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THEME | *The Engineered Gut: Use of Stem Cells and Tissue Engineering to Study Physiological Mechanisms and Disease Processes*

Epigenetic regulation of intestinal stem cell differentiation

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Verzi MP, Shivdasani RA. Epigenetic regulation of intestinal stem cell differentiation. *Am J Physiol Gastrointest Liver Physiol* 319: G189–G196, 2020. First published July 6, 2020; doi:10.1152/ajpgi.00084.2020.—To fulfill the lifelong need to supply diverse epithelial cells, intestinal stem cells (ISCs) rely on executing accurate transcriptional programs. This review addresses the mechanisms that control those programs. Genes that define cell behaviors and identities are regulated principally through thousands of dispersed enhancers, each individually <1 kb long and positioned from a few to hundreds of kilobases away from transcription start sites, upstream or downstream from coding genes or within introns. Wnt, Notch, and other epithelial control signals feed into these *cis*-regulatory DNA elements, which are also common loci of polymorphisms and mutations that confer disease risk. Cell-specific gene activity requires promoters to interact with the correct combination of signal-responsive enhancers. We review the current state of knowledge in ISCs regarding active enhancers, the nucleosome modifications that may enable appropriate and hinder inappropriate enhancer-promoter contacts, and the roles of lineage-restricted transcription factors.

differentiation; epigenetic control; stem cells; transcription factors; transcriptional regulation

Intestinal stem cells (ISCs) renew the epithelial lining continuously and throughout life, producing progeny that differentiate into absorptive enterocytes or various secretory cell types. The diversity of cells emanating from ISCs requires cessation of ISC-specific transcription and the initiation of discrete new transcriptional programs. Nature tackles the "one genome, many outcomes" problem through compartmental organization of chromatin, restricted access to transcription factors (TFs), and targeted nucleosome modifications. Cell-specific gene activity requires promoters to interact with the correct combination of signal-responsive enhancers. Here, we review the transcriptional basis of ISC differentiation through the lens of *cis*-regulatory element dynamics.

GENOME ORGANIZATION FOR CONTROLLED, CELL TYPE-SPECIFIC GENE ACTIVITY

Chromatin interaction and imaging assays reveal that each interphase chromosome occupies a unique domain (75), so genes interact with their cognate enhancers predominantly in *cis* and rarely, if ever, in *trans*. Chromosomes subdivide grossly into inactive heterochromatin and transcriptionally active euchromatin, with specific covalent histone modifications enriched in each compartment (Fig. 1). For example, heterochromatin may be methylated on lysine 9 (K9) and lysine 27 (K27) in the NH_2-terminal tails of histone H3 (H3K9me and H3K27me3), whereas active *cis*-elements are typically enriched for methyl (me) and acetyl (ac) groups on the same histone's K4 and K27 residues, respectively (68). In the next known unit of chromosome order, short- (across a few kb) and long- (>1 Mb) range DNA interactions occur largely within topological associating domains (TADs) (52). Promoters, thereby constrained to interact with the enhancers available within their respective TADs, do so through diverse transcription-dependent subdomain contacts (29). Nucleosomes, the smallest units of chromosome order, carry 146 bp of DNA wrapped around histone octamers, and TF activities require these octamers to be displaced, in part, by ATP-dependent chromatin remodeling enzymes (9). Individual enhancers generally span the distance corresponding to one or two nucleosomes and are enriched for sequence motifs that bind selected TFs (Fig. 1). Among the 1 million to 2 million potential enhancers encoded in mammalian genomes, tens of thousands are nucleosome-free in any cell type. TF actions that occur

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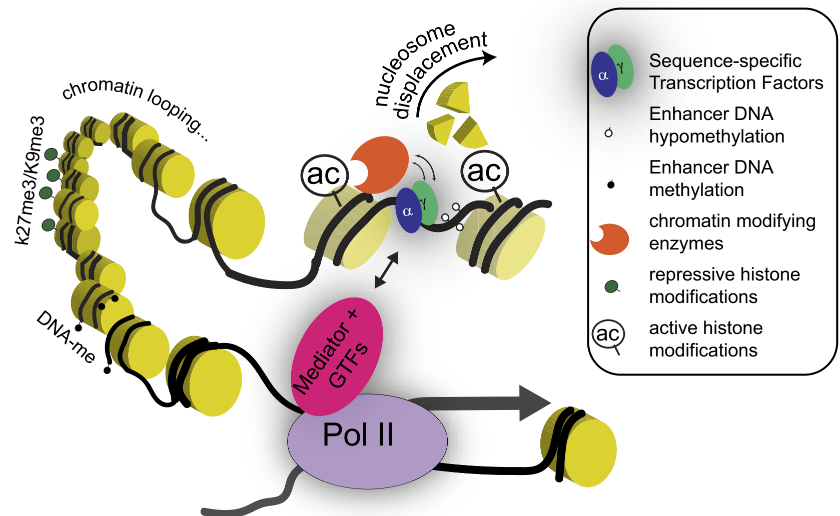

Fig. 1. Epigenetic regulatory mechanisms discussed in this review. Gene transcription is influenced by multiple regulatory inputs coordinated by sequence-specific transcription factors (TFs) at distal *cis*-regulatory elements (enhancers). Chromatin accessibility is established at these genomic regions by pioneer TFs and remodeling enzymes, which together displace nucleosomes. Other TF-recruited enzymes endow neighboring nucleosomes with covalent posttranslational modifications associated with gene activity (e.g., H3K27 acetylation shown here as "ac") or silencing (e.g., H3K27me3 and H3K9me3). In addition to carrying specific TF-binding motifs, enhancer DNA is hypomethylated at CpG dinucleotides, compared with inactive promoters or the genome background. Enhancers are thought to modulate transcription by forming three-dimensional loops that bring them close to RNA polymerase 2 (Pol II) and basal transcription machinery at target gene promoters. The protein complexes that bridge these loops contain Mediator and other general transcription factors (GTFs). Table 1 summarizes the current knowledge of these regulatory factors in the intestinal epithelium.

synchronously at subsets of these *cis*-elements confer tissue-specific identities and functions.

TFs deploy their DNA sequence specificity to compete with nucleosomes, bringing along chromatin-, histone- and DNA-modifying enzymes that contribute toward *cis*-regulatory activity (Fig. 1). The resulting activities enable identification and characterization of the full repertoire of enhancers active in a cell population. Nucleosome-depleted regions, synonymous with accessible or "open" chromatin, are sensitive to cleavage by DNase I or by transposase Tn5 in the assay for transposase-accessible chromatin (ATAC-seq) (11, 62), while those that immediately flank active enhancers usually carry covalent histone marks such as H3K4me1, H3K4me2, and H3K27ac (1). Chromatin immunoprecipitation and sequencing of the associated DNA fragments (ChIP-seq) identifies the sites that carry these canonical marks and bind specific TFs and ancillary proteins (4). Additionally, whereas most CpG dinucleotides in mammalian genomes are methylated (meCpG), those present in many active enhancers are not methylated to the same degree across cell populations, and whole-genome bisulfite sequencing (WGBS) identifies the cell type-restricted complement of hypomethylated enhancer DNA (60).

ATAC-seq, TF, or histone ChIP-seq, and WGBS data from ISCs, crypt progenitors, and mature villus cells capture transitions of active and inactive *cis*-elements that accompany, and may underlie, differentiation. These findings, which we review here, collectively inform current understanding of gene regulation during ISC differentiation and dedifferentiation. Of note, these approaches map putative enhancers, whose distant target genes and contributions toward their expression have not been validated experimentally. In general, however, enhancers so identified in intestinal and other cell populations lie near genes expressed in those cells (26, 37, 40, 69); even if some tissue-restricted enhancers are assigned incorrectly, aggregate statements are generally robust and accurate.

EPIGENETIC TRANSITIONS IN INTESTINAL CRYPT-VILLUS DIFFERENTIATION

Current challenges with ISC enhancers include understanding how they are chosen and maintained, and how they integrate external and cell-intrinsic signals to execute the ISC-specific transcriptional program and cell-specific functions. Molecular marker *Lgr5* allows isolation of murine ISCs to high purity (3), but cell yields are currently sufficient only to reveal open chromatin by ATAC-seq, hypomethylated DNA using WGBS, and selected histone modifications by ChIP-seq. Cell numbers remain limiting for robust ChIP-seq analysis of many TFs and for proximity-based DNA ligation methods to impute 3D genome organization. Accordingly, detailed understanding of ISC enhancers lags behind that of the sites active in mature villus and proliferative crypt cells (20). Cell numbers are also low in intestinal organoid cultures, and because organoid differentiation occurs to an unknown extent under nonphysiological conditions, here, we limit discussion to in vivo findings (Table 1).

Human Caco-2 cells partially mimic intestinal epithelial turnover, with replicating subconfluent cells resembling the ISC/progenitor pool and postconfluent cultures resembling differentiated cells. The histone mark H3K4me2 identifies some sites that are common to both cell states and additional thousands that are highly enriched in one or the other state; different DNA sequence motifs are enriched in the two groups of state-specific sites, suggesting that ISC and mature cell functions reflect combinatorial TF actions at distinct enhancers (69). Differences in enhancer states are less stark between mouse small intestine ISCs, crypt progenitors, and villus epithelial cells, where H3K4me1/2, H3K27ac, and open chromatin profiles are strikingly similar, especially between ISCs and the enterocyte lineage (37, 40). These findings imply that in vivo the *cis*-element repertoire for differentiated cell functions is largely established in Lgr5$^+$ ISCs and that differentiation into postmitotic enterocytes is dominated by activation of these

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Table 1. *Summary of epigenetic regulatory changes in the intestinal epithelium*

Epigenomic Feature	Intestinal Epithelial Process	References
3D chromatin looping	TADs are thought to be stable across cell types	
	Genome-wide 3D chromatin interactions have not been mapped in the intestine	
Chromatin accessiblity	Generally similar across intestinal epithelial cell populations	37, 40
	Secretory cells have specific accessible regions bound to secretory cell-specific TFs	32, 49
	These regions lose chromatin accessiblity when secretory cells dedifferentiate into ISCs	32
Histone Modifications		
H3K4me1/2, H3K27ac	Overall similar across intestinal cell lineages	37, 40
	Differences correlate with dynamic gene expression	69
H3K27me3	Associated with promoters of silent genes and largely stable across cell types. About 200 ISC-specific genes show increases H3K27me3 levels in differentiated cells	31
	Loss of H3K27 methyltransferase activity permits reexpression of developmentally silenced genes, but ISC-specific genes are not reactivated in mature cells.	31
DNA methylation	Majority of enhancers have similar methylation profiles across intestinal epithelial cell types	30, 35
	Small differences occur at a subset of cell type-specific genes	58

ISC, intestinal stem cells; TADS, topological associating domains; TFs, transcription factors; 3D, three-dimensional.

existing sites, with a smaller contribution from TFs generating access to new enhancers.

Adding to the evidence for limited enhancer dynamics, meCpG profiles defined by WGBS differ little between adult ISCs and mature villus enterocytes (30, 35) or colonocytes (73). Statistical comparisons of DNA hypomethylation are, however, less robust in areas of low CpG density, and another study found discernible meCpG differences at enhancers near 10% to 15% of ISC- and enterocyte-selective genes (58); thus, many intestinal enhancers are, indeed, dynamic. When the methyltransferase DNMT1 is absent from the intestine, meDNA is globally reduced, crypt cell proliferation is increased (22), and differentiation is notably impaired (73) in young mice. Additional loss of another methyltransferase, DNMT3B, results in lethality (21), a phenotype that is not established to reflect defective enhancer function. In contrast to the single-base resolution of WGBS, immunoprecipitation of hydroxymethylated DNA returns data at lower, ~150-bp resolution. The latter method identifies thousands of differences between crypt stem/progenitor and villus cell populations in hydroxymethylated DNA at coding regions, largely correlated with gene activity (39, 65). These changes during differentiation are mediated by the same enzymes that demethylate DNA, but only a minority localize at distant enhancers, where the role in gene regulation remains unclear.

Gene expression evolves rapidly during development, requiring enhancers to be recruited and decommissioned in quick succession. Enhancers active during development subsequently lose accessible chromatin, TF occupancy, and H3K27ac marks, but may retain hypomethylated DNA and trace H3K4me1 over scores to hundreds of cell divisions, well into adulthood (28). As a result, the complement of hypomethylated sites in adult intestinal cells encompasses not only active enhancers but also a majority of those used during organogenesis (30). Genomic regions that carry H3K4me1 but lack H3K27ac are regarded as "poised" for future activity (18). However, their numbers in short-lived intestinal epithelium exceed those of active $H3K27ac^{+}$ enhancers, and rather than poised *cis*-elements, this large pool of $H3K4me1^{+}$ $H3K27ac^{-}$ sites with hypomethylated DNA appears to be a vestige of fetal *cis*-regulatory activity (18).

ATAC-seq analysis of purified adult enteroendocrine and goblet cell precursors subsequently revealed thousands of genomic regions that are selectively open in these secretory cells (i.e., inaccessible in ISCs or enterocytes), lie near lineage-restricted genes, and carry DNA sequence motifs that bind secretory-cell TFs (32). A recent study verified the broad base of equally open chromatin among mouse intestinal epithelial cells, with additional sites detected in secretory cells (49). As a group, these putative enhancers lack H3K27ac and have little H3K4me1, so their apparent activity in the secretory lineage (32) challenges the idea that those histone marks are essential for *cis*-element function. Active enhancers lacking canonical histone marks have also been detected in embryonic stem cells (47) and experiments in other cells and species suggest that H3K4 methylation is dispensable for gene activity in vivo (19, 50). One provocative possibility is that the purpose of "active" histone modifications is not to influence *cis*-element activity per se but to flag them during cell division ("mitotic bookmarking") (36). In principle, postmitotic secretory cells may, therefore, dispense with certain histone marks, while retaining the open chromatin necessary for TF binding and enhancer activity.

TFS IN INTESTINAL CRYPT-VILLUS DIFFERENTIATION

ISCs must express TFs that recognize DNA sequences in the desired genomic sites and bear domains that interact with crucial signals from the Wnt, Notch, and other pathways. These TFs' dynamic interactions, with external signals on one hand and specific *cis*-regulatory elements on the other, collectively determine ISC responses such as cell division, remaining in or leaving the ISC pool, and differentiation into enterocyte or secretory progenitors (Fig. 2). TFs that uniquely support ISC

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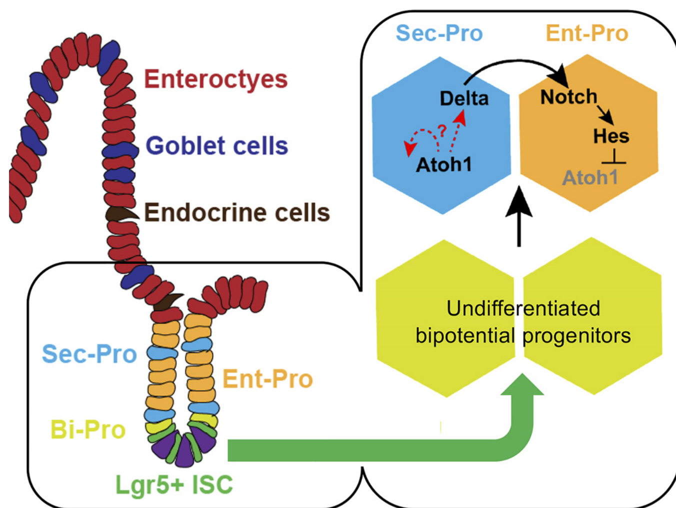

Fig. 2. Crucial cell fate decisions in intestinal epithelium. The choice between absorptive enterocytes (Ent) and secretory (Sec) cells is enacted in transient bipotential progenitor (Bi-Pro) cells very soon after intestinal stem cells (ISCs) leave the stem-cell compartment. This cell fate decision depends on signaling from cell-bound ligands of the Delta family to adjoining cells that carry Notch-family receptors. Notch signaling activates the transcriptional repressor Hes1, which tightly represses expression of the transcription factor (TF) gene *Atoh1*. Delta-positive cells increase *Atoh1* expression and adopt the Sec fate, while *Atoh1*-negative cells become enterocytes. [Adapted with permission from T.-H. Kim et al., *Nature* 506: 511–515, 2014.] Examples of TFs enriched in ISCs and different epithelial progenitors (Pro) are listed in Table 2.

functions are not as yet fully characterized. The Krüppel-like zinc-finger protein KLF5 is one of few crypt-restricted TFs (38), and the basic-helix-loop-helix factor ASCL2 is the only known TF tightly restricted to $Lgr5^+$ ISCs (66). The sequence motif for Krüppel-like TFs is particularly enriched among enhancers marked in ISCs, and depletion of KLF5 results in ISC failure, with precocious differentiation (38). Early findings suggested that *Ascl2* is similarly indispensable for ISC survival (66), but subsequent studies revealed only a subtle role in ensuring long-term integrity of the ISC compartment and a more overt requirement in allowing progenitors to dedifferentiate when ISCs are deficient (43, 57). Crucial transcriptional targets of KLF5 and ASCL2 have been hard to define, highlighting the technical challenges of ChIP-seq in ISC, and available data indicate that ASCL2 facilitates the intestine-specific response to Wnt signaling at many loci (57).

Enterocyte-active regions are most enriched for sequence motifs that bind AP-1, HNF4, CDX, KLF, and GATA-family TFs, and the epithelium expresses certain members of each family. Notable examples, such as the intestine-specifying homeodomain protein CDX2, its functional partners HNF4A and HNF4G, and region-specifying zinc-finger proteins GATA4 and GATA6, are expressed in most or all epithelial cells, including ISCs (5–7, 15, 54, 55, 69). CDX2 is present throughout the epithelium, but at least some target genes are different in ISCs and enterocytes (55). Absence of CDX2 profoundly impairs ISC replication, presumably acting through ISC-selective enhancers (55); additional absence of its homolog CDX1 blocks replication of both ISC and transit-amplifying crypt cells (69, 70). In contrast, TFs such as ATOH1, GFI1, SPDEF, NEUROG3, and NEUROD1 are restricted to single or all Sec cell types (25, 33, 40, 44, 59, 72) and likely interact selectively with Sec-restricted enhancers.

In summary, a handful of intestinal TFs (some restricted to ISCs, others expressed broadly) interact with available enhancers to fulfill ISC- and enterocyte-specific functions. Separately, ATOH1-mediated secretory differentiation activates thousands of lineage-restricted enhancers that presumably engage secretory cell-specific TFs to drive goblet, enteroendocrine, Paneth, and tuft cell identities and functions (Table 2). Additional ChIP, chromosomal conformation, and functional data are necessary to determine whether the panoply of intestinal TFs controls overlapping or distinct groups of enhancers and genes.

ROLES FOR GENE SILENCING

Gene regulation involves both activation and controlled repression. One histone modification classically associated with gene silencing, H3K27me3, is found predominantly near transcription start sites and is largely absent from distant enhancers (27). Its erasure reactivates certain developmentally silenced genes in many tissues and in embryonic stem cells (8, 31, 45). At least in the latter, chemical interference with RNA transcription causes H3K27me3 to appear at experimentally silenced promoters (51), raising the prospect that H3K27me3 does not "cause" transcriptional repression per se. Although thousands of transcripts are modulated during the crypt-villus transition in wild-type mice, genome-wide H3K27me3 distributions are substantively similar in ISCs and postmitotic villus cells, and gene activity correlates little with promoter H3K27me3 dynamics (31). Thus, the H3K27me3 profile is largely specific to the tissue and not to its constituent cell states, although ~200 ISC-selective genes, including *Lgr5* and *Ascl2*, acquire H3K27me3 locus-wide when cells exit the ISC compartment. Mouse intestines with epithelium-specific absence of the H3K27 methyltransferase complex PRC2 derepress hundreds of developmentally silenced genes within 3 or 4 days, but do not express H3K27me3-marked ISC genes in mature cells (31). Thus, when ISCs differentiate, PRC2-catalyzed H3K27me3 signifies, but does not cause, state-specific transcriptional silencing of canonical ISC genes.

Table 2. *Exemplary intestinal epithelial lineage-restricted transcription factors*

Transcription Factor
Expressed in all or most epithelial cells
Hnf4a
Hnf4g
Cdx2
Gata4
Gata6
Enriched in resting and regenerating ISCs
Ascl2
Enriched in enterocytes
Hes1
Klf5 (enriched in crypt cells, including ISCs)
Enriched in secretory-fated cells
Atoh1
Spdef
Gfi1
Neurog3(enriched in endocrine cells)
Neurod1(enriched in endocrine cells)
Klf4 (enriched in goblet cells)

ISC, intestinal stem cells.

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An unexpected finding after many days of PRC2 deficiency in adult intestines is extensive recovery of the archival memory encapsulated in hypomethylated developmental enhancers. Decommissioned fetal enhancers, which usually bear traces of H3K4me1 and ATAC sensitivity, reactivate sooner than embryonic enhancers, where reduced meCpG is the only known residual feature (30). Linked genes become expressed as a result of this developmental *cis*-element reactivation, but PRC2 activity being largely absent from adult or embryonic enhancers, this striking consequence of its absence must be indirect. Developmental TFs activated early as a direct result of promoter H3K27me3 erasure likely later reactivate the dormant tissue-specific fetal and embryonic enhancers (30).

AN EPIGENETIC BASIS FOR CRYPT CELL DEDIFFERENTIATION?

Intestinal crypts' ability to restore ablated Lgr5-positive ISCs is well recognized. "Reserve" stem cells located in the 4th to 6th crypt tiers (+4 cells) were regarded as a principal source of ISC recovery (46, 56), but the evidence now favors various crypt progenitor cells—recent progeny of Lgr5-positive ISC that survived ISC ablation—as the predominant source. Both secretory (10, 32, 34, 56, 63, 67, 71, 74) and absorptive (61) cells harbor the latent capacity to revert into ISCs, raising the question of what chromatin barriers these cells overcome to accommodate the altered state. An additional, intriguing possibility is that initial ISC differentiation is inherently unstable, with crypt base cells shuttling between ISC and post-ISC states until one stabilizes. As noted above, chromatin states signified by H3K4me1/2, H3K27ac, and H3K27me3 marks are substantively similar along the ISC-enterocyte continuum. This similarity may reflect a low chromatin barrier for toggling between cell states and, thus, explain the remarkable ability of specified progenitors to revert quickly into $Lgr5^+$ ISC; thus, both ISC differentiation and dedifferentiation may reflect TFs' alternative interactions with a broadly permissive chromatin landscape. The appeal of this idea notwithstanding, when Sec cells dedifferentiate in response to ISC ablation, the chromatin at thousands of putative Sec-restricted enhancers becomes inaccessible within 36 h to 48 h (32). Thus, these areas of open, unmarked chromatin in differentiated cells do not represent a barrier that cells find difficult to breach. Adoption of a "different" tissue's chromatin landscape is rare (e.g., gastric intestinal metaplasia), but chromatin transitions that occur naturally within the adult intestinal epithelium seem to reverse readily.

DELINATION OF INTESTINAL ENHANCERS AND THEIR INTERACTIONS WITH TFS

ISC functions depend on well-known signaling pathways, including Notch, Hippo, and, most famously, Wnt. However, the same pathways control diverse processes in nearly every organ, with vastly different outcomes arising through the same transcriptional effectors: TCF/β-catenin, RBPJK, and TEAD protein complexes for Wnt, Notch, and Hippo signaling, respectively. How do cells craft unique transcriptional responses to the same signal? One solution lies in the repertoire of enhancers available to the global effector complexes: chromatin is accessible at different sites in intestinal than in other cells. Consistent with this idea, one ChIP study of the Wnt-effector protein TCF7L2 in cell lines from different tissues found that only 1.6% of 116,000 binding sites were present in all six lines (23). Therefore, adult tissue responses to external signals are dictated in large part by the stable, tissue-specific enhancer repertoires established during development. The intestinal chromatin landscape is initiated, in part, through the actions of CDX2 in the early embryo (2, 42), then reshaped late in gestation toward a nearly mature chromatin landscape by concerted actions of CDX2 and HNF4 factors (14). BMP signaling is an important determinant of adult crypt-villus differentiation, suppressing expression of ISC-specific genes in crypts (48) and cooperating in the villus with HNF4 factors, via the BMP-effector SMAD4, to promote enterocyte differentiation (15).

FRONTIERS OF EPIGENETIC REGULATION IN ISCS

Much remains to be learned in biochemical terms about how *cis*-elements assimilate diverse cues to implement crypt- and villus-specific signal-responsive gene expression. For example, do the TFs that direct Wnt and BMP responses, such as CDX2 (55), ASCL2 (43, 57), and HNF4 (15), stabilize binding of the corresponding signal effectors at cognate enhancers? Are discrete enhancers strengthened as a result, and is that modulation manifested in the amplitude or the frequency of transcriptional bursts? Mapping of the intestinal enhancer repertoire paves the way to characterize chromatin dynamics in response to specific physiologic and pathologic signals.

The intestinal epithelium responds to changes in diet, age, microbes, and immune activation. In most cases, it is unknown whether these responses occur in mature or progenitor cells and whether they involve epigenetic reprogramming or TF interactions with a fixed epigenome. Chromatin accessibility is largely similar in the epithelium of conventionally raised and germ-free mice (12), although responses to specific microbes have not been investigated in detail, and another group identified limited differences in methylation of selected CpG residues in germ-free mice (73). Dietary perturbations produce dynamic metabolic changes that may alter the epigenome. For example, ketone bodies that accumulate during fasting may inhibit histone deacetylases and, thereby, promote ISC renewal (16). However, dietary and metabolic influences on the intestinal epigenome remain largely unexplored, as do the effects from acute inflammation, compromised barrier function, and chronic immune conditions. These questions merit mechanistic investigation layered upon the growing understanding of interactions between canonical intestinal TFs and cell-specific epigenome states summarized in this article. Finally, noncoding RNAs are believed to regulate and mediate certain epigenome changes (13, 41). The intestinal epithelium expresses hundreds of microRNAs, some very selectively compared with other endoderm-derived tissues (24), and their biological functions have been reviewed previously (53). Examples such as miR-31 are necessary for ISC activity and regeneration (64), but it remains unclear if those requirements represent direct regulation of target transcripts or some epigenome process. Further investigation in vivo or in organoid models will shed light on these questions and, more broadly, on diverse epigenetic bases for human intestinal disorders.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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AUTHOR CONTRIBUTIONS

M.P.V. and R.A.S. drafted manuscript; M.P.V. and R.A.S. edited and revised manuscript; M.P.V. and R.A.S. approved final version of manuscript.

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