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MicroRNAs Control Intestinal Epithelial Differentiation, Architecture, and Barrier Function

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Abstract

Background and Aims—While the importance of miRNA for the development and maintenance of several tissues is well established, its role in the intestine is unknown. Our aims were to determine the entire miRNA expression profile of the mammalian small intestine in a quantitative manner and to determine the contribution of miRNAs to intestinal development and homeostasis using genetic means.

Methods—We determined the complete miRNA transcriptome of the mouse intestinal epithelium using ultra-high throughput sequencing. We employed gene ablation of *Dicer1* to generate mice deficient for all miRNAs in the mouse intestine.

Results—miRNA abundance varies over a large dynamic range in the mammalian small intestine, from one read per million to 250,000. Of the 453 miRNA families identified, mmu-miR-192 and mmu-let-7 are the most highly expressed. Morphologically, the epithelium of *Dicer1*^{loxP/loxP}; Villin-Cre mutants mice is disorganized in both the small and large intestine, with a four-fold decrease in goblet cells in the colon and a dramatic increase in apoptosis in the crypts of both the large and small intestine. Furthermore, intestinal barrier function is dependent on the presence of miRNAs, and consequently *Dicer1* deficient mice display intestinal inflammation with lymphocyte and neutrophil infiltration.

Conclusion—We have identified all intestinal miRNAs and shown using gene ablation of *Dicer1* that miRNAs play a vital role in the differentiation and function of the intestinal epithelium.

Keywords

miRNA; Intestinal Epithelium; Epithelial Barrier Function

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The authors declare that no conflict of interest exists.

Author contribution:

LBK: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript

JS: analysis and interpretation of data

JRS: study concept and design; analysis and interpretation of data, drafting of the manuscript

JBK: acquisition of data

KHK: study concept and design; critical revision of the manuscript for important intellectual content; obtained funding; study supervision

Introduction

MicroRNAs (miRNAs) are 19–25 nt single-stranded RNA molecules that play a role in modulating the activity of thousands of genes. miRNAs can decrease expression of target mRNAs by binding to their 3'UTRs, leading to mRNA degradation, or by translational inhibition^{1, 2}. It has been proposed that more than one third of human genes are regulated by miRNAs³.

The synthesis of mature miRNAs is complex. Primary miRNAs (pri-miRNAs) are transcribed by RNA polymerase II and cropped by the RNase III-type enzyme Drosha to a precursor miRNA (pre-miRNA), which forms a ~70 nt stem-loop structure⁴. Pre-miRNAs are then exported from the nucleus and cleaved by the RNase III-type enzyme Dicer to form the mature and functional miRNA, which is loaded onto the Argonaute protein in the miRNA-containing RNA-induced silencing complex (RISC)⁴. Because Dicer is obligatory for miRNA processing, the inactivation of this single gene by conditional gene ablation has been utilized to study global miRNA function in several organ systems and cell types^{5–9}.

miRNAs have been shown to play central roles in several important developmental and disease states ranging from larva formation in *Drosophila* to regulation of cancer progression in humans. In the intestine, thus far the main focus has been on the role of miRNAs in colorectal cancer^{10, 11}. In addition, specific miRNAs have been implicated in ulcerative colitis and intestinal epithelial cell differentiation^{12–14}.

The precise and complete miRNA transcriptome of most organ systems is not known. Only a relatively shallow survey, with ~10 to 1,000 miRNA sequences per organ system, has been reported based on sequencing of cloned miRNAs¹⁵. Thus, only the most abundantly expressed miRNAs were captured in this study. The 'colorectal microRNAome' has been described using miRNA serial analysis of gene expression (miRAGE), which also had a very limited sequencing depth due to technical limitations¹⁶. Thus, a comprehensive atlas of miRNAs expressed in the intestinal epithelium has not yet been reported.

Here we aimed to determine the comprehensive and precise miRNA transcriptome of the small intestinal epithelium of the mouse using ultra-high throughput sequencing. We also evaluated the contribution of miRNAs to intestinal differentiation and function using conditional gene ablation of *Dicer1*, the gene encoding the obligatory miRNA-processing enzyme, in the epithelium of the small and large intestine.

Results

In order to define the miRNA transcriptome of the small intestinal epithelium, small RNAs from purified jejunal epithelia of adult wild-type mice were isolated and quantified using ultra-high throughput sequencing. The resulting sequence reads were aligned to known miRNA precursor genes, obtained from miRBase¹⁷, in order to assess the abundance of each mature miRNA. Next, we verified that these sequence reads represented miRNAs and not degraded mRNAs by aligning them to the RefSeq database. As shown in Figure 1A, less than 20% of reads in the miRNA size range aligned to mRNAs, while more than 90% matched to precursor miRNAs, indicating that our small RNA preparation was highly enriched for true miRNAs. In total, we generated 15.3 million trimmed reads in the range of 19–25 nts that aligned to precursor miRNAs. Using these reads, we found evidence for expression of 606 of the 1,094 (55.4%) known or predicted mature miRNAs that represent 573 distinct families and cover 399 of 574 (69.5%) pre-miRNAs. (Fig 1B, Supplemental Table 1). Included in the 573 miRNA families expressed in the small intestinal epithelium are those with known functions in intestinal disease, such as miR-194, and the miR-200 and let-7 families (Fig. 1B)^{11, 14, 18}.

The extraordinary dynamic range (about six orders of magnitude) of the technology used allowed us to detect and quantify miRNAs present in a few copies per million as well as those that contribute up to ~30 percent of the total miRNA pool, i.e. mmu-miR-192. Because of technical limitations of prior efforts, many of the miRNAs identified here had been missed in previous studies¹⁶. As the intestinal epithelium is made up of multiple cell types of varying abundance, without detailed in situ hybridization analysis for all 606 intestinal miRNAs we cannot determine if they are expressed uniformly in all intestinal epithelial cells, or whether they might be differentially expressed in rarer cell-types such as enteroendocrine cells or stem cells. Nevertheless, our complete miRNA profile of the small intestinal epithelium dramatically extends prior knowledge in this field, and represents a rich resource for future investigations.

Next we searched for evidence of novel miRNAs by analyzing clusters of ten or more reads aligned to the genome that did not overlap with known small RNA genes, e.g. tRNAs, SNORDS, etc. However, none of these clusters appeared to represent novel miRNAs. This suggests that miRBase is approaching coverage of all miRNAs. We did, however, find support for 94 mature miRNAs (based on a minimum of two trimmed reads in our collection) that have been predicted by their precursor miRNAs, but are not presently included in miRBase. In addition, when we analyzed the 187 pre-miRNAs with at least 100 aligned trimmed reads, 113 (60%) showed evidence of miRNA editing, predominantly at the 3' end (data not shown).

Next, we wanted to determine if and to what degree miRNAs contribute to the differentiation and viability of the intestinal epithelium. We derived mice lacking functional miRNAs in the intestinal epithelium by crossing *Dicer1*^{loxP/loxP} conditional mutant mice to Villin-Cre transgenic mice^{5, 19}. qPCR analysis confirmed the effective deletion of *Dicer1* in the small intestinal epithelium at both three and ten weeks of age (Fig. 2A). In addition, we confirmed the ablation of *Dicer1* at the functional level by determining the abundance of two intestinal miRNAs, mmu-miR-21 and mmu-let-7b (Fig. 2A). Both were dramatically but not completely reduced, reflecting residual *Dicer1* and miRNA expression in cell populations where the Villin-Cre transgene is silent, such as mesenchymal or immune cells. This notion is supported by the fact that expression of mmu-let-7b, which is thought to be present in all cell types, is reduced by a smaller percentage than mmu-miR-21, which is enriched in epithelial cells, in our mutant mice²⁰. Collectively, these results indicate that *Dicer1* is functionally ablated in the intestinal epithelium of *Dicer1*^{loxP/loxP}; Villin-Cre mice.

Dicer1^{loxP/loxP}; Villin-Cre mutants appeared normal at birth and were born in the expected Mendelian ratio (data not shown). Some severely affected mutants, approximately 10% of the total, did not survive to weaning and displayed severely stunted growth (data not shown). Mutant mice that survived fed normally but were significantly smaller than their littermate controls beginning at p10 (Fig. 2B,C). This growth impairment continued through weaning (p21), when the food source is switched from the mother's milk to starch-based chow. After approximately two weeks on normal chow, surviving mutants began to catch up in weight with their control littermates, becoming indistinguishable in size by seven weeks of age (Fig. 2B). In conjunction with impaired growth, pre-weaned pups had noticeably pale and loose stool. Oil-red-O staining on fecal smears from pre-weaned (p19) mutants showed the presence of large fat droplets, which were absent from control stool (Fig. 2D). Once weaned and subsisting on normal mouse chow, which has only 13.5% kcal from fat as compared to the 80% kcal from fat in mouse milk²¹, mutant mice no longer produced fatty stool (data not shown). However, when we placed *Dicer1*^{loxP/loxP}; Villin-Cre mice on a "Western Diet" of 45% kcal from fat, mutants again had markedly increased levels of fat in their stool as compared to controls, even after only two days on the diet (Fig. 2D). Thus,

Dicer1^{loxP/loxP}; Villin-Cre mutants have a severely impaired ability to process dietary triglycerides. In addition, adult mutants have ~20% higher percent mass of water in their stool as compared to controls, indicative of decreased water absorption in the colon (Fig. 2E).

The *Dicer1*-deficient intestine differs from that of controls not only functionally but also morphologically. In the small intestine, the villi appeared normal; however, the crypt zone was markedly expanded and the lamina propria appeared more cellular (Fig. 3A,B). In the colon, the regular crypt structure was disorganized in *Dicer1*-deficient mice, and a more densely packed lamina propria was present between crypts (Fig. 3C,D). There was also a four-fold decrease in the number of mucus filled goblet cells in the colon, which was verified by Alcian blue staining (Fig. 3I–K). Thus, epithelial miRNAs are necessary to maintain normal intestinal architecture and goblet cell number. There was also a drastic increase in the number of apoptotic cells as shown by TUNEL staining in the lower crypt zone of both the small and large intestine of *Dicer1* mutants as compared to controls (Fig. 3E–H).

Next, we aimed to link the intestinal phenotype of *Dicer1*-mutants to dysregulation of specific classes of mRNAs. While miRNAs affect protein translation, in many cases they also regulate mRNA abundance^{1, 2}. We employed oligonucleotide microarray analysis to identify differentially expressed protein-coding genes. We identified 3,156 differentially expressed genes in the small intestinal epithelium of *Dicer1* mutants (see Experimental Procedures for details, and Supplemental Fig. 2). Next, we identified enriched KEGG Pathways using Gene Set Enrichment Analysis (GSEA), and grouped the genes into categories (Fig. 4A, Supplemental Fig. 2)^{22–24}. The most significantly changed pathways in each category are listed in Supplemental Fig 3. Surprisingly, differentially expressed genes in immune pathways were the largest category of genes affected in *Dicer1*-deficient mice.

Based on our mRNA expression profiling data, we decided to further investigate immune pathways in *Dicer1* mutants. An important component of intestinal defense against luminal pathogens are neutrophils in the lamina propria. There was an increase in the number of neutrophils in the lamina propria in both the small and large intestine, with a more dramatic phenotype in the large intestine (Fig. 2A–D). Low magnification images demonstrate a dramatic increase in lymphoid nodules in the large intestine of mutants as compared to controls (Fig. 4B,C). In the large intestine of *Dicer1* mutant mice there was an increase in neutrophil number in the lamina propria at the base of the crypts as compared to controls, in addition to a small number seen infiltrating the colonic epithelium (Fig. 4F,G). Increased inflammation has been linked previously to loss of goblet cells²⁵, Makkink MK, 2002 #50 and is a likely explanation for the four-fold decrease in goblet cells in *Dicer1* mutants described above.

To investigate potential causes for the redistribution and increase in immune cells in *Dicer1*-deficient mice, we next analyzed epithelial maintenance and tight junctions in the small intestine of the mutants, as these act as part of the physical barrier that prevents luminal pathogens from entering the blood stream. Claudin-7, a component of tight junctions, was localized to the basolateral membrane of epithelial cells in the control large intestine, as has been described previously (Fig. 5A)²⁶. Claudin-7 was mislocalized in the large intestine of *Dicer1*^{loxP/loxP}; Villin-Cre mutants, with all cell surfaces staining positive for the protein (Fig. 5B). An important aspect of the intestinal barrier function is proper epithelial cell arrangement and organization. In the control large intestine, the epithelial nuclei are uniformly aligned parallel to the apical surface (Fig. 5A; insert). This was not the case, however, in the mutant colon, where the epithelial layer was disorganized and the nuclei distributed on different planes throughout the epithelium (Fig. 5B; insert). In addition to

Claudin-7, we investigated the localization and expression of Claudin-4, a functional component of tight junctions in the small intestine. In the control small intestine Claudin-4 is expressed in puncta, a majority of which line the apical membrane, with a few distributed on the intra-cellular and basolateral membranes (Fig. 5C). In contrast, in the small intestine of mutants there was a decrease in Claudin-4 positive tight junctions (Fig. 5D).

In order to determine the consequence of the observed epithelial disorganization in the *Dicer1* mutants, we measured the intestinal paracellular permeability. Lactulose and mannitol are non-digestible carbohydrates that cross the epithelium via paracellular routes and are thus useful tools to measure intestinal barrier function^{27, Travis S, 1992 #48}. We fed radioactively labeled mannitol and lactulose to three month old mice by gastric gavage and determined their rate of transfer to circulation by periodic sampling of peripheral blood. As shown in Figure 5E, the rate of epithelial crossing of both lactulose and mannitol was dramatically increased in *Dicer1*-deficient mice, clearly demonstrating decreased intestinal epithelial barrier function in these mice.

Discussion

In this study, we have provided the most comprehensive description of the miRNA transcriptome of the mammalian small intestinal epithelium to date, and established that miRNAs are essential for the proper differentiation of intestinal cell types and the maintenance of intestinal barrier function. Intestinal epithelium-specific *Dicer1*-deficient mice, which are devoid of all mature miRNAs, are growth retarded, unable to efficiently absorb fat in their diet, and have loose stool. Morphologically, *Dicer1* mutants display an expanded crypt zone in the small intestine and a decreased goblet cell number, predominantly observed in the large intestine.

On a genome-wide level, the largest functional group of genes differentially expressed in *Dicer1* mutants was that of genes involved in immune pathways. *Dicer1* mutants have a disorganized epithelium in both the small and large intestine and dramatically impaired epithelial barrier function. The increase in neutrophils in both the small and large intestine of *Dicer1*^{loxP/loxP}; Villin-Cre mutants, is a likely in response to this decreased barrier function.

Using microarray-based expression profiling, we identified several members of the Bone Morphogenetic Protein (BMP) gene family as significantly changed in *Dicer1* mutants (Supp. Fig. 3). Of these, three have known functions in intestinal epithelial cells and are predicted targets of miRNAs that we have identified as expressed in the intestinal epithelium. A decrease in BMP2, potentially targeted by mmu-miR-10a, has been shown to decrease apoptosis and cell-cell interaction in mature epithelial cells, suggesting a potential role as a tumor suppressor in colon cancer²⁸. Expression of another family member, BMP3, has not only been shown to be lost in various colon cancer cell lines, but hypermethylation of its promoter is reported in tissue samples from pre-cancerous polyps and malignant tumors^{28–30}. In addition to implications in colon cancer, expression of BMP7, potentially targeted by mmu-miR-22, mmu-miR-375, and mmu-miR-145*, another gene affected by the absence of *Dicer1*, has been shown to decrease the tissue damage in rats subjected to chemically induced colitis³¹. Not only are these BMP family members differentially expressed in our array study, they were all predicted targets of multiple miRNAs we identified in intestinal epithelial cells (Fig. 4H).

In addition to basic signaling molecules, differentially expressed transcription factors are also important candidate targets of miRNA regulation. Multiple members of the Krüppel-Like Factor family of Zinc-finger transcription factors were differentially expressed in *Dicer1* mutants (Fig. 4H). However, neither Klf10 nor Klf15 have known functions in

intestinal epithelial cells. These genes were, however, predicted targets of a miRNA expressed in the small intestine epithelium, mmu-miR-429. *Klf9* was up-regulated in *Dicer1* mutants, and is known to be down-regulated in the epithelium in human colon cancer, but its role in this tissue remains to be established, and is potentially targeted by mmu-miR-215 and mmu-miR-93³².

In summary, we have established that miRNAs play multiple important roles in the intestinal epithelium. This was not a foregone conclusion, because the function of hepatocytes, another endoderm derived cell type, is largely independent of microRNAs⁶. While previous studies have focused on the role of miRNAs in colon cancer, here we show new functional roles for miRNAs in epithelial organization and barrier function. Further studies are necessary to isolate individual roles of specific miRNAs in the intestinal epithelium and the genes they target, but this study identified the entire spectrum of their expression, providing the complete set of candidate miRNAs for further study.

Methods

Identification and quantification of miRNAs

The small intestinal epithelium was scraped from longitudinally sliced intestine of CD1 mice (n=4), and small RNAs were isolated using the *mirVana* miRNA kit (Ambion Cat# AM1560). Small RNAs were prepared for sequencing using the DGE-Small RNA sample prep kit (Illumina FC-102-1009). Clusters were generated (Illumina FC-103-1009) and sequenced on a Genome Analyzer II (Illumina FC-104-1003) using a read length of 36 nucleotides. The 3' Illumina adaptor sequence was matched against the end of each read, allowing for one, two or three mismatches as the match length increased. The adaptor sequence was trimmed and the reads grouped by length. The trimmed reads were then aligned to precursor miRNA sequence from miRBase (release 13.0), RefSeq sequence (downloaded from NCBI 10/28/2008) and the mouse genome (mm8, NCBI build 36) with up to 2 mismatches (as indicated below) using Illumina's ELAND aligner (from GA Pipeline v1.0). Because not all of the miRBase pre-miRNA sequences are annotated with both (or any) mature products, we imputed these features as necessary using the deepest loop or bulge to delimit the 5' arm from the 3' arm.

To compute expression levels, we grouped miRNA mature features into families when they shared a perfectly matching trimmed read with a length between 19 and 25 nt. This is a slight refinement of the miRBase families. For example, many members of the let-7 family can be distinguished by a single base difference, though miRBase groups them all together. Expression values were then computed as the total number of trimmed reads that align to any member of the family with up to two mismatches. When trimmed reads hit multiple families, the counts for those reads were spread across the families in proportion to the number of unambiguously assigned reads. The average expression was calculated as the weighted average of the reads per million of the two technical replicates (25% each) and the biological replicate (50%).

To identify novel miRNAs, we identified regions of the genome that contained at least ten overlapping trimmed reads and rejected those that overlapped RNA genes or other exons using the UCSC repeatmasker table (which includes many of the repetitive RNA genes) and the exons from the Ensembl table.

To identify instances of miRNA editing, we allowed up to two mismatches when aligning trimmed reads to pre-miRNAs, but considered only those trimmed reads that aligned to a single pre-miRNA. We required at least 100 total trimmed reads for a pre-miRNA to estimate the prevalence of editing.

Mice

The *Dicer1^{loxP}* mice³³ were a gift from Matthias Merckenschlager and the Villin-Cre mice¹⁹ were kindly shared with us by Deborah Gumucio. Genotyping was performed by PCR analysis using genomic DNA isolated from the tail tips of newborn mice. All procedures involving mice were conducted in accordance with approved Institutional Animal Care and Use Committee protocols.

Fecal Analysis

Fecal samples (n=6) were collected and smeared onto glass slides and stained with Oil-Red-O to identify the presence of triglycerides. Fecal water percent mass was measured by comparing the mass of fresh fecal samples (n=6) to their mass after 24hr drying time at 37°C.

Expression Analysis

Dicer mRNA expression was measured using quantitative RT-PCR, as previously described³⁴. miRNA levels were measured using the appropriate Taqman (Applied Biosystems) kits, using Sno202 as reference gene (Applied Biosystems: mir-21 4373090, let-7b 4373168, Sno202 4380914). (Primer sequences are available <http://www.med.upenn.edu/kaestnerlab/>)

Histological Analysis

Immunofluorescence was conducted as previously described³⁵ with the following antibodies: CD3 1:200 (rabbit anti-mouse Labvision cat # RM-9107), CD45R 1:1000 (Rat anti-mouse Pharmingen cat # 550786), and Claudin-7 1:200 (Rabbit anti-mouse Labvision cat # Rb-10284). TUNEL staining was done according to protocol of the Apoptag Plus peroxidase *In Situ* apoptosis kit (Millipore, S7101)

Microarray Analysis

Total RNA was isolated from small intestinal epithelium from control and *Dicer1^{loxP/loxP}*;Villin-Cre mice using the *mirVana* miRNA kit (Ambion Cat# AM1560). Fifty ng of each RNA sample (four pairs) was amplified and labeled using the Agilent QuickAmp kit. Samples were hybridized to the Agilent Whole Mouse genome array (4×44K). Of the four biological replicates, two were labeled with Cy3 (Test) and the other with Cy5 (Control) and the other two with Cy5 (Test) and Cy3 (Control), eliminating variations introduced by any dye bias. Labeled samples were purified using the CGH Cleanup Column (Invitrogen) and hybridized overnight to the Agilent 4X44 Whole Mouse Genome Array. After hybridization the arrays were washed and immediately scanned with the Agilent DNA microarray Scanner, Model G2565B (Agilent Technologies). Median intensities of each element on the array were captured with Agilent Feature Extraction version 10.5 (Agilent Technologies).

The data were normalized by the print tip loess method using the LIMMA (Linear models for microarray data) package in R as described³⁶, Gentleman, 2005 #2. For statistical analysis, genes were called differentially expressed using the Significance Analysis of Microarrays (SAM) one class response package with a false discovery rate (FDR) of 10%³⁷ and a minimum fold change of 1.5x. Genes marked as absent, i.e. with expression levels near background, were omitted. miRNA target predictions were downloaded from the miRBase website¹⁷.

Intestinal Permeability

Adult mice (n=4) were fasted 3 hrs and then gavaged with five hundred microliters of fluid containing unlabeled lactulose and mannitol (Sigma) at 5.5 mM each in water and ten μ Ci of [3 H] lactulose (1 mCi/mL) and five μ Ci of [14 C] mannitol (100 μ Ci/mL) (American Radiolabelled Chemicals). Fifteen microliters of plasma were collected via tail-vein at time-points of zero, thirty, sixty, ninety, and one hundred and eighty minutes and [3 H] lactulose and [14 C] mannitol radioactivity determined by liquid scintillation counting. Adapted from ³⁸.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The miRNA transcriptome of murine small intestinal epithelium

(A) Small RNAs between 19 and 25 nucleotides in length, the size of known miRNAs, align to both miRBase and the mouse genome (mm8) but not to mRNA sequences from RefSeq. (B) The fifteen most abundant miRNAs, with relative expression levels expressed as reads per million.

Figure 2. Mice with conditional ablation of *Dicer1* in the intestinal epithelium display impaired growth, fat absorption, and water retention
 (A) Cre/loxP-mediated gene ablation of *Dicer1* was verified by determination of expression levels of *Dicer1*, mmu-miR-21, and mmu-Let-7b in the intestinal epithelium of control (*Dicer1*^{loxP/+}) and mutant (*Dicer1*^{loxP/loxP};Villin-Cre) mice. (B) *Dicer1* mutants are growth retarded from ten to fifty days of age, but regain weight thereafter to catch up with controls when fed normal chow (* $p < .05$). (C) Size comparison of representative pre-weaned (p19) mutant and control littermate. (D) Oil-RedO staining of fecal smears showing fatty stool in mutant pups pre-weaning (p19), and adult mutants on high fat chow as compared to controls. (E) *Dicer1* mutants fail to absorb water in the colon, as evidenced by the high water content of their stool compared to control littermates (* $p < .05$).

Figure 3. Intestine-specific *Dicer1* mutants display an expanded crypt zone in the small intestine, an increase in apoptotic cells and a reduction in goblet cell number in the colon

Adult *Dicer1* mutants (B) have an expanded crypt zone (noted by *) in the small intestine (hematoxylin & eosin stained sections) as compared to littermate controls (A). The colonic crypts of adult *Dicer1* mutants (D) are disorganized compared to control mice (C). There is an increase in apoptotic cells, as shown by TUNEL staining, in both the small and large intestine of the mutants (F,H) as compared to controls (E,G). There is also a pronounced decrease in mucus-filled goblet cells in *Dicer1* mutants as compared to control in the colon, as shown by Alcian blue staining (I–K) (***) ($p < .001$).

Figure 4. Increased inflammation in the intestine of *Dicer1* mutants

(A) Global gene expression profiles of the small intestinal epithelium of *Dicer1* mutants and controls were determined using the Agilent Whole Mouse Genome Array. The differentially expressed genes were sorted into KEGG pathways using Gene Set Enrichment Analysis, and the differentially activated pathways combined into functional groups. Immune pathways made up a third of the differentially expressed genes. (B,C) Low magnification (4x) image of large intestine stained for B cells (CD45R) shows drastic increase in lymphoid nodules. (F,G) In the large intestine *Dicer1* mutants (G) there is an increase in the number of B cells as compared to controls (F).

Figure 5. *Dicer1* mutants exhibit a disorganized epithelium and decreased tight junctions leading to increased intestinal permeability

(A) Claudin-7 staining shows an extremely disorganized colonic epithelium of *Dicer1* mutants (B) as compared to controls (A) insert shows details at higher magnification. (C,D) Claudin-4 staining marks tight junctions in the small intestine. There is a decrease and disorganization of tight junctions in the small intestine of *Dicer1* mutants (D) as compared to controls (C). (E,F) *Dicer1* mutants have increased intestinal permeability as shown by lactulose (E) and mannitol (F) absorption (* $p > .05$, ** $p > .01$, *** $p > .0001$).

Table 1

Transcription Factors and Signaling Molecules Targeted by Intestinal miRNAs and Up-Regulated in Dicer1 Mutants

Transcription Factors and Signaling Proteins	mRNA Fold Change	Targeting miRNAs (Reads per million)
Tcfcp2l1	5.53	miR-25 (2313), miR-145 (9321)
Reg3g	4.52	miR-23a (1402), miR-23b (876), miR-130a (244), miR-130b (134)
Hoxb9	3.56	mir-150 (105)
Reg3b	2.73	miR-23a (1402), miR-23b (876), miR-805 (909)
Tnfaip3	2.09	miR-31 (1945)
Elf4	1.67	miR-22 (1099)
Onecut2	1.52	miR-33 (139), miR-375 (1244)