Role of DNA Methylation in the Development and Differentiation of Intestinal Epithelial Cells and Smooth Muscle Cells

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The mammalian intestine contains many different cell types but is comprised of 2 main cell types: epithelial cells and smooth muscle cells. Recent in vivo and in vitro evidence has revealed that various alterations to the DNA methylation apparatus within both of these cell types can result in a variety of cellular phenotypes including modified differentiation status, apoptosis, and uncontrolled growth. Methyl groups added to cytosines in regulatory genomic regions typically act to repress associated gene transcription. Aberrant DNA methylation patterns are often found in cells with abnormal growth/differentiation patterns, including those cells involved in burdensome intestinal pathologies including inflammatory bowel diseases and intestinal pseudo-obstructions. The altered methylation patterns being observed in various cell cultures and DNA methyltransferase knockout models indicate an influential connection between DNA methylation and gastrointestinal cell development and their response to environmental signaling. As these modified DNA methylation levels are found in a number of pathological gastrointestinal conditions, further investigations into uncovering the causative nature, and controlled regulation, of this epigenetic modification is of great interest.

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Key Words
Cell differentiation; DNA methylation; Intestinal mucosa; Muscle smooth
genetic mechanisms, especially DNA methylation, as both global and site-specific changes in DNA methylation levels are hallmarks of differentiating cells. Several recent in vivo studies have indeed found that DNA methylation levels change at various genomic sites during both development and differentiation of intestinal epithelium from Lgr5+ stem cells into fully mature ME. Many of these changes strongly correlate with DNA methylation levels found in human disease states such as inflammatory bowel diseases, and certain colorectal cancers. Variable and characteristic patterns of genomic DNA methylation that change under various phenotypic conditions are also found in another cell in the gastrointestinal (GI) tract: smooth muscle cells (SMC).

Surrounding the epithelium, and separated by a submucosal region, are 2 perpendicular layers of smooth muscle (circular and longitudinal) that produce peristaltic movement via calcium initiated, actin-myosin contractions when stimulated by neuronal input mediated through the pace making interstitial cells of Cajal. Unlike most terminally differentiated somatic cells, SMC have a unique plasticity in which they are able to transition between a mature, contractile state, and a synthetic, proliferative, more stem cell-like state. Of note, a similar type of dedifferentiation plasticity has recently been observed in villus epithelium. When comparing the 2 states in SMC, mature SMC express high levels of proteins necessary for contractility such as MYH11, ACTA2, and TAGLN and have low rates of proliferation while synthetic SMC have higher rates of proliferation, lower levels of contractile proteins, produce high levels of extracellular matrix and have lowered levels of the necessary microRNAs (miRNAs), miR-143/145. These synthetic SMC are no longer functionally contractile and proliferate in response to injury and begin to transition to a more differentiated state once the tissue repair has been accomplished. This plasticity and ability to proliferate is important for tissue repair but does carry with it the potential for SMC to improperly regulate the dynamic differentiation and growth process. Aberrant growth patterns of SMC once the tissue repair has been accomplished support the potential for SMC to improperly regulate the dynamic differentiation and growth process. As epithelial cells progress from IESC to ME, express Lgr5, have the capacity to become supportive Paneth cells, express Lgr5, have the capacity to become supportive Paneth cells, express Lgr5, have the capacity to become supportive Paneth cells, express Lgr5, have the capacity to become supportive Paneth cells, express Lgr5, have the capacity to become supportive Paneth cells.

### DNA Methylation in Intestinal Epithelium

#### Methylation Dynamics in Developing and Differentiating Intestinal Epithelial Cells

The GI tract begins its development at the gastrulation stage in early embryonic development. When DNA methylation is inhibited at this early developmental stage in zebrafish embryos through use of cytidine analogs (5-azacytidine, 5-aza and 5-aza-2'-deoxycytidine, 5-aza-dC), which non-selectively inhibit all DNMT isoforms and induce hypomethylation, gastrulation does not proceed and muscle progenitors do not organize as expected, indicating the shared importance of DNA methylation for both epithelial (endoderm) and muscle tissue (mesoderm). As it pertains to intestinal epithelium, there are 2 differentiation states of focus in relation to cellular identity: intestinal epithelial stem cells (IESC) and mature ME. IESC are found at the base of intestinal crypts in close contact to supportive Paneth cells, express Lgr5, have the capacity to become any functional intestinal epithelial cell and also have the potential to be the origin and driving force behind benign and malignant intestinal neoplasias as IESC are Wnt signaling responsive. In contrast, ME show little to no Lgr5 expression, strong Alpi expression and only show the ability to become proliferative upon selective elimination of Lgr5+ IESC. As epithelial cells progress from IESC to ME, there is a loss of Wnt signaling response and an equivalent increase in Bmp signaling response which, through SMAD1/SMAD4 activation, directly inhibits the transcription of genes necessary for proliferation. Many of these genes that have differential expression levels between IESC and ME, also have differential levels of methylation in varying genic elements. Approximately
14% of genes that were induced upon differentiation in intestinal epithelial cells, including ME markers Lct and Alpi, showed losses of methylation while stem cell related genes that lose expression upon differentiation, such as Lgr5 and Olfm4, showed increases in methylation with a majority of these methylation changes occurring in introns, for both gene categories. Additionally, both Lgr5 and Olfm4 have shown high levels of demethylation across their gene bodies in Lgr5+ cells that are not found in ME. The most common genomic regions of differential methylation between IESC and ME are introns, especially the first intron, where enhancer regions are often found. In fact, changes in methylation levels at enhancer regions in IESC can impact, and be impacted by, the binding of the Wnt responsive transcription factor, TCF4. Furthermore, in Dnmt1 & Dnmt3b ablated HCT116 colon cancer cells, 111 up-regulated genes lost methylation in enhancer regions with about 92% of those enhancers being found in introns, a pattern that has been observed in other cell types as well. In contrast, increased methylation at 3’ CpG islands correlates with an increase in related gene expression in both IESC and ME. Taken together, these data indicate that DNA methylation dynamics play a vital role in intestinal epithelial cell development but how these changes in methylation affect the overall expression of any given gene is specific to genomic and genic location.

**Cell Specific Knockout of Dnmt Isoforms Result in Developmental Time Point-dependent Phenotypes**

Dnmt3a and Dnmt1 are the most highly expressed Dnmt isoforms in both total intestinal mucosa (Fig. 1) and isolated epithelial cells. Elimination of Dnmt3a allowed for normal epithelial development while knockout of Dnmt1 altered epithelial differentiation and improper renewal of stem cell populations/crypt formation. Dnmt1 knockout results in differing phenotypes depending on whether the knockout occurs during embryonic development or in adulthood. When Dnmt1 was congenitally and cell-specifically knocked out of all intestinal epithelial cells (using VillinCre), progenitor cells severely reduced their proliferative potential, and had reduced methylation levels upstream of the promoters for genes induced by DNA damage (Cdkn1a, Chek2, Atm, and Mlh1) with only 35% of knockout pups surviving until weaning, a phenotype that is very similar to congenital knockout of Dnmt1 in smooth muscle. Surprisingly, when Dnmt1 is inducibly eliminated in adult mice (using VillinCreER), proliferative potential expands up the crypt, instead of being reduced, and cells show both genomic hypomethylation and an overall improper expression of differentiation markers that does not result in the premature death seen in embryonic Dnmt1 knockout mice. However, it was found that when Dnmt3b was also eliminated from the adult intestinal epithelium alongside Dnmt1 knockout, epithelial development and organization halts altogether as cells become apoptotic and proliferative potential is extinguished. These results suggest that DNMT3B, who’s expression is induced upon Dnmt1 knockout, has the ability to compensate for the maintenance of de novo methylation patterns lost upon Dnmt1 knockout, which has been observed for LINE1 sequences in embryonic stem cells. These results stress that expression, or lack thereof, of Dnmt isoforms can be a vital lynchpin at various stages of intestinal cell development (Table). Finally, it has also been shown that loss of methylation in adult intestinal epithelium occurs under germ-free conditions and methylation levels can be rescued upon fecal transplant, indicating an important developmental crosstalk between the microbiota and intestinal epithelia that requires further investigation.

**DNA Methylation Dynamics in Inflammatory Bowel Disease**

Inflammatory bowel disease (IBD) encompasses 2 related but different pathologies affecting the intestinal epithelium that both manifest in pro-inflammatory conditions: Crohn’s disease (CD) and ulcerative colitis (UC). In general, both IBD conditions arise in genetically susceptible individuals whose GI mucosa fails to maintain barrier integrity allowing for infiltration of microbiota and other environmental factors that initiate the recruitment of immune cells to the affected area causing the phenotypic inflammatory condition. UC is restricted to the colon while CD can occur anywhere along the GI tract. As both IBD diseases are of unknown etiology, most researchers in the field have turned their attention to establishing genetic, epigenetic, and environmental links to the disease states themselves through forward screening techniques. Both diseases states have commonalities in regards to symptom manifestation and genetic loci that are most often associated with immune regulation as well as a the dysbiosis of the gut microbiota. Many of the genes found to be commonly dysregulated, with correlating aberrant methylation patterns, in the genetics screens of UC affected tissue are associated with regulating apoptosis (Cdkn2a, Dapk, and Cdh1) and, in general, UC tissue shows overall genomic hypermethylation. Even though the number of genetic loci associated with IBD is now over 200, up from 163, these loci only seem to contribute to 13.1% of the variance seen in CD patients and 8.2% in UC patients. Thus, as genetic screens are not enough to explain the etiology of these conditions alone, it stands to reason that epigenetics plays some factor as epigenetic mechanisms...
Figure 1. DNA methyltransferase (DNMT) expression levels in intestinal tissue from mice and humans. (A) Using the Smooth Muscle Transcriptome Browser, we show the expression levels of various DNMTs in several intestinal cell types and tissues (J, jejunal; C, colonic; SM, smooth muscle tissue; SMC, smooth muscle cell; ICC, interstitial cells of Cajal; PαC, platelet-derived growth factor receptor α-positive [PDGFRα+] cell; Mu, mucosa tissue; and MuPαC, mucosal PDGFRα+ cell). Dnmt1a is the most highly expressed Dnmt isoform in colonic and jejunal smooth muscle tissue but this pattern is not consistent amongst all isolated cell types as JPαC/CPαC/CMu/CMuPαC express Dnmt1 more than Dnmt3a with Dnmt3b consistently being expressed the least amongst all cell types and tissues. While these expression levels are informative, they do not indicate necessity as Dnmt1 knockout causes the most detrimental phenotype in both intestinal epithelia and smooth muscle. (B) Expression levels of DNMT and 10-11 traslocation (TET) proteins in mice reveal that DNMT1 reduces its expression over time with a opposite pattern for DNMT3A (Adapted from Jorgensen et al). (C) Various diseased human tissue shows a dysregulation of DNMT1 and TET3 while other enzymes remain relatively stable in expression across conditions (Adapted from Jorgensen et al). FPKM, Fragments Per Kilobase Million; UBE, Ubiquitin-activating enzyme; M, marginal area; P, pouch.
are the lynchpin between genetics and environment. In 2013, authors Low, Mizoguchi and Mizoguchi produced a comprehensive and in-depth review on the intersection of DNA methylation and IBD, and as such, we will focus on research published since their publication, henceforth. As there are a myriad of cell types/tissues involved in the pathogenesis of IBD, studies involving DNA methylation changes are not restricted to a single cell type. Studies today tend to focus their attention on mucosal biopsies and blood samples, specifically immune cells, as both tissues are intricately linked to IBD. DNA methylation profiling of peripheral blood mononuclear cells show that 45% of differentially methylated positions found in CD samples were also found in UC samples, and 97% of differentially methylated positions in UC samples were also found in CD samples highlighting both the similarities and differences of the 2 conditions as it pertains to genomic methylation.

Additionally, levels of CpG methylation at individual loci, such as TIFAB, are dynamic based on the age of the patient with younger UC patients showing hypomethylation and older patients showing hypermethylation, again highlighting the situational dependent modulability of DNA methylation under IBD conditions. Recently, an intricately well-designed and seminal study showed, through the isolation of ileal and colonic intestinal epithelial cells from pediatric patients with IBD, that disease outcome could be predicted utilizing a unique machine learning approach that combines transcriptomics and methylomics. The multi-omics approach of this study is an encouraging diagnostic approach that is much more likely to give a personalized approach to this multi-faceted and individually-dependent disease state that may be transferable to disease states of SMC origin found in the GI tract.

DNA Methylation in Smooth Muscle

Much of the initial focus that has been given to DNA methylation in SMC has been centered on vascular (vSMC) or airway SMC (aSMC) as these cell types are associated with common, and costly, chronic conditions such as atherosclerosis and asthma, respectively. SMC have an uncommon ability, known as plasticity, to shift between a mature, contractile state and a more proliferative, synthetic condition that is dynamic based on environmental and genetic conditions. The more proliferative SMC state is phenotypically similar to that of the less-differentiated myofibroblasts through the production of extracellular matrix. This shift between states of differentiation has repeatedly been shown to be inextricably linked to, and often regulated by, changes in DNA methylation at various genomic elements. The multi-proliferative SMC state is phenotypically similar to that of the less-differentiated myofibroblasts through the production of extracellular matrix. This shift between states of differentiation has repeatedly been shown to be inextricably linked to, and often regulated by, changes in DNA methylation at various genomic elements. The multi-proliferative SMC state is phenotypically similar to that of the less-differentiated myofibroblasts through the production of extracellular matrix. This shift between states of differentiation has repeatedly been shown to be inextricably linked to, and often regulated by, changes in DNA methylation at various genomic elements.
of differentiation status, cytidine analogs consistently interfered with proper differentiation. Hepatic stellate cells were not able to transdifferentiate into myofibroblasts when exposed to 5-aza-dC.\textsuperscript{78} Cultured aSMC were not able to alter their phenotypic status when subjected to 5-aza-dC treatment even when the cells were induced to phenotypically switch by the addition of PDGF.\textsuperscript{46} Phenotypic switching in vSMC was revealed to be regulated by the presence of miR-1298 whose expression is directly dependent on levels of DNA methylation.\textsuperscript{77} In contrast to mature SMC, when cultured fibroblasts were exposed to 5-aza-dC they began to express higher levels of $\alpha$-SMA, indicating the maturing of fibroblasts towards mature SMC.\textsuperscript{44} Recently, high passage primary human intestinal SMC (iSMC), known for a lack of contractile protein expression compared to lower passage cells, were found to restore their contractile protein expression upon treatment of 5-aza.\textsuperscript{77} Collectively, these results emphasize the importance of DNA methylation to phenotypic switching in SMC of various origin. Unfortunately, the preponderance of these studies were done in vitro and not on iSMC, and thus, these conclusions needed to be tested in vivo for confirmation across experimental conditions. In order to rectify this, a murine model was employed to show the first in vivo evidence regarding the importance of DNA methylation in the development of iSMC.\textsuperscript{44} In a smooth muscle restricted (Myh11) manner, Dnmt1 was knocked out of the genome resulting in pups with a shortened GI, detrimental thinning of the GI tunica muscularis brought about by the loss of iSMC through apoptosis and significant reductions in mature smooth muscle marker expression. Global genomic methylation of smooth muscle tissue was reduced about 20\% with losses being found in promoters, exons and introns with introns having the largest reductions. These results are in contrast with the previously mentioned iSMC in vitro study\textsuperscript{77} that concludes that inhibition of DNA methylation through 5-aza increases the expression of $\alpha$-SMA and SM22-$\alpha$, both markers of mature SMC. The reconciliation of these diametrically opposed results from in vitro and in vivo iSMC (Table) is vitally important in order to assess the translatability of in vivo results into organisms themselves. A separate group found that when Tet2 was eliminated (a 5-mC deoxygenase that initiates the demethylation of cytosines)\textsuperscript{100,101} from the murine genome, vSMC do not retain the ability to attenuate necessary SMC growth following arterial injury,\textsuperscript{45} adding further evidence to the notion that regulation of the DNA methylation apparatus is vital to the ordered and highly coordinated processes of growth, development and differentiation in SMC. When considering that a number of GI pathologies find their etiology in the overgrowth of iSMC, and dysregulation of the DNA methylation apparatus in SMC often creates detrimental growth regulation, it is integral that the next steps in this field be steeped in illuminating the intersection between these interrelated phenomena.

**Phenotypic Parallels Between Smooth Muscle Cells and Epithelial Cells Resulting From Manipulation of Genomic DNA Methylation Levels**

While SMC and EC diverge in developmental lineages early in the embryo, they share the interdependent physiological goals of GI motility and absorption and thus the fate of one cell type will likely affect the other cell type’s homeostasis. Despite epithelial cells being endoderm derived and SMC mesoderm derived, they do share congruous characteristics. As previously mentioned, 5-aza will halt all gastrulation processes that would lead to the development of both mature SMC and epithelial cells in the absence of 5-aza,\textsuperscript{59} indicating both cell types require precise methylation patterns in order to properly develop. Additionally, when Dnmt1 is selectively eliminated from either epithelial cells or SMC, there is little change to any non-CpG methylation and a majority of the changes at CpG sites are contained within introns,\textsuperscript{54,43} which can contain regulatory enhancer regions for transcription factors such as TCF4 in epithelial cells\textsuperscript{41} or SRF in SMC\textsuperscript{13} that are necessary for differentiation. These studies reveal that SMC and epithelial cells both show about 35\% loss of overall genomic CpG methylation when Dnmt1 is selectively eliminated, with specific methylation losses occurring at pro-apoptotic genes, such as Nrk4a1 in SMC and Cdkn1a in epithelial cells, resulting in their increased expression and subsequent cell death. Growth patterns of both normal and neoplastic epithelial cells and SMC can be manipulated by interfering with DNA methylation. When Dnmt1 is eliminated from epithelial cells, proliferative potential expands to cells further up the crypt, genomic CpG methylation is reduced, and aberrant/apoptotic gene expression occurs, leading to lethality at various post-embryonic developmental stages which is exacerbated when combined with Dnmt3b knockout,\textsuperscript{15–17} a phenotype strikingly similar to that found in SMC selective Dnmt1 knockout.\textsuperscript{43} Furthermore, in neoplasias originating from epithelial cells (adenomas) or SMC (leiomyosarcomas), treatment with 5-aza has been shown to either reverse, or impede any further, proliferation or growth of these tissues,\textsuperscript{79,102} showing additional evidence of the parallel functional necessity of proper DNA methylation patterns in regulating both epithelial cell and SMC growth. Finally, both SMC and epithelial cells contain specific methylation signatures at various differentiation stages. Upon addition of 5-aza,
immature myofibroblasts lose promoter methylation of α-SMA, increasing expression, thus beginning the differentiation cascade into mature SMC, consistent with results from 5-aza addition to high-passage non-contractile SMC that regain strong α-SMA expression. Methylation signatures for epithelial cells can be specific to ileal/colonic location and these CpG methylation patterns only change in fetal epithelial cells while adult epithelial cells retain consistent methylation signatures, demonstrating that precise CpG methylation patterns are necessary for development of epithelial cells from embryonic stages but not necessarily for the proper functioning of adult cells. Further studies on both cultured and in vivo epithelial cells and SMC will elucidate more consequential correlations, and contrasts, that result from changes in DNA methylation.

Conclusion

The GI tract is a massively complex organ system that, in varying cell types, relies on careful regulation of DNA methylation patterns in order to develop or differentiate as needed. Careful, and conditionally dependent, coordination of DNA methylation patterns and locations is crucial to ensure necessary growth and differentiation as well as when to halt these same processes (Fig. 2). As aberrant DNA methylation patterns, or altered DNMT levels, have been useful in understanding and treating pathologies in the intestinal epithelium, similar strategies should be employed for SMC disease states in the GI tract.

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