discussion

The data in this chapter demonstrate that Hh signaling is a crucial regulator of

small intestinal homeostasis, with a clear role in the maintenance of smooth muscle

populations in the villus core and muscularis mucosa; this study is the first to directly

assess the development and maintenance of these important smooth muscle populations.

We show that Hh signaling is necessary and sufficient for villus smooth muscle

differentiation *in vivo*, and demonstrate in two different *in vitro* models that Hh signaling

can drive smooth muscle differentiation through activation of Myocardin. The effects of

Hh signaling on smooth muscle differentiation in 10T1/2 cells are cell autonomous, and

evidence supports the possibility that the activation of Myocardin by Hh signaling is

direct. These data provide the first evidence of the importance of Hh signaling in the

adult small intestine. Hh expression in the gastrointestinal tract is strongest during early

development, when Shh and Ihh ligand are expressed throughout the endoderm. This

expression is reduced during later development and expression of both Shh and Ihh is low

in the adult small intestine compared to early time points and the corpus of the stomach

(7). Nonetheless, these data demonstrate a clear requirement for Hh signaling during

adulthood, both in the regulation of crypt structure and in the maintenance of smooth

muscle populations. Given evidence that the highest levels of Hh signaling are inhibitory

to smooth muscle differentiation (2; 4), it is possible that the reduction in Hh signaling is

an important driving force in the development of smooth muscle populations close to the

epithelium; these populations develop during late gestation and early postnatal life, the

same time as Hh signaling is refined to the proliferative zone of the epithelium (7).

Hh signaling had previously been implicated in the control of smooth muscle populations in the GI tract (4-6) the urological system (2; 31) and vascular smooth muscle (32). The downstream effectors proposed for the effects of Hh have included direct signaling (6), patterning through modulation of Bmp4 expression (2; 4; 31), and maintenance of a Myocardin-expressing progenitor population (32). Our data suggest that in many cases the primary role for Hh signaling may be in the direct control of Myocardin expression. This model is supported by our data indicating that Hh signals directly activate smooth muscle differentiation of 10T1/2 cells in the presence of Noggin, without modulation of BMP ligand expression, and in a cell autonomous manner; together these findings provide additional evidence that Hh is directly capable of inducing smooth muscle differentiation. However, *in vivo*, it is likely that Hh activates the smooth muscle differentiation pattern via control of both Myocardin (in a cell autonomous manner) and of other factors, including Bmp4, which help prime or accelerate smooth muscle differentiation through pathways that parallel the action of Myocardin; for example, in the chick heart Hh signals are required for Myocardin activation whereas Nkx2.5 activation requires BMP signaling (33). The control of multiple parallel pathways by Hh could help to provide an explanation for the evidence that BMP signals are crucial for smooth muscle differentiation downstream of Hh in the GI tract (4) and kidney (31).

Apart from the role of Hh in the activation of SM differentiation through Myocardin, our data provide several additional insights into adult intestinal biology. Our

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finding that adult Hh inhibition leads to mislocalization of myofibroblasts, which are

themselves rich signaling centers (34) and produce Wnt ligands important in the

maintenance of epithelial proliferation, suggests that the intestinal crypt niche requires

functional Hh signaling and epithelial/mesenchymal crosstalk for proper maintenance;

this idea is emphasized by the expansion of crypts in the 12.4KVFHhip model. Recent

data have indicated that Hh signaling is also important in the regulation of colonic crypts

(35), further suggesting that epithelial/mesenchymal crosstalk is important in the

maintenance of stem cell niche *in vivo*. Indeed, the recent successful culture of intestinal

epithelial stem cells without mesenchyme required extensive addition of signaling

modulators to provide an environment supportive of culture, emphasizing the importance

of signaling crosstalk in the intestinal epithelial stem cell niche (36).

In addition, the finding that Hh signaling has no effect on lineage development in

the small intestinal epithelium is noteworthy; though it has recently been clearly

demonstrated that epithelial cells do not express Ptch1 and can not respond directly to Hh

signals (7), the question of whether Hh signaling has implication for the differentiation of

intestinal epithelial lineages remained of interest. Previous data had suggested that Hh

signaling was critical in the development of Paneth cells (9), and our models of Hh

modulation provided a direct opportunity to evaluate the role of Hh signaling in the

specification of epithelial lineages cells. These data suggest that modulation of Hh alone

is not capable of modifying Paneth cell populations; nor do we detect significant

differences in other epithelial populations in the context of modified Hh signaling, with

the exception of reduced absorptive surface in 12.4KVFHhip animals after crypt

expansion. Taken together, these data suggest that Hh impacts epithelial differentiation primarily through localization of myofibroblasts and other mesenchymal target cells, and that Hh signaling is not a major contributor to control of epithelial lineage specification.

Finally, our findings that 1) Hh appears to directly regulate Myocd, and 2) that this regulation occurs through a conserved intronic element may also provide insight into the potential role of this regulation in important developmental processes outside of the GI tract. Myocardin is critical in the development of cardiac and smooth muscle (17; 16; 14; 37). In addition, Hh has been implicated previously as a crucial modulator of early myocardial progenitors (38), and Myocd activation in the chick heart field requires the endoderm but appears to be independent of BMP signaling (33). This situation is very similar to the one observed in our studies, where Hh directly activates Myocd in a manner that does not require BMP signaling; together these data suggest a potential role for Hh in the activation of Myocardin during heart development, though this possibility must be directly tested. Second, our data raises the possibility that Hh may be involved in the patterning of mesoderm in Drosophila through activation of MRTF. The Hh-responsive enhancer we have identified is conserved to Drosophila, and MRTF is expressed in a temporal and spatial domain near a source of Hh (39), suggesting that Hh may be involved in this process. These possibilities will need to be formally tested, but our data suggest that the direct control of Myocardin by Hh should be examined in other systems.

Overall, these data highlight Hh signaling as an important regulator not just of development but also homeostasis in the GI tract, with clear roles in the regulation of

smooth muscle populations and crypt structure during adulthood. Smooth muscle mispatterning or dysfunction is a key aspect of several developmental and adult GI disorders, including VACTERL (10; 40), functional disorders (41), and mesenchymal tumors (42), suggesting that proper control of smooth muscle populations is required for proper GI function; our data implicate Hh and Myocd directly in this control. Given that villus smooth muscle provides a key mechanical component of the villus core (43; 44) and that mechanical forces play an important role in regulating cell biology and signal transduction in the GI tract (45), it is likely that dysfunction of this cell population may have important implications for the function of the GI tract in mouse or human as well. In addition, since Hh signaling is important in urinary, respiratory, and vascular smooth muscle, our data may have implications for development and pathology in multiple organs. Therefore, the data in this manuscript provide a novel, generalizable, conserved model for which to understand Hh-mediated smooth muscle differentiation.

Acknowledgements

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Chapter 3

Hedgehog is an anti-inflammatory epithelial signal for the intestinal lamina propria

Summary

<u>Background and Aims:</u> Hedgehog (Hh) signaling is a critical regulator of intestinal organogenesis, and emerging data have implicated the Hh pathway during homeostasis in the adult small intestine and colon. Here, we profiled genes and pathways modulated by Hh signaling in the intestinal mesenchyme and investigated the effects of chronic Hh ligand reduction *in vivo*.

<u>Methods:</u> We performed microarray analyses on whole small intestine from newborn transgenic animals over-expressing the pan-Hh inhibitor Hhip and on isolated intestinal mesenchyme cultured in the presence or absence of Hh ligand. GO term overrepresentation analysis, hierarchical clustering, and MeSH term filtration were performed. Regulated pathways were compared to phenotypes seen in an adult model of chronic Hh inhibition, 12.4KVFHhip (12.4KVil-Cre x 12.4KVil-flox-LacZ-flox-Hhip transgenic mice).

<u>Results:</u> The major pathways regulated by Hh signaling were muscle and inflammation-related; Hh ligand promoted pro-myogenic pathways and reduced expression of pro-

inflammatory molecules. In accordance with these findings, 6-10 month 12.4KVFHhip animals demonstrate villus smooth muscle loss and subsequent villus atrophy. Areas of villus loss become complicated by spontaneous inflammation, and 12.4KVFHhip animals exhibit significant wasting and death. The 12.4KVFHhip phenotype shares characteristics of human Celiac disease and IBD.

Conclusions: Hh ligand is a previously unrecognized epithelial modulator of the mesenchymal inflammatory milieu, playing an anti-inflammatory role. Acute modulation of Hh signals results in changes in inflammatory genes and pathways in intestinal mesenchyme. Chronic inhibition of Hh signaling in adult animals leads to spontaneous intestinal inflammation and death. Regulation of epithelial Hh levels may be an important mechanism to modulate tolerogenic vs. pro-inflammatory signaling in the small intestine.

Introduction

Functional intestinal immunity requires a precise balance of pro-inflammatory and tolerogenic influences to both protect against infectious disease and prevent aberrant inflammation. When inappropriate pro-inflammatory signals dominate, as is the case in Inflammatory Bowel Diseases (IBD) and Celiac disease, significant tissue damage results, leading to significant patient morbidity. IBD is a complex and multi-factorial condition, though genome-wide association studies in human populations have led to the identification of a number of candidate molecules that play a role in the two major sub-

types of IBD, Crohn's disease (CD) and ulcerative colitis (UC) (1). Some identified

susceptibility loci are apparently unique to CD (e.g ATG16L1 and NOD2), while others

are shared with CD and UC (IL23R, NKX2-3, STAT3, MST1, IL12B) (1; 2). Analysis

of the function of these associated loci, as well as those associated with abnormal

inflammation in mice including TLRs (3; 4), IL-1β, IL-6, TNF (5), and IL17/IL23 axis

(6; 7) has led to the hypothesis that IBD results from aberrant chronic activation of the

immune system in response to commensal flora or other stimuli.

The epithelium plays a critical role in the intestinal inflammatory response to

bacteria. Epithelial cells express pattern recognition receptors for bacteria both on their

cell surface (TLRs) and within their cytoplasm (NOD family members), and signaling

through these receptors activates NF-κB (8-10). Indeed, epithelial-specific inhibition of

the NF-κB pathway results in spontaneous inflammation and aberrant activation of

dendritic cells (4; 11). At least part of this epithelial cell dependent modulation of

dendritic cells is likely attributable to epithelially-expressed inflammatory signals; one

example of this class of signaling molecules is thymic stromal lymphopoietin or TSLP.

TSLP is induced by NF-κB and secreted by the epithelium; it exerts a tolerogenic effect

on myeloid cells (dendritic cells and macrophages) in the lamina propria (12-16).

However, other epithelial modulators of inflammation must exist, since dendritic cells

isolated from mice null for the TSLP receptor do not show defects in their ability to

activate regulatory T cells (17) and TSLP is not capable of completely replacing

epithelial cells as an anti-inflammatory modulator for intestinal myeloid populations (12).

Another epithelial signaling molecule recently linked to IBD is Hedgehog (18). Both Indian (Ihh) and Sonic (Shh) Hedgehog are proteins secreted by the intestinal epithelium that signal in a paracrine manner to the mesenchyme (19). Ihh and Shh are involved in a number of developmental patterning events in the intestine (20-22) and are also expressed in the adult GI tract (19). Among the target cells that respond to Hh signals in the adult colon are dendritic cells and macrophages (18). A recent study demonstrated that a non-synonymous SNP in GLI1, a downstream transcription factor and direct target of Hh signaling (23), is associated with IBD in three large European populations; the variant GLI1 protein shows reduced transactivation activity in transfected cells. In accordance with the idea that reduced Hh signals might predispose to inflammation, Gli1$^{+/-}$ mice demonstrate increased susceptibility to dextran sodium sulfate with markedly up-regulated IL23/IL17 signaling (18). Although the results of this study suggest the novel hypothesis that the adult intestinal epithelium might use Hh ligands to modulate the tolerogenic response of the lamina propria, the potential genetic targets of Hh signaling were not investigated further in that study.

The objectives of the current investigation were two fold: 1) to understand how a chronic reduction in Hh signals would impact intestinal health and 2) to learn more about the target genes and pathways that are downstream of Hh signals in the intestine. To accomplish the first goal, we carried out a long term analysis of a previously described bi-transgenic model of Hhip overexpression, 12.4KVil-Cre x 12.4KVil-flox-LacZ-flox-Hhip (which we call 12.4KVFHhip). This model allows analysis of the phenotypes arising from chronic reduction in Hh levels, as VFHhip animals show clear

downregulation of the Hh pathway by 1 month of life (Chapter 2). We find that these 12.4KVFHhip animals exhibit reduced villus smooth muscle early in life (Chapter 2), and later begin to display significant villus atrophy and loss. Regions of villus loss become complicated by spontaneous inflammation with characteristics similar to human inflammatory diseases. Between 6-10 months of age, 12.4KVFHhip animals develop rapid weight loss, wasting, dermatitis, and mortality.

To accomplish the second goal, we performed microarray analyses on small intestinal mesenchyme samples obtained from wild type mice to which purified Hh ligands were added. The results of this microarray indicate that Hh signaling is primarily pro-myogenic and anti-inflammatory in the small intestine. Furthermore, we established that ISEMF and myeloid populations in the small intestinal mesenchyme respond directly to Hh signals. Together, these data suggest an important role for Hh signaling as an important homeostatic mediator of small intestinal inflammation and suggest that chronically reduced Hh signaling play a role in intestinal inflammatory disease.

Materials and Methods

Microarray Design, Analysis, and Comparisons

Total RNA was prepared from mesenchyme cultured as described previously (Chapter 2) using Trizol (Invitrogen) following the manufacturer's protocol. Evaluation of RNA integrity, RNA labeling, and Chip hybridization were performed by the University of Michigan Affymetrix Microarray Core. RNA integrity was evaluated

utilizing the Agilent 2100 Bioanalyzer system. Samples were hybridized to Affymetrix

Mouse 430 microarray chips. After hybridization, expression values were normalized

using the RMA and LIMMA functions of Bioconductor toolset in R.   Fold changes were

calculated using the LIMMA function, and p values were calculated using the Student's

T-test. Comparisons used to identify differentially expressed genes are shown

schematically in Figure 3.1. Examination of overrepresented GO Terms and Functional

Annotation Clustering analyses were performed using DAVID (http://david.abcc.

ncifcrf.gov/) using described methods (24). Categorization of overrepresented GO Terms

was obtained using the GO Terms Classification Counter (http://www.animalgenome.org/

bioinfo/tools/countgo/). MeSH filtration for identification of potential cellular

enrichment was performed using the MeSH Anatomy filter in Bibliosphere (Genomatix).

Hierarchical clustering analysis was performed after RMA normalization using the K

means clustering (25) and Hierarchical clustering (26) functions of MultiExperiment

Viewer (http://www.tm4.org/mev.html).

E18.5 Intestinal Mesenchyme Culture

E18.5 intestinal mesenchyme was cultured and treated with Hh ligand as

previously described (Chapter 2). For localization of cell types studied, mesenchyme was

cultured on collagen-coated coverslips and staining was performed using previously

described methods (Chapter 2). CD11b-positive cells were identified using goat anti-

Cd11b (Abcam, 1:500) and Alexa Fluor rat anti-goat FITC (Molecular Probes).

Development and Analysis of 12.4KVFHhip animals

12.4KVil-Cre x 12.4KVil-flox-LacZ-flox-Hhip animals were designed as previously described (Chapter 2). For histology, intestinal tissue was prepared by fixation in 4% paraformaldehyde at 4°C overnight, followed by dehydration and infiltration with paraffin. 8μm sections were stained with Haematoxlyin and Eosin (H&E). For detection of the intestinal absorptive surface, alkaline phosphatase was visualized using the Vector Red Alkaline Phosphatase Kit (Vector Inc) following the manufacturer's protocol. For immunofluorescence, tissue was dissected in cold PBS and fixed for 30 minutes in 4% PFA at 4°C. After a brief rinse in PBS, the tissue was equilibrated overnight in 30% Sucrose/PBS at 4°C and then embedded in OCT and frozen on dry ice.

Immunohistochemistry was performed on 8-10 μm frozen sections. Sections were dried, rinsed in PBS, blocked for 1h in 10% normal goat serum, 0.1% BSA and 0.3% Triton-X in TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl). Antibodies used were: hamster anti-CD3 (Serotec, 1:500), rat anti-CD19 (Serotec, 1:500), goat anti-CD11b (Abcam 1:500), FITC-conjugated anti-CD11c (Abcam 1:250), and rabbit anti–β-galactosidase (a gift of J. Douglas Engel, Department of Cell and Developmental Biology, University of Michigan; 1:2000). All antibody staining was performed overnight at 4°C, followed by incubation Alexa Fluor secondary antibodies from Molecular Probes, 1:1000 for one hour. Nuclei were counterstained with DAPI, and slides were mounted in Prolong Gold Antifade Reagent (Invitrogen). Digital images were taken using an Olympus FV500 confocal microscope at 600x for 0.30μm optical sectioning.

A

105 upregulated probesets
and 300 downregulated probesets
Table 3.1

62 upregulated probesets
and 135 downregulated probesets
Table 3.2

Shh

Ihh

Vehicle

Vehicle

Commonly
Regulated

34 upregulated probesets (Table 3.3)
104 downregulated probesets (Table 3.4)

B

358 upregulated probesets
Table 3.9

Vehicle

Mesenchyme

102 downregulated probesets
Table 3.9

C

36 probesets
Table 3.12

Down with culture
Up with Hh

Vehicle vs
Mesenchyme

Shh vs
Vehicle

Ihh vs
Vehicle

Up with culture
Down with Hh

78 probesets
Table 3.14

Figure 3.1: Microarray comparisons for identification of potential Hh target genes and pathways. A) Identification of genes regulated by Hh signaling in isolated intestinal mesenchyme. Gene expression in isolated mesenchyme after addition of Shh or Ihh ligand was compared to gene expression after treatment with Vehicle. We identified genes regulated by Shh (Table 3.1) or Ihh (Table 3.2) as well as genes regulated by both Shh and Ihh (Tables 3.3 and 3.4). B) Identification of genes altered by removal of epithelium and transition to culture. We compared gene expression in freshly isolated mesenchyme (25) and mesenchyme after 72 hours in culture, and identified 460 probesets with >5 Fold change (Table 3.9). C) Identification of genes regulated by transition to culture and treatment with Hh ligand. We identified probesets that were upregulated by culture and downregulated by addition of Hh, as well as those downregulated by culture and upregulated by Hh ligand addition. The genes in these sets represent likely direct or indirect Hh target genes.

Results

Adult Inhibition of Hh signals leads to villus loss, spontaneous inflammation, and mortality

Because recent data suggest that Hh signals are important in the immune regulation of the adult colon (18), we wished to examine the long-term effects of moderate Hh inhibition in the adult intestine. We utilized a recently described double transgenic model: 12.4KVillin-Cre x 12.4KVillin-flox-LacZ-flox-Hhip (Chapter 2). In these mice (which we call 12.4KVFHhip), Hhip expression begins perinatally, several days later than in the previously described 12.4KVil-Hhip transgenic founders (20). QPCR evaluation of Hh pathway activity in these mice shows that by 1 month of age *Gli1* expression is reduced to approximately 30% of normal expression, indicating significant pathway inhibition (Chapter 2). We have reported that over the first 3 months of life, 12.4KVFHhip mice exhibit loss of villus smooth muscle, mislocalization of intestinal subepithelial myofibroblasts and expansion of the proliferative crypt region of the epithelium (Chapter 2).   We further aged 12.4KVFHhip animals and examined them for intestinal alterations over a period of 6-10 months. At 6 months of age, all 12.4KVFHhip animals examined appeared to be healthy and grossly indistinguishable from littermate controls. However, on histological examination at this time, 12.4KVFHhip animals display areas of clear villus atrophy and patches of villus loss (Figure 3.2A-F).
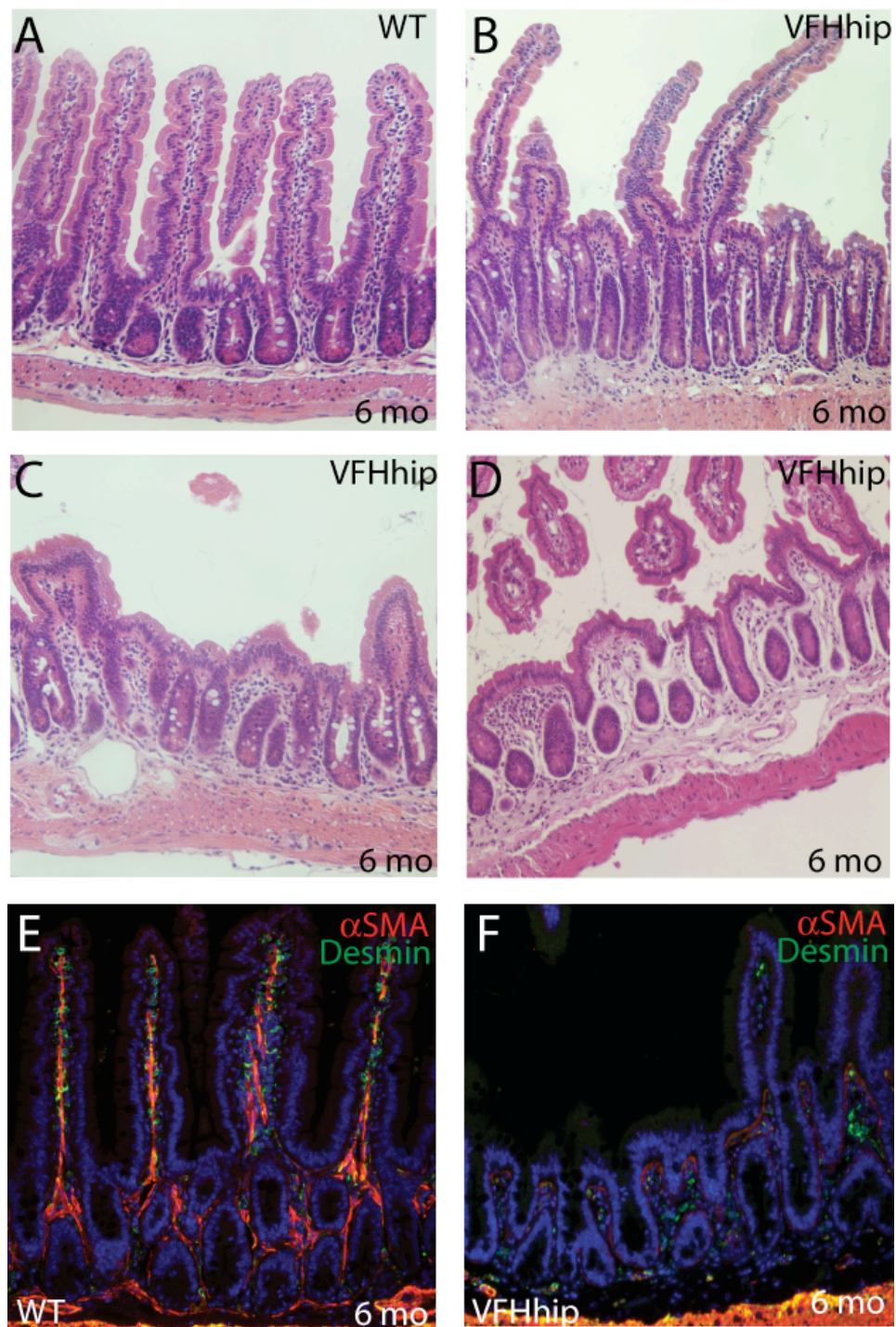
Figure 3.2: Chronic inhibition of Hh signaling leads to villus atrophy. A-C) While 6 month old single transgenic littermates exhibit normal villus structure, 6 month old 12.4KVFHhip animals show significant villus loss with crypt expansions. D) Regions of villus loss in 12.4KVFHhip animals become complicated by inflammation as early as 6 months of age. E, F) αSMA/Desmin double staining for visualization of myoblasts (green), myofibroblasts (red), and differentiated smooth muscle (yellow). 12.4KVFHhip animals have lost all villus smooth muscle and display discontinuity of the muscularis mucosa by 6 months of age (see also Chapter 2).

Over the course of the following 4 months, 12.4KVFHhip animals stochastically developed significant wasting, culminating in death. By 10 months of age, the entire cohort of 12.4KVFHhip animals (N=10) developed wasting and diarrhea and subsequently died (Figure 3.3A); none of the single transgenic littermates housed with 12.4KVFHhip animals died during this time (N=14). Also of note was a significant prevalence of dermatitis amongst 12.4KVFHhip animals. By 10 months of age (or at the time of death), 8/10 12.4KVFHhip animals had developed visible dermatitis, whereas only 4/15 single transgenic littermates showed dermatitis at 10 months of age (Figure 3.3B); examination of this dermatitis revealed deposition of IgA in the inflamed skin of some 12.4KVFHhip animals (Figure 3.4G-H; N=3/5 12.4KVFHhip, 0/5 WT). We proceeded to examine intestinal phenotypes in 12.4KVFHhip animals and WT littermates over this time period.

By 10 months of age, villus loss was prominent in the small intestines of 12.4KVFHhip animals (Figure 3.4A-D). We also found that regions of villus loss were often complicated by inflammation in the lamina propria (Figures 3.2D and 3.4A-D). Inflammation ranged from infiltration of immune cells in the lamina propria to clear ulceration with inflammation that involved the muscularis externa (Figure 3.4D); the extent and size of the lesions varied both within and between animals. Lesions were identified throughout the entire small intestine, from duodenum to ileum. Of particular note were several lesions that showed extensive villus atrophy, crypt hyperplasia, mucous cell expansion, and lamina propria inflammation (Figure 3.4C); these lesions bore a striking resemblance to those found in human Celiac disease. In addition, these regions
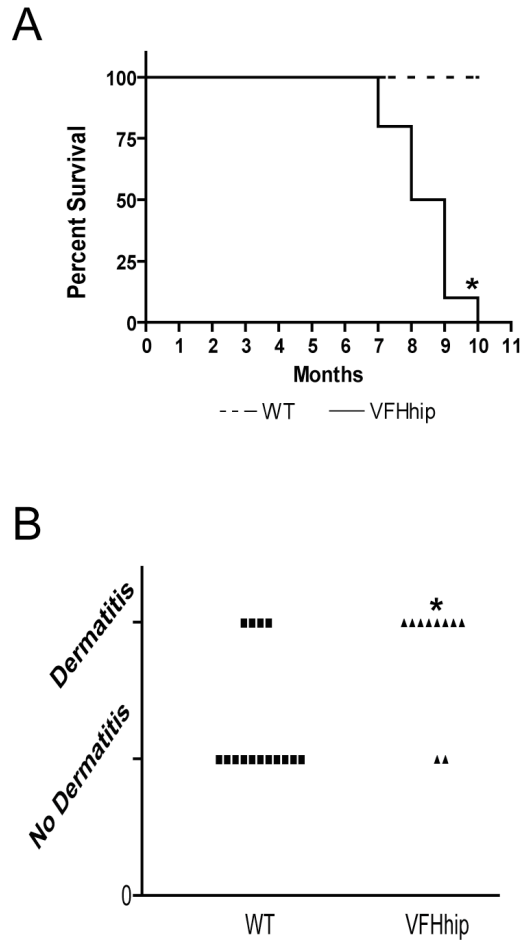
Figure 3.3: Villus loss leads to wasting, death, and dermatitis. A)
12.4KVFHhip animals show marked mortality between 6 and 10 months of age,
while single transgenic littermates housed in the same cages do not die during this
period. B) 12.4KVFHhip animals develop dermatitis at significantly increased rate
compared to littermates. * = p < 0.05.

of villus atrophy and inflammation display a striking lack of differentiated enterocytes

(Figure 3.4E-F), providing potential insight into the clinical malabsorptive syndrome

displayed by 12.4KVFHhip animals. Together, our data indicate that Hh signaling

impacts inflammatory pathways in the mesenchyme and long term inhibition of intestinal

Hh signaling is associated with villus loss, inflammation, and severe inflammatory

disease.
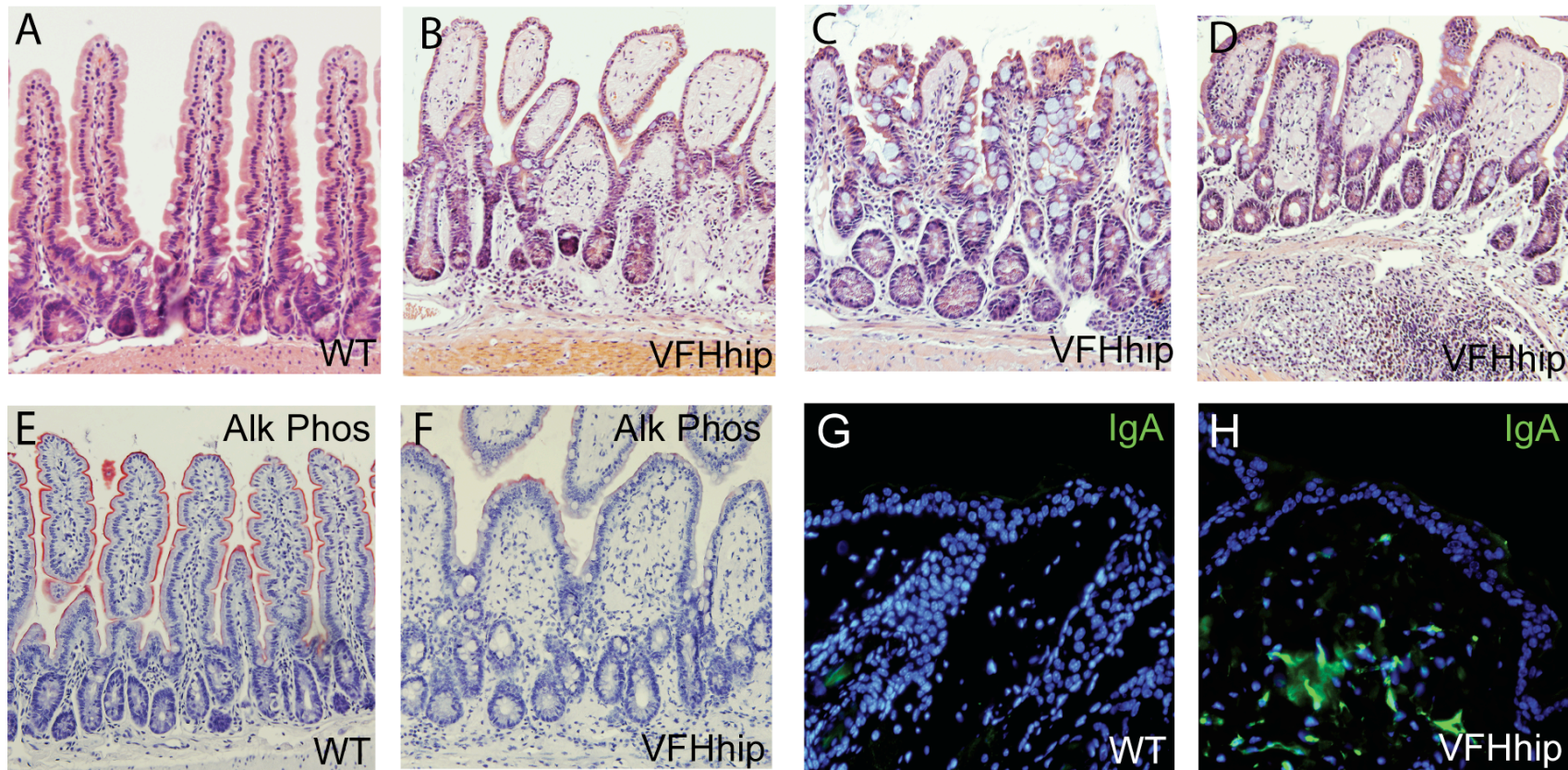
Figure 3.4: 12.4KVFHhip animals develop spontaneous small intestinal inflammation. A-D) 12.4KVFHhip animals develop spontaneous small intestinal inflammatory disease with villus blunting and crypt expansion between 6 and 10 months of age. Inflammation ranges from moderate (B) to severe (C, D), and can be transmural (D). E, F) Areas of villus atrophy and inflammation in 12.4KVFHhip animals show loss of functional absorptive surface as measured by expression of the intestinal enterocyte marker Alkaline Phosphatase. G, H) Dermatitis in 12.4KVFHhip exhibits depositions of IgA, while dermatitis observed in WT animals does not.

Hh Treatment inhibits expression of inflammatory genes in isolated mesenchyme

Our analysis of VFHhip late phenotypes indicated that chronic reduction in Hh

signaling led to the development of spontaneous inflammatory disease in mice. It did not

address, however, whether the development of this inflammatory disease was due directly

to modulation of Hh signals or whether the tissue changes seen as a result of Hh

inhibition (e.g. barrier breaks during villus atrophy) predisposed to inflammatory disease.

Therefore, to test whether Hh signals could directly modify inflammatory pathways in

perinatal intestinal mesenchyme, we isolated and cultured intestinal mesenchyme from

E18.5 wild type mice (Citation 20 and Chapter 2). Since the epithelium is the primary

source of Hh signals during this period of intestinal organogenesis (19), cultured whole

mesenchyme in the absence of epithelium quickly loses Hh signal transduction as

measured by expression of the direct target gene and Hh transcription factor Gli1, and the

Hh pathway can be robustly reactivated within 24 hours of addition of recombinant Shh

or Ihh ligand to the mesenchymal cultures (Chapter 2 Figure 2.3). In addition, these

mesenchymal cultures contain the major components of the intestinal lamina propria,

including smooth muscle cells, smooth muscle precursors, and ISEMFs (Chapter 2,

Figure 2.3) as well as myeloid and lymphoid immune lineages (data not shown). These

attributes suggested that isolated mesenchyme is an appropriate model to identify genes

and pathways that are modulated by Hh signaling.

Intestinal mesenchyme was isolated, cultured for 48 hours without passage, and

then exposed to recombinant Shh or Ihh ligand (or vehicle) for 24 hours. The direct Hh

target *Gli1* was induced approximately 1.5 fold (p ≤ 0.05) with both Shh and Ihh treatment, and we utilized this cutoff to assess potential Hh targets. Probeset expression values that were modulated greater than 1.5 fold (and p ≤ 0.05) by the addition of ligand compared to vehicle in isolated cultured mesenchyme were therefore identified. Unexpectedly, we identified more downregulated (300) than upregulated (105) probesets (Table 3.1) with Shh treatment; this finding was also true for Ihh treatment (Table 3.2). There was significant overlap of probesets regulated in Shh and Ihh treated cultures (75 common downregulated targets and 27 common upregulated targets, Tables 3.3 and 3.4); this finding further validates the genes associated with these probesets as likely downstream targets (either direct or indirect) of Hh signaling.

We carefully examined the array data for evidence of a differential response to Shh vs. Ihh. Twenty-five potential differential targets with a fold difference of greater than 1.2 and p ≤ 0.05 were identified; we utilized a reduced cutoff of 1.2 fold in order to stringently assess differentially regulated targets. However, careful individual analysis of all of these potential targets indicated that each was regulated with both Shh and Ihh treatment in the same direction though with subtle expression differences (data not shown); these differences may therefore be a result of recombinant ligand activity *in vitro* rather than true differential activation by Shh and Ihh ligand. Thus, we found no solid evidence for a differential response to Shh vs. Ihh by intestinal mesenchyme in these studies.
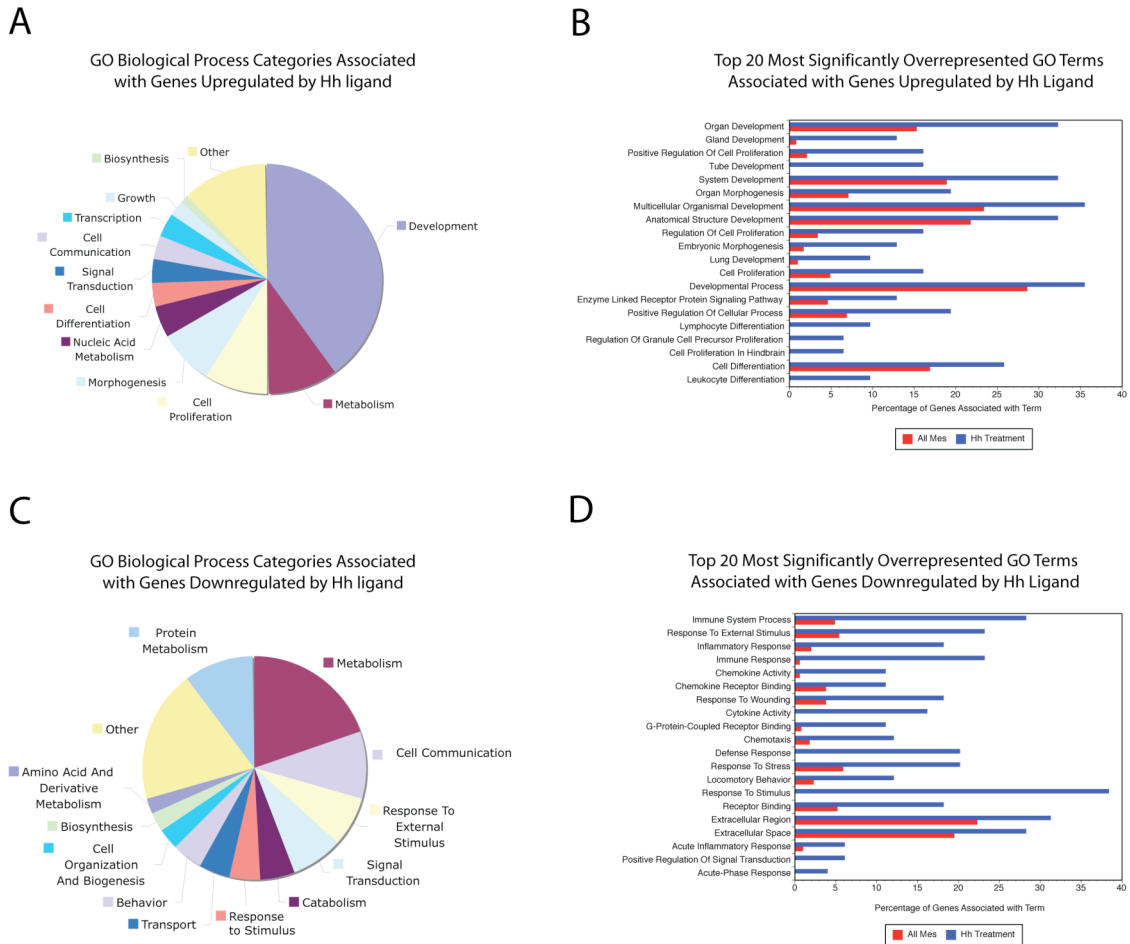
Figure 3.5: Ihh and Shh regulate developmental and inflammatory pathways in isolated intestinal mesenchyme. A) GO Biological Process categories associated with probesets upregulated by both Shh and Ihh ligand in intestinal mesenchyme. B) Top overrepresented GO terms associated with probesets upregulated by Hh. Upregulated probesets are associated with development. Associations include lung and hindbrain development, two organs where Hh signaling is important for differentiation. C) GO Biological categories associated of genes downregulated by both Shh and Ihh ligand in the intestinal mesenchyme. D.) Top overrepresented GO terms associated with probesets downregulated by Hh. Downregulated probesets are associated with immune and inflammatory signaling (see also Table 3.7).

Because of the overlap between the Shh and Ihh targets, we proceeded to examine the

properties of those targets that were significantly regulated by both Shh and Ihh in

cultured mesenchyme. DAVID Functional Annotation analysis revealed that genes

upregulated by both ligands are associated strongly with developmental processes (Figure

3.5A-B and Table 3.9); MeSH filtration demonstrated that genes upregulated by Hh

ligands were reported to be expressed in muscle and other mesenchymal cell types (Table

3.10). These findings suggest Hh signals are important in the development of muscle, in

accordance with the role of Hh signaling in development in the intestine (21; 22; 20) (see

also Chapter 2 of this thesis). Development of white blood cells was another top process

identified by the DAVID analysis, and several of the upregulated genes are expressed in

lymphocytes (Table 3.10), suggesting that Hh signals may play a role in the control of

immune cell specification in the intestine.

Strikingly, genes downregulated by both Shh and Ihh signaling were strongly

associated with pro-inflammatory function (Figure 3.5C-D); major intestinal

inflammatory players such as *Il-1β, IL-6*, and several key CC and CXC class chemokines

are downregulated by Hh signaling (Table 3.8). In addition, several key players in the

maturation and function of myeloid immune lineages, including *CD11b/Itgam* and *CD14*,

are downregulated in response to Hh signals. These results suggested that Hh signaling

might directly affect immune lineages in the small intestine (Table 3.11). Indeed, MeSH

filtration reveals that the majority of genes regulated by Hh are expressed in myeloid and

other immune cell types, bolstering the conclusion that the activation of Hh signaling in

intestinal mesenchyme regulates inflammatory processes in these cells (Table 3.12).

<u>Hh signals are sufficient to blunt the inflammatory response present in intestinal</u>

<u>mesenchyme following removal of the intestinal epithelium and entry to culture</u>

In order to better contextualize the gene expression changes found in isolated

mesenchyme after response to Hh signaling, we sought to profile the changes associated

with the transition of mesenchyme to culture. For this purpose, we utilized data from a

previous microarray examining gene expression in freshly isolated E18.5 mesenchyme

(27). We compared gene expression in this set of three microarray samples with three

chips hybridized with cDNA from mesenchyme treated with vehicle (the control set for

the Hh treatment analysis above; the cultured, vehicle-treated mesenchyme had been in

culture for a total of 72 hours). This comparison revealed 358 upregulated probesets and

76 downregulated probesets (Table 3.9) with expression changes greater than 5 fold in

cultured mesenchyme. We chose the cutoff of 5 fold for this comparison to identify the

subset of all regulated processes that were mostly strongly modified by the transition to

culture. In fact, over 7000 genes were changed greater than 2 fold during culture, with

the vast majority of these genes (~6000) demonstrating changes between 2 and 3 fold.

Importantly, *Gli1* (-7.3 fold)*, Ptch1* (-4.3 fold)*,* and *Hhip* (-12.5 fold), which are

all direct early target genes of Hh signaling (23; 28; 29), were each downregulated in

cultured mesenchyme, providing further evidence that removal of epithelium causes

reduced activity of the Hh pathway (Table 3.9). DAVID Functional Annotation analysis

revealed that the major upregulated gene categories during entry to culture include

inflammatory response, cytokine signaling and positive regulation of growth and

proliferation (Figure 3.6 and Table 3.10). Major downregulated processes included

muscle and extracellular matrix related-pathways (Table 3.11).
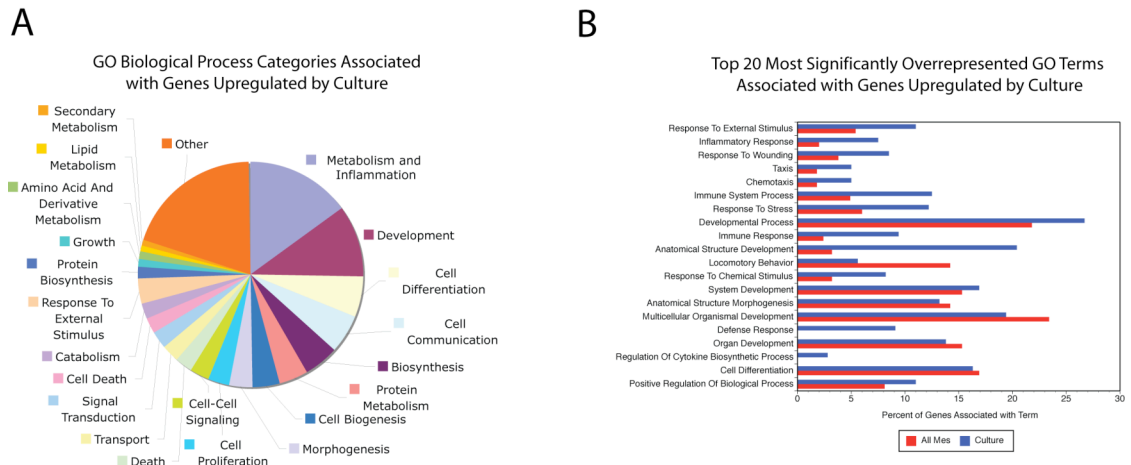
Figure 3.6: Removal of epithelium and transition to culture causes proliferation, inflammation, and decreased differentiation of intestinal mesenchyme. A) GO Biological Process Categories Associated with genes upregulated by culture. B) Top overrepresented GO terms of probesets upregulated by the transition to culture. Inflammation, stress response, and proliferation are the most upregulated processes.

Removal of the epithelium and culture of the mesenchyme is likely to modulate

more than just Hh signaling in the mesenchyme, since the epithelium is the source of a

number of signals in addition to Hh that can modify mesenchymal cell response (27). We

therefore examined the extent to which Hh signaling alone was capable of altering the

direction of pathway regulation (i.e. could Hh cause a gene that was downregulated by

culture to be instead upregulated), and directly compared those genes which were

regulated by transition to culture in one direction with those genes which were modified

by both Shh and Ihh ligand in the alternate direction. We predicted that this comparison

was likely to identify specifically those processes that were regulated by Hh signaling (with or without the aid of other epithelial factors).

First, we identified those genes that were downregulated by transition to culture (and removal of the source of Hh ligand) and upregulated by the addition of both Shh and Ihh. This cohort of genes (Table 3.12) includes the known direct Hh targets *Ptch1* and *Gli1*, as well as the recently identified likely direct target *Myocd* (Chapter 2). These findings indicate that this group of genes is likely to contain other novel direct Hh targets. Functional analysis with DAVID indicates that the major process related to this group of genes is organ development (Table 3.13); muscle development, leukocyte differentiation, and the smoothened signaling pathway are also associated with the genes in this group (data not shown).

Next, we identified those genes that were upregulated by transition to culture and downregulated by the addition of Hh ligand (Figure 3.7). Hierarchical clustering analysis identified two clusters of genes with low expression in isolated mesenchyme, upregulation during the process of culture, and downregulation with addition of Hh ligand; direct comparison of genes upregulated by culture and downregulated by Hh ligand expression identifies a subset of these genes that are most strongly associated with regulation both during culture and after Hh addition (Figure 3.7A). These genes (Table 3.14) are clearly pro-inflammatory (Table 3.15 and Figure 3.7B-C); many of the most strongly regulated genes in mesenchyme upon induction to culture are then markedly downregulated by addition of Hh ligand. This group includes *IL-1b*, *IL-6*, CC and CXC

chemokines, TLRs, and cell surface markers expressed on myeloid cells including

CD11b and *CD14*. These data strongly suggest that a) removal of the epithelium has a

pro-inflammatory effect on the mesenchyme, b) Hh acts as an anti-inflammatory signal,

and c) that Hh ligand alone can replace some of the anti-inflammatory activity provided

by the epithelium.
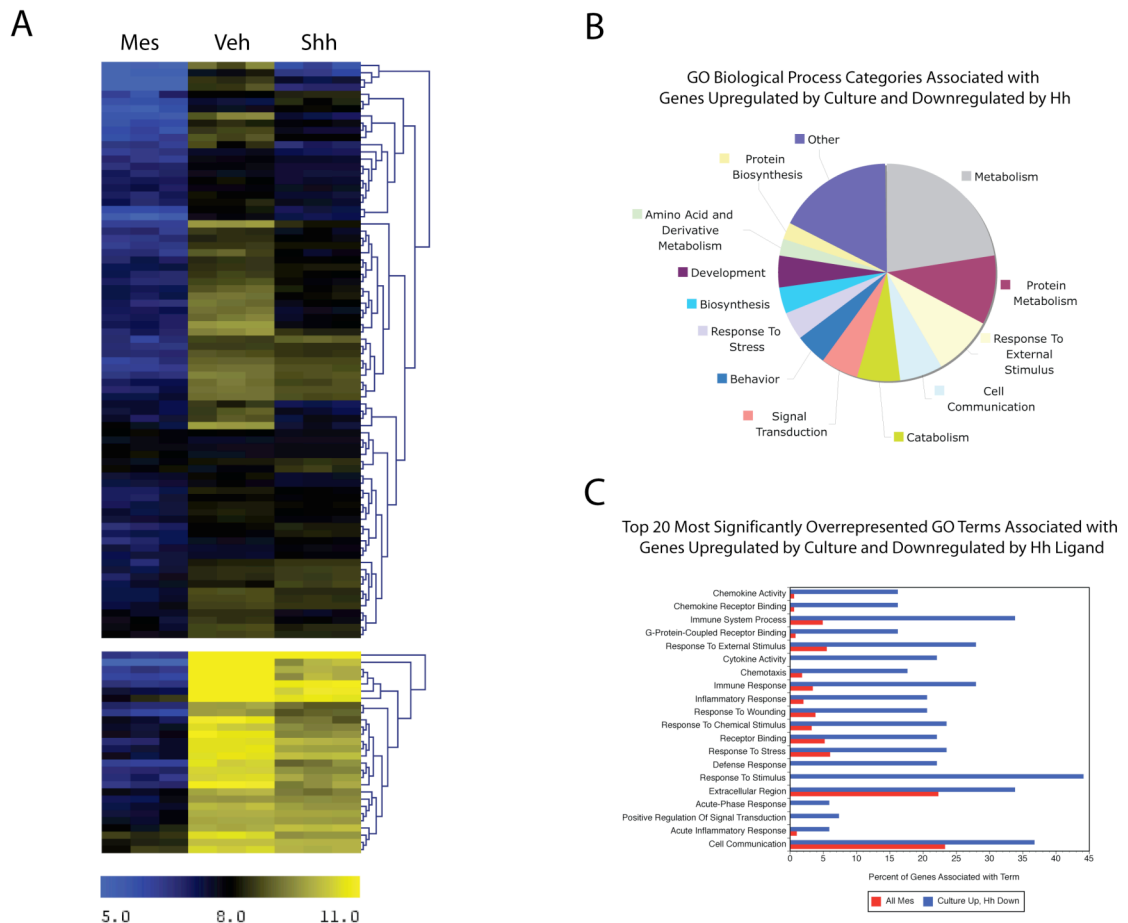
Figure 3.7: Shh and Ihh signaling drive differentiation and inhibit inflammation in isolated mesenchyme. A) K mean and hierarchical clustering analysis of gene expression from freshly isolated mesenchyme (Mes), cultured mesenchyme (Veh), and Hh ligand-treated cultured mesenchyme (Shh). Gene expression level is indicated by color, with blue indicating low expression, black moderate expression, and yellow high expression. Two clusters of genes that were low in freshly isolated mesenchyme, more highly expressed in cultured mesenchyme, and reduced with Hh ligand expression were visualized. B,C) Genes upregulated by culture and downregulated by Hh are overwhelmingly associated with inflammation and immune response.

Small Intestinal Myeloid Immune Lineages Respond Directly to Hh Signaling

One striking finding of these microarray data was a clear association of Hh-regulated genes with expression in myeloid cell lineages. Myeloid cells have been reported to respond directly to Hh signals in the context of the spleen (30) and colon (18), but intestinal myeloid lineages have specific characteristics (12; 31; 32), and we wished to assess whether myeloid cells responded to Hh signals in the small intestine. To this end, we utilized Gli1$^{+/LacZ}$ animals, which express β-galactosidase under the control of the Gli1 locus. Because Gli1 is a direct Hh target expressed only in cells that respond to Hh signaling (33), β-galactosidase expression is a sensitive readout of Hh response in these animals. We examined β-galactosidase expression in Cd19-positive B cells, Cd3-positive T cells, and Cd11b and Cd11c-positive myeloid lineages. As shown in Figure 3.8, Cd11b and Cd11c positive myeloid lineages in the small intestine respond directly to Hh signals and express β-galactosidase from the *Gli1* locus (Figure 3.8A-F), but lymphocyte populations do not appear to respond to Hh signals under homeostatic conditions (Figure 3.8A-B and data not shown). Myeloid lineages are known to be immunomodulatory in the small intestine (12) and these findings, in combination with our 12.4KVFHhip and microarray data, suggest that Hh signals may be acting to promote the creation or function of tolerogenic myeloid populations in the small intestine.
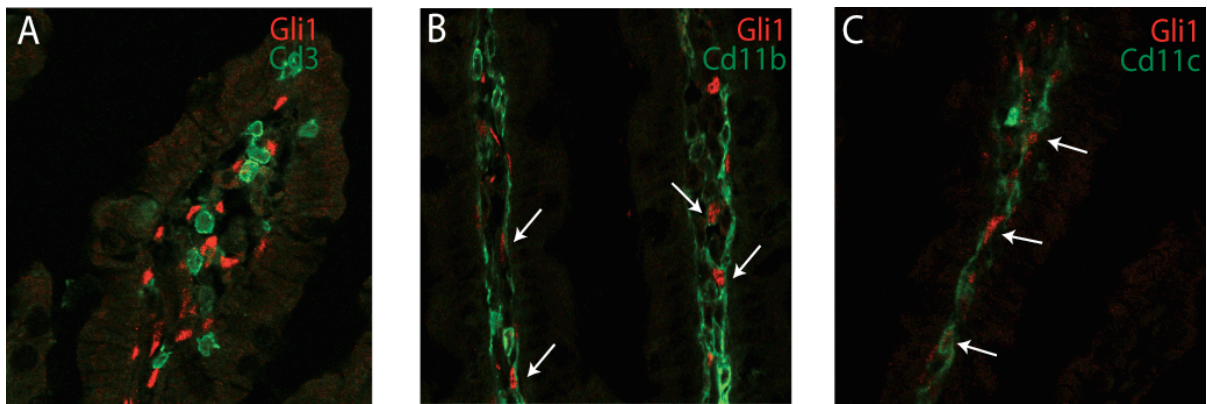
Figure 3.8: Small intestine myeloid lineages respond directly to Hh signaling. Co-staining of LacZ in Gli1+/LacZ animals with Cd3 (A), Cd11b (B), or Cd11c (C). Nuclei detected with anti-LacZ antibody are shown in red, and cell surface stainings for immune lineages are in green. Images shown are representative .30μm confocal slices taken at 600x. Cd11b- and Cd11c-positive myeloid lineages respond to Hh signals and express LacZ (arrows) while Cd3 positive lymphoid lineages do not.

Discussion

The data in this chapter demonstrate that Hh signaling is an important epithelial modulator of inflammatory signaling in the small intestinal lamina propria. Microarray analyses of Shh-or Ihh-treated isolated E18.5 mesenchyme all showed immune response and inflammatory pathways among the top regulated processes. In addition, these studies demonstrate that exogenous Hh alone can modulate the strong pro-inflammatory response seen in cultured mesenchyme, suggesting that Hh signals act in an anti-inflammatory manner in the small intestine. In accordance with this hypothesis, analysis of adult 12.4KVFHhip animals demonstrates that chronic reduction of Hh signals in the adult intestine leads to villus loss, spontaneous inflammation, and death. Taken together, these data provide the first direct evidence implicating epithelial Hh signals in modulation of inflammation in the small intestine, and provide insight into the role of this developmental signaling pathway in immune homeostasis in the gut.

One of the key trends to emerge from our microarray experiments was the observation that Hh signals stimulate the expression of genes involved in the development and function of smooth muscle cells, including *Myocd, Igf1*, and *Fgfr2*. This is an important observation in concert with extensive data indicating that Hh signals are crucial in smooth muscle development in the GI tract (21; 22; 20), lung (34), bladder (35), ureter (36), and vasculature (37). In addition, recent data from our group indicate that Hh signals stimulate development of villus and muscularis mucosa smooth muscle through direct activation of Myocardin (Chapter 2). The fact that Myocardin and several other smooth muscle-related genes were also identified in our microarray experiments emphasizes the importance of Hh signals in the specification of smooth muscle cells. In addition, comparison of the putative positive Hh target list identified in our studies to that derived from the Shh^{-/-} lung (34), which also has a clear reduction in smooth muscle, reveals a substantial overlap (Table 3.16), suggesting that at least some of the genes regulated by Hh during the specification of smooth muscle are common in multiple Hh-expressing organs.

The other major trend of our microarray studies was the unexpected identification of Hh signals as a key regulator of immune pathways in the intestine. It is worth noting that Hh ligand has been implicated in other *in vitro* studies as a pro-inflammatory modulator (38), though a later study suggests that this effect may be due to contamination of recombinant Hh ligand with pro-inflammatory impurities such as endotoxin (39). If in fact recombinant Hh ligands contain pro-inflammatory contaminants, the fact that our

data show that Hh ligands act in an anti-inflammatory manner in our *in vitro* intestinal mesenchyme cultures despite such potential contamination is remarkable.

Given the role of Hh in fate determination in multiple systems, it is tempting to speculate that the changes in expression that we interpret as modification of inflammatory signals actually represent modulation of the fate or activity of ISEMF or myeloid lineages. The major pathways identified in our expression data are clearly associated with myeloid cell innate immunity, and our data demonstrate that myeloid cells respond directly to Hh signaling in the small intestine. Myeloid cells are key determinants of inflammation in the intestine (12), exerting regulatory control and communicating with other cell types to maintain proper immune homeostasis. Differential populations of myeloid cells are pro- or anti-inflammatory, and one intriguing possibility is that Hh signals help to create or maintain a proper balance of tolerogenic versus pro-inflammatory myeloid populations in the small intestine; this possibility will need to be formally explored in future studies. Additionally, ISEMFs have been shown to secrete inflammatory mediators (40), and are responsive to Hh signaling throughout life (19). Reduction in Hh signaling in late gestation (20) or postnatally (Chapter 2) leads to mislocalization of ISEMFs. Such changes in localization or function stimulated by reduced Hh may provoke altered inflammatory signaling from this regulatory population. Together, the myeloid and ISEMF populations provide the best candidates for the cellular targets that receive Hh signals intended to regulate immune response and inflammation.

Recent studies in the colon suggest that reduction in Hh signal transduction predisposes to inflammation in both mouse and human (18). Our results emphasize the point that functional Hh signals are required to maintain a tolerogenic milieu in the context of the mammalian gut. The stochastic nature of the development of significant inflammatory disease in 12.4KVFHhip suggests that an unknown stimulus is needed to begin the inflammatory process. This stimulus may be the aberrant response to normal trauma in the absence of functional Hh signaling, a barrier breach due to villus loss, or another trigger. Regardless of the precise etiology, Hh signaling is clearly required to protect from such an event as single transgenic littermates of 12.4KVFHhip animals survive without inflammatory disease.

The inflammatory phenotype seen in 12.4KVFHhip animals shares similarities with both human Crohn's disease and Celiac disease. The inflammation is patchy and can be transmural, characteristics similar to Crohn's. More strikingly, 12.4KVFHhip animals exhibit villus atrophy, crypt hyperplasia, and profound inflammation, mirroring a Celiac-like phenotype. Moreover, the wasting disease experienced by older 12.4KVFHhip animals may be the result of malnutrition secondary to lost absorptive surface after villus loss and inflammation; reduced absorptive surface is a hallmark of Celiac disease in humans. Additionally, the prevalence of dermatitis in 12.4KVFHhip animals parallels the high incidence of dermatitis herpetiformis in human Celiac disease patients (41). While the dermatitis in 12.4KVFHhip animals may be a result of malabsorption or malnutrition, some 12.4KVFHhip skin lesions demonstrate IgA deposition, a key finding in Celiac-related dermatitis in humans. Finally, loss of smooth muscle may be a first step

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in the development of inflammation in 12.4KVFHhip animals. Likewise, in humans with Celiac disease, smooth muscle populations are affected; anti-smooth muscle antibodies are often found in Celiac patients and may help identify a subset of those patients who are particularly susceptible to advanced disease (42).

In addition, the fact that 12.4KVFHhip animals develop "spontaneous" inflammation is of particular interest. Spontaneous small intestinal inflammation is rare, though it has been observed in a few other models, including older IL-10 knockout mice (43) and in OCTN2 null animals, which also display villus atrophy (44). Notably, little small intestinal inflammation is seen in other models that develop spontaneous colitis (4; 45), suggesting that the mechanisms underlying inflammatory disease in the colon and small intestine are different in some important respects. Hh, however, has been implicated in both colonic (18) and now small intestinal inflammatory regulation. This is an intriguing finding in light of significant recent studies demonstrating that some human susceptibility loci are associated with Celiac disease and Ulcerative Colitis; in particular, polymorphism in the IL2/IL21 region of the genome confers increased susceptibility to both UC and Celiac disease (46). The finding that Hh signals appear to be important in both the small intestine and colon as well as the fact 12.4KVFHhip animals develop small intestinal inflammation with characteristics similar to both Celiac and IBD suggest that Hh signals may impact a process common to the pathogenesis of Celiac and UC.

Overall, the data presented here provide novel evidence that Hh signaling is an important anti-inflammatory signal in the small intestine. The inflammatory milieu in the

small intestine is specialized, and many studies have shown that tolerogenic signals from both the stroma and epithelium are critical in modulating the tolerogenic response of the small intestine innate immune system (12; 47; 48). The emerging role of the Hh signaling pathway as important modulator of inflammation identifies an additional cellular signaling molecule from the epithelium as an important factor in balancing the inflammatory response of the mesenchyme. It is possible, of course, that the role of Hh signals as an anti-inflammatory modulator may require other intestine-specific signals. However, these microarray studies show that Hh alone can alter inflammatory signaling in isolated mesenchyme. Lack of this proper homeostatic influence may predispose to a disordered inflammatory response even in the presence of an otherwise normal immune system and may contribute to human gastrointestinal disease.

Acknowledgements

Table 3.1: Shh Regulated Genes in Isolated Mesenchyme

Probe ID	Gene Symbol	Description	FC
1428853_at	Ptch1	Patched Homolog 1	2.66
1419692_a_at	Ltc4S	Leukotriene C4 Synthase	2.4
1417597_at	Cd28	Cd28 Antigen	2.32
1430570_at	Kynu	Kynureninase (L-Kynurenine Hydrolase)	2.26
1457871_at	Colec10	Collectin Sub-Family Member 10	2.23
1449038_at	Hsd11B1	Hydroxysteroid 11-Beta Dehydrogenase 1	2.18
1460000_at	Shisa3	Shisa homolog 3 (xenopus laevis)	2.14
1425978_at	Myocd	Myocardin	2.09
1439663_at	Ptch1	Patched Homolog 1	2.05
1450019_at	Cx3Cr1	Chemokine (C-X3-C) Receptor 1	2.05
1434152_at	Apol7C	Apolipoprotein L 7c	2.01
1431385_a_at	Mbtps1	Membrane-Bound Transcription Factor Peptidase, Site 1	1.97
1419662_at	Ogn	Osteoglycin	1.94
1439517_at	Mysm1	Myb-like, SWIRM and MPN domains 1	1.93
1435290_x_at	H2-Aa	Histocompatibility 2, Class Ii Antigen A, Alpha	1.91
1445991_at	Lhfp	Lipoma Hmgic Fusion Partner	1.9
1448303_at	Gpnmb	Glycoprotein (Transmembrane) Nmb	1.89
1424808_at	Lama4	Laminin, Alpha 4	1.88
1435399_at	Synpo2	Synaptopodin 2	1.88
1429272_a_at	Apol7A	Apolipoprotein L 7A	1.86
1455224_at	Angptl1	Angiopoietin-Like 1	1.86
1453496_at	Fam162b	Family with sequence similarity 162, member B	1.85
1434528_at	Aard	Alanine and Arginine rich domain containing protein	1.85
1419519_at	Igf1	Insulin-Like Growth Factor 1	1.84
1426179_a_at	Twsg1	Twisted Gastrulation Homolog 1 (Drosophila)	1.84
1433492_at	Epb4.1L2	Erythrocyte Protein Band 4.1-Like 2	1.83
1437540_at	Mcoln3	Mucolipin 3	1.83
1419476_at	Adamdec1	Adam-Like, Decysin 1	1.82
1455050_at	E130203B14Rik	Riken Cdna E130203B14 Gene	1.82
1445938_at	5930427L02Rik	Riken Cdna 5930427L02 Gene	1.77
1455930_at	N/A	N/A	1.75
1458121_at	A430107O13Rik	Riken Cdna A430107O13 Gene	1.75
1427489_at	Itga8	Integrin Alpha 8	1.73
1457038_at	Frem2	Fras1 Related Extracellular Matrix Protein 2	1.71
1424733_at	P2ry14	Purinergic Receptor P2Y, G-Protein Coupled, 14	1.7
1421895_at	Eif2S3x	Eukaryotic Translation Initiation Factor 2, Subunit 3, Structural Gene X-Linked	1.7
1455318_at	Timd4	T-Cell Immunoglobulin And Mucin Domain Containing 4	1.7
1420610_at	Prkacb	Protein Kinase, Camp Dependent, Catalytic, Beta	1.69
1429466_s_at	Aph1B/1C	Anterior Pharynx Defective 1B Homolog (C. Elegans)	1.69
1417672_at	Slc4A10	Solute Carrier Family 4, Sodium Bicarbonate Cotransporter-Like, Member 10	1.69

1437401_at	Igf1	Insulin-Like Growth Factor 1	1.68
1430427_a_at	Pcdh18	Protocadherin 18	1.67
1440862_at	Tmem181	Transmembrane protein 181	1.65
1452014_a_at	Igf1	Insulin-Like Growth Factor 1	1.65
1421168_at	Abcg3	Atp-Binding Cassette, Sub-Family G (White), Member 3	1.65
1449178_at	Pdlim3	Pdz And Lim Domain 3	1.64
1428549_at	Ccdc3	Coiled-Coil Domain Containing 3	1.64
1420965_a_at	Enc1	Ectodermal-Neural Cortex 1	1.63
1419592_at	Unc5C	Unc-5 Homolog C (C. Elegans)	1.63
1421792_s_at	Trem2	Triggering Receptor Expressed On Myeloid Cells 2C	1.63
1445427_at	Nlk	Nemo Like Kinase	1.63
1443996_at	Na	Na	1.63
1415824_at	Scd2	Stearoyl-Coenzyme A Desaturase 2	1.63
1421137_a_at	Pkib	Protein Kinase Inhibitor Beta, Camp Dependent, Testis Specific	1.62
1446682_at	Zswim6	Zinc Finger, Swim Domain Containing 6	1.62
1450020_at	Cx3Cr1	Chemokine (C-X3-C) Receptor 1	1.62
1429072_at	1110001D15Rik	Riken Cdna 1110001D15 Gene	1.61
1434413_at	Igf1	Insulin-Like Growth Factor 1	1.61
1447227_at	Slc40A1	Solute Carrier Family 39 (Iron-Regulated Transporter), Member 1	1.61
1419289_a_at	Syngr1	Synaptogyrin 1	1.6
1427038_at	Penk1	Preproenkephalin 1	1.6
1421933_at	Cbx5	Chromobox Homolog 5 (Drosophila Hp1A)	1.6
1423326_at	Entpd1	Ectonucleoside Triphosphate Diphosphohydrolase 1	1.6
1422967_a_at	Tfrc	Transferrin Receptor	1.6
1441172_at	Aff3	Af4/Fmr2 Family, Member 3	1.59
1439567_at	Tbx3	T-Box 3	1.59
1450881_s_at	Gpr137B	G Protein-Coupled Receptor 137B	1.59
1422341_s_at	Lypla3	Lysophospholipase 3	1.59
1433489_s_at	Fgfr2	Fibroblast Growth Factor Receptor 2	1.59
1430984_at	Azin1	Antizyme Inhibitor 1	1.58
1443208_at	Gpr137B	G Protein-Coupled Receptor 137B	1.58
1416111_at	Cd83	Cd83 Antigen	1.58
1455900_x_at	Tgm2	Transglutaminase 2, C Polypeptide	1.57
1439256_x_at	Gpr137B	G Protein-Coupled Receptor 137B	1.57
1420847_a_at	Fgfr2	Fibroblast Growth Factor Receptor 2	1.57
1457047_at	N/A	N/A	1.57
1432304_a_at	9030624J02Rik	Riken Cdna 2610029J22 Gene	1.57
1450939_at	Entpd1	Ectonucleoside Triphosphate Diphosphohydrolase 1	1.56
1420699_at	Clec7A	C-Type Lectin Domain Family 7, Member A	1.56
1424265_at	Npl	N-Acetylneuraminate Pyruvate Lyase	1.56
1434909_at	Rragd	Ras-Related Gtp Binding D	1.55
1435903_at	Cd300A	Cd300A Antigen	1.55
1435134_at	Aadacl1	Arylacetamide Deacetylase-Like 1	1.54
1450061_at	Enc1	Ectodermal-Neural Cortex 1	1.54
1416416_x_at	Gstm1	Glutathione S-Transferase, Mu 1	1.54
1429338_a_at	Nol9	Nucleolar Protein 9	1.53

1439255_s_at	Gpr137B	G Protein-Coupled Receptor 137B	1.53
1438183_x_at	Sord	Sorbitol Dehydrogenase	1.53
1449751_at	Slc6A6	Solute Carrier Family 6 (Neurotransmitter Transporter, Taurine), Member 6	1.53
1440080_at	Srpk2	Serine/Arginine-Rich Protein Specific Kinase 2	1.53
1453956_a_at	Pftk1	Pftaire Protein Kinase 1	1.53
1415948_at	Creg1	Cellular Repressor Of E1A-Stimulated Genes 1	1.52
1424007_at	Gdf10	Growth Differentiation Factor 10	1.52
1417399_at	Gas6	Growth Arrest Specific 6	1.52
1423630_at	Cygb	Stellate Cell Activation Associated Protein	1.51
1452528_a_at	Nkx2-3	Nk2 Transcription Factor Related, Locus 3 (Drosophila)	1.51
1459731_at	N/A	N/A	1.51
1422646_at	Mga	Max Gene Associated	1.51
1421899_a_at	Mr1	Major Histocompatibility Complex, Class I-Related	1.51
1438661_a_at	Arf2	Adp-Ribosylation Factor 2	1.51
1417012_at	Sdc2	Syndecan 2	1.51
1416023_at	Fabp3	Fatty Acid Binding Protein 3, Muscle And Heart	1.51
1432344_a_at	Aplp2	Amyloid Beta (A4) Precursor-Like Protein 2	1.51
1423072_at	6720475J19Rik	Riken Cdna 6720475J19 Gene	1.5
1450051_at	Atrx	Alpha Thalassemia/Mental Retardation Syndrome X-Linked Homolog (Human)	1.5
1451406_a_at	Pcsk5	Proprotein Convertase Subtilisin/Kexin Type 5	-1.5
1437318_at	Pak3	P21 (Cdkn1A)-Activated Kinase 3	-1.5
1439747_at	Ptges	Prostaglandin E Synthase	-1.5
1447179_at	Fam108c	Family with sequence similarity 108, member C	-1.51
1433956_at	Cdh5	Cadherin 5	-1.51
1425526_a_at	Prrx1	Paired Related Homeobox 1	-1.51
1453783_at	6330411E07Rik	Riken Cdna 6330411E07 Gene	-1.51
1440773_at	Fbxo8	F-Box Only Protein 8	-1.51
1450798_at	Tnxb	Tenascin X	-1.51
1441547_at	Nfia	Nuclear Factor I/A	-1.51
1452352_at	Ctla2B	Cytotoxic T Lymphocyte-Associated Protein 2 Beta	-1.51
1439048_at	Cadm2	Cell adhesion molecule 2	-1.51
1420499_at	Gch1	Gtp Cyclohydrolase 1	-1.51
1456251_x_at	Tspo	Benzodiazepine Receptor, Peripheral	-1.51
1449453_at	Bst1	Bone Marrow Stromal Cell Antigen 1	-1.51
1416686_at	Plod2	Procollagen Lysine, 2-Oxoglutarate 5-Dioxygenase 2	-1.52
1454037_a_at	Flt1	Fms-Like Tyrosine Kinase 1	-1.52
1440615_at	Dusp16	Dna Segment, Chr 6, Erato Doi 213, Expressed	-1.52
1456661_at	Jarid2	Jumonji, At Rich Interactive Domain 2	-1.52
1424375_s_at	Gimap4	Gtpase, Imap Family Member 4	-1.52
1426818_at	Soat1	Sterol O-Acyltransferase 1	-1.52
1422694_at	Ttyh1	Tweety Homolog 1 (Drosophila)	-1.52
1434089_at	Synpo	Synaptopodin	-1.52
1427313_at	Ptgir	Prostaglandin I Receptor (Ip)	-1.52
1448562_at	Upp1	Uridine Phosphorylase 1	-1.52
1431424_at	2810055G20Rik	Riken Cdna 2810055G20 Gene	-1.52
1431689_at	5830400J07Rik	Riken Cdna 5830400J07 Gene	-1.53

1448600_s_at	Vav3	Vav 3 Oncogene	-1.53
1416432_at	Pfkfb3	6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3	-1.53
1435144_at	Fyb	Fyn Binding Protein	-1.53
1439622_at	Rassf4	Ras association (RalGDS/AF-6) domain family member 4	-1.53
1435264_at	Emilin2	Elastin Microfibril Interfacer 2	-1.53
1431109_at	Prr16	Proline rich 16	-1.53
1422978_at	Cybb	Cytochrome B-245, Beta Polypeptide	-1.53
1423311_s_at	Tpbg	Trophoblast Glycoprotein	-1.53
1431394_a_at	Lrrk2	Leucine-Rich Repeat Kinase 2	-1.53
1428942_at	Mt2	Metallothionein 2	-1.53
1433638_s_at	Hoxd8	Homeo Box D8	-1.54
1434322_at	Micall2	MICAL-like 2	-1.54
1424524_at	1200002N14Rik	Riken Cdna 1200002N14 Gene	-1.54
1426159_x_at	Tcrb-V13	T-Cell Receptor Beta, Variable 13	-1.54
1418133_at	Bcl3	B-Cell Leukemia/Lymphoma 3	-1.54
1418911_s_at	Acsl4	Acyl-Coa Synthetase Long-Chain Family Member 4	-1.54
1428777_at	Spred1	Sprouty Protein With Evh-1 Domain 1, Related Sequence	-1.54
1417680_at	Kcna5	Potassium Voltage-Gated Channel, Shaker-Related Subfamily, Member 5	-1.54
1434719_at	A2M	Alpha-2-Macroglobulin	-1.54
1452365_at	Csgalnact1	Chondroitin sulfate N-acetylgalactosaminyltransferase 1	-1.54
1428392_at	Rassf2	Ras Association (Ralgds/Af-6) Domain Family 2	-1.54
1453595_at	2900064B18Rik	Riken Cdna 2900064B18 Gene	-1.54
1429693_at	Dab2	Disabled Homolog 2 (Drosophila)	-1.55
1460250_at	Sostdc1	Sclerostin Domain Containing 1	-1.55
1441150_x_at	Mbd1	Methyl-Cpg Binding Domain Protein 1	-1.55
1427932_s_at	1200016E24Rik	Riken Cdna 1200016E24 Gene	-1.55
1436199_at	Trim14	Tripartite Motif-Containing 14	-1.55
1457231_at	Hif1A	Hypoxia Inducible Factor 1, Alpha Subunit	-1.55
1435852_at	Spred3	Sprouty-Related, Evh1 Domain Containing 3	-1.55
1449222_at	Ebi3	Epstein-Barr Virus Induced Gene 3	-1.55
1449871_at	Tbx18	T-Box18	-1.55
1437218_at	Fn1	Fibronectin 1	-1.56
1418015_at	Pum2	Pumilio 2 (Drosophila)	-1.56
1446276_at	N/A	N/A	-1.56
1455965_at	Adamts4	A Disintegrin-Like And Metallopeptidase (Reprolysin Type) With Thrombospondin Type 1 Motif, 4	-1.56
1419100_at	Serpina3N	Serine (Or Cysteine) Peptidase Inhibitor, Clade A, Member 3N	-1.56
1434553_at	Tmem56	transmembrane protein 56	-1.56
1420768_a_at	Dhx58	DEXH (Asp-Glu-X-His) box polypeptide 58	-1.56
1422782_s_at	Tlr3	Toll-Like Receptor 3	-1.56
1452179_at	Phf17	Phd Finger Protein 17	-1.57
1456288_at	Slfn5	Schlafen 5	-1.57

1427844_a_at	Cebpb	Ccaat/Enhancer Binding Protein (C/Ebp), Beta	-1.57
1448069_at	Tm4Sf1	Transmembrane 4 Superfamily Member 1	-1.57
1438948_x_at	Tspo	Benzodiazepine Receptor, Peripheral	-1.58
1425226_x_at	Tcrb-V13	T-Cell Receptor Beta, Variable 13	-1.58
1418511_at	Dpt	Dermatopontin	-1.58
1437473_at	Maf	Avian Musculoaponeurotic Fibrosarcoma (V-Maf) As42 Oncogene Homolog	-1.58
1439773_at	Ly6E	Lymphocyte Antigen 6 Complex, Locus E	-1.59
1415943_at	Sdc1	Syndecan 1	-1.59
1436293_x_at	D1Ertd471E	Dna Segment, Chr 1, Erato Doi 471, Expressed	-1.59
1452244_at	6330406I15Rik	Riken Cdna 6330406I15 Gene	-1.59
1417065_at	Egr1	Early Growth Response 1	-1.6
1420250_at	N/A	N/A	-1.6
1436999_at	5033414K04Rik	Riken Cdna 5033414K04 Gene	-1.6
1417795_at	Chl1	Cell Adhesion Molecule With Homology To L1Cam	-1.6
1418901_at	Cebpb	Ccaat/Enhancer Binding Protein (C/Ebp), Beta	-1.6
1450112_a_at	Gas2	Growth Arrest Specific 2	-1.6
1416286_at	Rgs4	Regulator Of G-Protein Signaling 4	-1.6
1419427_at	Csf3	Colony Stimulating Factor 3 (Granulocyte)	-1.6
1421852_at	Kcnk5	Potassium Channel, Subfamily K, Member 5	-1.6
1458268_s_at	Igfbp3	Insulin-Like Growth Factor Binding Protein 3	-1.6
1423267_s_at	Itga5	Integrin Alpha 5 (Fibronectin Receptor Alpha)	-1.6
1428680_at	Cds1	Cdp-Diacylglycerol Synthase 1	-1.61
1449591_at	Casp4	Caspase 4, Apoptosis-Related Cysteine Peptidase	-1.61
1435748_at	Gda	Guanine Deaminase	-1.61
1419060_at	Gzmb	Granzyme B	-1.61
1419699_at	Scgb3A1	Secretoglobin, Family 3A, Member 1	-1.61
1456212_x_at	Socs3	Suppressor Of Cytokine Signaling 3	-1.61
1452117_a_at	Fyb	Fyn Binding Protein	-1.61
1424617_at	Ifi35	Interferon-Induced Protein 35	-1.61
1423100_at	Fos	Fbj Osteosarcoma Oncogene	-1.61
1440070_at	Chat	Choline Acetyltransferase	-1.62
1455606_at	Bc004022	Cdna Sequence Bc004022	-1.62
1441855_x_at	Cxcl1	Chemokine (C-X-C Motif) Ligand 1	-1.62
1456274_at	C230071H18Rik	Riken Cdna C230071H18 Gene	-1.62
1449027_at	Rhou	Ras Homolog Gene Family, Member U	-1.62
1417426_at	Srgn	Proteoglycan 1, Secretory Granule	-1.62
1447863_s_at	Nr4A2	Nuclear Receptor Subfamily 4, Group A, Member 2	-1.63
1423062_at	Igfbp3	Insulin-Like Growth Factor Binding Protein 3	-1.63
1418547_at	Tfpi2	Tissue Factor Pathway Inhibitor 2	-1.63
1416129_at	Errfi1	Erbb Receptor Feedback Inhibitor 1	-1.64
1434342_at	S100B	S100 Protein, Beta Polypeptide, Neural	-1.64
1450672_a_at	Trex1	Three Prime Repair Exonuclease 1	-1.64
1458683_at	Sirpb1	Sirp-Beta B	-1.64
1418344_at	Tmem8	Transmembrane Protein 8 (Five Membrane-Spanning Domains)	-1.64
1417813_at	Ikbke	Inhibitor Of Kappab Kinase Epsilon	-1.64
1423407_a_at	Fbln2	Fibulin 2	-1.64

1423519_at	Fam108c	Family with sequence similarity 108, member C	-1.64
1452294_at	Pcdh1	Protocadherin 1	-1.64
1437673_at	Wnt5A	Wingless-Related Mmtv Integration Site 5A	-1.65
1429743_at	6720468P15Rik	Riken Cdna 6720468P15 Gene	-1.65
1419569_a_at	Isg20	Interferon-Stimulated Protein	-1.66
1437939_s_at	Ctsc	Cathepsin C	-1.66
1450387_s_at	Ak3L1	Adenylate Kinase 3 Alpha-Like 1	-1.66
1434484_at	1100001G20Rik	Riken Cdna 1100001G20 Gene	-1.66
1436100_at	Sh2D5	Sh2 Domain Containing 5	-1.66
1430357_at	H3F3B	H3 Histone, Family 3A	-1.66
1449143_at	Rtp4	Receptor Transporter Protein 4	-1.66
1453678_at	Mbd1	Methyl-Cpg Binding Domain Protein 1	-1.67
1417859_at	Gas7	Growth Arrest Specific 7	-1.67
1419759_at	Abcb1A	Atp-Binding Cassette, Sub-Family B (Mdr/Tap), Member 1A	-1.67
1436919_at	Trp53I11	Trp53 Inducible Protein 11	-1.67
1426858_at	Inhbb	Inhibin Beta-B	-1.67
1450034_at	Stat1	Signal Transducer And Activator Of Transcription 1	-1.68
1429909_at	Na	Na	-1.68
1421207_at	Lif	Leukemia Inhibitory Factor	-1.68
1449468_at	St6Galnac5	St6 (Alpha-N-Acetyl-Neuraminyl-2,3-Beta-Galactosyl-1,3)-N-Acetylgalactosaminide Alpha-2,6-Sialyltransferase 5	-1.68
1428909_at	A130040M12Rik	Riken Cdna A130040M12 Gene	-1.68
1422868_s_at	Gda	Guanine Deaminase	-1.69
1435137_s_at	1200016E24Rik	Riken Cdna 1200016E24 Gene	-1.69
1427091_at	Znfx1	Zinc finger, NFX1-type containing 1	-1.69
1422470_at	Bnip3	Bcl2/Adenovirus E1B Interacting Protein 1, Nip3	-1.71
1420760_s_at	N/A	N/A	-1.71
1460116_s_at	Spred1	Sprouty Protein With Evh-1 Domain 1, Related Sequence	-1.71
1416238_at	Tie1	Tyrosine Kinase Receptor 1	-1.71
1460482_at	3110047P20Rik	Riken Cdna 3110047P20 Gene	-1.71
1420401_a_at	Ramp3	Receptor (Calcitonin) Activity Modifying Protein 3	-1.72
1460218_at	Cd52	Cd52 Antigen	-1.72
1451756_at	Flt1	Fms-Like Tyrosine Kinase 1	-1.72
1455034_at	Nr4A2	Nuclear Receptor Subfamily 4, Group A, Member 2	-1.73
1416576_at	Socs3	Suppressor Of Cytokine Signaling 3	-1.73
1416855_at	Gas1	Growth Arrest Specific 1	-1.73
1455030_at	Ptprj	Protein Tyrosine Phosphatase, Receptor Type, J	-1.74
1426340_at	Slc1A3	Solute Carrier Family 1 (Glial High Affinity Glutamate Transporter), Member 3	-1.74
1435454_a_at	Bc006779	Cdna Sequence Bc006779	-1.74
1460197_a_at	Steap4	Steap Family Member 4	-1.75
1449340_at	Sostdc1	Sclerostin Domain Containing 1	-1.76
1419758_at	Abcb1A	Atp-Binding Cassette, Sub-Family B (Mdr/Tap), Member 1A	-1.76
1418499_a_at	Kcne3	Potassium Voltage-Gated Channel, Isk-Related Subfamily, Gene 3	-1.76

1460011_at	Cyp26B1	Cytochrome P450, Family 26, Subfamily B, Polypeptide 1	-1.76
1419684_at	Ccl8	Chemokine (C-C Motif) Ligand 8	-1.76
1456786_at	Ldb2	Lim Domain Binding 2	-1.77
1452911_at	Spred1	Sprouty Protein With Evh-1 Domain 1, Related Sequence	-1.77
1439902_at	C5Ar1	Complement Component 5A Receptor 1	-1.77
1448728_a_at	Nfkbiz	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Zeta	-1.77
1417266_at	Ccl6	Chemokine (C-C Motif) Ligand 6	-1.78
1422046_at	Itgam	Integrin Alpha M	-1.78
1453757_at	Herc5	Hect Domain And Rld 5	-1.78
1420464_s_at	Pira1/2/3/4/6	Paired-Ig-Like Receptor A3	-1.78
1450750_a_at	Nr4A2	Nuclear Receptor Subfamily 4, Group A, Member 2	-1.78
1419132_at	Tlr2	Toll-Like Receptor 2	-1.79
1426772_x_at	Tcrb-V13	T-Cell Receptor Beta, Variable 13	-1.79
1436778_at	Cybb	Cytochrome B-245, Beta Polypeptide	-1.8
1450586_at	Bdkrb1	Bradykinin Receptor, Beta 1	-1.8
1444531_at	Sod2	Superoxide Dismutase 2, Mitochondrial	-1.8
1426937_at	6330406I15Rik	Riken Cdna 6330406I15 Gene	-1.8
1417122_at	Vav3	Vav 3 Oncogene	-1.8
1452418_at	1200016E24Rik	Riken Cdna 1200016E24 Gene	-1.81
1417633_at	Sod3	Superoxide Dismutase 3, Extracellular	-1.82
1450764_at	Aoah	Acyloxyacyl Hydrolase	-1.82
1450448_at	Stc1	Stanniocalcin 1	-1.83
1418879_at	Fam110c	Family with sequence similarity 110, member C	-1.83
1451564_at	Parp14	Poly (ADP-ribose) polymerase family, member 14	-1.83
1418932_at	Nfil3	Nuclear Factor, Interleukin 3, Regulated	-1.84
1435541_at	Btc	Betacellulin, Epidermal Growth Factor Family Member	-1.84
1424302_at	Lilrb3	Leukocyte Immunoglobulin-Like Receptor, Subfamily B (With Tm And Itim Domains), Member 3	-1.84
1454976_at	Sod2	Superoxide Dismutase 2, Mitochondrial	-1.85
1420249_s_at	Ccl6	Chemokine (C-C Motif) Ligand 6	-1.86
1425854_x_at	Tcrb-V13	T-Cell Receptor Beta, Variable 13	-1.86
1418842_at	Hcls1	Hematopoietic Cell Specific Lyn Substrate 1	-1.87
1448610_a_at	Sod2	Superoxide Dismutase 2, Mitochondrial	-1.88
1420804_s_at	Clec4D	C-Type Lectin Domain Family 4, Member D	-1.89
1421830_at	Ak3L1	Adenylate Kinase 3 Alpha-Like 1	-1.9
1416298_at	Mmp9	Matrix Metallopeptidase 9	-1.91
1440481_at	Stat1	Signal Transducer And Activator Of Transcription 1	-1.91
1460469_at	Tnfrsf9	Tumor Necrosis Factor Receptor Superfamily, Member 9	-1.92
1422041_at	Pilrb1	Paired Immunoglobin-Like Type 2 Receptor Beta	-1.92
1439925_at	Tm4Sf1	Transmembrane 4 Superfamily Member 1	-1.92
1417876_at	Fcgr1	Fc Receptor, Igg, High Affinity I	-1.93
1428660_s_at	Tor3A	Torsin Family 3, Member A	-1.95
1448291_at	Mmp9	Matrix Metallopeptidase 9	-1.96
1417483_at	Nfkbiz	Nuclear Factor Of Kappa Light Polypeptide Gene	-1.98

Enhancer In B-Cells Inhibitor, Zeta

1420380_at	Ccl2	Chemokine (C-C Motif) Ligand 2	-1.98
1426112_a_at	Cd72	Cd72 Antigen	-1.99
1425407_s_at	Clec4A2/B1	C-Type Lectin Domain Family 4, Member A2	-1.99
1439819_at	Ctsc	Cathepsin C	-2.01
1418937_at	Dio2	Deiodinase, Iodothyronine, Type Ii	-2.01
1448025_at	Sirpb1	Sirp-Beta B	-2.01
1460259_s_at	Clca1/A2	Chloride Channel Calcium Activated ½	-2.02
1440926_at	Flt1	Fms-Like Tyrosine Kinase 1	-2.02
1452205_x_at	Tcrb-V13	T-Cell Receptor Beta, Variable 13	-2.02
1417193_at	Sod2	Superoxide Dismutase 2, Mitochondrial	-2.03
1440342_at	N/A	N/A	-2.04
1445804_at	Nav3	Neuron Navigator 3	-2.05
1448775_at	N/A	N/A	-2.06
1424921_at	Bst2	Bone Marrow Stromal Cell Antigen 2	-2.06
1451161_a_at	Emr1	Egf-Like Module Containing, Mucin-Like, Hormone Receptor-Like Sequence 1	-2.08
1435560_at	Itgal	Integrin Alpha L	-2.09
1460454_at	Glod5	Glyoxalase domain containing 5	-2.1
1449252_at	Fam110c	Family with sequence similarity 110, member C	-2.11
1416593_at	Glrx	Glutaredoxin	-2.13
1457644_s_at	Cxcl1	Chemokine (C-X-C Motif) Ligand 1	-2.14
1457404_at	Nfkbiz	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Zeta	-2.14
1418847_at	Arg2	Arginase Type Ii	-2.17
1418480_at	Ppbp	Chemokine (C-X-C Motif) Ligand 7	-2.17
1427327_at	Pilra	paired immunoglobin-like type 2 receptor alpha	-2.17
1438841_s_at	Arg2	Arginase Type Ii	-2.18
1424775_at	Oas1A	2'-5' Oligoadenylate Synthetase 1A	-2.2
1421228_at	Ccl7	Chemokine (C-C Motif) Ligand 7	-2.21
1436779_at	Cybb	Cytochrome B-245, Beta Polypeptide	-2.23
1452408_at	N/A	N/A	-2.23
1448881_at	Hp	Haptoglobin	-2.24
1419420_at	St6Galnac5	St6 (Alpha-N-Acetyl-Neuraminyl-2,3-Beta-Galactosyl-1,3)-N-Acetylgalactosaminide Alpha-2,6-Sialyltransferase 5	-2.25
1430770_at	3110080E11Rik	Riken Cdna 3110080E11 Gene	-2.26
1460603_at	Samd9L	Est Aa175286	-2.28
1429184_at	Gvin1	Gtpase, Very Large Interferon Inducible 1	-2.28
1429413_at	Cpm	Carboxypeptidase M	-2.28
1448494_at	Gas1	Growth Arrest Specific 1	-2.28
1451905_a_at	Mx1	Myxovirus (Influenza Virus) Resistance 1	-2.3
1419728_at	Cxcl5	Chemokine (C-X-C Motif) Ligand 5	-2.36
1419463_at	Clca2	Chloride Channel Calcium Activated 2	-2.36
1451426_at	Dhx58	DEXH (Asp-Glu-X-His) box polypeptide 58	-2.37
1444344_at	Fgf10	Fibroblast Growth Factor 10	-2.38
1416592_at	Glrx	Glutaredoxin	-2.41

1425528_at	Prrx1	Paired Related Homeobox 1	-2.41
1424998_at	Emr4	Egf-Like Module Containing, Mucin-Like, Hormone Receptor-Like Sequence 4	-2.43
1428034_a_at	Tnfrsf9	Tumor Necrosis Factor Receptor Superfamily, Member 9	-2.43
1421134_at	Areg	Amphiregulin	-2.44
1418126_at	Ccl5	Chemokine (C-C Motif) Ligand 5	-2.45
1416560_at	Slc13A3	Solute Carrier Family 13 (Sodium-Dependent Dicarboxylate Transporter), Member 3	-2.52
1421256_at	Gzmc	Granzyme C	-2.53
1452349_x_at	Mnda	Myeloid Cell Nuclear Differentiation Antigen	-2.54
1419282_at	Ccl12	Chemokine (C-C Motif) Ligand 12	-2.55
1449366_at	Mmp8	Matrix Metallopeptidase 8	-2.57
1443698_at	Fbxo39	F-Box Protein 39	-2.57
1423954_at	C3	Complement Component 3	-2.59
1422013_at	Clec4A2	C-Type Lectin Domain Family 4, Member A2	-2.6
1424727_at	Ccr5	Chemokine (C-C Motif) Receptor 5	-2.6
1417314_at	Cfb	Complement Factor B	-2.67
1422203_at	Slc18A3	Solute Carrier Family 18 (Vesicular Monoamine), Member 3	-2.67
1434372_at	Aw112010	Expressed Sequence Aw112010	-2.69
1429987_at	9930013L23Rik	Riken Cdna 6330404C01 Gene	-2.74
1449994_at	Epgn	Epithelial Mitogen	-2.75
1421008_at	Rsad2	Radical S-Adenosyl Methionine Domain Containing 2	-2.78
1448898_at	Ccl9	Chemokine (C-C Motif) Ligand 9	-2.81
1424339_at	Oasl1	2'-5' Oligoadenylate Synthetase-Like 1	-2.81
1419209_at	Cxcl1	Chemokine (C-X-C Motif) Ligand 1	-2.9
1435330_at	Pyhin1	Pyrin and HIN domain family, member 1	-2.94
1435040_at	Irak3	Interleukin-1 Receptor-Associated Kinase 3	-2.94
1420591_at	Gpr84	G Protein-Coupled Receptor 84	-3.01
1453009_at	Cpm	Carboxypeptidase M	-3.02
1417268_at	Cd14	Cd14 Antigen	-3.09
1451563_at	Emr4	Egf-Like Module Containing, Mucin-Like, Hormone Receptor-Like Sequence 4	-3.09
1417936_at	Ccl9	Chemokine (C-C Motif) Ligand 9	-3.24
1417244_a_at	Irf7	Interferon Regulatory Factor 7	-3.25
1450484_a_at	Tyki	Thymidylate Kinase Family Lps-Inducible Member	-3.29
1449399_a_at	Il1B	Interleukin 1 Beta	-3.33
1416783_at	Tac1	Tachykinin 1	-3.44
1436058_at	Rsad2	Radical S-Adenosyl Methionine Domain Containing 2	-3.44
1449984_at	Cxcl2	Chemokine (C-X-C Motif) Ligand 2	-3.45
1439774_at	Prrx1	Paired Related Homeobox 1	-3.47
1427747_a_at	Lcn2	Lipocalin 2	-3.48
1438148_at	Cxcl3	Gene Model 1960, (Ncbi)	-3.55
1427381_at	Irg1	Immunoresponsive Gene 1	-3.57
1420330_at	Clec4E	C-Type Lectin Domain Family 4, Member E	-3.68
1449025_at	Ifit3	Interferon-Induced Protein With Tetratricopeptide	-3.71

		Repeats 3	
1450297_at	Il6	Interleukin 6	-4.24
1438377_x_at	Slc13A3	Solute Carrier Family 13 (Sodium-Dependent Dicarboxylate Transporter), Member 3	-4.24
1458297_s_at	Marco	Macrophage Receptor With Collagenous Structure	-4.33
1431591_s_at	Isg15	Interferon, Alpha-Inducible Protein	-4.37
1423555_a_at	Ifi44	Interferon-Induced Protein 44	-4.42
1418191_at	Usp18	Ubiquitin Specific Peptidase 18	-4.53
1421009_at	Rsad2	Radical S-Adenosyl Methionine Domain Containing 2	-4.69
1449498_at	Marco	Macrophage Receptor With Collagenous Structure	-4.94
1419709_at	Stfa3	Stefin A1	-4.97
1450826_a_at	Saa3	Serum Amyloid A 3	-5.32
1450783_at	Ifit1	Interferon-Induced Protein With Tetratricopeptide Repeats 1	-5.53
1450808_at	Fpr1	Formyl Peptide Receptor 1	-6.25
1419549_at	Arg1	Arginase 1, Liver	-6.84
1450291_s_at	Ms4A4C	Membrane-Spanning 4-Domains, Subfamily A, Member 4C	-7.89
1422953_at	Fpr-Rs2	Formyl Peptide Receptor, Related Sequence 2	-10.69
1419082_at	Serpinb2	Serine (Or Cysteine) Peptidase Inhibitor, Clade B, Member 2	-10.73

Table 3.2: Ihh Regulated Genes in Isolated Mesenchyme

Probe ID	Gene Symbol	Description	FC
1428853_at	Ptch1	Patched Homolog 1	2.91
1439663_at	Ptch1	Patched Homolog 1	2.64
1419692_a_at	Ltc4S	Leukotriene C4 Synthase	2.5
1425978_at	Myocd	Myocardin	2.49
1449038_at	Hsd11B1	Hydroxysteroid 11-Beta Dehydrogenase 1	2.37
1434152_at	Apol7C	Apolipoprotein L 7c	2.31
1435290_x_at	H2-Aa	Histocompatibility 2, Class Ii Antigen A, Alpha	2.28
1430570_at	Kynu	Kynureninase (L-Kynurenine Hydrolase)	2.27
1417597_at	Cd28	Cd28 Antigen	2.22
1441172_at	Aff3	Af4/Fmr2 Family, Member 3	2.11
1433933_s_at	Slco2B1	Solute Carrier Organic Anion Transporter Family, Member 2B1	2.09
1429272_a_at	Apol7A	apolipoprotein L 7a	2.08
1450020_at	Cx3Cr1	Chemokine (C-X3-C) Receptor 1	2.07
1427351_s_at	Igh-6	Immunoglobulin Heavy Chain 6 (Heavy Chain Of Igm)	2.06
1450019_at	Cx3Cr1	Chemokine (C-X3-C) Receptor 1	2.06
1449058_at	Gli1	Gli-Kruppel Family Member Gli1	1.98
1455489_at	Lrrtm2	Leucine Rich Repeat Transmembrane Neuronal 2	1.91
1444487_at	Lrat	Lecithin-Retinol Acyltransferase (Phosphatidylcholine-Retinol-O-Acyltransferase)	1.91
1455224_at	Angptl1	Angiopoietin-Like 1	1.88
1437787_at	Lrrtm2	Leucine Rich Repeat Transmembrane Neuronal 2	1.83
1455050_at	E130203B14Rik	Riken Cdna E130203B14 Gene	1.81
1435399_at	Synpo2	Synaptopodin 2	1.77
1438858_x_at	H2-Aa	Histocompatibility 2, Class Ii Antigen A, Alpha	1.77
1452431_s_at	H2-Aa	Histocompatibility 2, Class Ii Antigen A, Alpha	1.76
1437401_at	Igf1	Insulin-Like Growth Factor 1	1.74
1447227_at	Slc40A1	Solute Carrier Family 39 (Iron-Regulated Transporter), Member 1	1.74
1427038_at	Penk1	Preproenkephalin 1	1.74
1422903_at	Ly86	Lymphocyte Antigen 86	1.74
1415824_at	Scd2	Stearoyl-Coenzyme A Desaturase 2	1.73
1453841_at	2310050P20Rik	Riken Cdna 2310050P20 Gene	1.73
1444045_at	A430107O13Rik	Riken Cdna A430107O13 Gene	1.73
1419219_at	Cyp4F18	Cytochrome P450, Family 4, Subfamily F, Polypeptide 18	1.72
1433719_at	Slc9A9	Solute Carrier Family 9 (Sodium/Hydrogen Exchanger), Isoform 9	1.71
1458746_at	A430107O13Rik	Riken Cdna A430107O13 Gene	1.7
1434413_at	Igf1	Insulin-Like Growth Factor 1	1.69
1416111_at	Cd83	Cd83 Antigen	1.69
1428922_at	1200009O22Rik	Riken Cdna 1200009O22 Gene	1.69
1419905_s_at	Hpgd	Hydroxyprostaglandin Dehydrogenase 15 (Nad)	1.68
1458121_at	A430107O13Rik	Riken Cdna A430107O13 Gene	1.68
1421899_a_at	Mr1	Major Histocompatibility Complex, Class I-Related	1.68
1445929_at	Pdcd5	Programmed Cell Death 5	1.66

1419476_at	Adamdec1	Adam-Like, Decysin 1	1.63
1455318_at	Timd4	T-Cell Immunoglobulin And Mucin Domain Containing 4	1.63
1419519_at	Igf1	Insulin-Like Growth Factor 1	1.63
1421168_at	Abcg3	Atp-Binding Cassette, Sub-Family G (White), Member 3	1.61
1423153_x_at	Eg214403	Complement Component Factor H	1.6
1425519_a_at	Cd74	Cd74 Antigen (Invariant Polypeptide Of Major Histocompatibility Complex, Class Ii Antigen-Associated)	1.6
1454877_at	Sertad4	Serta Domain Containing 4	1.6
1417025_at	H2-Eb1	Histocompatibility 2, Class Ii Antigen E Beta	1.59
1452528_a_at	Nkx2-3	Nk2 Transcription Factor Related, Locus 3 (Drosophila)	1.57
1427489_at	Itga8	Expressed Sequence Ai447669	1.57
1440879_at	Abca9	Atp-Binding Cassette Transporter Sub-Family A Member 9	1.56
1431353_at	Pabpc4l	poly(A) binding protein, cytoplasmic 4-like	1.55
1421851_at	Mtap1B	Microtubule-Associated Protein 1 B	1.52
1448700_at	G0S2	G0/G1 Switch Gene 2	1.52
1453496_at	Fam162b	Family with sequence similarity 162, member B	1.52
1450648_s_at	H2-Ab1	Histocompatibility 2, Class Ii Antigen A, Beta 1	1.52
1422263_at	Bdkrb2	Bradykinin Receptor, Beta 2	1.52
1420965_a_at	Enc1	Ectodermal-Neural Cortex 1	1.51
1447345_at	N/A	N/A	1.51
1420847_a_at	Fgfr2	Fibroblast Growth Factor Receptor 2	1.51
1426225_at	Rbp4	Retinol Binding Protein 4, Plasma	1.5
1448728_a_at	Nfkbiz	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Zeta	-1.5
1455393_at	Cp	Ceruloplasmin	-1.5
1416296_at	Il2Rg	Interleukin 2 Receptor, Gamma Chain	-1.51
1422317_a_at	Il1Rl1	Interleukin 1 Receptor-Like 1	-1.51
1449143_at	Rtp4	Receptor Transporter Protein 4	-1.51
1452279_at	Cfp	Properdin Factor, Complement	-1.51
1455859_at	A330021E22Rik	Riken Cdna A330021E22 Gene	-1.53
1436368_at	Slc16A10	Solute Carrier Family 16 (Monocarboxylic Acid Transporters), Member 10	-1.53
1450798_at	Tnxb	Tenascin X	-1.54
1428942_at	Mt2	Metallothionein 2	-1.55
1450744_at	Ell2	Elongation Factor Rna Polymerase Ii 2	-1.56
1417495_x_at	Cp	Ceruloplasmin	-1.56
1425951_a_at	Clec4N	C-Type Lectin Domain Family 4, Member N	-1.56
1449156_at	Ly9	Lymphocyte Antigen 9	-1.56
1420250_at	Na	Na	-1.57
1422243_at	Fgf7	Fibroblast Growth Factor 7	-1.58
1418937_at	Dio2	Deiodinase, Iodothyronine, Type Ii	-1.58
1460197_a_at	Steap4	Steap Family Member 4	-1.58
1418879_at	Fam110c	Family with sequence similarity 110, member C	-1.59
1436999_at	5033414K04Rik	Riken Cdna 5033414K04 Gene	-1.6
1445687_at	Gm885	Gene Model 885, (Ncbi)	-1.6
1417494_a_at	Cp	Ceruloplasmin	-1.61

1428909_at	A130040M12Rik	Riken Cdna A130040M12 Gene	-1.61
1418511_at	Dpt	Dermatopontin	-1.62
1419627_s_at	Clec4N	C-Type Lectin Domain Family 4, Member N	-1.62
1417483_at	Nfkbiz	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Zeta	-1.62
1448734_at	Cp	Ceruloplasmin	-1.62
1429743_at	6720468P15Rik	Riken Cdna 6720468P15 Gene	-1.62
1452117_a_at	Fyb	Fyn Binding Protein	-1.63
1420499_at	Gch1	Gtp Cyclohydrolase 1	-1.63
1423267_s_at	Itga5	Integrin Alpha 5 (Fibronectin Receptor Alpha)	-1.63
1416295_a_at	Il2Rg	Interleukin 2 Receptor, Gamma Chain	-1.66
1448377_at	Slpi	Secretory Leukocyte Peptidase Inhibitor	-1.66
1449454_at	Bst1	Bone Marrow Stromal Cell Antigen 1	-1.68
1452205_x_at	Tcrb-V13	T-Cell Receptor Beta, Variable 13	-1.68
1419569_a_at	Isg20	Interferon-Stimulated Protein	-1.68
1458683_at	Sirpb1	Sirp-Beta B	-1.69
1418945_at	Mmp3	Matrix Metallopeptidase 3	-1.7
1417929_at	Slc7A8	Solute Carrier Family 7 (Cationic Amino Acid Transporter, Y+ System), Member 8	-1.7
1418465_at	Ncf4	Neutrophil Cytosolic Factor 4	-1.7
1434484_at	1100001G20Rik	Riken Cdna 1100001G20 Gene	-1.7
1430704_at	Irak3	Interleukin-1 Receptor-Associated Kinase 3	-1.71
1444531_at	Sod2	Superoxide Dismutase 2, Mitochondrial	-1.71
1426112_a_at	Cd72	Cd72 Antigen	-1.72
1417266_at	Ccl6	Chemokine (C-C Motif) Ligand 6	-1.72
1420464_s_at	Pira1/2/3/4/6	Paired-Ig-Like Receptor A2	-1.73
1417813_at	Ikbke	Inhibitor Of Kappab Kinase Epsilon	-1.73
1422177_at	Il13Ra2	Interleukin 13 Receptor, Alpha 2	-1.74
1449982_at	Il11	Interleukin 11	-1.74
1418480_at	Ppbp	Chemokine (C-X-C Motif) Ligand 7	-1.75
1438405_at	Fgf7	Fibroblast Growth Factor 7	-1.75
1421207_at	Lif	Leukemia Inhibitory Factor	-1.76
1420361_at	Slc11A1	Solute Carrier Family 11 (Proton-Coupled Divalent Metal Ion Transporters), Member 1	-1.76
1422776_at	Serpinb8	Serine (Or Cysteine) Peptdiase Inhibitor, Clade B, Member 8	-1.77
1422978_at	Cybb	Cytochrome B-245, Beta Polypeptide	-1.77
1448494_at	Gas1	Growth Arrest Specific 1	-1.79
1441855_x_at	Cxcl1	Chemokine (C-X-C Motif) Ligand 1	-1.8
1444344_at	Fgf10	Fibroblast Growth Factor 10	-1.82
1454976_at	Sod2	Superoxide Dismutase 2, Mitochondrial	-1.83
1449453_at	Bst1	Bone Marrow Stromal Cell Antigen 1	-1.83
1425528_at	Prrx1	Paired Related Homeobox 1	-1.85
1422041_at	Pilrb1	Paired Immunoglobin-Like Type 2 Receptor Beta	-1.85
1448881_at	Hp	Haptoglobin	-1.86
1453757_at	Herc5	Hect Domain And Rld 5	-1.87
1439774_at	Prrx1	Paired Related Homeobox 1	-1.89
1435560_at	Itgal	Integrin Alpha L	-1.91
1424302_at	Lilrb3	Leukocyte Immunoglobulin-Like Receptor, Subfamily B (With Tm And Itim Domains), Member 3	-1.91

1420413_at	Slc7A11	Solute Carrier Family 7 (Cationic Amino Acid Transporter, Y+ System), Member 11	-1.92
1418842_at	Hcls1	Hematopoietic Cell Specific Lyn Substrate 1	-1.92
1448291_at	Mmp9	Matrix Metallopeptidase 9	-1.93
1449222_at	Ebi3	Epstein-Barr Virus Induced Gene 3	-1.93
1452408_at	Na	Na	-1.93
1418930_at	Cxcl10	Chemokine (C-X-C Motif) Ligand 10	-1.93
1436778_at	Cybb	Cytochrome B-245, Beta Polypeptide	-1.94
1425407_s_at	Clec4A2/B1	C-Type Lectin Domain Family 4, Member A2	-1.94
1420249_s_at	Ccl6	Chemokine (C-C Motif) Ligand 6	-1.95
1440342_at	N/A	N/A	-1.96
1422046_at	Itgam	Integrin Alpha M	-1.98
1417193_at	Sod2	Superoxide Dismutase 2, Mitochondrial	-1.99
1416871_at	Adam8	A Disintegrin And Metallopeptidase Domain 8	-2.01
1416298_at	Mmp9	Matrix Metallopeptidase 9	-2.03
1451161_a_at	Emr1	Egf-Like Module Containing, Mucin-Like, Hormone Receptor-Like Sequence 1	-2.03
1450764_at	Aoah	Acyloxyacyl Hydrolase	-2.04
1418847_at	Arg2	Arginase Type Ii	-2.07
1448610_a_at	Sod2	Superoxide Dismutase 2, Mitochondrial	-2.08
1419282_at	Ccl12	Chemokine (C-C Motif) Ligand 12	-2.12
1416593_at	Glrx	Glutaredoxin	-2.12
1421008_at	Rsad2	Radical S-Adenosyl Methionine Domain Containing 2	-2.14
1438841_s_at	Arg2	Arginase Type Ii	-2.19
1457644_s_at	Cxcl1	Chemokine (C-X-C Motif) Ligand 1	-2.22
1436779_at	Cybb	Cytochrome B-245, Beta Polypeptide	-2.27
1424921_at	Bst2	Bone Marrow Stromal Cell Antigen 2	-2.27
1419728_at	Cxcl5	Chemokine (C-X-C Motif) Ligand 5	-2.3
1421228_at	Ccl7	Chemokine (C-C Motif) Ligand 7	-2.32
1420380_at	Ccl2	Chemokine (C-C Motif) Ligand 2	-2.32
1422013_at	Clec4A2	C-Type Lectin Domain Family 4, Member A2	-2.35
1416592_at	Glrx	Glutaredoxin	-2.4
1448239_at	Hmox1	Heme Oxygenase (Decycling) 1	-2.43
1427327_at	Pilra	Expressed Sequence Av021745	-2.45
1448898_at	Ccl9	Chemokine (C-C Motif) Ligand 9	-2.51
1435040_at	Irak3	Interleukin-1 Receptor-Associated Kinase 3	-2.51
1419132_at	Tlr2	Toll-Like Receptor 2	-2.62
1450297_at	Il6	Interleukin 6	-2.62
1427747_a_at	Lcn2	Lipocalin 2	-2.72
1419209_at	Cxcl1	Chemokine (C-X-C Motif) Ligand 1	-2.74
1418126_at	Ccl5	Chemokine (C-C Motif) Ligand 5	-2.74
1417936_at	Ccl9	Chemokine (C-C Motif) Ligand 9	-2.77
1424339_at	Oasl1	2'-5' Oligoadenylate Synthetase-Like 1	-2.79
1416783_at	Tac1	Tachykinin 1	-2.83
1436058_at	Rsad2	Radical S-Adenosyl Methionine Domain Containing 2	-2.84
1417314_at	Cfb	Complement Factor B	-2.86
1420804_s_at	Clec4D	C-Type Lectin Domain Family 4, Member D	-2.86
1424998_at	Emr4	Egf-Like Module Containing, Mucin-Like, Hormone Receptor-Like Sequence 4	-2.88

1416560_at	Slc13A3	Solute Carrier Family 13 (Sodium-Dependent Dicarboxylate Transporter), Member 3	-2.95
1421009_at	Rsad2	Radical S-Adenosyl Methionine Domain Containing 2	-3.07
1417268_at	Cd14	Cd14 Antigen	-3.25
1449399_a_at	Il1B	Interleukin 1 Beta	-3.32
1423954_at	C3	Complement Component 3	-3.43
1420591_at	Gpr84	G Protein-Coupled Receptor 84	-3.71
1449366_at	Mmp8	Matrix Metallopeptidase 8	-3.76
1451563_at	Emr4	Egf-Like Module Containing, Mucin-Like, Hormone Receptor-Like Sequence 4	-3.8
1423555_a_at	Ifi44	Interferon-Induced Protein 44	-4.09
1438377_x_at	Slc13A3	Solute Carrier Family 13 (Sodium-Dependent Dicarboxylate Transporter), Member 3	-4.78
1449984_at	Cxcl2	Chemokine (C-X-C Motif) Ligand 2	-4.85
1419549_at	Arg1	Arginase 1, Liver	-5.1
1420330_at	Clec4E	C-Type Lectin Domain Family 4, Member E	-5.59
1419709_at	Stfa3	Stefin A1	-5.9
1450826_a_at	Saa3	Serum Amyloid A 3	-6.15
1438148_at	Cxcl3	Gene Model 1960, (Ncbi)	-6.99
1427381_at	Irg1	Immunoresponsive Gene 1	-7.68
1450808_at	Fpr1	Formyl Peptide Receptor 1	-9.37
1419082_at	Serpinb2	Serine (Or Cysteine) Peptidase Inhibitor, Clade B, Member 2	-11.82
1422953_at	Fpr-Rs2	Formyl Peptide Receptor, Related Sequence 2	-14.53
1458297_s_at	Marco	Macrophage Receptor With Collagenous Structure	-16.06
1449498_at	Marco	Macrophage Receptor With Collagenous Structure	-17.03

Table 3.3: Shh and Ihh Common Up-regulated Genes

Probe ID	Gene Symbol	Description	Ihh FC	Shh FC
1428853_at	Ptch1	Patched Homolog 1	2.91	2.66
1439663_at	Ptch1	Patched Homolog 1	2.64	2.05
1419692_a_at	Ltc4S	Leukotriene C4 Synthase	2.50	1.63
1425978_at	Myocd	Myocardin	2.49	2.09
1449038_at	Hsd11B1	Hydroxysteroid 11-Beta Dehydrogenase 1	2.37	2.18
1434152_at	Apol7C	Riken Cdna 2210421G13 Gene	2.31	2.01
1435290_x_at	H2-Aa	Histocompatibility 2, Class Ii Antigen A, Alpha	2.28	1.91
1430570_at	Kynu	Kynureninase (L-Kynurenine Hydrolase)	2.27	2.26
1417597_at	Cd28	Cd28 Antigen	2.22	2.32
1441172_at	Aff3	Af4/Fmr2 Family, Member 3	2.11	1.59
1429272_a_at	Apol7A/7C	Riken cDNA 2210421G13 Gene	2.08	1.86
1450020_at	Cx3Cr1	Chemokine (C-X3-C) Receptor 1	2.07	1.62
1450019_at	Cx3Cr1	Chemokine (C-X3-C) Receptor 1	2.06	2.05
1449058_at	Gli1	GLI-Kruppel family member GLI1	1.98	1.48
1455224_at	Angptl1	Angiopoietin-Like 1	1.88	1.86
1455050_at	E130203B14Rik	Riken Cdna E130203B14 Gene	1.81	1.82
1435399_at	Synpo2	Synaptopodin 2	1.77	1.88
1437401_at	Igf1	Insulin-Like Growth Factor 1	1.74	1.68
1447227_at	Slc40A1	Solute Carrier Family 39 (Iron-Regulated Transporter), Member 1	1.74	1.61
1427038_at	Penk1	Preproenkephalin 1	1.74	1.60
1415824_at	Scd2	Stearoyl-Coenzyme A Desaturase 2	1.73	1.63
1434413_at	Igf1	Insulin-Like Growth Factor 1	1.69	1.61
1416111_at	Cd83	Cd83 Antigen	1.69	1.58
1458121_at	A430107O13Rik	Riken Cdna A430107O13 Gene	1.68	1.75
1421899_a_at	Mr1	Major Histocompatibility Complex, Class I-Related	1.68	1.51
1419476_at	Adamdec1	Adam-Like, Decysin 1	1.63	1.82
1455318_at	Timd4	T-Cell Immunoglobulin And Mucin Domain Containing 4	1.63	1.70
1419519_at	Igf1	Insulin-Like Growth Factor 1	1.63	1.84
1421168_at	Abcg3	Atp-Binding Cassette, Sub-Family G (White), Member 3	1.61	1.65
1452528_a_at	Nkx2-3	Nk2 Transcription Factor Related, Locus 3 (Drosophila)	1.57	1.51
1427489_at	Itga8	Expressed Sequence Ai447669	1.57	1.73
1453496_at	Fam162b	Family with sequence similarity 162, member B	1.52	1.85
1420965_a_at	Enc1	Ectodermal-Neural Cortex 1	1.51	1.63
1420847_a_at	Fgfr2	Fibroblast Growth Factor Receptor 2	1.51	1.57

Table 3.4: Shh and Ihh Common Downregulated Genes

Probe ID	Gene Symbol	Description	Ihh FC	Shh FC
1449498_at	Marco	Macrophage Receptor With Collagenous Structure	-17.03	-4.94
1458297_s_at	Marco	Macrophage Receptor With Collagenous Structure	-16.06	-4.33
1422953_at	Fpr-Rs2	Formyl Peptide Receptor, Related Sequence 2	-14.53	-10.69
1419082_at	Serpinb2	Serine (Or Cysteine) Peptidase Inhibitor, Clade B, Member 2	-11.82	-10.73
1450808_at	Fpr1	Formyl Peptide Receptor 1	-9.37	-6.25
1427381_at	Irg1	Immunoresponsive Gene 1	-7.68	-3.57
1438148_at	Cxcl3	Gene Model 1960, (Ncbi)	-6.99	-3.55
1450826_a_at	Saa3	Serum Amyloid A 3	-6.15	-5.32
1419709_at	Stfa3	Stefin A1	-5.90	-4.97
1420330_at	Ccl2	Chemokine (C-C Motif) Ligand 2	-5.59	-3.68
1419549_at	Arg1	Arginase 1, Liver	-5.10	-6.84
1449984_at	Cxcl2	Chemokine (C-X-C Motif) Ligand 2	-4.85	-3.45
1438377_x_at	Slc13A3	Solute Carrier Family 13 (Sodium-Dependent Dicarboxylate Transporter), Member 3	-4.78	-4.24
1423555_a_at	Ifi44	Interferon-Induced Protein 44	-4.09	-4.42
1451563_at	Emr4	Egf-Like Module Containing, Mucin-Like, Hormone Receptor-Like Sequence 4	-3.80	-3.09
1449366_at	Mmp8	Matrix Metallopeptidase 8	-3.76	-2.57
1420591_at	Gpr84	G Protein-Coupled Receptor 84	-3.71	-3.01
1423954_at	C3	Complement Component 3	-3.43	-2.59
1449399_a_at	Il1B	Interleukin 1 Beta	-3.32	-3.33
1417268_at	Cd14	Cd14 Antigen	-3.25	-3.09
1421009_at	Rsad2	Radical S-Adenosyl Methionine Domain Containing 2	-3.07	-4.69
1416560_at	Slc13A3	Solute Carrier Family 13 (Sodium-Dependent Dicarboxylate Transporter), Member 3	-2.95	-2.52
1424998_at	Emr4	Egf-Like Module Containing, Mucin-Like, Hormone Receptor-Like Sequence 4	-2.88	-2.43
1420804_s_at	Clec4D	C-Type Lectin Domain Family 4, Member D	-2.86	-1.89
1417314_at	Cfb	Complement Factor B	-2.86	-2.67
1436058_at	Rsad2	Radical S-Adenosyl Methionine Domain Containing 2	-2.84	-3.44
1416783_at	Tac1	Tachykinin 1	-2.83	-3.44
1424339_at	Oasl1	2'-5' Oligoadenylate Synthetase-Like 1	-2.79	-2.81
1417936_at	Ccl9	Chemokine (C-C Motif) Ligand 9	-2.77	-3.24
1418126_at	Ccl5	Chemokine (C-C Motif) Ligand 5	-2.74	-2.45
1419209_at	Cxcl1	Chemokine (C-X-C Motif) Ligand 1	-2.74	-2.90
1427747_a_at	Lcn2	Lipocalin 2	-2.72	-3.48
1450297_at	Il6	Interleukin 6	-2.62	-4.24
1419132_at	Tlr2	Toll-Like Receptor 2	-2.62	-1.79
1435040_at	Irak3	Interleukin-1 Receptor-Associated Kinase 3	-2.51	-2.94
1448898_at	Ccl9	Chemokine (C-C Motif) Ligand 9	-2.51	-2.81
1427327_at	Pilra	Expressed Sequence Av021745	-2.45	-2.17
1416592_at	Glrx	Glutaredoxin	-2.40	-2.41

1422013_at	Clec4A2	C-Type Lectin Domain Family 4, Member A2	-2.35	-2.60
1420380_at	Pira1/2/3/4/6	Paired-Ig-Like Receptor A2	-2.32	-1.98
1421228_at	Ccl7	Chemokine (C-C Motif) Ligand 7	-2.32	-2.21
1419728_at	Cxcl5	Chemokine (C-X-C Motif) Ligand 5	-2.30	-2.36
1424921_at	Bst2	Bone Marrow Stromal Cell Antigen 2	-2.27	-2.06
1436779_at	Cybb	Cytochrome B-245, Beta Polypeptide	-2.27	-2.23
1457644_s_at	Cxcl1	Chemokine (C-X-C Motif) Ligand 1	-2.22	-2.14
1438841_s_at	Arg2	Arginase Type Ii	-2.19	-2.18
1421008_at	Rsad2	Radical S-Adenosyl Methionine Domain Containing 2	-2.14	-2.78
1416593_at	Glrx	Glutaredoxin	-2.12	-2.13
1419282_at	Ccl12	Chemokine (C-C Motif) Ligand 12	-2.12	-2.55
1448610_a_at	Sod2	Superoxide Dismutase 2, Mitochondrial	-2.08	-1.88
1418847_at	Arg2	Arginase Type Ii	-2.07	-2.17
1450764_at	Aoah	Acyloxyacyl Hydrolase	-2.04	-1.82
1451161_a_at	Emr1	Egf-Like Module Containing, Mucin-Like, Hormone Receptor-Like Sequence 1	-2.03	-2.08
1416298_at	Mmp9	Matrix Metallopeptidase 9	-2.03	-1.91
1417193_at	Sod2	Superoxide Dismutase 2, Mitochondrial	-1.99	-2.03
1422046_at	Itgam	Integrin Alpha M	-1.98	-1.78
1440342_AT	NA	NA	-1.96	-2.04
1420249_s_at	Ccl6	Chemokine (C-C Motif) Ligand 6	-1.95	-1.86
1425407_s_at	Clec4B1	C-Type Lectin Domain Family 4, Member A2	-1.94	-1.99
1436778_at	Cybb	Cytochrome B-245, Beta Polypeptide	-1.94	-1.80
1452408_AT	Gpr31c	G protein-coupled receptor 31, D17Leh66c region	-1.93	-2.23
1449222_at	Ebi3	Epstein-Barr Virus Induced Gene 3	-1.93	-1.55
1448291_at	Mmp9	Matrix Metallopeptidase 9	-1.93	-1.96
1418842_at	Hcls1	Hematopoietic Cell Specific Lyn Substrate 1	-1.92	-1.87
1424302_at	Lilrb3	Leukocyte Immunoglobulin-Like Receptor, Subfamily B (With Tm And Itim Domains), Member 3	-1.91	-1.84
1435560_at	Itgal	Integrin Alpha L	-1.91	-2.09
1439774_at	Prrx1	Paired Related Homeobox 1	-1.89	-3.47
1453757_at	Herc5	Hect Domain And Rld 5	-1.87	-1.78
1448881_at	Hp	Haptoglobin	-1.86	-2.24
1422041_at	Pilrb1	Paired Immunoglobin-Like Type 2 Receptor Beta	-1.85	-1.92
1425528_at	Prrx1	Paired Related Homeobox 1	-1.85	-2.41
1449453_at	Bst1	Bone Marrow Stromal Cell Antigen 1	-1.83	-1.51
1454976_at	Sod2	Superoxide Dismutase 2, Mitochondrial	-1.83	-1.85
1444344_at	Fgf10	Fibroblast Growth Factor 10	-1.82	-2.38
1441855_x_at	Cxcl1	Chemokine (C-X-C Motif) Ligand 1	-1.80	-1.62
1448494_at	Gas1	Growth Arrest Specific 1	-1.79	-2.28
1422978_at	Cybb	Cytochrome B-245, Beta Polypeptide	-1.77	-1.53
1421207_at	Lif	Leukemia Inhibitory Factor	-1.76	-1.68
1418480_at	Cxcl7	Chemokine (C-X-C Motif) Ligand 7	-1.75	-2.17
1417813_at	Ikbke	Inhibitor Of Kappab Kinase Epsilon	-1.73	-1.64
1420464_s_at	Lilrb3	Leukocyte Immunoglobulin-Like Receptor, Subfamily B (With Tm And Itim Domains),	-1.73	-1.78

		Member 3		
1417266_at	Ccl6	Chemokine (C-C Motif) Ligand 6	-1.72	-1.78
1426112_a_at	Cd72	Cd72 Antigen	-1.72	-1.99
1444531_at	Sod2	Superoxide Dismutase 2, Mitochondrial	-1.71	-1.80
1434484_at	1100001G20Rik	Riken Cdna 1100001G20 Gene	-1.70	-1.66
1458683_at	Sirpb1	Sirp-Beta B	-1.69	-1.64
1419569_a_at	Isg20	Interferon-Stimulated Protein	-1.68	-1.66
1452205_x_at	Tcrb-V13	T-Cell Receptor Beta, Variable 13	-1.68	-2.02
1423267_s_at	Itga5	Integrin Alpha 5 (Fibronectin Receptor Alpha)	-1.63	-1.60
1420499_at	Gch1	Gtp Cyclohydrolase 1	-1.63	-1.51
1452117_a_at	Fyb	Fyn Binding Protein	-1.63	-1.61
1429743_at	6720468P15Rik	Riken Cdna 6720468P15 Gene	-1.62	-1.65
1417483_at	Nfkbiz	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Zeta	-1.62	-1.98
1418511_at	Dpt	Dermatopontin	-1.62	-1.58
1428909_at	A130040M12Rik	Riken Cdna A130040M12 Gene	-1.61	-1.68
1436999_at	5033414K04Rik	Riken Cdna 5033414K04 Gene	-1.60	-1.60
1418879_at	Fam110c	Family with sequence similarity 110, member C	-1.59	-1.83
1460197_a_at	Steap4	Steap Family Member 4	-1.58	-1.75
1418937_at	Dio2	Deiodinase, Iodothyronine, Type Ii	-1.58	-2.01
1420250_at	Clec4E	C-Type Lectin Domain Family 4, Member E	-1.57	-1.60
1428942_at	Mt2	Metallothionein 2	-1.55	-1.53
1450798_at	Tnxb	Tenascin X	-1.54	-1.51
1449143_at	Rtp4	Receptor Transporter Protein 4	-1.51	-1.66
1448728_a_at	Nfkbiz	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Zeta	-1.50	-1.77

Table 3.5: DAVID Functional Annotation Clusters for Genes Upregulated by Shh and Ihh

Functional Cluster	Enrichment Score	Top Term		Top Term FE	Top Term P Value	Top Term FDR
1	2.78	GO:0035295	Tube Development	15.52	2.28E-04	4.35E-01
2	1.60	INTERPRO: IPR013783	Immunoglobulin-Like Fold	6.29	1.92E-03	3.66E+00
3	1.24	GO:0030098	Cytokine Activity	17.50	1.16E-02	2.00E+01
4	1.19	UP_SEQ_FEATURE	Disulfide Bond	2.37	1.80E-02	3.19E+01
5	1.17	GO:0048513	Carbohydrate Binding	4.41	1.41E-04	2.69E-01
6	1.13	SP_PIR_KEYWORDS	Transmembrane Protein	7.57	1.34E-02	1.89E+01
7	0.89	GO:0044249	Cellular Biosynthetic Process	3.17	6.06E-02	6.97E+01
8	0.78	GO:0048598	Embryonic Morphogenesis	12.80	3.18E-03	5.90E+00
9	0.75	GO:0044238	Primary Metabolic Process	1.39	7.52E-02	7.76E+01
10	0.64	GO:0006955	Immune Response	4.51	5.15E-02	6.36E+01

Table 3.5: DAVID Functional Annotation Clusters for Genes Upregulated by both Shh and Ihh. DAVID analysis reveals that Development, Ig Fold, and Immune response are the top categories associated with genes upregulated by Shh and Ihh in intestinal mesenchyme. A representative highly significant term from each of the top 10 clusters identified by the DAVID Functional Annotating Clustering tool is shown. The Cluster Enrichment Score refers to the enrichment of all terms in that cluster.

Table 3.6: MeSH Anatomy Classes Associated with Genes Upregulated by Shh and Ihh Ligand

MeSH Anatomy Term	ID	Class Total	Observed	Expected	ZScore
Jurkat Cells	A11.251.210.190.495	4810	120	19.60	22.79
Musculoskeletal System	A02	52477	470	213.84	18.13
Myoblasts	A11.872.645	1071	41	4.36	17.58
Muscle, Skeletal	A10.690.552.500	9857	144	40.17	16.51
T-Lymphocytes	A15.382.490.555.567.569	61493	496	250.58	16.14
Muscle Cells	A11.620	6956	113	28.35	16.00
Myocytes, Smooth Muscle	A11.620.520	1682	47	6.85	15.38
Osteoblasts	A11.329.629	4788	87	19.51	15.35
Bone and Bones	A02.835.232	7558	114	30.80	15.09
Muscles	A02.633	38509	338	156.92	14.83

Table 3.6: MeSH Anatomy Term Association of Upregulated by Shh and Ihh. MeSH Term Association analysis in Bibliosphere shows that the majority of genes upregulated by Shh and Ihh ligand are associated with muscle. Genes were ranked by Z score; more positive Z scores show higher enrichment or association with the data set.

Table 3.7: DAVID Functional Annotation Clusters for Genes Downregulated by Shh and Ihh

Functional Cluster	Enrichment Score	Top Term		Top Term FE	Top Term P Value	Top Term FDR
1	12.22	GO:0009605	Response To External Stimulus	10.52	5.31E-17	1.01E-13
2	10.81	GO:0002376	Immune System Process	7.40	3.36E-17	6.43E-14
3	8.98	GO:0005125	Cytokine Activity	15.54	6.26E-14	1.11E-10
4	3.54	GO:0042221	Response To Chemical Stimulus	7.15	3.20E-09	6.12E-06
5	2.30	GO:0030246	Carbohydrate Binding	5.96	3.50E-04	6.22E-01
6	1.78	GO:0007159	Leukocyte Adhesion	61.89	9.95E-04	1.88E+00
7	1.74	GO:0008201	Heparin Binding	10.86	3.04E-02	4.23E+01
8	1.67	GO:0009967	Positive Regulation Of Signal Transduction	12.84	9.52E-05	1.82E-01
9	1.54	KEGG_PATHWAY	Toll-Like Receptor Signaling Pathway	6.67	1.58E-03	1.96E+00
10	1.44	SP_PIR_KEYWORDS	Innate Immunity	13.44	2.05E-02	2.74E+01

Table 3.7: DAVID Functional Annotation Clusters for Genes Downregulated by both Shh and Ihh. DAVID analysis reveals that Immune response, Cytokine Signaling, and Innate Immune Recognition are the top processes associated with genes downregulated in intestinal mesenchyme by both Shh and Ihh. A representative highly significant term from each of the top 10 clusters identified by the DAVID Functional Annotating Clustering tool is shown. The Cluster Enrichment Score refers to the enrichment of all terms in that cluster.

Table 3.8: MeSH Anatomy Classes Associated with Genes Downregulated by Hh Ligand

MeSH Anatomy Term	ID	Class Total	Observed	Expected	ZScore
Phagocytes	A15.382.680	45352	5825	1561.86	112.87
Myeloid Cells	A11.627	55489	6462	1910.97	109.63
Mononuclear Phagocyte System	A15.382.812	42555	5339	1465.54	105.68
Monocytes	A11.148.580	13950	2366	480.42	88.28
Macrophages	A11.733.397	23963	2916	825.25	75.15
Immune System	A15.382	149225	9831	5139.10	73.46
Hematopoietic System	A15.378	31345	3264	1079.48	68.96
Hemic and Immune Systems	A15	169140	10436	5824.95	68.81
Connective Tissue Cells	A11.329	79419	5251	2735.08	51.45
Leukocytes	A15.145.229.637	113779	6806	3918.39	50.49

Table 3.8: MeSH Anatomy Term Association of Downregulated by Shh and Ihh. MeSH Term Association analysis in Bibliosphere shows that the majority of genes downregulated by Shh and Ihh ligand immune cells, particulary myeloid cell lineages. The second most common cell association is with connective tissue cells; this category includes ISEMFs. Genes were ranked by Z score; more positive Z scores show higher enrichment or association with the data set.

Table 3.9: Genes Regulated in Cultured Mesenchyme after 72 hours in Culture

Probe ID	Gene Symbol	Description	FC
1438148_at	Cxcl3	Chemokine (C-X-C motif) ligand 3	129.37
1419431_at	Ereg	Epiregulin	90.19
1418480_at	Cxcl7	Chemokine (C-X-C Motif) Ligand 7	68.47
1449153_at	Mmp12	Matrix Metallopeptidase 12	68.42
1419549_at	Arg1	Arginase 1, Liver	59.23
1421134_at	Areg	Amphiregulin	42.56
1419463_at	Clca2	Chloride Channel Calcium Activated 2	41.62
1449498_at	Marco	Macrophage Receptor With Collagenous Structure	39.77
1417266_at	Ccl6	Chemokine (C-C Motif) Ligand 6	37.74
1417262_at	Ptgs2	Prostaglandin-Endoperoxide Synthase 2	37.33
1450826_a_at	Saa3	Serum Amyloid A 3	37.24
1458943_at	Hgf	Hepatocyte Growth Factor	35.92
1419728_at	Cxcl5	Chemokine (C-X-C Motif) Ligand 5	34.61
1420394_s_at	Lilrb4	Leukocyte Immunoglobulin-Like Receptor, Subfamily B, Member 4	33.46
1449254_at	Spp1	Secreted Phosphoprotein 1	31.2
1420249_s_at	Ccl6	Chemokine (C-C Motif) Ligand 6	29.83
1418945_at	Mmp3	Matrix Metallopeptidase 3	28.78
1420804_s_at	Clec4D	C-Type Lectin Domain Family 4, Member D	28.39
1434046_at	Aa467197	Expressed Sequence Aa467197	25.93
1419473_a_at	Cck	Cholecystokinin	24.92
1417256_at	Mmp13	Matrix Metallopeptidase 13	24.66
1451718_at	Plp1	Proteolipid Protein (Myelin) 1	24.37
1419149_at	Serpine1	Serine (Or Cysteine) Peptidase Inhibitor, Clade E, Member 1	23.89
1448303_at	Gpnmb	Glycoprotein (Transmembrane) Nmb	23.7
1425951_a_at	Clec4N	C-Type Lectin Domain Family 4, Member N	23.56
1420450_at	Mmp10	Matrix Metallopeptidase 10	22.73
1426808_at	Lgals3	Lectin, Galactose Binding, Soluble 3	21.12
1419209_at	Cxcl1	Chemokine (C-X-C Motif) Ligand 1	20.9
1417263_at	Ptgs2	Prostaglandin-Endoperoxide Synthase 2	20.55
1427747_a_at	Lcn2	Lipocalin 2	20.16
1438841_s_at	Arg2	Arginase Type Ii	19.82
1448898_at	Pigf	Phosphatidylinositol Glycan, Class F	19.33
1438160_x_at	Slco4A1	Solute Carrier Organic Anion Transporter Family, Member 4A1	19.13
1417936_at	Ccl9	Chemokine (C-C Motif) Ligand 9	18.7
1420992_at	Ankrd1	Ankyrin-Like Repeat Protein	17.87
1419082_at	Serpinb2	Serine (Or Cysteine) Peptidase Inhibitor, Clade B, Member 2	17.54
1429987_at	9930013L23Rik	Riken cDNA 9930013L23 gene	17.28
1424938_at	Steap1	Six Transmembrane Epithelial Antigen Of The Prostate 1	17.07
1438377_x_at	Slc13A3	Solute Carrier Family 13 (Sodium-Dependent Dicarboxylate Transporter), Member 3	17.03

1449982_at	Il11	Interleukin 11	16.78
1437578_at	Clca2	Chloride Channel Calcium Activated 2	16.75
1450297_at	Il6	Interleukin 6	15.66
1426858_at	Inhbb	Inhibin Beta-B	15.59
1420380_at	Ccl2	Chemokine (C-C Motif) Ligand 2	15.54
1449340_at	Sostdc1	Sclerostin Domain Containing 1	15.38
1419561_at	Ccl3	Chemokine (C-C Motif) Ligand 3	15.29
1459253_at	Arrdc3	Arrestin Domain Containing 3	14.83
1425468_at	Plp1	Proteolipid Protein (Myelin) 1	14.56
1417795_at	Chl1	Cell Adhesion Molecule With Homology To L1Cam	14.5
1443253_at	Na	Na	14.12
1438405_at	Fgf7	Fibroblast Growth Factor 7	13.85
1449984_at	Cxcl2	Chemokine (C-X-C Motif) Ligand 2	13.13
1422851_at	Hmga2	High Mobility Group At-Hook 2	12.95
1442082_at	C3Ar1	Complement Component 3A Receptor 1	12.9
1451798_at	Il1Rn	Interleukin 1 Receptor Antagonist	12.77
1418248_at	Gla	Galactosidase, Alpha	12.73
1455034_at	Nr4A2	Nuclear Receptor Subfamily 4, Group A, Member 2	12.69
1456524_at	Nrg1	Neuregulin 1	12.35
1460667_at	U90926	Cdna Sequence U90926	12.32
1418937_at	Dio2	Deiodinase, Iodothyronine, Type Ii	12.3
1418847_at	Arg2	Arginase Type Ii	11.75
1439902_at	C5Ar1	Complement Component 5A Receptor 1	11.73
1429413_at	Cpm	Carboxypeptidase M	11.69
1442884_at	Hgf	Hepatocyte Growth Factor	11.66
1455299_at	Vgll3	vestigial like 3 (Drosophila)	11.41
1452352_at	Ctla2B	Cytotoxic T Lymphocyte-Associated Protein 2 Beta	11.37
1416967_at	Sox2	Sry-Box Containing Gene 2	11.32
1420991_at	Ankrd1	Ankyrin-Like Repeat Protein	11.28
1458297_s_at	Marco	Macrophage Receptor With Collagenous Structure	11.14
1418203_at	Pmaip1	Phorbol-12-Myristate-13-Acetate-Induced Protein 1	11.04
1442018_at	Btg1	B-cell translocation gene 1, anti-proliferative	11
1451828_a_at	Acsl4	Acyl-Coa Synthetase Long-Chain Family Member 4	10.92
1419627_s_at	Clec4N	C-Type Lectin Domain Family 4, Member N	10.82
1419758_at	Abcb1A	Atp-Binding Cassette, Sub-Family B (Mdr/Tap), Member 1A	10.74
1420699_at	Clec7A	C-Type Lectin Domain Family 7, Member A	10.73
1436530_at	Rp23-430I21.1	Novel Protein Similar To Extracellular Proteinase Inhibitor Expi	10.56
1453009_at	Cpm	Carboxypeptidase M	10.54
1457231_at	Hif1A	Hypoxia Inducible Factor 1, Alpha Subunit	10.52
1423547_at	Lyzs	Lysozyme	10.46
1438186_at	Pdlim5	Pdz And Lim Domain 5	10.32
1430792_at	Na	Na	10.3
1457373_at	Cdh19	Cadherin 19, Type 2	10.26
1456344_at	8030463A06Rik	Riken Cdna 8030463A06 Gene	10.2
1418162_at	Tlr4	Toll-Like Receptor 4	10.18
1434342_at	S100B	S100 Protein, Beta Polypeptide, Neural	10.08
1422317_a_at	Il1Rl1	Interleukin 1 Receptor-Like 1	10.08

1453593_at	Vgll3	vestigial like 3 (Drosophila)	10.08
1435477_s_at	Fcgr2B	Fc Receptor, Igg, Low Affinity Iib	10.03
1449414_at	Zfp53	Zinc Finger Protein 53	9.98
1443921_at	Ranbp3L	RAN binding protein 3-like	9.97
1427844_a_at	Cebpb	Ccaat/Enhancer Binding Protein (C/Ebp), Beta	9.91
1442827_at	Tlr4	Toll-Like Receptor 4	9.89
1436996_x_at	Lyz	P Lysozyme Structural	9.89
1433531_at	Ankrd13C	Ankyrin Repeat Domain 13C	9.86
1433966_x_at	Asns	Asparagine Synthetase	9.77
1451006_at	Xdh	Xanthine Dehydrogenase	9.68
1426471_at	Zfp52	Zinc Finger Protein 52	9.6
1458648_at	Au042950	Expressed Sequence Au042950	9.49
1430596_s_at	Vgll3	vestigial like 3 (Drosophila)	9.38
1457404_at	Nfkbiz	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Zeta	9.38
1419483_at	C3Ar1	Complement Component 3A Receptor 1	9.34
1416077_at	Adm	Adrenomedullin	9.33
1438097_at	Rab20	Rab20, Member Ras Oncogene Family	9.3
1458318_at	3110047M12Rik	Riken Cdna 3110047M12 Gene	9.17
1416871_at	Adam8	A Disintegrin And Metallopeptidase Domain 8	9.12
1430700_a_at	Pla2G7	Phospholipase A2, Group Vii (Platelet-Activating Factor Acetylhydrolase, Plasma)	9.07
1422573_at	Ampd3	Amp Deaminase 3	9.01
1439256_x_at	Gpr137B	G Protein-Coupled Receptor 137B	8.99
1436279_at	Slc26A7	Solute Carrier Family 26, Member 7	8.89
1439426_x_at	Lyz	P Lysozyme Structural	8.86
1458934_at	D5Ertd505E	Dna Segment, Chr 5, Erato Doi 505 , Expressed	8.61
1426472_at	Zfp52	Zinc Finger Protein 52	8.59
1446071_at	Steap2	Six Transmembrane Epithelial Antigen Of Prostate 2	8.56
1438773_at	Steap2	Six Transmembrane Epithelial Antigen Of Prostate 2	8.56
1424270_at	Dclk1	Double Cortin And Calcium/Calmodulin-Dependent Protein Kinase-Like 1	8.51
1439255_s_at	Gpr137B	G Protein-Coupled Receptor 137B	8.46
1417483_at	Nfkbiz	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Zeta	8.39
1448239_at	Hmox1	Heme Oxygenase (Decycling) 1	8.38
1422875_at	Cd84	Cd84 Antigen	8.21
1419599_s_at	Ms4a6d	Membrane-spanning 4-domains, subfamily A, member 6D	8.13
1452179_at	Phf17	Phd Finger Protein 17	8.12
1417268_at	Cd14	Cd14 Antigen	8.09
1450750_a_at	Nr4A2	Nuclear Receptor Subfamily 4, Group A, Member 2	8.05
1455660_at	Csf2Rb	Colony Stimulating Factor 2 Receptor, Beta 1, Low-Affinity (Granulocyte-Macrophage)	8.04
1442489_at	Agfg1	ArfGAP with FG repeats 1	8.03
1451866_a_at	Hgf	Hepatocyte Growth Factor	8
1418911_s_at	Acsl4	Acyl-Coa Synthetase Long-Chain Family Member 4	7.99
1448291_at	Mmp9	Matrix Metallopeptidase 9	7.98
1449670_x_at	Gpr137B	G Protein-Coupled Receptor 137B	7.97
1446886_at	Usp3	Ubiquitin Specific Peptidase 3	7.94

1424542_at	S100A4	S100 Calcium Binding Protein A4	7.91
1450355_a_at	Capg	Capping Protein (Actin Filament), Gelsolin-Like	7.87
1420697_at	Slc15A3	Solute Carrier Family 15, Member 3	7.82
1427682_a_at	Egr2	Early Growth Response 2	7.76
1436329_at	Egr3	Early Growth Response 3	7.75
1457164_at	Trpa1	Transient Receptor Potential Cation Channel, Subfamily A, Member 1	7.74
1449280_at	Esm1	Endothelial Cell-Specific Molecule 1	7.69
1438265_at	Wwc2	Dna Segment, Chr 8, Erato Doi 594, Expressed	7.67
1436892_at	Spred2	Sprouty-Related, Evh1 Domain Containing 2	7.65
1457753_at	Tlr13	Toll-Like Receptor 13	7.6
1422615_at	Map4K4	Nck Interacting Kinase	7.6
1443589_at	Dxertd242E	Dna Segment, Chr X, Erato Doi 242, Expressed	7.59
1448894_at	Msr2	Macrophage Scavenger Receptor 2	7.48
1429327_at	Sdccag1	Serologically Defined Colon Cancer Antigen 1	7.44
1448818_at	Wnt5A	Wingless-Related Mmtv Integration Site 5A	7.41
1424638_at	Cdkn1A	Cyclin-Dependent Kinase Inhibitor 1A (P21)	7.38
1448831_at	Angpt2	Angiopoietin 2	7.36
1431609_a_at	Acp5	Acid Phosphatase 5, Tartrate Resistant	7.35
1457304_at	Jarid2	jumonji, AT rich interactive domain 2	7.34
1428562_at	2210403K04Rik	Riken Cdna 2210403K04 Gene	7.29
1448025_at	Sirpb1	Sirp-Beta B	7.29
1455883_a_at	Lrrtm1	Leucine Rich Repeat Transmembrane Neuronal 1	7.22
1442340_x_at	Cyr61	Cysteine Rich Protein 61	7.19
1460003_at	Ai956758	Expressed Sequence Ai956758	7.15
1443426_at	Sltm	Safb-Like, Transcription Modulator	7.14
1449360_at	Csf2Rb2	Colony Stimulating Factor 2 Receptor, Beta 2, Low-Affinity (Granulocyte-Macrophage)	7.11
1419598_at	Ms4A6D	Membrane-Spanning 4-Domains, Subfamily A, Member 6D	7.1
1459977_x_at	Cox10	Cox10 Homolog, Cytochrome C Oxidase Assembly Protein, Heme A: Farnesyltransferase (Yeast)	7.1
1419759_at	Abcb1A	Atp-Binding Cassette, Sub-Family B (Mdr/Tap), Member 1A	7.1
1455785_at	Kcna1	Potassium Voltage-Gated Channel, Shaker-Related Subfamily, Member 1	7.02
1431110_at	Plxdc2	Plexin domain containing 2	7.02
1438109_at	Clca5	Chloride Channel Calcium Activated 5	7.02
1425379_at	Hgf	Hepatocyte Growth Factor	7.01
1419665_a_at	Nupr1	Nuclear Protein 1	7
1426366_at	Eif2C2	Eukaryotic Translation Initiation Factor 2C, 2	6.98
1435749_at	Gda	Guanine Deaminase	6.98
1415899_at	Junb	Jun-B Oncogene	6.98
1446700_at	Abl2	V-Abl Abelson Murine Leukemia Viral Oncogene 2 (Arg, Abelson-Related Gene)	6.98
1444176_at	Atp6V0D2	ATPas, H+ transporting, lysosomal Vo subunit D2	6.95
1436790_a_at	Sox11	Sry-Box Containing Gene 11	6.94
1426037_a_at	Rgs16	Regulator Of G-Protein Signaling 16	6.94
1427135_at	Sfrs12	Splicing Factor, Arginine/Serine-Rich 12	6.93
1421694_a_at	Vcan	Chondroitin Sulfate Proteoglycan 2	6.92

1421308_at	Car13	Carbonic Anhydrase 13	6.84
1439774_at	Prrx1	Paired Related Homeobox 1	6.84
1439618_at	Pde10A	Phosphodiesterase 10A	6.83
1448576_at	Il7R	Interleukin 7 Receptor	6.8
1435313_at	Cd200R4	Cd200 Receptor 4	6.8
1433047_at	5330430B06Rik	Riken Cdna 5330430B06 Gene	6.8
1416298_at	Mmp9	Matrix Metallopeptidase 9	6.78
1419754_at	Myo5a	Myosin VA	6.78
1424649_a_at	Tspan8	Tetraspanin 8	6.77
1437372_at	Cpsf6	Cleavage And Polyadenylation Specific Factor 6	6.76
1445804_at	Nav3	Neuron Navigator 3	6.76
1447839_x_at	Adm	Adrenomedullin	6.76
1449037_at	Crem	Camp Responsive Element Modulator	6.74
1427539_a_at	Zwint	Zw10 Interactor	6.74
1422557_s_at	Mt1	Metallothionein 1	6.74
1426937_at	6330406I15Rik	Riken Cdna 6330406I15 Gene	6.69
1448617_at	Cd53	Cd53 Antigen	6.69
1445717_at	E130108L08Rik	Riken Cdna E130108L08 Gene	6.68
1452114_s_at	Igfbp5	Insulin-Like Growth Factor Binding Protein 5	6.68
1438719_at	Map3k2	Mitogen-activated protein kinase kinase kinase 2	6.67
1438519_at	Klhl24	Kelch-Like 24 (Drosophila)	6.66
1457122_at	Na	Na	6.65
1456506_at	Prpf38B	Prp38 Pre-Mrna Processing Factor 38 (Yeast) Domain Containing B	6.62
1446196_at	Hmga2	High Mobility Group At-Hook 2	6.62
1444344_at	Fgf10	Fibroblast Growth Factor 10	6.6
1450744_at	Ell2	Elongation Factor Rna Polymerase Ii 2	6.58
1436326_at	Rora	Rar-Related Orphan Receptor Alpha	6.55
1450781_at	Hmga2	High Mobility Group At-Hook 2	6.49
1440177_at	9630027E11	Hypothetical Protein 9630027E11	6.49
1442168_at	A230071A22Rik	Riken Cdna A230071A22 Gene	6.46
1419356_at	Klf7	Kruppel-Like Factor 7 (Ubiquitous)	6.42
1453540_at	5430404G13Rik	Riken Cdna 5430404G13 Gene	6.41
1428834_at	Dusp4	Dual Specificity Phosphatase 4	6.39
1457424_at	Eya1	Eyes Absent 1 Homolog (Drosophila)	6.39
1446509_at	Smox	Spermine Oxidase	6.36
1428909_at	A130040M12Rik	Riken Cdna A130040M12 Gene	6.36
1444620_at	Tcf12	Transcription Factor 12	6.35
1420411_a_at	Pi4K2B	Phosphatidylinositol 4-Kinase Type 2 Beta	6.32
1429841_at	Megf10	multiple EGF-like-domains 10	6.31
1450871_a_at	Bcat1	Branched Chain Aminotransferase 1, Cytosolic	6.3
1449310_at	Ptger2	Prostaglandin E Receptor 2 (Subtype Ep2)	6.28
1445512_at	Na	Na	6.22
1440563_at	2010111I01Rik	Riken Cdna 2300006M17 Gene	6.21
1422953_at	Fpr-Rs2	Formyl Peptide Receptor, Related Sequence 2	6.21
1444175_at	Arfgef1	Adp-Ribosylation Factor Guanine Nucleotide-Exchange Factor 1(Brefeldin A-Inhibited)	6.2
1443088_at	9930031P18Rik	Riken Cdna 9930031P18 Gene	6.19

1429427_s_at	Tcf7L2	Transcription Factor 7-Like 2, T-Cell Specific, Hmg-Box	6.17
1419420_at	St6Galnac5	St6 (Alpha-N-Acetyl-Neuraminyl-2,3-Beta-Galactosyl-1,3)-N-Acetylgalactosaminide Alpha-2,6-Sialyltransferase 5	6.16
1418930_at	Cxcl10	Chemokine (C-X-C Motif) Ligand 10	6.16
1426951_at	Crim1	Cysteine Rich Transmembrane Bmp Regulator 1 (Chordin Like)	6.14
1427381_at	Irg1	Immunoresponsive Gene 1	6.12
1450586_at	Bdkrb1	Bradykinin Receptor, Beta 1	6.09
1453698_at	6030451C04Rik	Riken Cdna 6030451C04 Gene	6.08
1419004_s_at	Bcl2A1A	B-Cell Leukemia/Lymphoma 2 Related Protein A1A	6.08
1456204_at	Snhg8	small nucleolar RNA host gene (non-protein coding) 8	6.07
1455227_at	Aadacl1	Arylacetamide Deacetylase-Like 1	6.07
1422046_at	Itgam	Integrin Alpha M	6.06
1430353_at	Glis3	Glis Family Zinc Finger 3	6.05
1419647_a_at	Mbp	Myelin Basic Protein	6.03
1437939_s_at	Ctsc	Cathepsin C	6.02
1436293_x_at	Ildr2	immunoglobulin-like domain containing receptor 2	6
1448995_at	Cxcl4	Chemokine (C-X-C Motif) Ligand 4	5.99
1438855_x_at	Tnfaip2	Tumor Necrosis Factor, Alpha-Induced Protein 2	5.99
1444531_at	Sod2	Superoxide Dismutase 2, Mitochondrial	5.98
1421326_at	Csf2Rb	Colony Stimulating Factor 2 Receptor, Beta 1, Low-Affinity (Granulocyte-Macrophage)	5.98
1448748_at	Plek	Pleckstrin	5.97
1429775_a_at	Gpr137B	G Protein-Coupled Receptor 137B	5.97
1440565_at	Zbtb20	Zinc Finger And Btb Domain Containing 20	5.94
1437633_at	Ankrd11	ankyrin repeat domain 11	5.94
1419254_at	Mthfd2	Methylenetetrahydrofolate Dehydrogenase (Nad+ Dependent), Methenyltetrahydrofolate Cyclohydrolase	5.92
1441115_at	Rnf125	ring finger protein 125	5.91
1416953_at	Ctgf	Connective Tissue Growth Factor	5.91
1459670_at	Hnrpll	Heterogeneous Nuclear Ribonucleoprotein L-Like	5.91
1415844_at	Syt4	Synaptotagmin Iv	5.91
1435645_at	Mmd	Monocyte To Macrophage Differentiation-Associated	5.9
1442109_at	Fubp1	Far Upstream Element (Fuse) Binding Protein 1	5.86
1459635_at	Dlg1	Discs, Large Homolog 1 (Drosophila)	5.85
1437230_at	Kcna1	Potassium Voltage-Gated Channel, Shaker-Related Subfamily, Member 1	5.85
1427225_at	Epn2	Epsin 2	5.85
1457562_at	Rps6Kb1	Ribosomal Protein S6 Kinase, Polypeptide 1	5.83
1455965_at	Adamts4	A Disintegrin-Like And Metallopeptidase (Reprolysin Type) With Thrombospondin Type 1 Motif, 4	5.82
1428025_s_at	Pitpnc1	Phosphatidylinositol Transfer Protein, Cytoplasmic 1	5.82
1449169_at	Has2	Hyaluronan Synthase 2	5.81
1449036_at	Rnf128	Ring Finger Protein 128	5.8
1418912_at	Plxdc2	Plexin Domain Containing 2	5.79
1433782_at	Cldn12	Claudin 12	5.78
1442027_at	Nbeal1	Neurobeachin like 1	5.78
1457823_at	Cyr61	Cysteine Rich Protein 61	5.76

1423635_at	Bmp2	Bone Morphogenetic Protein 2	5.76
1424573_at	Tmed5	Transmembrane Emp24 Protein Transport Domain Containing 5	5.75
1451289_at	Dclk1	Double Cortin And Calcium/Calmodulin-Dependent Protein Kinase-Like 1	5.75
1421818_at	Bcl6	B-Cell Leukemia/Lymphoma 6	5.75
1450780_s_at	Hmga2	High Mobility Group At-Hook 2	5.73
1459917_at	Ggnbp2	Gametogenetin binding protein 2	5.71
1445687_at	Gm885	Gene Model 885, (Ncbi)	5.69
1436821_at	Plcxd3	Phosphatidylinositol-Specific Phospholipase C, X Domain Containing 3	5.66
1420342_at	Gdap10	Ganglioside-Induced Differentiation-Associated-Protein 10	5.65
1443517_at	Fam178a	Family with sequence similarity 178, member A	5.62
1425597_a_at	Qk	Quaking	5.61
1425528_at	Prrx1	Paired Related Homeobox 1	5.61
1434955_at	March1	Membrane-Associated Ring Finger (C3Hc4) 1	5.57
1452366_at	Csgalnact1	Chondroitin sulfate N-acetylgalactosaminyltransferase 1	5.57
1450678_at	Itgb2	Integrin Beta 2	5.56
1450448_at	Stc1	Stanniocalcin 1	5.55
1455859_at	A330021E22Rik	Riken Cdna A330021E22 Gene	5.55
1424296_at	Gclc	Glutamate-Cysteine Ligase, Catalytic Subunit	5.54
1418901_at	Cebpb	Ccaat/Enhancer Binding Protein (C/Ebp), Beta	5.52
1438506_s_at	Pdss1	Prenyl (Solanesyl) Diphosphate Synthase, Subunit 1	5.51
1429527_a_at	Plscr1	Phospholipid Scramblase 1	5.51
1435133_at	Ugcg	Udp-Glucose Ceramide Glucosyltransferase	5.5
1424333_at	Rg9Mtd1	Rna (Guanine-9-) Methyltransferase Domain Containing 1	5.5
1436893_a_at	March7	Membrane-Associated Ring Finger (C3Hc4) 7	5.49
1428636_at	Steap2	Six Transmembrane Epithelial Antigen Of Prostate 2	5.45
1418314_a_at	A2Bp1	Ataxin 2 Binding Protein 1	5.45
1447227_at	Slc40A1	Solute Carrier Family 39 (Iron-Regulated Transporter), Member 1	5.44
1460227_at	Timp1	Tissue Inhibitor Of Metalloproteinase 1	5.42
1435640_x_at	A130040M12Rik	Riken Cdna A130040M12 Gene	5.42
1460147_at	A730009E18Rik	Riken Cdna A730009E18 Gene	5.41
1442125_at	Krit1	Krit1, Ankyrin Repeat Containing	5.39
1420401_a_at	Ramp3	Receptor (Calcitonin) Activity Modifying Protein 3	5.37
1455899_x_at	Socs3	Suppressor Of Cytokine Signaling 3	5.37
1448213_at	Anxa1	Annexin A1	5.35
1454236_a_at	Ppp4R1L	Riken Cdna C030004A17 Gene	5.34
1447863_s_at	Nr4A2	Nuclear Receptor Subfamily 4, Group A, Member 2	5.34
1456927_at	Mast2	Microtubule Associated Serine/Threonine Kinase 2	5.33
1445235_at	Ythdf3	Yth Domain Family 3	5.32
1416681_at	Ube3A	Ubiquitin Protein Ligase E3A	5.31
1451941_a_at	Fcgr2B	Fc Receptor, Igg, Low Affinity Iib	5.29
1441407_at	Rod1	Rod1 Regulator Of Differentiation 1 (S. Pombe)	5.29
1457191_at	Na	Na	5.29

1417492_at	Ctsb	Cathepsin B	5.29
1421307_at	Car13	Carbonic Anhydrase 13	5.29
1418678_at	Has2	Hyaluronan Synthase 2	5.28
1420464_s_at	Pira3	Paired-Ig-Like Receptor A3	5.28
1457297_at	Mef2A	Myocyte Enhancer Factor 2A	5.28
1422860_at	Nts	Neurotensin	5.27
1421408_at	Igsf6	Immunoglobulin Superfamily, Member 6	5.27
1443394_at	1700006J14Rik	Riken Cdna 1700006J14 Gene	5.27
1456212_x_at	Socs3	Suppressor Of Cytokine Signaling 3	5.26
1418476_at	Crlf1	Cytokine Receptor-Like Factor 1	5.26
1422243_at	Fgf7	Fibroblast Growth Factor 7	5.26
1451348_at	Depdc6	Dep Domain Containing 6	5.26
1426875_s_at	Srxn1	Neoplastic Progression 3	5.25
1424507_at	Rin1	Ras And Rab Interactor 1	5.23
1423086_at	Npc1	Niemann Pick Type C1	5.23
1449221_a_at	Rrbp1	ribosome binding protein 1	5.23
1436203_a_at	1110059G02Rik	Riken Cdna 1110059G02 Gene	5.21
1417601_at	Rgs1	Regulator Of G-Protein Signaling 1	5.21
1457712_at	Chd8	Chromodomain Helicase Dna Binding Protein 8	5.21
1428804_at	Mfap3L	Microfibrillar-Associated Protein 3-Like	5.2
1455374_at	Kcnj3	Potassium Inwardly-Rectifying Channel, Subfamily J, Member 3	5.2
1453124_at	Tnpo3	Transportin 3	5.19
1437279_x_at	Sdc1	Syndecan 1	5.18
1438126_at	Exoc5	Exocyst Complex Component 5	5.18
1451827_a_at	Nox4	Nadph Oxidase 4	5.18
1439189_at	Fnip2	Folliculin interacting protein 2	5.17
1438824_at	Slc20A1	Solute Carrier Family 20, Member 1	5.17
1446130_at	Na	Na	5.16
1434996_at	Slc25A16	Solute Carrier Family 25 (Mitochondrial Carrier, Graves Disease Autoantigen), Member 16	5.15
1440342_at	Na	Na	5.15
1421882_a_at	Elavl2	Elav (Embryonic Lethal, Abnormal Vision, Drosophila)-Like 2 (Hu Antigen B)	5.13
1440104_at	Ranbp2	Ran Binding Protein 2	5.13
1459668_at	Tank	Traf Family Member-Associated Nf-Kappa B Activator	5.12
1425682_a_at	Tprkb	Tp53Rk Binding Protein	5.12
1459371_at	Eif4E	Eukaryotic Translation Initiation Factor 4E	5.11
1456975_at	Taok1	TAO kinase 1	5.1
1422168_a_at	Bdnf	Brain Derived Neurotrophic Factor	5.1
1456574_at	Zfp800	zinc finger protein 800	5.09
1427256_at	Vcan	Chondroitin Sulfate Proteoglycan 2	5.07
1435331_at	Pyhin1	Expressed Sequence Ai447904	5.07
1453313_at	Sesn3	Sestrin 3	5.06
1427257_at	Vcan	Chondroitin Sulfate Proteoglycan 2	5.06
1452483_a_at	Cd44	Cd44 Antigen	5.05
1449252_at	Fam110c	family with sequence similarity 110, member C	5.04
1458299_s_at	Nfkbie	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Epsilon	5.04

1431056_a_at	Lpl	Lipoprotein Lipase	5.04
1421228_at	Ccl7	Chemokine (C-C Motif) Ligand 7	5.04
1418932_at	Nfil3	Nuclear Factor, Interleukin 3, Regulated	5.03
1440085_at	Eda2R	Ectodysplasin A2 Isoform Receptor	5.02
1449249_at	Pcdh7	Protocadherin 7	5.02
1440461_at	Fam49b	family with sequence similarity 49, member B	5.01
1418350_at	Hbegf	Heparin-Binding Egf-Like Growth Factor	5
1443827_x_at	Bc004044	Cdna Sequence Bc004044	5
1436614_at	March3	Membrane-Associated Ring Finger (C3Hc4) 3	5
1418986_a_at	Uxt	Ubiquitously Expressed Transcript	5
1428361_x_at	Hba-A1	Hemoglobin Alpha, Adult Chain 1	-5.02
1419674_a_at	Dpep1	Dipeptidase 1 (Renal)	-5.03
1419663_at	Ogn	Osteoglycin	-5.12
1418511_at	Dpt	Dermatopontin	-5.13
1457038_at	Frem2	Fras1 Related Extracellular Matrix Protein 2	-5.15
1437528_x_at	A730017C20Rik	protein phosphatase 4, regulatory subunit 1-like	-5.17
1454830_at	Fbn2	Fibrillin 2	-5.21
1450648_s_at	H2-Ab1	Histocompatibility 2, Class Ii Antigen A, Beta 1	-5.22
1451675_a_at	Alas2	Aminolevulinic Acid Synthase 2, Erythroid	-5.28
1418278_at	Apoc3	Apolipoprotein C-Iii	-5.32
1417789_at	Ccl11	Small Chemokine (C-C Motif) Ligand 11	-5.38
1434354_at	Maob	Monoamine Oxidase B	-5.4
1452107_s_at	Npnt	Nephronectin	-5.4
1419232_a_at	Apoa1	Apolipoprotein A-I	-5.43
1439019_at	Fras1	Fraser Syndrome 1 Homolog (Human)	-5.49
1450344_a_at	Ptger3	Prostaglandin E Receptor 3 (Subtype Ep3)	-5.51
1443299_at	Pdlim3	Pdz And Lim Domain 3	-5.55
1456335_at	Gm106	Gene Model 106, (Ncbi)	-5.58
1448553_at	Myh7	Myosin, Heavy Polypeptide 7, Cardiac Muscle, Beta	-5.65
1443783_x_at	H2-Aa	Histocompatibility 2, Class Ii Antigen A, Alpha	-5.71
1425475_at	Col4A5	Procollagen, Type Iv, Alpha 5	-5.76
1426442_at	Gpm6A	Glycoprotein M6A	-5.79
1449939_s_at	Dlk1	Delta-Like 1 Homolog (Drosophila)	-5.84
1453128_at	Lyve1	Extra Cellular Link Domain-Containing 1	-5.84
1416468_at	Aldh1A1	Aldehyde Dehydrogenase Family 1, Subfamily A1	-5.9
1452913_at	Pcp4L1	Purkinje Cell Protein 4-Like 1	-5.91
1433837_at	8430408G22Rik	Riken Cdna 8430408G22 Gene	-5.94
1425519_a_at	Cd74	Cd74 Antigen (Invariant Polypeptide Of Major Histocompatibility Complex, Class Ii Antigen-Associated)	-6.03
1419400_at	Mttp	Microsomal Triglyceride Transfer Protein	-6.09
1422846_at	Rbp2	Retinol Binding Protein 2, Cellular	-6.15
1417614_at	Ckm	Creatine Kinase, Muscle	-6.19
1438532_at	Hmcn1	hemicentin 1	-6.27
1428781_at	Dmkn	Dermokine	-6.28
1439479_at	Lct	Lactase	-6.29
1421426_at	Hhip	Hedgehog-Interacting Protein	-6.35
1449465_at	Reln	Reelin	-6.35

1457429_s_at	Gm106	Gene Model 106, (Ncbi)	-6.48
1417025_at	H2-Eb1	Histocompatibility 2, Class Ii Antigen E Beta	-6.49
1437355_at	Zcchc5	Zinc Finger, Cchc Domain Containing 5	-6.52
1425505_at	Mylk	Myosin, Light Polypeptide Kinase	-6.59
1452106_at	Npnt	Nephronectin	-6.6
1419426_s_at	Ccl21c	Chemokine (C-C Motif) Ligand 21A	-6.67
1455280_at	Frem1	Fras1 Related Extracellular Matrix Protein 1	-6.68
1419233_x_at	Apoa1	Apolipoprotein A-I	-6.68
1437871_at	Pgm5	Phosphoglucomutase 5	-6.68
1435290_x_at	H2-Aa	Histocompatibility 2, Class Ii Antigen A, Alpha	-6.76
1438858_x_at	H2-Aa	Histocompatibility 2, Class Ii Antigen A, Alpha	-6.82
1452431_s_at	H2-Aa	Histocompatibility 2, Class Ii Antigen A, Alpha	-6.84
1426511_at	Susd2	Sushi Domain Containing 2	-6.86
1417917_at	Cnn1	Calponin 1	-6.87
1446685_at	Loc436089	Similar To Matrilin 1, Cartilage Matrix Protein	-6.92
1425476_at	Col4A5	Procollagen, Type Iv, Alpha 5	-7
1455540_at	Cps1	Carbamoyl-Phosphate Synthetase 1	-7.04
1435796_at	Wscd2	WSC domain containing 2	-7.27
1434237_at	Upk3B	Uroplakin 3B	-7.28
1455201_x_at	Apoa1	Apolipoprotein A-I	-7.3
1449058_at	Gli1	Gli-Kruppel Family Member Gli1	-7.35
1457871_at	Colec10	Collectin Sub-Family Member 10	-7.46
1415927_at	Actc1	Actin, Alpha, Cardiac	-7.47
1427866_x_at	Hbb-b2	Hemoglobin Beta Chain Complex	-7.51
1434927_at	Hspb7	Heat Shock Protein Family, Member 7 (Cardiovascular)	-7.53
1421421_at	Angptl1	Angiopoietin-Like 1	-7.53
1425504_at	Mylk	Myosin, Light Polypeptide Kinase	-7.71
1420693_at	Myom1	Myomesin 1	-7.77
1456741_s_at	Gpm6A	Glycoprotein M6A	-7.89
1451721_a_at	H2-Ab1	Histocompatibility 2, Class Ii Antigen A, Beta 1	-7.92
1450990_at	Gpc3	Glypican 3	-8.19
1425253_a_at	Madcam1	Mucosal Vascular Addressin Cell Adhesion Molecule 1	-8.35
1429379_at	Lyve1	Extra Cellular Link Domain-Containing 1	-8.5
1448962_at	Myh11	Myosin, Heavy Polypeptide 11, Smooth Muscle	-8.56
1417851_at	Cxcl13	Chemokine (C-X-C Motif) Ligand 13	-8.6
1437933_at	Hhip	Hedgehog-Interacting Protein	-8.88
1438884_at	Shisa3	shisa homolog 3 (Xenopus laevis)	-9.02
1436665_a_at	Ltbp4	latent transforming growth factor beta binding protein 4	-9.14
1427345_a_at	Sult1A1	Sulfotransferase Family 1A, Phenol-Preferring, Member 1	-9.37
1417184_s_at	Hbb-B2	Hemoglobin Beta Chain Complex	-9.56
1417761_at	Apoa4	Apolipoprotein A-Iv	-10.1
1437260_at	Mmrn1	Multimerin 1	-10.42
1448764_a_at	Fabp1	Fatty Acid Binding Protein 1, Liver	-10.79
1426731_at	Des	Desmin	-10.84
1417714_x_at	Hba-A1	Hemoglobin Alpha, Adult Chain 1	-11.01

1438840_x_at	Apoa1	Apolipoprotein A-I	-11.09
1436504_x_at	Apoa4	Apolipoprotein A-Iv	-11.45
1459072_at	Na	Na	-11.82
1455224_at	Angptl1	Angiopoietin-Like 1	-12.08
1417556_at	Fabp1	Fatty Acid Binding Protein 1, Liver	-12.19
1422598_at	Casq1	Calsequestrin 1	-12.26
1438083_at	Hhip	Hedgehog-Interacting Protein	-12.39
1455277_at	Hhip	Hedgehog-Interacting Protein	-12.46
1454159_a_at	Igfbp2	Insulin-Like Growth Factor Binding Protein 2	-13.35
1449396_at	Aoc3	Amine Oxidase, Copper Containing 3	-13.58
1449178_at	Pdlim3	Pdz And Lim Domain 3	-13.64
1429072_at	1110001D15Rik	Riken Cdna 1110001D15 Gene	-14.96
1418438_at	Fabp2	Fatty Acid Binding Protein 2, Intestinal	-15.19
1454881_s_at	Upk3B	Uroplakin 3B	-17.4
1435399_at	Synpo2	Synaptopodin 2	-18.17
1460214_at	Pcp4	Purkinje Cell Protein 4	-23.83
1424967_x_at	Tnnt2	Troponin T2, Cardiac	-31.65
1422644_at	Sh3bgr	Sh3-Binding Domain Glutamic Acid-Rich Protein	-31.99
1427053_at	Abi3bp	ABI gene family, member 3 (NESH) binding protein	-33.45
1427054_s_at	Abi3bp	ABI gene family, member 3 (NESH) binding protein	-35.86
1418726_a_at	Tnnt2	Troponin T2, Cardiac	-43.17

Table 3.10: DAVID Functional Annotation Clusters for Genes Upregulated by Culture

Functional Cluster	Enrichment Score	Top Term		Top Term FE	Top Term P Value	Top Term FDR
1	8.91	SP_PIR_KEYWORDS	Secreted	2.85	1.78E-11	2.77E-08
2	8.39	GO:0009605	Response To External Stimulus	4.68	1.05E-13	2.00E-10
3	5.61	GO:0032502	Developmental Process	1.86	1.78E-09	3.39E-06
4	5.16	GO:0008083	Growth Factor Activity	6.32	1.11E-07	1.97E-04
5	4.38	GO:0001871	Pattern Binding	8.34	1.90E-07	3.38E-04
6	3.43	GO:0042089	Cytokine Biosynthetic Process	7.65	2.18E-05	4.16E-02
7	3.05	GO:0030154	Cell Differentiation	1.87	9.86E-06	1.88E-02
8	2.60	GO:0008283	Cell Proliferation	2.34	1.30E-03	2.46E+00
9	2.59	GO:0045595	Regulation Of Cell Differentiation	4.39	9.23E-05	1.76E-01
10	2.56	GO:0040007	Growth	3.51	9.88E-05	1.89E-01

Table 3.10: DAVID Functional Annotation Clusters for Genes Upregulated by Culture. DAVID analysis reveals that inflammation, cytokine signaling, growth factor activity, and control of cellular proliferation and differentiation are the most upregulated processes in cultured intestinal mesenchyme compared to freshly isolated mesenchyme. A representative highly significant term from each of the top 10 clusters identified by the DAVID Functional Annotating Clustering tool is shown. The Cluster Enrichment Score refers to the enrichment of all terms in that cluster.

Table 3.11: DAVID Functional Annotation Clusters for Genes Downregulated by Culture

Functional Cluster	Enrichment Score		Top Term	Top Term FE	Top Term P Value	Top Term FDR
1	5.91	GO:0044421	Extracellular Region Part	3.38	1.42E-09	2.18E-06
2	4.81	GO:0031012	Extracellular Matrix	10.12	1.86E-08	2.85E-05
3	3.71	GO:0048856	Anatomical Structure Development	3.32	1.82E-08	3.49E-05
4	3.46	SP_PIR_KEYWORDS	Extracellular Matrix	10.52	9.50E-06	1.47E-02
5	2.67	INTERPRO: IPR006210	EGF	9.45	8.82E-05	1.70E-01
6	2.49	GO:0003012	Muscle System Process	19.99	1.16E-06	2.21E-03
7	2.38	INTERPRO: IPR000463	Cytosolic Fatty-Acid Binding	48.08	1.68E-03	3.21E+00
8	2.30	GO:0008009	Chemokine Activity	27.99	3.70E-04	6.57E-01
9	2.02	GO:0002495	Antigen Processing And Presentation Of Peptide Antigen Via MHC Class Ii	43.79	9.48E-05	1.81E-01
10	1.72	GO:0008289	Lipid Binding	5.98	1.06E-04	1.89E-01

Table 3.11: DAVID Functional Annotation Clusters for Genes Downregulated by Culture. DAVID analysis reveals regulation of the extracellular matrix, EGF signaling, muscle differentiation, chemokine activity and MHC Class II antigen presentation are the most downregulated processes in cultured intestinal mesenchyme compared to freshly isolated mesenchyme. A representative highly significant term from each of the top 10 clusters identified by the DAVID Functional Annotating Clustering tool is shown. The Cluster Enrichment Score refers to the enrichment of all terms in that cluster.

Table 3.12: Genes Downregulated by Culture and Upregulated by Hh ligand

Probe ID	Gene Symbol	Description	Vehicle v Mes FC	Ihh v Vehicle FC	Shh v Vehicle FC
1435399_at	Synpo2	Synaptopodin 2	-18.17	1.77	1.88
1455224_at	Angptl1	Angiopoietin-Like 1	-12.08	1.88	1.86
1449058_at	Gli1	GLI-Kruppel family member GLI1	-7.35	1.98	1.48
1435290_x_at	H2-Aa	Histocompatibility 2, Class Ii Antigen A, Alpha	-6.76	2.28	1.91
1428853_at	Ptch1	Patched Homolog 1	-4.27	2.91	2.66
1419476_at	Adamdec1	Adam-Like, Decysin 1	-3.73	1.63	1.82
1425978_at	Myocd	Myocardin	-3.54	2.49	2.09
1439663_at	Ptch1	Patched Homolog 1	-3.22	2.64	2.05
1427489_at	Itga8	Expressed Sequence Ai447669	-2.98	1.57	1.73
1455050_at	E130203B14Rik	Riken Cdna E130203B14 Gene	-2.75	1.81	1.82
1415824_at	Scd2	Stearoyl-Coenzyme A Desaturase 2	-2.41	1.73	1.63
1452528_a_at	Nkx2-3	Nk2 Transcription Factor Related, Locus 3 (Drosophila)	-2.3	1.57	1.51
1427038_at	Penk1	Preproenkephalin 1	-2.14	1.74	1.60

Table 3.13: DAVID Functional Annotation Clusters for Genes Down with Culture and Up with Hh

Functional Cluster	Enrichment Score	Top Term		Top Term FE	Top Term P Value	Top Term FDR
1	0.83	GO:0048513	Organ Development	6.35	6.83E-04	1.30E+00
2	0.71	SP_PIR_KEYWORDS	Secreted	4.51	1.13E-01	8.46E+01
3	0.61	GO:0043169	Cation Binding	2.31	1.09E-01	8.72E+01
4	0.21	GO:0016021	Integral To Membrane	1.33	4.60E-01	1.00E+02

Table 3.13: DAVID Functional Annotation Clusters for Genes Downregulated by Culture and Upregulated by Addition of Hh Ligand. DAVID analysis reveals that genes that are downregulated by culture and upregulated by Hh ligand are strongly associated with development. This cluster additionally includes myogenesis and transcription factor term annotations. A representative highly significant term from each of the clusters identified by the DAVID Functional Annotating Clustering tool is shown. The Cluster Enrichment Score refers to the enrichment of all terms in that cluster.

Table 3.14: Genes Upregulated by Culture and Downregulated by Hh ligand

Probe ID	Gene Symbol	Description	Vehicle vs Mes FC	Ihh vs Vehicle FC	Shh vs Vehicle FC
1438148_at	Cxcl3	Chemokine (C-X-C motif) Ligand 3	129.37	-6.99	-3.55
1418480_at	Cxcl7	Chemokine (C-X-C Motif) Ligand 7	68.47	-1.75	-2.17
1419549_at	Arg1	Arginase 1, Liver	59.23	-5.10	-6.84
1449498_at	Marco	Macrophage Receptor With Collagenous Structure	39.77	-17.03	-4.94
1417266_at	Ccl6	Chemokine (C-C Motif) Ligand 6	37.74	-1.72	-1.78
1450826_a_at	Saa3	Serum Amyloid A 3	37.24	-6.15	-5.32
1419728_at	Cxcl5	Chemokine (C-X-C Motif) Ligand 5	34.61	-2.30	-2.36
1420249_s_at	Ccl6	Chemokine (C-C Motif) Ligand 6	29.83	-1.95	-1.86
1420804_s_at	Clec4D	C-Type Lectin Domain Family 4, Member D	28.39	-2.86	-1.89
1419209_at	Cxcl1	Chemokine (C-X-C Motif) Ligand 1	20.9	-2.74	-2.90
1427747_a_at	Lcn2	Lipocalin 2	20.16	-2.72	-3.48
1438841_s_at	Arg2	Arginase Type Ii	19.82	-2.19	-2.18
1448898_at	Ccl9	Chemokine (C-C Motif) Ligand 9	19.33	-2.51	-2.81
1417936_at	Ccl9	Chemokine (C-C Motif) Ligand 9	18.7	-2.77	-3.24
1419082_at	Serpinb2	Serine (Or Cysteine) Peptidase Inhibitor, Clade B, Member 2	17.54	-11.82	-10.73
1438377_x_at	Slc13A3	Solute Carrier Family 13 (Sodium-Dependent Dicarboxylate Transporter), Member 3	17.03	-4.78	-4.24
1450297_at	Il6	Interleukin 6	15.66	-2.62	-4.24
1420380_at	Pira1/2/3/4/6	Paired-Ig-Like Receptor A2	15.54	-2.32	-1.98
1449984_at	Cxcl2	Chemokine (C-X-C Motif) Ligand 2	13.13	-4.85	-3.45
1418937_at	Dio2	Deiodinase, Iodothyronine, Type Ii	12.3	-1.58	-2.01
1418847_at	Arg2	Arginase Type Ii	11.75	-2.07	-2.17
1458297_s_at	Marco	Macrophage Receptor With Collagenous Structure	11.14	-16.06	-4.33
1417483_at	Nfkbiz	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Zeta	8.39	-1.62	-1.98
1417268_at	Cd14	Cd14 Antigen	8.09	-3.25	-3.09
1448291_at	Mmp9	Matrix Metallopeptidase 9	7.98	-1.93	-1.96
1439774_at	Prrx1	Paired Related Homeobox 1	6.84	-1.89	-3.47
1416298_at	Mmp9	Matrix Metallopeptidase 9	6.78	-2.03	-1.91
1444344_at	Fgf10	Fibroblast Growth Factor 10	6.6	-1.82	-2.38
1428909_at	A130040M12Rik	Riken Cdna A130040M12 Gene	6.36	-1.61	-1.68
1422953_at	Fpr-Rs2	Formyl Peptide Receptor, Related Sequence 2	6.21	-14.53	-10.69
1427381_at	Irg1	Immunoresponsive Gene 1	6.12	-7.68	-3.57
1422046_at	Itgam/Cd11b	Integrin Alpha M (Cd11b)	6.06	-1.98	-1.78
1444531_at	Sod2	Superoxide Dismutase 2, Mitochondrial	5.98	-1.71	-1.80
1425528_at	Prrx1	Paired Related Homeobox 1	5.61	-1.85	-2.41

1420464_s_at	Lilrb3	Leukocyte Immunoglobulin-Like Receptor, Subfamily B (With Tm And Itim Domains), Member 3	5.28	-1.73	-1.78
1440342_AT	N/A	N/A	5.15	-1.96	-2.04
1421228_at	Ccl7	Chemokine (C-C Motif) Ligand 7	5.04	-2.32	-2.21
1424302_at	Lilrb3	Leukocyte Immunoglobulin-Like Receptor, Subfamily B (With Tm And Itim Domains), Member 3	4.95	-1.91	-1.84
1460197_a_at	Steap4	Steap Family Member 4	4.71	-1.58	-1.75
1448610_a_at	Sod2	Superoxide Dismutase 2, Mitochondrial	4.7	-2.08	-1.88
1452408_at	Gpr31c	G protein-coupled receptor 31, D17Leh66c region	4.66	-1.93	-2.23
1450808_at	Fpr1	Formyl Peptide Receptor 1	4.56	-9.37	-6.25
1436778_at	Cybb	Cytochrome B-245, Beta Polypeptide	4.44	-1.94	-1.80
1449366_at	Mmp8	Matrix Metallopeptidase 8	4.44	-3.76	-2.57
1435040_at	Irak3	Interleukin-1 Receptor-Associated Kinase 3	4.43	-2.51	-2.94
1420330_at	Ccl2	Chemokine (C-C Motif) Ligand 2	4.3	-5.59	-3.68
1452117_a_at	Fyb	Fyn Binding Protein	4.24	-1.63	-1.61
1448728_a_at	Nfkbiz	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Zeta	4.15	-1.50	-1.77
1416592_at	Glrx	Glutaredoxin	4.08	-2.40	-2.41
1428942_at	Mt2	Metallothionein 2	3.91	-1.55	-1.53
1449399_a_at	Il1B	Interleukin 1 Beta	3.88	-3.32	-3.33
1454976_at	Sod2	Superoxide Dismutase 2, Mitochondrial	3.88	-1.83	-1.85
1422978_at	Cybb	Cytochrome B-245, Beta Polypeptide	3.82	-1.77	-1.53
1421009_at	Rsad2	Radical S-Adenosyl Methionine Domain Containing 2	3.8	-3.07	-4.69
1427327_at	Pilra	Expressed Sequence Av021745	3.76	-2.45	-2.17
1419709_at	Stfa3	Stefin A1	3.64	-5.90	-4.97
1436058_at	Rsad2	Radical S-Adenosyl Methionine Domain Containing 2	3.54	-2.84	-3.44
1418879_at	Fam110c	Family with sequence similarity 110, member C	3.53	-1.59	-1.83
1417193_at	Sod2	Superoxide Dismutase 2, Mitochondrial	3.49	-1.99	-2.03
1451161_a_at	Emr1	Egf-Like Module Containing, Mucin-Like, Hormone Receptor-Like Sequence 1	3.27	-2.03	-2.08
1436779_at	Cybb	Cytochrome B-245, Beta Polypeptide	3.22	-2.27	-2.23
1417813_at	Ikbke	Inhibitor Of Kappab Kinase Epsilon	3.05	-1.73	-1.64
1422013_at	Clec4A2	C-Type Lectin Domain Family 4, Member A2	3.04	-2.35	-2.60
1420250_at	Clec4E	C-Type Lectin Domain Family 4, Member E	2.85	-1.57	-1.60
1458683_at	Sirpb1	Sirp-Beta B	2.71	-1.69	-1.64
1416593_at	Glrx	Glutaredoxin	2.66	-2.12	-2.13
1419282_at	Ccl12	Chemokine (C-C Motif) Ligand 12	2.58	-2.12	-2.55
1449222_at	Ebi3	Epstein-Barr Virus Induced Gene 3	2.32	-1.93	-1.55
1418126_at	Ccl5	Chemokine (C-C Motif) Ligand 5	2.29	-2.74	-2.45

1425407_s_at	Clec4B1	C-Type Lectin Domain Family 4, Member A2	2.24	-1.94	-1.99
1448881_at	Hp	Haptoglobin	2.21	-1.86	-2.24
1452205_x_at	Tcrb-V13	T-Cell Receptor Beta, Variable 13	2.19	-1.68	-2.02
1424921_at	Bst2	Bone Marrow Stromal Cell Antigen 2	2.19	-2.27	-2.06
1420499_at	Gch1	Gtp Cyclohydrolase 1	2.13	-1.63	-1.51
1419132_at	Tlr2	Toll-Like Receptor 2	2.12	-2.62	-1.79
1457644_s_at	Cxcl1	Chemokine (C-X-C Motif) Ligand 1	2.1	-2.22	-2.14
1418842_at	Hcls1	Hematopoietic Cell Specific Lyn Substrate 1	2.03	-1.92	-1.87
1420591_at	Gpr84	G Protein-Coupled Receptor 84	2.01	-3.71	-3.01

Table 3.15: DAVID Functional Annotation Clusters for Genes Up with Culture and Down with Hh

Functional Cluster	Enrichment Score		Top Term	Top Term FE	Top Term P Value	Top Term FDR
1	8.89	GO:0008009	Chemokine Activity	79.64	6.62E-17	2.00E-13
2	1.88	KEGG_PATHWAY	Toll-Like Receptor Signaling Pathway	8.89	3.84E-04	4.78E-01
3	1.88	SP_PIR_KEYWORDS	Immune Response	9.69	1.64E-03	2.52E+00
4	1.62	GO:0043123	Positive Regulation Of IKκB Kinase/NF-κB Cascade	21.60	8.16E-03	1.45E+01
5	1.53	GO:0004871	Signal Transducer Activity	1.75	1.08E-02	1.76E+01
6	0.98	GO:0009310	Amine Catabolic Process	14.16	1.83E-02	2.97E+01

Table 3.15: DAVID Functional Annotation Clusters for Genes Upregulated by Culture and Downregulated by Shh and Ihh ligand. DAVID analysis reveals that inflammation, cytokine and chemokine signaling, and the Toll-like Receptor/NF-κB signaling pathway are processes which are downregulated by Hh signaling that are otherwise induce in cultured mesenchyme. A representative highly significant term from each of the top clusters identified by the DAVID Functional Annotating Clustering tool is shown. The Cluster Enrichment Score refers to the enrichment of all terms in that cluster.

Table 3.16: Genes Commonly Regulated in Shh-/- lung and Hh-treated Mesenchyme				
Gene Symbol	Description	Shh-/- lung FC	Ihh Treated Mes FC	Shh Treated Mes FC
Ptch	Patched Homolog 1	-2.33	2.91	2.66
Gli1	Gli-Kruppel Family member Gli1	-1.6	1.98	1.48
Enc1	Ectodermal-neural cortex 1	-1.55	1.51	1.63
Adamdec1	ADAM-like, Decysin 1	-3.82	1.63	1.82
Myocd (Srfcp)	Myocardin	-1.61	2.49	2.09

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Chapter 4

Analysis of germline GLI1 variation implicates Hedgehog signaling in the regulation of intestinal inflammatory pathways[*]

Summary

Background: Ulcerative colitis (UC) and Crohn's disease (CD) are polygenic chronic inflammatory bowel diseases (IBD) of high prevalence that are associated with considerable morbidity. The Hedgehog (HH) signaling pathway plays vital roles in gastrointestinal tract development, homeostasis and malignancy. We identified a germline variation in *GLI1* (within the IBD2 linkage region, 12q13) in patients with IBD. Since this IBD-associated variant encodes a GLI1 protein with reduced function and our expression studies demonstrated down-regulation of the HH response in IBD, we tested whether mice with reduced Gli1 activity demonstrate increased susceptibility to chemically induced colitis.

Methods and Findings: Using a gene-wide haplotype-tagging approach, germline *GLI1* variation was examined in three independent populations of IBD patients and healthy controls from Northern Europe (Scotland, England and Sweden) totalling over 5000 individuals. On log-likelihood analysis, *GLI1* was associated with IBD, predominantly UC, in Scotland and England (p<0.0001). A non-synonymous SNP (rs2228226C→G), in exon 12 of *GLI1* (Q1100E) was strongly implicated, with

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pooled odds ratio of 1.194 (C.I.=1.09-1.31, p=0.0002). *GLI1* variants were tested in vitro for transcriptional activity in luciferase assays. Q1100E falls within a conserved motif near the C-terminus of GLI1; the variant GLI protein exhibited reduced transactivation function in vitro. In complementary expression studies, we noted the colonic HH response, including GLI1, PTCH and HHIP, to be down-regulated in patients with UC. Finally, *Gli1*$^{+/lacZ}$ mice were tested for susceptibility to DSS-induced colitis. Clinical response, histology and expression of inflammatory cytokines and chemokines were recorded. *Gli1*$^{+/lacZ}$ mice rapidly developed severe intestinal inflammation, with considerable morbidity and mortality compared with wild-type. Local myeloid cells were shown to be direct targets of HH signals and cytokine expression studies revealed robust up-regulation of IL-12, IL-17 and IL-23 in this model.

Conclusions: HH signaling through GLI1 is required for appropriate modulation of the intestinal response to acute inflammatory challenge. Reduced GLI1 function predisposes to a heightened myeloid response to inflammatory stimuli, potentially leading to IBD.

INTRODUCTION

Ulcerative colitis (UC; MIM 191390) and Crohn's disease (CD; MIM 266600) are chronic, relapsing, inflammatory bowel diseases (IBD) of high prevalence (200-400 cases per 100,000 in N Europe and N America (7)) and are associated with considerable morbidity. Precise aetio-pathogenetic mechanisms are not understood but several lines of evidence implicate the central importance of a dysregulated host response to intestinal bacteria (8). Epidemiological data, detailed molecular studies, and recent genome-wide association studies strongly suggest that UC and CD are related polygenic diseases that share some susceptibility loci (*IL-23R*, *IL-12B*, and *NKX2.3*), but differ at others: *NOD2*, *ATG16L1* and *IRGM* are specific CD genes; the *ECM1* locus is associated with UC (9-16). The IBD2 locus (OMIM 601458) on chromosome 12q13 was first identified in a UK genome-wide linkage scan (peak LOD score 5.47 at D12S83) (17) involving both UC and CD patients. Later studies showed that IBD2 contributes significantly to UC, notably extensive disease, but perhaps in a lesser way to CD susceptibility (18; 19).

A strong candidate gene that maps to the IBD2 locus is *GLI1*, one of three related GLI transcription factors that transduce secreted hedgehog (HH) signals. HH signaling is key in gut development, homeostasis and malignancy, but has not been carefully studied in IBD(20). In developing intestine, Sonic (SHH) and Indian Hedgehog (IHH) provide a paracrine signal from epithelium to the mesenchymally expressed receptor patched (PTCH) (21; 22). The binding of HH to PTCH releases the membrane protein smoothened (SMO) from inhibition and allows HH signal transduction through the zinc finger transcription factors GLI1, GLI2, and GLI3 to

direct tissue patterning and cell fate (see 14, 16 and citations therein). Chronic injury, inflammation and repair are critical aspects of IBD, and thus it is pertinent that the HH pathway is centrally involved in these processes in several other tissues, including muscle (21), liver (23; 24)), and lung(25; 26). Indeed, HH signaling may play a central role in the inflammatory response since SHH is critical for T lymphocyte development(27), adult human CD4+ T cell activation (6; 28), and myeloid cell maturation in the spleen (29). Furthermore, SHH has recently been suggested to be a direct transcriptional target of NF-κB (30). Dysregulation of components of the HH pathway has also been noted in inflammatory diseases of the gut, including Barrett's esophagus, chronic gastritis and IBD (31). Using microarray gene expression analyses of colonoscopic biopsies, we recently demonstrated that GLI1 expression is greater in the distal compared with the proximal colon, and is downregulated in the intestinal mucosa in inflamed UC compared with non-inflamed samples (32).

Taken together, these studies suggested a possible association between GLI1 and IBD susceptibility, and we set out to directly test this possibility in both human disease and murine model systems. Our main initial objectives were to ascertain whether germline *GLI1* variation was associated with IBD and to describe the expression of HH signaling components in colonic inflammation in man. These data led us to hypothesize that a reduced dosage of functional GLI1 protein might play an important role in colonic inflammation. To test this directly, we challenged *Gli1*$^{+/lacZ}$ mice and their wild type (WT) littermates with dextran sodium sulphate (DSS) to induce acute intestinal inflammation, and monitored clinical and histological parameters, cytokine profiles and cellular targets of HH signaling in vivo. Together, these studies demonstrate that functional GLI1 activity and intact HH signaling plays

a crucial role in modulating the intestinal response to inflammatory stimuli in mouse and man.

METHODS

Written, informed consent was obtained from all patients and controls. The study protocol was approved by the Lothian Research and Ethics Committee (LREC-2000/4/192 and 2004/S1103/22); Cambridge (LREC-01/418; MREC-03/5/012) and Regional Ethics Committee, Karolinska Institutet.

Subjects and Samples

Subjects for genotyping

Scotland: The population consisted of 817 IBD cases (474 UC; 335 CD; 8 IBDU) and 1374 healthy controls (Table 4.1). Diagnosis was made by clinical, radiological and histopathological means, adhering to the criteria of Lennard-Jones (33). All IBD patients attended the clinic at the Western General Hospital (Edinburgh, Scotland), a tertiary referral centre for IBD in south-east Scotland. The population was 98.5% white, non-Jewish. The median age at diagnosis was 31.0 (IQR 23.3-46.0). Controls were recruited from across Scotland; median age was 50.0 (IQR 43.0-55.0), ethnicity >99% white, non-Jewish, and 48.7% male sex.

Cambridge, England: 1748 unrelated Caucasian IBD patients (Lennard-Jones criteria) of north European origin attending IBD clinics in East Anglia, UK were recruited comprising 928 UC, 737 CD, and 83 with IBDU. The population was >99% white, non-Jewish. The 589 ethnically and geographically matched healthy controls were

previously recruited in East Anglia for the European Prospective Investigation of Nutrition and Cancer (EPIC). Median age of controls was 60 years.

Sweden: 288 UC and 205 CD patients (Lennard-Jones criteria) were recruited from various hospitals in Stockholm County. 281 Swedish HC were healthy volunteers from the Karolinska University Hospital staff (n=170) and orthopaedic day-case surgery patients (n=111) with no previous medical conditions.   Cases and controls were >99% white, non-Jewish.

Phenotyping. Phenotypic data were collected by patient questionnaire, interview, and case note review, and classified according to Montreal criteria (34) (Table 4.1).

Genotyping

Scotland and Sweden: Genomic DNA was extracted from blood samples using a modified salting-out technique as previously described, and Nucleon kits.   Genotypes were derived using the Taqman system for allelic discrimination; the assays were available from Applied Biosystems as Taqman SNP Genotyping Assays (7900HT sequence detection system; Applied Biosystems), except for SNPs rs10783819, rs3809114, rs507562, rs542278, rs730560, rs1669296, rs775322 which were genotyped on the Illumina platform. The accuracy of each Taqman assay was checked by repeat analysis in 5% of cases, with 100% concordance. Genotype distributions in all populations were consistent with Hardy-Weinberg Equilibrium (HWE) (p>0.01) for all SNPs. Genotypes, in cases for the four tSNPs that could not be derived by Taqman were obtained by direct sequencing.

Cambridge: DNA was extracted using Nucleon kits. Genotyping of CD cases and controls was performed using the Taqman biallelic discrimination system using an

ABI 7900HT analyser (Applied Biosystems). Genotyping of UC cases was performed

using a 1536 SNP Golden Gate bead array (Illumina). Concordance between

platforms was assessed by genotyping 92 UC cases for SNPs rs2228224 and

rs2228226 with concordance rates of 100% and 97.9% respectively and no evidence

of systematic bias between platforms. Genotype distributions in case and control

populations were consistent with HWE (p>0.01) for all SNPs.

Gene expression by microarray and QPCR

The cohort of patients used in the microarray studies consisted of 67 patients

with UC, 53 with CD and 31 healthy controls (HC). For UC patients, 8 were

recruited at diagnosis (treatment naïve), 18 had active disease and 41 quiescent

disease; 10 were on systemic corticosteroid therapy, 11 on immunosuppressants

(azathioprine, mercaptopurine or methotrexate) and 40 on 5-amino salicylic acid (5-

ASA) therapy. 4/53 patients with CD were on corticosteroids, 13/53 on

immunosuppressants and 21/53 on a 5-ASA. 8/31 HC had non-IBD inflammation: 2

scattered lymphoid aggregates with history of gastroenteritis, 2 microscopic colitis, 1

pseudomembranous colitis, 1 diverticulitis, 1 amoebiasis and 1 eosinophilic infiltrate.

The assessment of RNA quality and integrity was performed for the microarray

experiments as follows: the amplified cRNA was purified using the RNeasy Mini Kit

protocol (Qiagen) and 1 µl of amplified cRNA was quantified using the NanoDrop

ND-1000 Spectrophotometer. The distribution of log intensities for each sample was

plotted and outlier samples (i.e. greaterthan 2 standard deviations from the mean)

were excluded from analysis. For additional demographics, phenotyping, and full

methodology pertaining to the generation of the microarray dataset and QPCR see

Noble et al (32).

Induction of DSS-induced colitis and histological analysis

Groups of two to four wild type $Gli1^{+/lacZ}$ or wild type littermate controls in

mixed cages (bred at least 3 generations onto a C57BL/6 background) were

administered 3% DSS in drinking water for 4-6 days. The amount of DSS consumed

was not significantly different between WT and $Gli1^{+/lacZ}$ animals (data not shown).

$Gli1^{+/lacZ}$ animals tended to weigh more than their WT littermates (mean = 28g for

$Gli1^{+/lacZ}$ animals and 24g for WT animals). Therefore, DSS-treated weight matched

WT C57Bl/6 animals (n= 4) were also tested along with $Gli1^{+/lacZ}$ animals and WT

littermates. No differences were evident between WT littermates and weight matched

C57Bl/6. All animals were monitored daily for diarrhoea, bloody stool, and weight

loss. Clinical scoring was as follows: 0 = no symptoms, 1 = diarrhoea, 2 = bloody

stool, 4 = severe rectal bleeding and morbidity to the point of immobility/death. For

histology, a segment of large intestine tissue of equal length and location for all

animals was fixed overnight in 4% paraformaldehyde, dehydrated, infiltrated with

paraffin, and sectioned at 5μm. The remaining colonic tissue was taken for RNA

analysis or frozen sectioning and Gli1-LacZ expression analysis. Slides were stained

with haematoxylin/eosin and scored histologically by a gastrointestinal pathologist

(HA) blinded to the source of the tissue. Colitis scores were calculated using

published methods (5).

Gli1-LacZ immunohistochemistry

Colonic tissue was dissected out in cold PBS and fixed for 30 minutes in 4% PFA at 4°C. After a brief rinsing in PBS, the tissue was incubated in 30% Sucrose/PBS at 4°C over night and then embedded in OCT and frozen on dry ice. Immunohistochemistry was performed on 8-10 μm frozen sections. Sections were dried, rinsed in PBS, blocked for 1h in 10% normal goat serum, 0.1% BSA and 0.3% Triton-X in TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl). Antibodies used were: hamster anti-CD3 (Serotec, 1:500), rat anti-CD19 (Serotec, 1:500), goat anti-CD11b (Abcam 1:500), FITC-conjugated anti-CD11c (Abcam 1:250), and rabbit anti–β-galactosidase (a gift of J. Douglas Engel, Department of Cell and Developmental Biology, University of Michigan; 1:2000). All antibody staining was performed over night at 4°C, followed by incubation with fluorophore-labeled Alexafluor secondary antibodies from Molecular Probes, 1:1000 for one hour. Nuclei were counterstained with DAPI, and slides were mounted in Prolong Gold Antifade Reagant (Invitrogen). Images were taken using an Olympus BX51 at 200x for immunofluorescence and an Olympus FV500 confocal microscope at 1000x for 0.30μm optical sectioning.

GLI1 Protein Mutagenesis, Luciferase Assay and Immunofluorescence.

GLI1 E1100 was amplified from Image Clone #3531657, and cloned into pCMV-Tag2b, adding a N-terminal FLAG motif. GLI1 Q1100 was obtained by inducing a point mutation using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's protocol. Plasmids encoding 8xGli-Luciferase, m8x Gli-Luciferase (mutated Gli sites) and Gli2ΔN were gifts of Dr.

Andrzej Dlugosz (University of Michigan). 293T cells were plated in 12 well plates, transfected with 0.7μg/well transcription factor, 0.4μg/well reporter plasmid, and 2ng/well pRL-TK Renilla (Promega) and analyzed for luciferase expression 36 hours after transfection using the Dual-Luciferase Reporter Kit (Promega) following the manufacturer's protocol. Firefly luciferase expression was normalized by well to Renilla, and fold changes were calculated by comparing to 8xGli-Luciferase transfected alone. Statistical analysis was performed using the Student's t-test. For immunofluorescence, 293T cells grown on collagen-coated coverslips were transfected with 1.5μg/well of transcription factor and analyzed after 36 hours. FLAG-tagged GLI1 isoforms were detected with mouse-anti-FLAG (Sigma) and anti-mouse AlexaFlour488 (Molecular Probes) both at 1:1000.

Cytokine Expression QPCR

Colonic tissue was carefully cleaned of all fatty tissue and whole colonic mRNA was collected using Trizol, followed by RNA clean-up with DNase digestion using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using the iScript cDNA synthesis kit (Biorad), and SybrGreen QPCR was performed on a Biorad iCycler. Expression levels were normalized to GAPDH, and statistical analysis was performed using the Student's T-test.

Statistical analysis

Haplotype frequencies of the tSNPs were inferred using the expectation-maximization algorithm and these used to test whether haplotype frequencies were

different in cases and controls as implemented in the EH and PM programmes. The

test statistic $2*(In(Lcase) + In(Lcontrol) – In(Lcase/Lcontrol))$, which has a χ^2

distribution with n-1 degrees of freedom (where n = number of possible haplotypes)

was calculated and empirical p values obtained by permuting the data 10,000 times.

Haplotypes were examined and linkage disequilibrium between individual SNPs (r^2)

calculated using the Haploview programme v3.2 (www.hapmap.org). Individual SNP

analysis was performed using χ^2 or Fisher's exact test, where appropriate, with two-

tailed p-values given and odds ratios (OR) presented with 95% confidence intervals

(C.I.). The meta-analysis of SNP rs2228226 was performed using the Mantel-

Haenszel method using a fixed effects model (R-software package). Details for

calculation of false positive report probability are provided in supplementary

methods. Expression profiles were analysed using Mann-Whitney U test and

Kruskal-Wallis test, assuming a non-parametric distribution of all datasets (GraphPad

Prism 4, GraphPad Software Inc.).

RESULTS

<u>Gene-wide variation in *GLI1* is associated with IBD and attributable to a non-synonymous SNP (rs2228226) in the Scottish population</u>

Four multi-marker tagging single nucleotide polymorphisms (tSNPs; $r^2 \geq 0.8$) were identified (rs3817474, rs2228225, rs2228224, and rs2228226) to describe haplotypic variation of *GLI1*, detecting haplotypes of a frequency >1%. We genotyped these 4 tSNPs in a Scottish IBD population consisting of 474 UC and 335 CD cases, and 1364 well-matched healthy controls (Table 4.1). We then used a model-free analysis (35) to test the association of *GLI1* and IBD susceptibility. In the Scottish population, we demonstrate a highly significant association in IBD (p<0.0001) and UC (p<0.0001), and an association with CD of borderline significance (p=0.03). On analysis of individual estimated haplotype frequencies in Haploview, 3 common haplotypes were described (A to C; Table 4.2). We confirmed that this effect was confined to the *GLI1* gene by genotyping an additional 7 haplotype-tagging SNPs, chosen from Phase II HapMap data, to tag neighbouring blocks, in 166 CD and 170 UC patients. This confirmed the presence of a *GLI1* spanning haplotype block that did not extend into neighbouring genes (*INHBE* and *ARHGAP9*) (Figure 4.1).

The association on haplotype testing and log-likelihood analysis was largely attributable to a non-synonymous SNP in exon 12 of *GLI1* (rs2228226C$\rightarrow$G; tSNP$_4$). rs2228226 was associated with IBD (allelic frequency OR=1.23, C.I. 1.07-1.40, p=0.0026; homozygotes OR=1.56, C.I. 1.15-2.11, p=0.0047), CD (allelic frequency OR=1.30, C.I. 1.08-1.55, p=0.0053, homozygotes OR=1.79, C.I. 1.21-2.65,
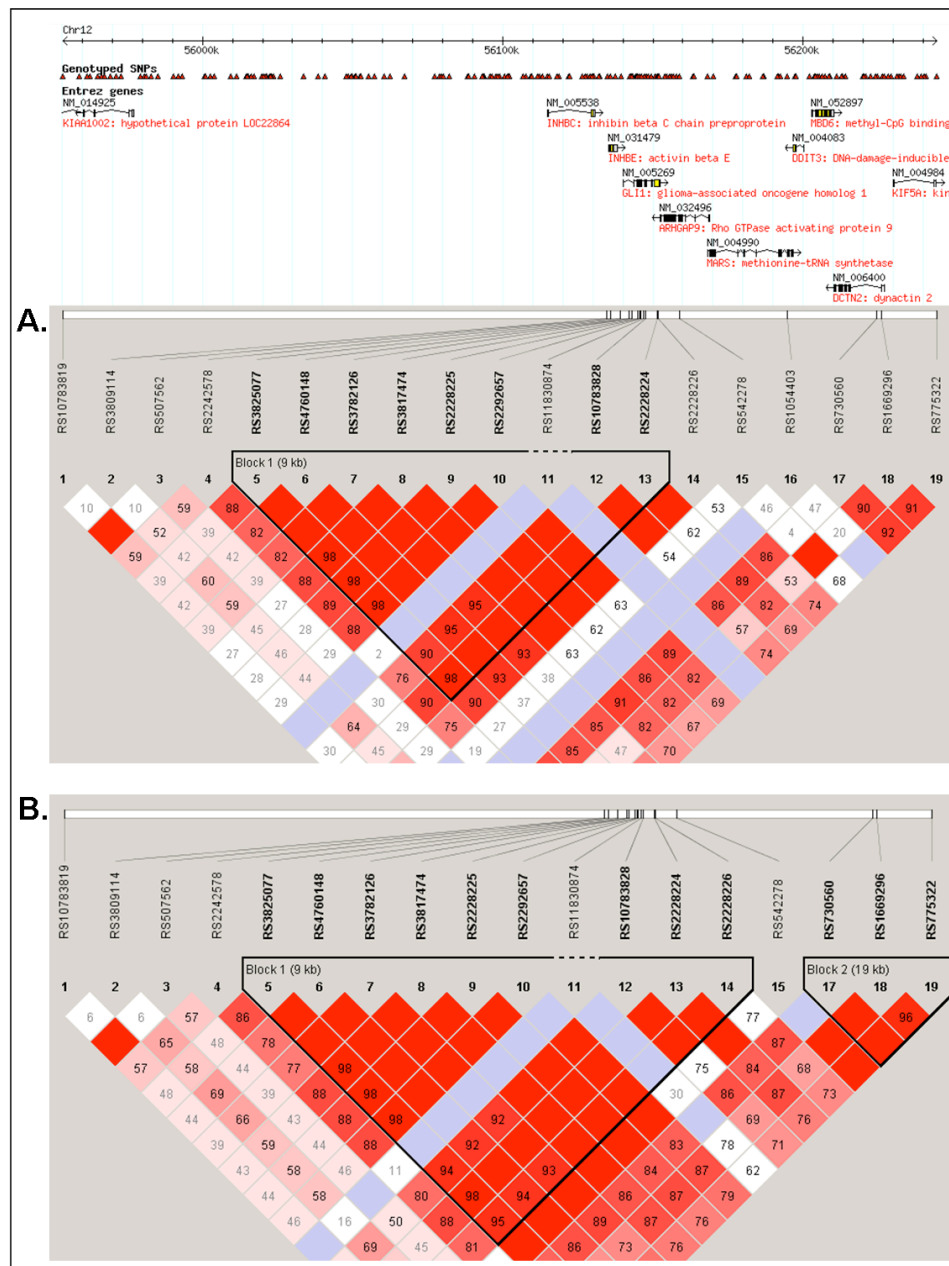
Figure 4.1. The haplotype structure in *GLI1* and surrounding haplotype blocks are presented in Haploview from genotyping in UC (A) and CD (B). There is a haplotype block spanning the *GLI1* gene that does not extend into neighbouring genes. LD is described here by D′ values.

p=0.0048) and UC (allelic frequency OR=1.19, C.I. 1.01-1.39, p=0.04; homozygotes

OR=1.41, C.I. 0.98-2.03, p=0.079) (Table 4.3 and Table 4.4). These data suggest an

allele specific dose response with a greater odds ratio for homozygotes than

heterozygote patients. Mutation screening of the *GLI1* coding regions by direct

sequencing failed to identify any novel SNPs. There was no association between 7

additional *GLI1* variants from dbSNP and IBD (Table 4.4). Despite tight linkage

disequilibrium (LD) across *GLI1* (Figure 4.1), there was notably less LD between

rs2228226 and other tSNPs ($r^2 = 0.7$) in both cases and controls, and this is likely to

provide the explanation for the lack of association in neighbouring SNPs.

Replication of *GLI1* association in two independent North European IBD cohorts and
meta-analysis

We then sought to replicate these findings in other populations. In a large IBD

panel from Cambridge, England (n=928 UC, 737 CD, 83 IBDU and 589 HC)

association with *GLI1* was replicated by log-likelihood analysis in IBD (p=0.009) and

UC (p<0.0001). rs2228226 was associated with IBD (OR 1.17, C.I. 1.00-1.36,

p=0.042) and UC (OR 1.21, C.I. 1.03-1.42, p=0.017) but not CD in this population

(Table 4.3). As in Scotland, there was no association with tSNPs$_{1-3}$. Minor allelic

frequencies differed slightly from the Scottish population; this difference is in keeping

with that noted for a number of SNPs analysed for population stratification in the

WTCCC study (9). In the smaller Swedish cohort (n=770), there was a consistent

trend to association of rs2228226 with IBD (OR 1.14, C.I. 0.90-1.45) (Table 4.3)

although this did not reach significance. However, the allelic frequencies in cases and

controls were very similar in Scotland and Sweden indicating that the lack of

statistical significance in the latter cohort is due to insufficient statistical power.

There were no consistent genotype-phenotype associations across the populations,

including stratification for colonic IBD.
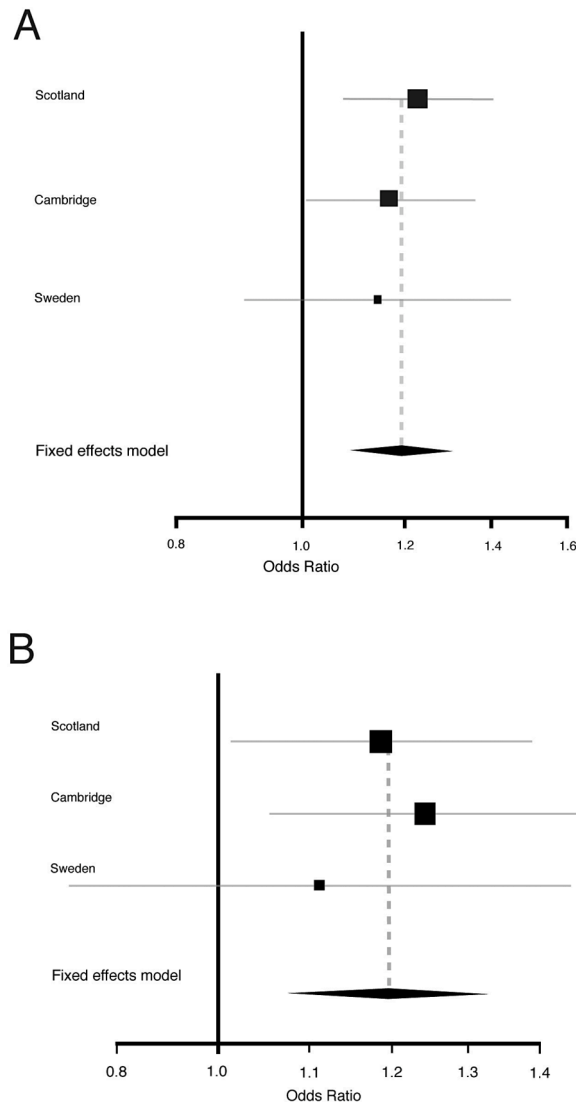
**Figure 4.2. Meta-analysis of non-synonymous *GLI1* SNP rs2228226 (tSNP$_4$) in Scotland,
Cambridge and Sweden using Mantel-Haenszel method.** (n=5352 individuals). A) Inflammatory
bowel disease versus healthy controls. B) Ulcerative colitis versus healthy controls. There was no
evidence of heterogeneity in the contribution of rs2228226 between the 3 cohorts (p=0.825).
Confidence intervals for individual populations are represented by horizontal lines and population sizes
by square boxes. The diamond represents the pooled odds ratio (fixed effect model) with 95%
confidence interval delineated by the diamond's width.

A meta-analysis of Scottish, English and Swedish data for rs2228226, using the Mantel-Haenszel method with a fixed effects model on IBD versus healthy controls (OR 1.194, C.I. 1.089-1.309, p=0.0002) (Figure 4.2A) confirmed the association of this variant in Northern Europe, achieving criteria for significance in a gene-centric study (36; 37). The meta-analysis was repeated separately for UC cases versus healthy controls with a similar effect size noted (OR 1.196, C.I. 1.077-1.327, p=0.0008) (Figure 4.2B).

Recognising the current problem with the publication of false positive findings in genetic association studies we estimated the probability that the association with disease risk found in the meta-analysis of *GLI1* SNP rs2228226 represents a true (rather than false positive) association by adopting the false positive report probability (FPRP) approach described by Wacholder (38). This gives an estimated probability that these findings represent a true finding of at least 92% (FPRP < 0.08). This method is designed to avoid over-interpretation of statistically significant findings that are not likely to signify a true positive. In our study, this parameter gives clear support to our interpretation of these data.

The GLI1 variant encoded by rs2228226 is functionally deficient in activating GLI-responsive transcription in vitro

rs2228226C→G is a missense mutation in exon 12 of *GLI1*, encoding a change from glutamine to glutamic acid (Q1100E). The mutation falls within a well conserved motif at the C-terminus of mammalian GLI1 proteins, near a recognized
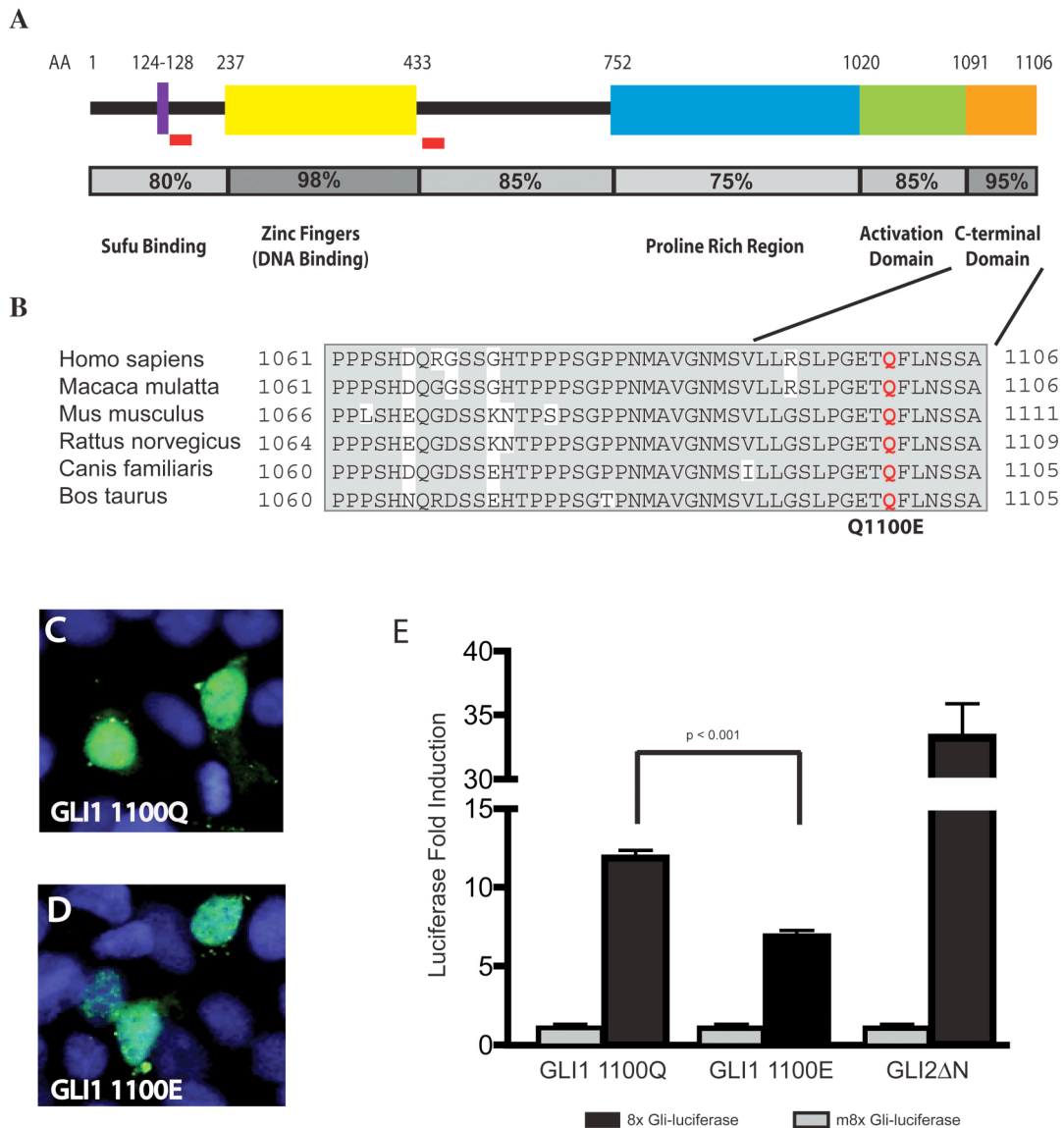
Figure 4.3. Q1100E disrupts a conserved region of the GLI1 protein and reduces GLI1 transcriptional activity. **A)** Conservation of known functional domains in the Gli1 protein. Previously described Sufu binding, DNA binding, and transactivation domains (1-3) are shown schematically. Amino acid conservation of each domain is represented numerically and by shading of the bar below the domain. Red boxes indicate regions known to regulate GLI1 protein stability (4). The conserved C-terminal domain that includes Q1100E is adjacent to a known transactivation domain. **B)** Alignment of the C-terminus of mammalian Gli1 proteins. This region (AA 1080-1106) is highly conserved in mammalian lineages. **C, D)** GLI1 Q1100 and E1100 have similar cellular localization in 293T cells. **E)** GLI1 E1100 is deficient in driving activation of the 8xGli-Luciferase reporter compared to GLI1 Q1100. GLI2ΔN is a strong activator of 8xGli-Luciferase and serves as a positive control for GLI1 activation. The m8xGli-Luciferase construct contains 8 mutant Gli binding sites and serves as a negative control. Data are shown from 6 triplicate experiments done using two different plasmid preparations (N=18). Bar heights indicate mean fold activation above baseline, and error bars indicate SEM. p values were calculated using the Student's t-test.

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transactivation domain (Figure 4.3A) (1). The Q1100 residue is itself 100%

conserved in all mammals examined (Figure 4.3B). In order to evaluate the

functional consequences of the Q1100E mutation, we transfected either GLI1 Q1100

or the variant GLI1 E1100 into 293T cells. No significant differences in level of

expression or cellular localization were detected between these GLI1 variants; both

proteins were readily detectable in the nucleus of transfected cells (Figure 4.3C-D).

Blinded quantitation of cellular localization demonstrated a range of cytoplasmic and

nuclear localization that was similar for both GLI1 variants (data not shown). We

further evaluated the ability of each variant to activate the well-characterized GLI

reporter 8xGli-Luciferase (39). We utilized Gli2ΔN, a very strong activator of 8xGli-

Luciferase, as a positive control for GLI1 transcriptional activity (40). While both

GLI1 variants activated 8xGli-Luciferase above baseline, WT GLI1 Q1100 was two-

fold more efficient as a transcriptional activator than the variant GLI1 E1100 (Figure

4.3E).

Hedgehog pathway activity is dysregulated in colonic inflammation.

Extended in silico analysis of microarray data demonstrates that mRNA transcripts of

PTCH and HHIP, along with HH protein, are greater in the distal compared with the

proximal colon in man, mirroring the expression gradient of GLI1 previously reported

(32) (Figure 4.4A-B). GLI1, PTCH and HHIP are pathway response elements whose

expression levels predict pathway activity (20; 22) GLI1 (p=0.0003), PTCH

(p=0.002), and HHIP (p=0.0003) were lower in inflamed UC compared with HC from

equivalent location (Figure 4.4C). IHH was lower in UC regardless of inflammation

(p=0.02). GLI1 expression was lower in CD than HC (p=0.004) irrespective of
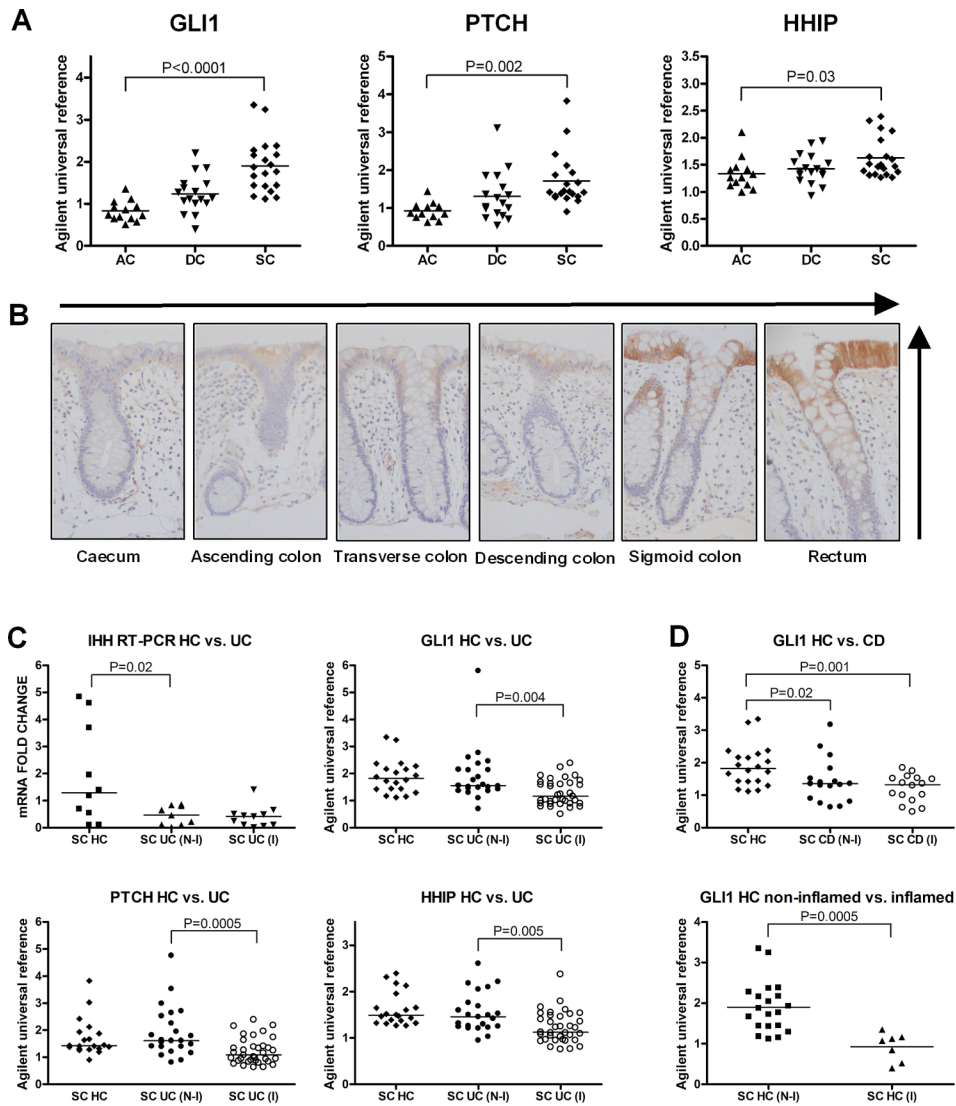
**Figure 4.4. Expression of hedgehog (HH) signaling components in the healthy human adult colon (HC) and ulcerative colitis (UC). A)** Patched **(**PTCH), Hedgehog-interacting protein (HHIP), and GLI1 mRNA levels increase along the length of the healthy adult colon, from ascending colon (AC) to descending colon (DC) and sigmoid colon (SC). **B)** HH protein expression in terminally differentiated enterocytes at the luminal surface, is greater in the distal colon compared with the proximal (vertical and horizontal arrows represent these gradients). **C)** Quantitative analysis of mRNA levels of Indian hedgehog (IHH), PTCH, GLI1, and HHIP in UC compared with non-inflamed HC (HC N-I). To account for the gradients identified along the length of the healthy colon (**a-b**), the data from SC only are shown. QPCR data is presented for IHH as this gene was not present on the Agilent microarray chip. **D)** GLI1 expression in CD versus HC (N-I) and non-IBD inflammation (HC I) versus HC. Disease specimens are sub-categorised into non-inflamed (N-I) and inflamed (I) tissues. There was no change in levels of DHH, PTCH2, GLI2, GLI3, SUFU or DISP1 in either UC or CD compared with HC, or in non-IBD inflammation (data not shown). Analysis of SHH mRNA demonstrated a mild increase in expression levels related to inflammation that is consistent with the known expression of SHH in inflammatory cells (6). Individual data points are plotted with horizontal lines representing the medians for each dataset. P-values presented are derived from Kruskal-Wallis test, comparing levels in AC, DC and SC, and from Mann-Whitney U-tests (UC vs. HC (N-I)).

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inflammatory status (Figure 4.4E), a noteworthy finding given that *GLI1* variation was also associated with CD in Scotland. PTCH was lower in inflamed CD compared with non-inflamed CD and HC (p=0.007) (Figure 4.5). GLI1 and PTCH were both lower in non-IBD inflammation versus HC (Figure 4.4D and Figure 4.6). Treatment for IBD (5-amino salicylic acid, thiopurines and corticosteroids) did not influence expression of GLI1 (Figure 4.7). These data demonstrate overall down-regulation of HH pathway activity, including GLI1, PTCH, and HHIP, in areas of colonic inflammation.
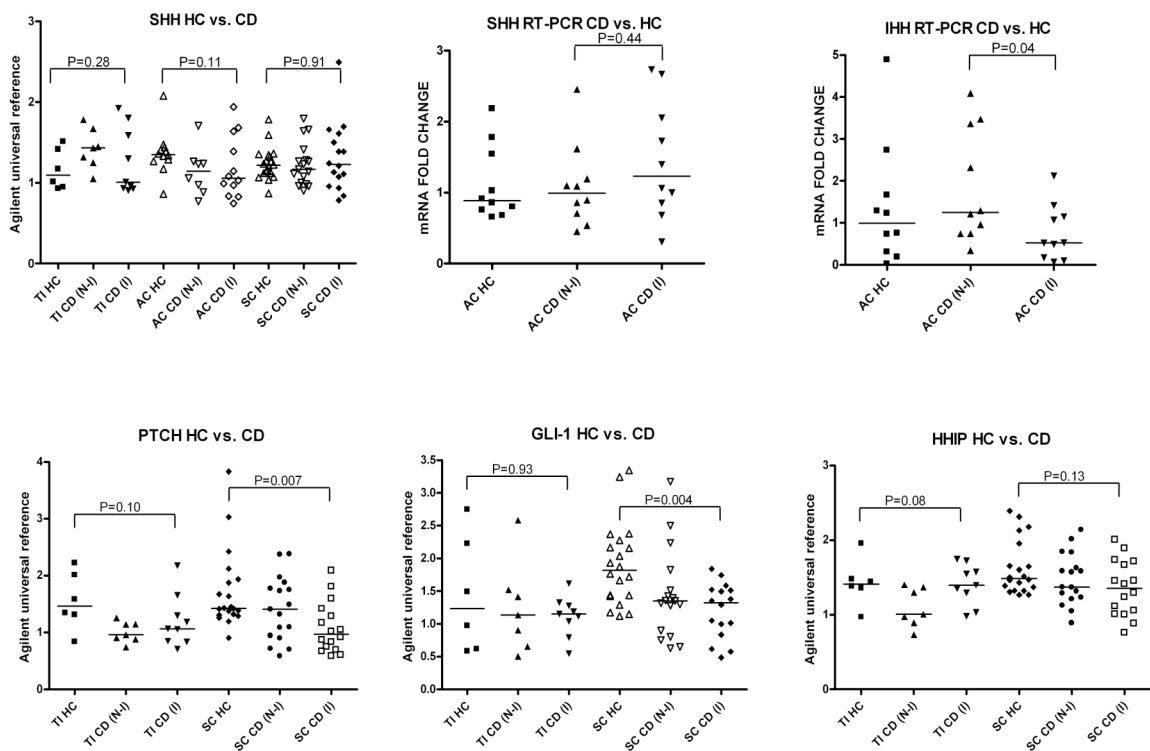
Figure 4.5. Quantitative analysis of mRNA levels by microarray and RT-PCR of Sonic hedgehog (SHH), GLI1, Patched (PTCH), and Hedgehog-interacting protein (HHIP) in Crohn's disease (CD) compared with non-inflamed healthy controls (HC). Disease specimens are taken from the terminal ileum (TI), ascending colon (AC) and sigmoid colon (SC), and are sub-categorised into non-inflamed (N-I) and inflamed (I) tissues. P-values represent Kruskal-Wallis analysis of HC, CD (N-I) and CD (I) for each anatomical location.
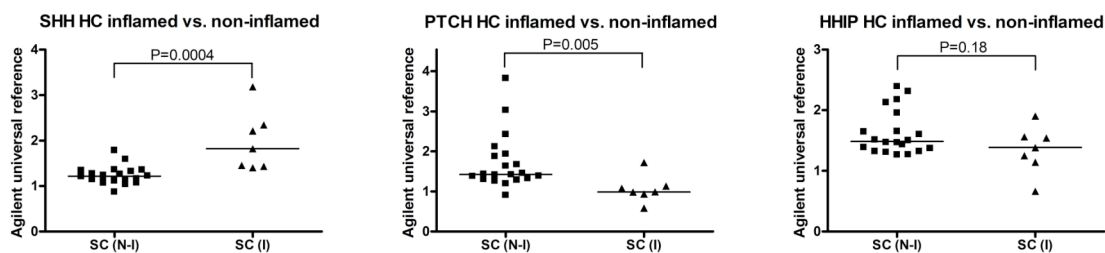
Figure 4.6. Analysis of Sonic hedgehog (SHH), Patched (PTCH), and Hedgehog-interacting protein (HHIP) mRNA levels by microarray in colonoscopic biopsies from the sigmoid colon (SC) of non-IBD patients with inflammation (I) compared with non-inflamed healthy controls (NI).
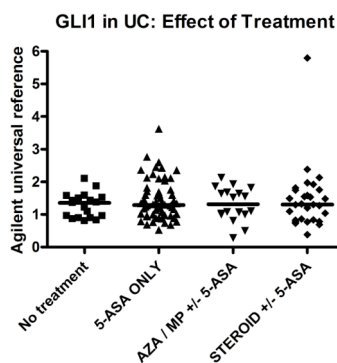

Figure 4.7. Analysis of GLI1 expression in UC by treatment. Data are presented for UC patients on no treatment (Rx), on a 5-amino salicylic acid (5-ASA) only, on azathioprine (AZA) or mercaptopurine (MP) with or without a 5-ASA and on steroids (oral and topical) with or without a 5-ASA. Individual data points are represented with horizontal lines representing medians. There was no difference between any of the groups on analysis by Mann-Whitney U testing.

<u>*Gli1*$^{+/lacZ}$ animals exhibit mortality and heightened morbidity in response to intestinal</u>

<u>inflammation induced by 3% DSS treatment</u>

In vitro analysis of the GLI1 1100E variant demonstrated a 50% deficiency in transactivation function compared to WT GLI1, and our genetic analysis demonstrated an allele-specific dosage response, suggesting that a moderate reduction in GLI1 function may predispose to intestinal inflammatory disease. To specifically test this hypothesis, we treated *Gli1*$^{+/lacZ}$ mice (41) and their WT littermates with 3% DSS to induce acute intestinal inflammation.

Gli1$^{+/lacZ}$ animals were rapidly and severely affected by DSS treatment. After 6 days, 4/9 had died, and 3 of the survivors demonstrated severe morbidity, with significant rectal bleeding and almost complete immobility (Figure 4.8A). In contrast, no WT animals (N=14) died, all were mobile and showed less morbidity on days 5 and 6 after treatment. *Gli1*$^{+/lacZ}$ animals developed bloody diarrhoea and significant weight loss by day 4, whereas WT animals did not develop clinical signs or measurable weight loss until day 6 (Figure 4.8B-C). The rapid onset of weight loss, clinical signs, and mortality in *Gli1*$^{+/lacZ}$ animals was potentially indicative of extensive colonic inflammation, leading us to directly assess the histology of *Gli1*$^{+/lacZ}$ and WT colons after DSS administration.
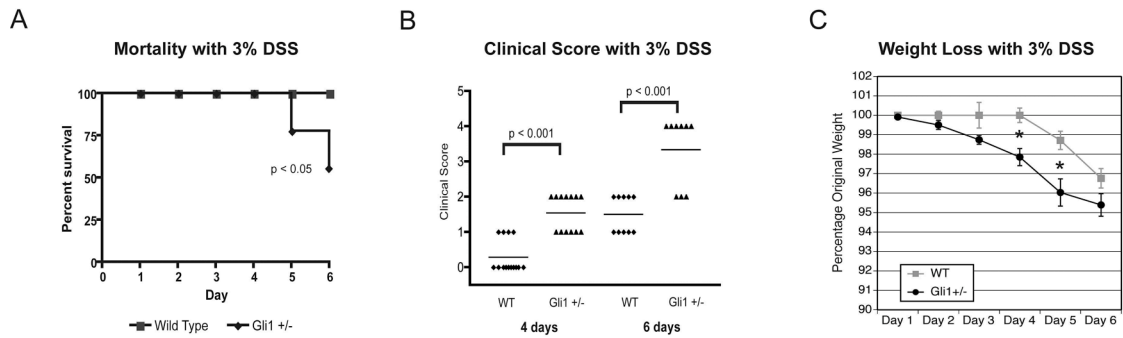
Figure 4.8. *Gli1^{+/lacZ}* animals show mortality, severe clinical symptoms, and profound weight loss after DSS treatment. A) Kaplan-Meier survival curve. WT animals are 100% viable over the 6 day treatment period (N=14). Nearly 50% of *Gli1^{+/lacZ}* animals (4/9) die in response to 3% DSS treatment for 6 days. **B)** *Gli1^{+/lacZ}* animals display markedly more severe symptoms than WT animals after 4 or 6 days of 3% DSS treatment. 1=diarrhoea, 2=bloody diarrhoea, 4=severe bleeding/death. Each dot represents an individual animal and the solid line shows the mean observation in each cohort. **C)** *Gli1^{+/lacZ}* animals (N=9) have lose weight more rapidly than their WT littermates (N=10). * = p <0.05 by Student's t-test.

Gli1^{+/lacZ} animals develop more severe colonic pathology than WT littermates in response to DSS treatment

Untreated WT and *Gli1^{+/lacZ}* animals had intact colonic mucosa; no clinical or histological signs of inflammatory disease were present prior to DSS treatment (Figure 4.9A-B). After 4 days of DSS treatment, WT colons exhibited evidence of inflammatory change but with few destructive lesions (Figure 4.9C), while extensive inflammatory infiltration and destructive colonic ulcers were prominent in *Gli1^{+/lacZ}* mice (Figure 4.9D). After 6 days, the number, size, and invasiveness of inflammatory lesions (Figure 4.9E-G) were significantly greater in *Gli1^{+/lacZ}* animals, and the overall colitis score (5) (Figure 4.9H) was significantly greater. Taken together, these results demonstrate that the loss of a single *Gli1* allele leads to increased sensitivity to DSS treatment as reflected by severe intestinal inflammatory pathology and clinical signs.

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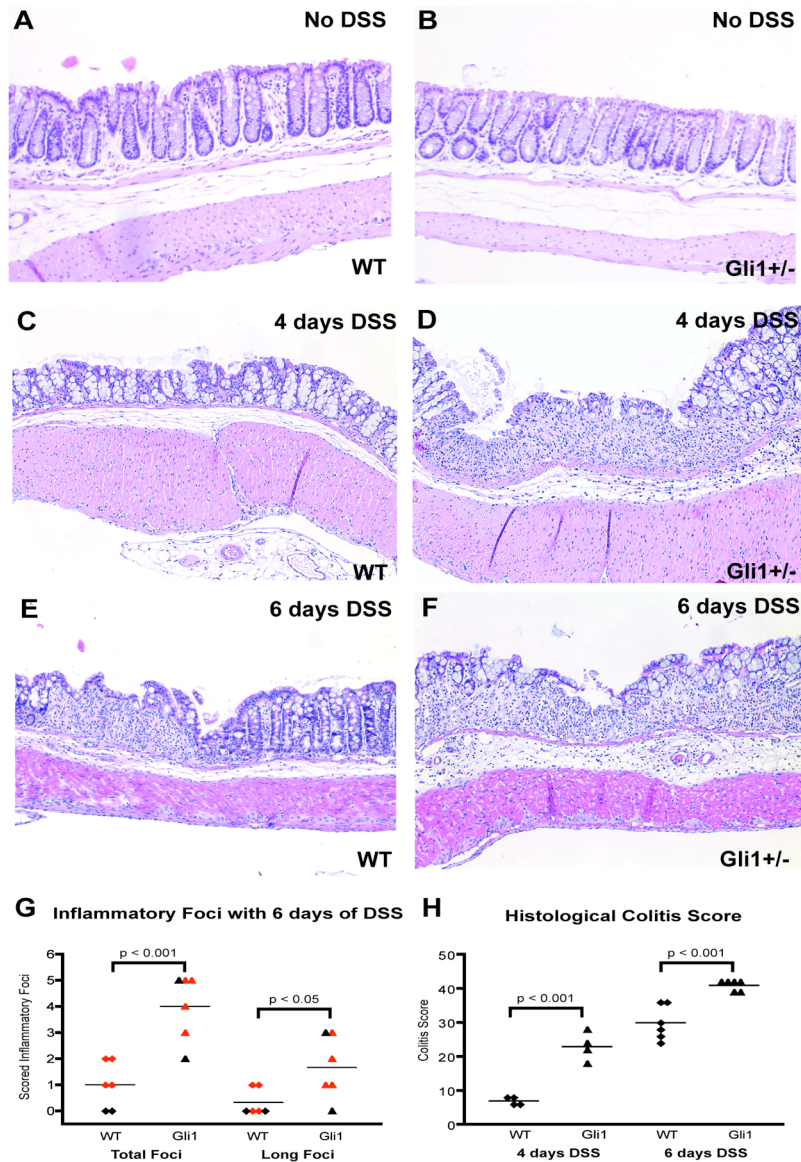
FIGURE 4.9. *Gli1$^{+/lacZ}$* **animals demonstrate more severe intestinal inflammation than WT littermates in response to DSS treatment. A, B)** WT and *Gli1$^{+/lacZ}$* animals demonstrate normal thickness and structure of colonic mucosa in the absence of DSS. **C)** WT animals (N=4) exhibit mild colonic inflammation but do not develop substantial epithelial or ulcerative inflammatory pathology within 4 days of DSS treatment. **D)** *Gli1$^{+/lacZ}$* animals (N=4) develop significant inflammatory infiltration, epithelial damage, and ulceration within 4 days of DSS treatment. **E)** WT animals (N=14) demonstrate ulceration following DSS treatment. **F)** *Gli1$^{+/lacZ}$* animals develop profound intestinal inflammation in response to 3% DSS treatment, with severe epithelial damage in long stretches of their colonic mucosa (N=9). **G)** Blinded histological scoring of colonic damage after 6 days of DSS treatment. Standard lengths of tissue from the mid colon and distal descending colon were scored in each animal. *Gli1$^{+/lacZ}$* animals (N=6) have more overall inflammatory foci and more long foci (10+ crypt units affected) than WT animals (N=6). Each dot represents the number of observed foci in an individual animal; the solid line shows the mean observation in each cohort. Red dots indicate the animals that were analyzed for cytokine expression. **H)** Comprehensive colitis scoring (5) of WT and *Gli1$^{+/lacZ}$* animals after 4 and 6 days of DSS treatment.

Intestinal myeloid cells respond directly to HH signals

We previously demonstrated that HH signaling is paracrine in murine intestine and colon (42). Given that $Gli1^{+/lacZ}$ animals contain a copy of bacterial β-galactosidase under the control of the Gli1 promoter, they represent an excellent model to confirm this observation and identify cell types that are Hh-responsive (i.e. LacZ positive) before and during inflammatory disease. These studies revealed that no autocrine Hh signaling was detectable in untreated adult colon or following inflammatory stress; colonic epithelial cells do not express Gli1 or respond to Hh signals during colonic homeostasis or in areas of either mild or severe epithelial perturbation (Figure 4.10 and data not shown). We did, however, detect a large population of Hh-responsive cells within the inflammatory infiltrate of DSS-induced ulcers after 4 days of DSS treatment in $Gli1^{+/lacZ}$ animals (Figure 4.11D-F). Both lymphocytes (6; 28) and myeloid cells (29) have recently been shown to respond to Hh signals outside of the GI tract. We therefore investigated whether these populations were responding in the adult colon.

We detected CD3-positive T lymphocytes, CD11b-positive myeloid cells, and CD11c-positive dendritic cells responding to Hh signaling in resting colon without DSS treatment (Figure 4.10A-C). After 4 days of DSS treatment, significant numbers of Hh-responsive myeloid and dendritic cells were seen in inflammatory ulcers (Figure 4.10D-F). We did not detect Hh response amongst the infiltrating T cells in DSS-induced ulcers (Figure 4.10D). Examination of CD19-postive B lymphocytes in resting colon and after both 4 and 6 days of 3% DSS treatment revealed no clear Hh response in this population (data not shown). Taken together, these data suggest that

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myeloid cells, including dendritic cells, are direct targets of Hh signaling in the colon both during homeostasis and after inflammatory stress. This finding, in association with the findings above, implies that direct Hh signaling may play a role in innate immune function in the colon.
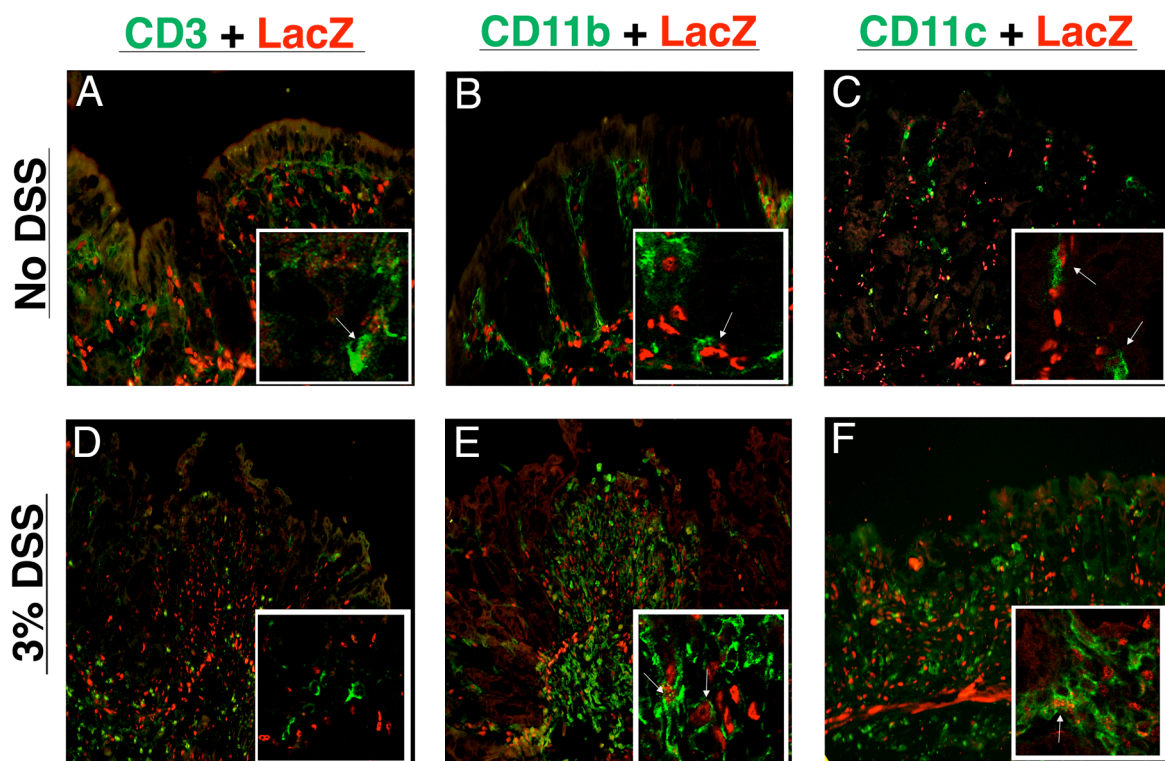

Figure 4.10: Intestinal myeloid cells respond directly to Hh signaling during homeostasis and inflammation. **A-C)** No DSS treatment. **D-F)** 4 days of 3% DSS. **A, D)** Few CD3+ T lymphocytes respond to Hh signaling during colonic homeostasis. Infiltrating T cells in DSS-induced ulcers do not express Gli1 or respond to Hh signalling. **B, E)** Many but not all CD11b+ myeloid cells respond to Hh signalling and express LacZ. After 4 days of DSS, ulcers in Gli1 are filled with myeloid cells, many of which respond to Hh signals. **C, F)** CD11c+ dendritic cells respond directly to Hh signals during homeostasis and inflammation. Nuclear DAPI staining was performed but has been omitted to allow clear visualization of co-expression of nuclear LacZ and cell surface markers.

<u>*Gli1*^{+/lacZ} animals have dramatically increased IL-23p19 and pro-inflammatory</u>

<u>cytokine expression after DSS treatment.</u>

Overall, unchallenged WT and *Gli1*^{+/lacZ} animals have a similar cytokine and chemokine profile as determined by QPCR examination of whole colonic tissue (Figure 4.11A). There are, however, several molecules (Cxcl2, Cxcl5, Cxcl10, and Arg1) that are modestly increased in *Gli1*^{+/lacZ} animals, and a small decrease in IL-6 expression; these changes may indicate a subtle difference in the inflammatory milieu of *Gli1*^{+/lacZ} animals at baseline. More strikingly, after 6 days of DSS *Gli1*^{+/lacZ} animals demonstrate robustly upregulated expression of several pro-inflammatory cytokines and chemokines compared to WT (Figure 4.11B). When baseline expression values are compared to values after DSS challenge for each genotype (Figure 4.11C), robust expression and upregulation of T_H1 cytokines, including IFNγ, is detectable in *Gli1*^{+/lacZ} animals (Figure 4.11B-C). We did not detect a significant difference in TGFβ and IL-10 between *Gli1*^{+/lacZ} and WT animals, suggesting that down-regulation of anti-inflammatory cytokines was not the primary mechanism of increased inflammation in this model.

The most highly expressed cytokine after DSS treatment in *Gli1*^{+/lacZ} animals was IL-23p19, a molecule that drives differentiation of T_H17 lymphocytes, key mediators of inflammation in several systems, including IBD (43; 44). IL-12 and IL-17, cytokines closely associated with IL-23, were also robustly upregulated in *Gli1*^{+/lacZ} animals. In fact, this entire pathway was highly upregulated from the baseline in *Gli1*^{+/lacZ} animals compared to WT littermates (Figure 4.11B-C). These findings are particularly interesting since the IL-23 pathway has recently been

strongly implicated in IBD pathogenesis both in humans (10) and mice (44). Taken together, these cytokine data confirm that animals lacking one genomic copy of Gli1 develop significantly increased inflammatory disease compared to WT littermates in response to the same inflammatory challenge.
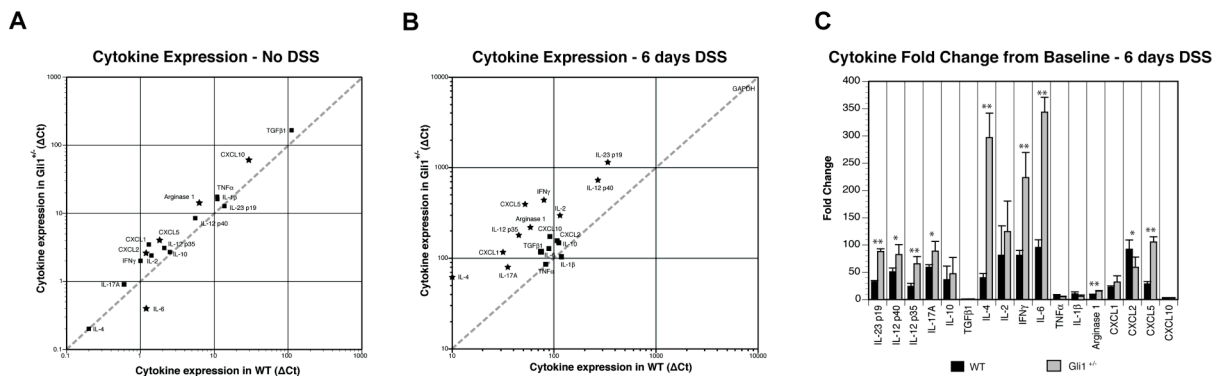
Figure 4.11. Cytokine analysis of *Gli1*^{+/lacZ} and WT mice after DSS treatments demonstrates robust pro-inflammatory cytokine activation. A, B) Cytokine and chemokine expression normalized to GAPDH is plotted on the Y-axis for *Gli1*^{+/lacZ} mice, and on the X-axis for WT animals. Gene expression levels that are statistically different are shown with stars. The dotted diagonal trend line indicates identical expression levels between WT and *Gli1*^{+/lacZ} mice. **A)** Cytokine and chemokine expression in WT and *Gli1*^{+/lacZ} animals (N=3) without DSS treatment. Cytokine and chemokine expression is low and very similar between WT and *Gli1*^{+/lacZ} animals. **B)** Cytokine expression in WT and *Gli1*^{+/lacZ} animals (N=4) after 6 days of 3% DSS treatment. Many pro-inflammatory cytokines and chemokines are significantly more highly expressed in *Gli1*^{+/lacZ} animals compared to WT. Note the difference in axes in panels A and B. **C)** Fold change of cytokine expression from baseline to inflamed for WT and *Gli1*^{+/lacZ} animals. We detect dramatic upregulation of T_h17 and T_h1 pathway cytokines and chemokines in *Gli1*^{+/lacZ} animals. * = $p < 0.05$ ** = $p < 0.01$ by the Student's t-test.

DISCUSSION

The data presented here provide the first evidence that intact HH signaling is critical in the mammalian gut response to inflammatory challenge, and suggest that reduced GLI1 function is implicated in IBD pathogenesis. We confirm that the HH signaling pathway is downregulated in colonic inflammation in man. We identify a specific *GLI1* variant that is highly associated with UC/IBD, and demonstrate that the variant protein is functionally deficient as a transcriptional activator in vitro. Finally, we demonstrate that mice with a 50% reduction in *Gli1* exhibit a heightened intestinal inflammatory response to DSS with significant upregulation of the IL-23 pathway. Not only do these findings have clear implications for the understanding of IBD pathogenesis and suggest potential for therapeutic intervention, they are the first clear description of a functional role for HH signaling and GLI1 in bowel inflammation.

The inherited variation in the *GLI1* gene that we have detected is associated with IBD and UC, in both Scotland and England, with findings for rs2228226 confirmed by meta-analysis of over 5000 individuals, with odds ratio of 1.19. Evidence for an effect in CD is seen in the present study, but the predominant effect is clearly related to UC, consistent with the reported IBD2 linkage studies (17; 19). The magnitude of this association is entirely in line with the effect size noted in a number of recent studies of complex disease genetics, including CD (45), colorectal cancer (46), and Celiac disease (47). The level of significance attained satisfies suggested criteria of $p < 10^{-4} - 10^{-6}$ for gene-centric studies (36; 37). Population heterogeneity has been previously described at the IBD2 linkage region (17; 19; 48; 49). The three Northern European populations studied here have previously demonstrated similar contribution of other IBD susceptibility genes / loci, including NOD2 and IBD5 (50).

Whilst our resequencing efforts identify rs2228226 as the only coding variant associated with IBD, further deep re-sequencing will be important in the future as the haplotype analysis and log-likelihood analyses raise the possibility that other germ-line variants may also contribute to IBD risk. These need be explored formally – specifically the role of intronic variants, long-range promoter effects and / or copy number variation. In this context, several complex disease genes, including NOD2 (14; 15), have multiple independent mutations conferring disease risk, some disease genes have no causative mutations within coding sequences (e.g. *IRGM* in CD (16; 51)), and synonymous SNPs may be associated with functional effects (52).

rs2228226C→G encodes a change from glutamine to glutamic acid (Q1100E). Our in vitro data demonstrates that GLI1 1100E is a subfunctional transcriptional activator compared to WT GLI1, though it is appropriately synthesized and localized. The Q1100E mutation causes a significant charge change in a conserved region directly adjacent to the known transactivation region of GLI1; this change could directly modify transactivation activity, disrupt the structure of the transactivation domain, or affect protein stabilization(4), decreasing activity. Our murine data confirm that reduction in GLI1 activity has significant consequences for the inflammatory response.

Intestinal HH signaling in mouse and man is exclusively paracrine: HH ligands in the epithelium signal to the HH response network (PTCH, GLI1, HHIP) in the mesenchyme (42; 53; 54). This has been demonstrated during development (42), homeostasis (55), malignancy (54) and now, in this report, in inflammation. The human expression data presented here also supports the relevance of the HH pathway

to colonic inflammation in man. GLI1 expression, a reliable indicator of pathway activity, is down-regulated in all forms of colonic inflammation examined (UC, CD and non-IBD inflammation). Whilst embryonic HH pathway activity is reportedly recapitulated in diverse organs / tissues in response to acute injury and inflammatory challenge (21; 23-26), GLI1 expression is notably decreased in psoriasis, a disease of the epithelial border that shares some pathogenic features with IBD (56).

The localisation of HH protein demonstrated here is similar to that shown for IHH by van den Brink and colleagues by in situ hybridisation (57). However, along with other commentators, we would urge caution in the use of the commercially-available HH pathway antibodies presently available (22). We note further that the expression analysis of disease samples in humans is inherently limited in that it only depicts a complex process at a late time point in established disease. Nonetheless, in combination with studies utilizing models (such as the $Gli1^{+/lacZ}$ mouse) to directly correlate HH response with inflammatory challenge, these studies provide useful information to help elucidate the aetiology of human inflammatory disease.

$Gli1^{+/lacZ}$ animals, which have only 50% of the WT level of Gli1, develop severe and rapid inflammation in response to DSS, despite having normal histological structure and a largely WT cytokine and chemokine profile prior to DSS treatment. These data, to our knowledge, provide the first description of a phenotype for reduced Gli1 function (41), and demonstrate the key role that a full complement of Gli1 plays in protection from inflammatory disease. Our in vitro data demonstrates that GLI1 E1100 is capable of activating some Gli response, suggesting that under homeostatic conditions, GLI1 E1100 could perform adequately. Similar to the situation in

Gli1^{+/lacZ} animals, however, under conditions of inflammatory stress, GLI1 E1100 can only function at 50% of the level of WT GLI1. Whether the predisposition to inflammation in these systems is a direct result of lowered HH signal transduction within HH-responsive inflammatory cells or reflects the effect of lower HH signals on other stromal target cells that, in turn, release signals that impact the integrity of the epithelial layer or the inflammatory phenotype of resident immune cells is not yet clear, and will be an important area of future study. Furthermore, we believe that it will be important to determine if other primary (e.g. germ-line variation in other HH pathway genes and the HH interactome, epigenetic changes, and gene-environmental interactions) or secondary phenomena (e.g. due to alterations in commensal flora) in the human / murine intestine alter the transduction of Hedgehog signals in response to acute and chronic inflammatory challenge.

We have additionally shown that the HH pathway may directly modify the innate immune response through signaling to myeloid target cells. This finding is in accord with recent data demonstrating a crucial role for Hh signaling in myeloid cell maturation in the spleen (29). Interestingly, myeloid cell populations are known to modify the intestinal inflammatory milieu through a significant impact on the IL-23/IL-17 pathway (58), a pathway that we find to be clearly and distinctly dysregulated in *Gli1*^{+/lacZ} inflammation. Taken together, these data raise the exciting possibility that the normal role of HH signaling is to promote a tolerogenic phenotype in mucosal myeloid cells. In the face of reduced HH signal transduction in these cells, minor inflammatory stimuli may trigger robust inflammatory responses. Future studies directly investigating this hypothesis by specifically testing the effects of Hh

signaling on intestinal myeloid cells will be crucial to the evolution of a more complete understanding the role of Hh in intestinal inflammation.

Limitations of the study

A number of issues meriting further work emerge from the present study. Firstly, deep re-sequencing of the entire *GLI1* gene is required to identify additional variants that contribute to the overall association noted for the gene. Secondly, the in vivo examination of reduced Gli1 function is presently limited to heterozygous mice. Despite the robust phenotype observed in these mice following acute inflammatory challenge, these findings need to be extended to homozygous null mutant animals ($Gli1^{-/-}$). Thirdly, additional animal models of colitis, particularly of chronic disease, need to be examined; for example, trinitrobenzene sulfonic acid (TNBS) induced colitis or adoptive transfer of $CD4^+CD45RB^{high}$ T cells into recombinase-activating gene (*rag*)-1 deficient ($rag1^{-/-}$) mice. Finally, we have not yet directly examined the phenotype of reduced GLI1 function in antigen presenting cells in vitro.

Conclusions

A key paradigm emerging in complex disease genetics is that gene discovery may lead to fundamental shifts in the direction of basic and translational research efforts. Recently, it is apparent that these insights may have greater significance than the index gene discovery itself, especially when one considers the small contribution each locus / variant makes towards disease susceptibility. IBD has provided several recent examples. Notably, the discoveries of *NOD2* and *IL-23R* have launched academic and pharmaceutical research efforts into innate immunity and Th17 signaling, whereas *ATG16L1* and *IRGM* have focused much attention on the role of

autophagy in CD pathogenesis. In the present study, our genetic studies led in turn to detailed observations of expression in established human disease as well as a mechanistic study in the mouse. We believe that these combined findings have the potential to open entirely novel lines of enquiry that may have great consequences for the understanding of inflammatory bowel disease pathogenesis and potentially for inflammatory pathways in other hedgehog-expressing organs (e.g. skin, lung, bone).

In conclusion, we demonstrate here for the first time the dysregulation of a developmental signaling pathway in IBD. Furthermore, we show that these effects are in part genetically determined, with evidence implicating *GLI1* as an IBD2 gene, and identification of a specific variant with reduced transcriptional activity. The functional relevance of Gli1 is demonstrated by the severe intestinal inflammation that develops in the face of a 50% reduction in Gli1 concentration in an established mouse model of colitis. Taken together, these data strongly argue that tolerance to inflammatory stimuli requires a fully functional HH signal transduction network. With HH signaling modulators under active investigation in a number of clinical trials (http://www.curis.com and http://www.clinicaltrials.gov), the data provide promise for the development of novel therapeutic strategies. Since the use of HH inhibitors has recently been advocated for the treatment of certain tumours that secrete HH, our finding that reduced HH signaling has inflammatory consequences suggests that inflammatory status should be carefully followed during upcoming Phase II trials (59).

Acknowledgements:

	Scottish Panel	*Cambridge Panel*	*Swedish Panel*
Total number	**2191** (1374 HC; 474 UC; 335 CD; 8 IBDU)	**2337** (589 HC; 928 UC; 737 CD; 83 IBDU)	**774** (281 HC; 288 UC; 205 CD; 0 IBDU)
Sex - % male	HC 48.7% UC 52.0% CD 39.6%	HC 45.0% UC 52.6% CD 37.0%	HC 45.3% UC 57.3% CD 49.8%
Median age at diagnosis/ recruitment -years (IQR)	HC 50.0 (43.0-55.0) UC 34.1 (25.2-49.9) CD 27.8 (20.8-41.1)	HC 60.0 (53.0-69.0) UC 36.7 (26.84-50.35) CD 26.1 (20.3-37.2)	HC 47.6 (36.4-60.7) UC 28.6 (20.6-41.6) CD 24.5 (19.1-36.7)
CD location			
Terminal ileum (L1)	35.9%	31.3%	17.4%
Colon (L2)	36.5%	36.5%	47.9%
Ileocolon (L3)	25.1%	30.3%	32.6%
Upper GI disease (L4)	2.5%	1.9%	2.1%
UC location			
Proctitis (E1)	17.3%	14.8%	10.4%
Left-sided colitis (E2)	40.5%	34.1%	49.5%
Extensive colitis (E3)	42.2%	51.1%	40.1%
CD 5 year behaviour			
Inflammatory (B1)	64.8%	52.3%	70.9%
Stricturing (B2)	14.8%	35.2%	21.5%
Penetrating (B3)	20.3%	12.5%	7.5%
Perianal involvement (p)	17.4%	24.4%	6.4%

Table 4.1. Detailed demographics and phenotypic data on Scottish, Cambridge and Swedish IBD population. HC – healthy control; CD – Crohn's disease; UC – ulcerative colitis; IBDU – colonic IBD type unclassified.

A.

	tSNP$_1$	tSNP$_2$	tSNP$_3$	tSNP$_4$	IBD n=809	UC n=474	CD n=335	HC n=1374	IBD vs. HC p-value OR (C.I.)	UC vs. HC p-value OR (C.I.)	CD vs. HC p-value OR (C.I.)
HAPLOTYPE											
A	1	1	1	1	59.2%	59.6%	58.7%	60.9%	0.26 0.93 (0.82-1.05)	0.50 0.95 (0.81-1.10)	0.30 0.91 (0.76-1.08)
B	2	2	2	2	33.9%	32.5%	35.9%	30.7%	**0.036** 1.16 (1.01-1.32)	0.32 1.09 (0.93-1.28)	**0.012** 1.26 (1.06-1.51)
C	2	2	2	1	4.9%	4.8%	5.1%	6.9%	**0.014** 0.71 (0.54-0.93)	**0.031** 0.68 (0.49-0.96)	0.13 0.74 (0.51-1.07)
Log-likelihood p-value									**< 0.0001**	**< 0.0001**	**0.03**

B.

	tSNP$_1$	tSNP$_2$	tSNP$_3$	tSNP$_4$	IBD n=1748	UC n=928	CD n=737	HC n=589	IBD vs. HC p-value OR (C.I.)	UC vs. HC p-value OR (C.I.)	CD vs. HC p-value OR (C.I.)
HAPLOTYPE											
A	1	1	1	1	62.8%	64.5%	60.4%	63.3%	0.94 0.99 (0.87-1.13)	0.48 1.06 (0.91-1.23)	0.25 0.91 (0.78-1.06)
B	2	2	2	2	29.7%	30.8%	28.2%	27.7%	0.14 1.12 (0.97-1.29)	0.067 1.16 (0.99-1.36)	0.61 1.05 (0.89-1.24)
C	2	2	2	1	5.3%	4.7%	6.1%	6.9%	**0.049** 0.76 (0.58-0.99)	**0.0093** 0.66 (0.49-0.90)	0.44 0.88 (0.65-1.19)
Log-likelihood p-value									**0.009**	**< 0.0001**	**0.002**

C.

	tSNP$_1$	tSNP$_2$	tSNP$_3$	tSNP$_4$	IBD n=493	UC n=288	CD n=205	HC n=281	IBD vs. HC p-value OR (C.I.)	UC vs. HC p-value OR (C.I.)	CD vs. HC p-value OR (C.I.)
HAPLOTYPE											
A	1	1	1	1	58.2%	58.6%	57.6%	57.0%	0.68 1.05 (0.85-1.30)	0.62 1.07 (0.84-1.36)	0.86 1.03 (0.79-1.35)
B	2	2	2	2	35.1%	34.4%	36.1%	34.2%	0.81 1.03 (0.83-1.29)	0.97 1.01 (0.78-1.29)	0.55 1.10 (0.83-1.44)
C	2	2	2	1	6.7%	7.0%	6.3%	8.8%	0.17 0.75 (0.50-1.10)	0.32 0.78 (0.50-1.21)	0.16 0.68 (0.41-1.12)
Log-likelihood p-value									0.13	0.41	0.19

Table 4.2. *GLI1* **haplotype frequencies in inflammatory bowel disease (IBD), ulcerative colitis (UC), Crohn's disease (CD) and healthy controls (HC) in A. Scotland, B. Cambridge, England, and C. Sweden.** tSNP$_1$: rs3817474; tSNP$_2$: rs2228225; tSNP3: rs2228224; tSNP$_4$: rs2228226. Differences between IBD, CD, UC and HC frequencies are shown, calculated by χ^2 test, with two-sided p-value, odds ratio (OR) and 95% confidence intervals (C.I.). Estimated haplotype frequencies were calculated using Haploview, vers. 3.2. Log-likelihood p-values (calculated on the PM/EH platform with 10,000 permutations and 15 degrees of freedom) are given for each analysis.

	HC		IBD			UC			CD		
	N	%	N	%	p value OR (C.I.)	N	%	p value OR (C.I.)	N	%	p value OR (C.I.)
Scotland	1374	30.3	884	34.8	0.0026 1.23 (1.07-1.40)	474	33.9	0.042 1.19 (1.01-1.39)	335	36.1	0.0053 1.30 (1.08-1.55)
Cambridge	589	26.4	1737	29.6	0.042 1.17 (1.00-1.36)	928	30.8	0.017 1.21 (1.03-1.42)	737	27.9	0.40 1.08 (0.90-1.28)
Sweden	281	30.6	493	35.0	0.27 1.14 (0.90-1.45)	288	34.4	0.43 1.11 (0.90-1.45)	205	35.9	0.24 1.19 (0.90-1.58)

Table 4.3. Minor allelic frequencies for *GLI1* non-synonymous SNP rs2228226 (tSNP4) in Scottish, English, and Swedish healthy controls (HC), inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC). Odds ratios and two-tailed p-values are given for χ^2 analysis of IBD vs. HC, UC vs. HC and CD vs. HC in each of these three populations. Meta-analysis of these data are presented in Figure 4.2. Frequencies of estimated haplotypes in all three populations and the full genotype data for the Scottish population are detailed in Tables 4.2 and 4,4.

SNP	dbSNP ID & Position	Location	Allele 1/2		UC N=474	CD N=335	HC N=1374*	UC vs. HC P-value OR (C.I.)	CD vs. HC P-value OR (C.I.)
1	rs2242578 56139420	Promoter	C/G	*U/K*	*48 (8.7%)*	*44 (9.9%)*	*30 (8.1%)*	22 vs 11 P=0.18 OR 0.74 (0.48-1.14) 2 vs 1 P=0.40 OR 0.92 (0.75-1.13)	22 vs 11 P=0.57 OR 0.87 (0.55-1.36) 2 vs 1 P=0.96 OR 1.01 (0.82-1.25)
				CC CG GG	221 (44.1%) 225 (44.9%) 55 (11.0%)	164 (40.9%) 189 (47.1%) 48 (12.0%)	151 (44.4%) 138 (40.6%) 51 (15.0%)		
				C G	667 (66.6%) 335 (33.4%)	517 (64.5%) 285 (35.5%)	440 (64.7%) 240 (35.3%)		
2	rs3825077 56142281	Intron 1/2	T/C	*U/K*	*51 (9.3%)*	*34 (7.6%)*	*26 (7.0%)*	22 vs 11 P=0.51 OR 0.86 (0.56-1.32) 2 vs 1 P=0.94 OR 1.00 (0.82-1.22)	22 vs 11 P=0.91 OR 1.03 (0.66-1.60) 2 vs 1 P=0.59 OR 1.06 (0.86-1.31)
				TT TC CC	190 (38.2%) 249 (50.0%) 59 (11.8%)	153 (37.2%) 201 (48.9%) 57 (13.9%)	141 (41.0%) 152 (44.2%) 51 (14.8%)		
				T C	629 (63.2%) 367 (36.8%)	507 (61.7%) 315 (38.3%)	434 (63.1%) 254 (36.9%)		
3	rs4760148 56143043	Intron 1/2	G/C	*U/K*	*65 (11.8%)*	*40 (9.0%)*	*20 (5.4%)*	22 vs 11 P=0.63 OR 0.88 (0.55-1.41) 2 vs 1 P=0.55 OR 1.07 (0.87-1.32)	22 vs 11 P=0.72 OR 1.12 (0.70-1.79) 2 vs 1 P=0.27 OR 1.13 (0.91-1.41)
				GG GC CC	209 (43.2%) 231 (47.7%) 44 (9.1%)	175 (43.2%) 183 (45.2%) 47 (11.6%)	171 (48.9%) 138 (39.4%) 41 (11.7%)		
				G C	649 (67.0%) 319 (33.0%)	533 (65.8%) 277 (34.2%)	480 (68.6%) 220 (31.4%)		
4	rs3782126 56143200	Intron 1/2	T/C	*U/K*	*49 (8.9%)*	*56 (12.6%)*	*19 (5.1%)*	22 vs 11 P=0.55 OR 0.86 (0.54-1.37) 2 vs 1 P=0.67 OR 1.05 (0.86-1.29)	22 vs 11 P=1.00 OR 0.99 (0.61-1.62) 2 vs 1 P=0.29 OR 1.13 (0.91-1.40)
				TT TC CC	222 (44.4%) 233 (46.6%) 45 (9.0%)	165 (42.4%) 185 (47.6%) 39 (10.0%)	173 (49.3%) 137 (39.0%) 41 (11.7%)		
				T C	677 (67.7%) 323 (32.3%)	515 (66.2%) 263 (33.8%)	483 (68.8%) 219 (31.2%)		
5 *tSNP 1*	**rs3817474 56145102**	**Intron 4/5**	**A/G**	*U/K*	*26 (5.4%)*	*32 (9.6%)*	*95 (6.9%)*	**22 vs 11 P=0.81 OR 0.94 (0.67-1.33) 2 vs 1 P=0.71 OR 1.03 (0.88-1.21)**	**22 vs 11 P=0.49 OR 1.17 (0.80-1.71) 2 vs 1 P=0.25 OR 1.12 (0.93-1.14)**
				AA AG GG	**163 (35.9%) 232 (51.1%) 59 (13.0%)**	**105 (34.8%) 150 (49.7%) 47 (15.6%)**	**506 (39.6%) 577 (45.2%) 194 (15.2%)**		
				A G	**558 (61.5%) 350 (38.5%)**	**360 (59.6%) 244 (40.4%)**	**1589 (62.2%) 965 (37.8%)**		
6 *tSNP 2*	**rs2228225 56145698**	**Exon 6**	**A/G**	*U/K*	*37 (7.7%)*	*4 (1.2%)*	*91 (6.6%)*	**22 vs 11 P=0.96 OR 1.02 (0.74-1.42) 2 vs 1 P=0.67 OR 1.04 (0.89-1.21)**	**22 vs 11 P=0.46 OR 1.16 (0.81-1.67) 2 vs 1 P=0.28 OR 1.10 (0.93-1.31)**
				AA AG GG	**161 (36.2%) 220 (49.4%) 64 (14.4%)**	**118 (35.2%) 162 (48.4%) 55 (16.4%)**	**504 (39.3%) 577 (45.0%) 202 (15.7%)**		
				A G	**542 (60.9%) 348 (39.1%)**	**398 (59.4%) 272 (40.6%)**	**1585 (61.8%) 981 (38.2%)**		
7	rs2292657 56146199	Intron 7/8	A/G	*U/K*	*39 (7.1%)*	*38 (8.5%)*	*26 (7.0%)*	22 vs 11 P=1.00 OR 1.01 (0.66-1.53) 2 vs 1 P=0.44 OR 1.09 (0.89-1.33)	22 vs 11 P=0.82 OR 1.05 (0.68-1.63) 2 vs 1 P=0.39 OR 1.10 (0.89-1.36)
				AA AG GG	186 (36.5%) 256 (50.2%) 68 (13.3%)	149 (36.6%) 201 (49.4%) 57 (14.0%)	146 (42.4%) 145 (42.2%) 53 (15.4%)		
				A G	628 (61.6%) 392 (38.4%)	499 (61.3%) 315 (38.7%)	437 (63.5%) 251 (36.5%)		
8	rs11830874 56147239	Intron 8/9	T/C	*U/K*	*32 (5.8%)*	*29 (6.5%)*	*16 (4.3%)*	22 vs 11 N/A 2 vs 1 P=0.075 OR 0.53 (0.24-1.18)	22 vs 11 N/A 2 vs 1 P=0.61 OR 1.27 (0.64-2.52)
				TT TC CC	506 (97.9%) 11 (2.1%) 0 (0%)	404 (97.1%) 12 (2.9%) 0 (0%)	340 (96.0%) 14 (4.0%) 0 (0%)		
				T C	1023 (98.9%) 11 (1.1%)	820 (98.6%) 12 (1.4%)	694 (98.0%) 14 (2.0%)		
9	rs10783828 56147751	Intron 9/10	G/A	*U/K*	*48 (8.7%)*	*58 (13.0%)*	*23 (6.2%)*	22 vs 11 P=0.81 OR 0.93 (0.59-1.49) 2 vs 1 P=0.35 OR 1.11 (0.90-1.37)	22 vs 11 P=0.47 OR 1.20 (0.75-1.92) 2 vs 1 P=0.13 OR 1.19 (0.95-1.48)
				GG GA AA	225 (44.9%) 229 (45.7%) 47 (9.4%)	172 (44.4%) 169 (43.7%) 46 (11.9%)	179 (51.6%) 128 (36.9%) 40 (11.5%)		
				G A	679 (67.8%) 323 (32.2%)	513 (66.3%) 261 (33.7%)	486 (70.0%) 208 (30.0%)		
10 *tSNP 3*	**rs2228224 56151588**	**Exon 12**	**A/G**	*U/K*	*14 (2.9%)*	*14 (4.1%)*	*101 (7.4%)*	**22 vs 11 P=0.62 OR 0.91 (0.65-1.26) 2 vs 1 P=0.98 OR 1.00 (0.86-1.17)**	**22 vs 11 P=0.21 OR 1.28 (0.89-1.83) 2 vs 1 P=0.091 OR 1.17 (0.98-1.39)**
				AA AG GG	**175 (37.2%) 230 (48.9%) 65 (13.8%)**	**109 (33.6%) 158 (48.8%) 57 (17.6%)**	**506 (39.7%) 560 (44.0%) 207 (16.3%)**		
				A G	**580 (61.7%) 360 (38.3%)**	**376 (58.0%) 272 (42.0%)**	**1572 (61.7%) 974 (38.3%)**		
11 *tSNP 4*	**rs2228226 56152088**	**Exon 12**	**C/G**	*U/K*	*9 (1.9%)*	*10 (2.9%)*	*110 (8.0%)*	**22 vs 11 P=0.079 OR 1.41 (0.98-2.03) 2 vs 1 P=0.040 OR 1.19 (1.01-1.39)**	**22 vs 11 P=0.0048 OR 1.79 (1.21-2.65) 2 vs 1 P=0.0053 OR 1.30 (1.08-1.55)**
				CC CG GG	**205 (43.2%) 217 (45.7%) 53 (11.2%)**	**137 (41.5%) 148 (44.8%) 45 (13.6%)**	**610 (48.3%) 542 (42.9%) 112 (8.9%)**		
				C G	**627 (66.0%) 323 (34.0%)**	**422 (63.9%) 238 (36.1%)**	**1762 (69.7%) 766 (30.3%)**		

Table 4.4. Details of the 11 *GLI1* SNPs genotyped in the Scottish population, including the 4 tagging SNPs (tSNPs) used for haplotype analysis. *n=370 for SNPs 1-4 and 7-9. U/K = unknown.

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Chapter 5

Summary, Conclusions, and Future Directions

The work described in this thesis is focused on the function of Hh signaling in adult intestinal homeostasis. Utilizing transgenic and knockout mouse models with altered levels of Hh signaling in the intestine, we have examined the impact of Hh on specific mesenchymal target cell populations that are critical for intestinal function. Work presented in Chapter 2 demonstrates a critical role for Hh signaling in the specification and maintenance of intestinal smooth muscle populations, and findings presented in Chapters 3 and 4 identify a novel role for Hh signaling as an anti-inflammatory epithelial signal in both the small intestine and colon. In addition, work in this thesis has carefully examined Hh responding cells in the intestine and colon, leading to the identification of intestine-resident myeloid lineages as a major Hh-responsive population. Together these results reveal that in the adult intestine, Hh signaling is an important epithelial signal that exerts continuous control over villus smooth muscle cells, ISEMFs and myeloid cells. Furthermore, reducing the level of Hh signals to these cell populations leads to intestinal pathology. In this chapter I will discuss the importance of these findings and consider future strategies to continue the elucidation of the role of Hh signaling in myogenesis, inflammation, and homeostasis in the intestine.

<u>Hh signals are critical for homeostasis of the adult gastrointestinal tract</u>

At the outset of the studies described in this thesis, the role of Hh signaling in the adult intestine was unclear. A potential role for Hh in the context of adult colonic stem cell regulation (1) and intestinal Paneth cell differentiation (2) in mice had been proposed; one study had also examined Hh during inflammatory disease in humans (3). However, no mechanistic examination of the roles of Hh signaling had been carried out, and even the direction of Hh signaling in the adult intestine (autocrine, paracrine, or both) was very controversial. The data presented here and in a recent study from our laboratory (4) demonstrate that Hh signals in the antral stomach, intestine and colon are exclusively paracrine from E10 through adulthood, an important finding that allows identification of Hh direct target populations and provides the opportunity to design mechanistic studies to identify direct Hh target genes. These data are also in accord with a more recent study that re-examines Hh signaling in cancer and implicates Hh as an important paracrine mediator of cancer in multiple organs including the colon (5).

In addition to confirming a paracrine signaling path, data presented in this thesis provide evidence that certain mesenchymal cells (including myofibroblasts, smooth muscle, and myeloid cells) are highly sensitive to the levels of Hh ligand in the small intestine and colon. The transgenic studies in Chapter 2 and 3 demonstrate clearly that modulation of Hh signaling has critical implications for tissue patterning in the small intestine; this is true both for increased Hh signaling, demonstrated by expansion of smooth muscle in 12.4KVil-Ihh animals, and for inhibition of Hh signaling, which leads

to villus and muscularis mucosa smooth muscle loss, crypt expansion, villus atrophy, and spontaneous small intestinal inflammation in VFHhip animals. These studies are the first to provide direct evidence that proper levels of Hh signaling are required for tissue homeostasis in the small intestine. While these studies were underway, an additional study was published describing an important role for Hh signals in maintenance of adult colonic crypt populations (6). Together, these data provide substantial evidence that Hh signals are required not just for embryonic tissue patterning (7-9) but also during adulthood. Moreover, these data demonstrate that cells of the lamina propria appear to be highly attuned to the level of Hh signals and that modulation of these levels can alter mesenchymal gene expression and lead to pathology.

<u>Hh signals direct smooth muscle differentiation through activation of Myocardin</u>

The data presented in Chapter 2 demonstrate that Hh signals are necessary and sufficient for the induction of the smooth muscle populations of the muscularis mucosa and villus core. While it was well known that Hh signals were important in patterning smooth muscle in the GI tract and other organs (7-12), the specific pathway activated by Hh that promotes smooth muscle induction was poorly understood. Indeed, a significant literature suggested that BMP or other signals might be important inducers of smooth muscle downstream of Hh signaling; and the function of Hh was thought to be largely related to control of these other signals (7; 10). The data in Chapter 2 and our recent examination of Hh-responsive cell populations in the intestine (4) demonstrate clearly that smooth muscle cells in the intestine respond directly to Hh signals, indicating that Hh

signaling is directly capable of inducing smooth muscle. Indeed, Hh ligand and an activated form of Gli2 (13) are both clearly capable of driving smooth muscle induction in the 10T1/2 mesenchymal cell line, and this activity does not appear to require BMP signaling. Hh appears to exert this effect through the direct activation of Myocardin expression in the small intestine. Myocardin is a master regulator of smooth muscle differentiation, and has been extensively studied in vascular and cardiac muscle (14-18). Several mechanisms have been described for modulating the expression (19) or activity (17) of Myocardin; our studies implicating Hh as a direct regulator of Myocardin add an additional layer of regulatory control upstream of Myocardin expression, and raise the question of whether Hh is involved in the activation of Myocardin in other contexts.

While our data demonstrate that the activation of Myocardin is an important aspect of the myogenic pathway downstream of Hh signals, several critical questions remain. First, to what extent is Myocardin activity actually required for the development of smooth muscle downstream of Hh signals? What would happen if Myocardin expression were reduced or absent in the context of normal Hh signals? Would Hh activate alternative smooth muscle pathways, or is Myocardin strictly required for Hh-mediated myogenesis? FoxF1 and Nkx2.3, two other putative Hh target genes, appear to be expressed in smooth muscle and may be important in the differentiation of SM populations in the intestine (20-22); these factors may provide redundancy with Myocardin downstream of Hh signals. In addition, it would be of significant interest to examine whether overexpression of Myocardin can rescue the phenotype of VFHhip animals, or cause expansion of smooth muscle in the presence of normal Hh signaling.

Studies have suggested that Myocardin activates a subset of smooth muscle genes, but is not sufficient for complete smooth muscle differentiation, at least in the context of embryonic stem cells (23), so the possibility remains that Myocardin acts together with other regulators of smooth muscle in the GI tract. Studies addressing this issue will be technically demanding; while smooth muscle specific promoters exist (24), Myocardin is expressed very early in smooth muscle differentiation and regulates smooth muscle specific genes (14) and global Myocardin knockout animals die of vascular insufficiency before induction of the gut tube, precluding intestinal studies in this model (18). Thus a conditional knockout strategy would be necessary. The Gli1Cre-ER mouse could be one potential tool to accomplish this. On the other hand, overexpression of Myocardin is likely possible, though targeting to the proper cell type (i.e. Desmin-positive/αSMA-negative smooth muscle precursors) would be a challenge.

A second important challenge is to understand the extent to which Myocardin is regulated by Hh signals in other contexts. For example, Hh is critically required for smooth muscle induction in the early muscularis externa (7-9), and it will be important to directly evaluate the role of Myocardin activation during this process. In addition, Hh signals have been implicated in the specification of the heart (25; 26) and vascular smooth muscle (11), two areas where Myocardin activity is known to be critically involved in myogenesis. In fact, recent studies have shown that activation of Myocardin in the early chick heart requires the endoderm but does not require BMP signals (27); this regulation therefore appears superficially similar to the one identified by our studies in the GI tract, and it is tempting to speculate that Hh may be involved in activation of

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Myocardin during cardiovascular development as well. Directly assessing the importance of Hh in the control of *Myocd* in these and other myogenic processes related to either Myocardin or Hh will be a crucial area of future investigation.

Finally, it will be important to confirm and extend the observation that Myocardin appears to be a direct target of Hh signaling. The intronic putative enhancer element we have identified appears to be functionally conserved from *Drosophila* to Human, and our data indicate that this element is potentially capable of regulating Myocardin expression. However, further testing and characterization of this element should begin in mouse, including ChIP analysis to confirm that Gli transcription factors bind the putative regulatory region in vivo. In addition, creation of transgenic mice where this element drives the expression of β-Galactosidase would help to formally demonstrate the functionality of this element. Thereafter, it would be of interest to understand whether the enhancer is in fact functionally conserved, and test the ability of orthologous enhancers to drive expression in other systems; one particularly attractive model is *Drosophila*, where MRTF (the fly orthologue of Myocd) is important in the migration of larval mesoderm (28). Taken with the studies described above, these approaches would allow evaluation of the generalizability of the observations made regarding this pathway in the GI tract to other processes.

<u>Hh is a novel anti-inflammatory epithelial signal in the intestine and colon</u>

The data presented in Chapters 3 and 4 of this thesis suggest that Hh signals provide a critical anti-inflammatory signal during homeostasis in the adult intestine and colon. Our data demonstrate that functional Hh signals are required for protection from inflammatory disease in the intestine, and that chronic inhibition of Hh signaling leads to the development of spontaneous inflammation in VFHhip animals. Further, we implicate Hh signals directly in the regulation of inflammatory pathways in the intestinal lamina propria. Finally, we identify a novel GLI1 variant that causes predisposition to inflammatory disease in humans and acts as a subfunctional transcription factor in vitro, and demonstrate that lack of a single genomic copy of Gli1 predisposes mice to the development of severe colitis in response to inflammatory stimuli. Taken together, these data provide the first evidence that Hh signals act in an anti-inflammatory manner, and tie Hh signals to the regulation of major inflammatory signals including the IL-1β, IL-6, and IL-17/23 pathways.

The finding that Hh is an important modulator of inflammation was unanticipated at the start of the work described in this thesis, and numerous specific questions remain regarding the mechanism by which Hh signals are modulating the inflammatory milieu of the mammalian intestine. In addition, the combination of in vitro and in vivo modeling employed in these studies provides multiple directions for future investigation. It will therefore be necessary to identify the best path to continue to elucidate the mechanism by which Hh regulates inflammation in the GI tract.

First, it will be crucial to identify the cell types that are involved in the regulation of inflammation by Hh. As described in Chapter 3, ISEMF and myeloid immune populations provide the best candidates. Both cell types are Hh responsive ((4; 9) and Chapter 3 and 4 of this thesis), and both have been implicated in regulation of the immune response in the intestine. ISEMFs both respond to and express many pro-inflammatory cytokines (29), and have been implicated as antigen-presenting cells (APCs), with robust expression of MHC Class II cell surface molecules (30). Hh signaling clearly has significant impact on ISEMF populations, and both developmental (9) and adult inhibition of Hh signals is sufficient to modulate their localization and, presumably, their activity. Myeloid lineages are regulators of immune homeostasis in the intestine (31; 32), and have been implicated particularly in modulating the balance between pro-inflammatory and tolerogenic differentiation of lymphocytes.

In sum, then, the majority of APCs in the intestine respond directly to Hh signals. It will therefore be important to differentiate the effects of the CD45-positive immune lineages (including myeloid cells) from the CD45-negative stroma, including myofibroblasts; this could be achieved by FACS sorting of mesenchymal cultures (which contain both ISEMF and myeloid cells – Chapters 2 and 3) for CD45, isolation of primary myeloid cells (32), or evaluation of ISEMF cells lines. Thereafter, these cells can be treated with Hh ligand and evaluated for gene expression, differentiation state (by examination of cell surface markers for subtypes of myeloid cells), or through functional assays such as programming of naïve T-cells (31; 32). Also of interest would be cell type

specific knockouts of Hh pathway members; recent data suggests that hematopoietic differentiation can proceed in the absence of Hh signaling (33), but the function of different immune lineages in the absence of Hh signaling requires further elucidation.

In addition, some of these studies can be performed utilizing bone marrow transplantation in Gli1$^{+/LacZ}$ animals. Transplantation of Gli1$^{+/LacZ}$ bone marrow into WT host will isolate Hh-response deficient immune cells in the context of a WT epithelial and stromal compartment, directly assessing the role of immune cell Hh response in susceptibility to DSS-mediated colitis. The converse experiment (transplantation of WT bone marrow into Gli1$^{+/LacZ}$ animals) will allow examination of the importance of stromal Hh response in the context of a WT immune system. In the end, it is likely that both the stromal and immune Hh responses will be important in the regulation of inflammation downstream of Hh, but these approaches will provide the opportunity to study the isolated contributions of different cellular populations.

Once the further characterization of cellular response to Hh signals is complete, it would be of interest to attempt to understand the role Hh signaling plays in the modification of gene expression in these cell types. For example, Hh may directly mediate gene expression changes, or may modulate expression levels of cohorts of genes by regulating fate transitions through control of other transcriptional or signaling molecules. In either case, identification of those genes that are direct Hh targets will be of cardinal importance. To this end, we have begun comparison of the targets identified in the microarray experiments described in Chapter 3 with a novel Gli-responsive

enhancer prediction algorithm based on the computational method SCORE (34). This computational strategy was developed by Aaron Udager, a graduate student in our group. This algorithm allows stratification of putative Hh targets into likely direct and indirect targets; those targets that contain a nearby putative Gli-responsive enhancer can be considered potentially directly regulated by Hh signals. In the future it will be critical to test the enhancer elements predicted by this algorithm to identify bona fide direct Hh targets involved in immune function, and ask specifically how Hh regulates these targets and what significance these genes have in the control of inflammation downstream of Hh signaling.

Another important future goal will be to begin to separate the developmental and adult roles of Hh signaling in the control of intestinal inflammation. Next to nothing is known about the development and specification of intestinal innate immunity, though Hh signals have been implicated as crucial in the development of myeloid lineages in the thymus (35), and CD11b-positive cells in mesenchyme cultures appear to co-express LacZ in mesenchymal cultures made from Gli1$^{+/LacZ}$ small intestine (data not shown). Therefore, it is of significant interest both to study the general development of innate immune cells and structures in the intestine and colon as well as the role of Hh signaling in the patterning and fate decisions of these cells during development.

Others in our laboratory have recently obtained interesting results regarding the potential role of Hedgehog in the development of innate immunity in the fetal gut. Xing Li and Aaron Udager showed that at E16, there is a sudden, dramatic, and coordinated

up-regulation of over 1000 genes in the intestinal epithelium. The genes that are up-regulated are primarily those that endow intestinal epithelial cells with their unique functional attributes in absorption and metabolism (Li et al, submitted). Very interestingly, one of the few epithelial genes that is down-regulated at this time is Shh. The seven-fold decrease observed in Shh expression leads to a dramatic decrease in the expression of the entire Hh pathway in the associated intestinal mesenchyme (*Gli1, Hhip, Ptch1*, as well as the recently described co-receptors *Gas1, Boc* and *Cdo*). Importantly, this down-regulation of Hh signaling coincides with intestinal epithelial maturation; this is a physiological reduction but not total ablation of Hh signals in the intestine. Hh levels in the stomach domain remain high. Concomitant with this intestinal maturation and reduction in Hh signals, we see up-regulation of only a few mesenchymal genes. Strikingly, DAVID analysis indicates that these few genes have largely pro-inflammatory function (Li et al., submitted). This physiological event is particularly interesting in light of the findings presented in this thesis, which suggest a sensitivity of the mesenchyme to Hh ligand concentration and predict that lowering Hh signals promotes an inflammatory mesenchymal response. Perhaps this physiological lowering of Hh that occurs during intestinal development helps to prepare the mesenchyme for its role in immune surveillance.

In addition, much remains to be investigated regarding the adult role of Hh in immune and inflammatory control. One issue with the approaches undertaken in Chapters 3 and 4 of this thesis is the difficulty in precisely identifying the stimulus for the observed inflammatory changes. In the VFHhip model, the development of

inflammatory disease requires chronic Hh inhibition but occurs stochastically, suggesting a random stimulus that causes inflammatory disease in the context of Hh inhibition. In the Gli1$^{+/LacZ}$ model, the stimulus is acute (DSS treatment) but the animals have been missing a genomic copy of Gli1 throughout embryogenesis and life. While no phenotype has been reported in Gli1 null animals (36), it is unclear whether a subtle difference in tissue form or function exists prior to the stress. It is therefore critical to examine a model of acute Hh reduction and assay whether short term Hh inhibition is sufficient to induce susceptibility to inflammation (spontaneous or otherwise); this could be achieved by combining the 12.4K-Villin-flox-LacZ-flox-Hhip allele with the intestine specific Villin-CreERT2 (37), and induction with tamoxifen. The success of this approach however, is dependent upon the degree of recombination that can be achieved. It would be of interest to then test the susceptibility of these animals to both acute (i.e. DSS) and chronic (i.e. IL-10 knockout (38)) inflammatory stimuli.

Developmental Signaling Networks Can Regulate Adult Homeostasis

While this study interrogates Hh signaling directly, our finding that Hh signals are critically important in adult homeostasis in the GI tract has implications for other signaling pathways. In the intestine, Hh signals are involved in modulation of both Wnt (through modification of localization and/or activity of ISEMF populations) and BMP signaling (at least through modification of BMP-4 expression). The phenotypes we have identified downstream of Hh signaling are likely sequelae not only of changes directly caused by altered Hh signaling, but also of modification of these secondary signals. This

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is most likely the case for the expansion of crypts seen in VFHhip animals, as ISEMF

populations are rich sources of Wnt ligand (9; 29) and mislocalized ISEMFs line the

expanded crypt of the VFHhip intestine.   Our microarray studies in Chapter 3 also

demonstrate that modification of Hh signaling causes secondary changes in the

expression of several signaling molecules and modulators, both of traditional signaling

pathways (*Igf-1, Fgf-7*) as well as modulators of inflammatory signaling (*IL-1b, IL-6*, CC

and CXC chemokines); many of these molecules are also expressed at altered levels in

Gli1$^{+/LacZ}$ animals stressed by DSS. It is likely that direct modification of these other

pathways would have implications for intestinal homeostasis; indeed, Wnt signals appear

to be crucial modulators of intestinal proliferation and specification during adulthood

(39-41), and modification of BMP signals has important implications for the development

of GI cancers (42). Overall, the cell-cell crosstalk that occurs both during intestinal

development and following maintenance of intestinal homeostasis clearly has

implications for pathological response. The richness of this cellular communication

network makes unraveling the key initiators a very difficult challenge. One way to begin

to address this is to profile the specific target genes downstream from each signaling

pathway, and to attempt to integrate these expression data with each other and with

phenotypic data from experimental models to develop a holistic picture of signaling. The

data in this thesis and the recent development of a computational strategy to identify

potential Hh target genes (described above) provide a starting point for this type of

analysis, and we are beginning to make some progress in this regard.

Not only does Hh appear to regulate secondary signaling, but our studies have also identified several transcription factors that are putative targets of Hh signaling. Myocardin is clearly downstream of Hh signaling, and while more studies will be required to truly characterize the responsive element of the Myocd locus, studies described in Chapter 2 demonstrate that *Myocd* is upregulated with similar kinetics to other direct Hh targets, likely through the action of a Gli-responsive intronic enhancer element. Our microarray studies also identify several other potentially Hh-responsive transcription factors, including Nkx2.3, Enc1, and Prrx1. Given that Hh regulates the expression, activity, or localization of these secondary signaling molecules and transcription factors, it is tempting to consider Hh signals in the intestinal epithelium at the head of a complex gene regulatory network that influences cells of the lamina propria. In such a scenario, Hh would activate, repress, or otherwise modify signaling and transcriptional modulators in order to direct large-scale tissue changes or cell fate decisions. Networks of this type have been examined during development (43); however, to date such networks have not been developed for understanding of complex disease processes such as inflammation. The VFHhip model and 12.4kVil-Ihh models may provide the opportunity to further elucidate the role of Hh in the control of such a regulatory network during homeostasis and the development of inflammatory disease in VFHhip animals.

<u>Conclusion</u>

In conclusion, our data place Hh signaling at the head of an important gene regulatory network required for intestinal homeostasis, and suggest that dysregulation of this developmental signaling pathway during adulthood predisposes to pathology. Our results implicate Hh signaling as an important regulator of intestinal smooth muscle, crypt structure, and inflammatory response, and provide evidence that Hh signals modulate numerous gene targets (directly and indirectly) in these pathways. These findings may have implications for understanding the pathogenesis of human disorders including Celiac disease and IBD, and may provide avenues for future therapeutic intervention.

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