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Role of MicroRNA-155 in a Mouse Model of Colon Cancer.

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ROLE OF MICRORNA-155 IN A MOUSE MODEL OF COLON CANCER.

by

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ABSTRACT

Colorectal cancer remains the third most common malignancy and the fourth most common cause of cancer mortality worldwide. Dysregulated miRNA levels are associated with several types of malignancies and may serve as important biomarkers and/or therapeutic targets in colorectal cancer. We examined the role of miRNA-155 on tumorigenesis and associated symptoms using a well-characterized mouse model of colorectal cancer. C57BL/6 wild-type mice and miRNA-155-/- mice (n=7-13 group) were given a single injection of AOM (10mg/Kg) followed by 3 cycles of DSS (2% in the water for 1 week followed by 2 weeks of plain water). A C57BL/6 wild-type group that did not receive AOM/DSS treatment was also included as a comparison (n=5). Mice were monitored twice weekly for body weight changes and symptom severity. Prior to sacrifice, body composition measurements were taken. At sacrifice, colon tissue, spleen, and adipose tissue were harvested. miRNA-155-/- mice had significantly fewer tumors than wild-type mice. In addition, miRNA-155-/- mice exhibited a lower symptom severity score and a greater body weight gain than wild-type mice over the course of the experiment. In general, wild-type mice had a reduction in fat mass and percent body fat as well as fat pad weights compared to the disease-free controls but miRNA-155 completely offset this effect. If these findings can be clinically translated, miRNA-155 may lead to an effective clinical biomarker and/or therapeutic target in colorectal cancer.

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CHAPTER 1

INTRODUCTION

Colorectal cancer remains a significant global health concern; despite advances in screening, surgery and treatment and recent reductions in mortality, it remains the third most common malignancy and the fourth most common cause of cancer mortality worldwide [6, 7, 14, 18]. In the US alone in 2013, it is estimated that over 142,000 new cases of colorectal cancer will be diagnosed and that there will be over 50,000 deaths from the disease. These statistics can be attributed largely to the lack of uptake of effective screening modalities (especially colonoscopy) that are available for prevention, detection, and diagnosis,[23, 24] and the fact that colorectal cancer symptoms are rare in the early stages so non-screening-detected cancers tend to present in late stage when treatments are ineffective[13]. Identifying new targets that may play a role in the development and progression of colorectal cancer is of significant public health concern, not only from a treatment point of view but also for the development of potential biomarkers that can be used for assessment and diagnosis of the disease [15].

Mammalian miRNAs are noncoding RNA oligonucleotides that have emerged as potent regulators of gene expression [20]. Consequently, dysregulated miRNA levels are associated with several types of malignancies [3, 15, 21]. For example, in a recent report miRNA-155 was significantly higher in breast cancer tissue than in corresponding non-tumor tissue and high miRNA-155 expression was correlated with higher tumor grade, advanced tumor stage and lymph node metastasis [4]. And further, the disease-free and

overall survival rates of the high miRNA-155 group were significantly lower than those of the low microRNA-155 group [4]. Another study reported that expression of miRNA-155 promoted the proliferation of breast cancer cells in vitro as well as the development of tumors in nude mice [8]. While there is accumulating evidence to suggest a role for miRNA-155 in the development of breast cancer, there are relatively fewer studies that report a link between miRNA-155 and colorectal cancer. A recent report, however, does indicate that miRNA-155 is increased in human colorectal cancer samples indicating that it may be involved in the progression and development of colorectal cancer [22] but to date there have been no studies done in animals to implicate a causal relationship.

The purpose of this study was to examine the role of miRNA-155 on the development of colorectal cancer. For this, we used a well-established mouse model of colorectal cancer, the azoxymethane (AOM) + dextran sodium sulfate (DSS) chemically-induced model [12, 16, 17]. This model recapitulates the phases of initiation and progression of the tumor that occurs in humans [12, 16, 17] . A miRNA-155 knockout mouse was used to examine the role of miRNA-155 on colorectal cancer. We hypothesized that lack of miRNA-155 would lead to a decrease in sickness symptoms along with a reduction in tumorigenesis. In this report, we demonstrate that miRNA-155 plays a necessary role in the development of colorectal cancer given our findings of a reduction in tumor number in miRNA-155 knockout mice. This miRNA may serve as an important biomarker and/or therapeutic target in colorectal cancer.

CHAPTER 2

MATERIALS AND METHODS

Animals. C57BL/6 wild-type mice and miRNA-155-/- mice on a C57BL/6 background were bred in the University of South Carolina's Center for Colon Cancer Research (CCCR) Mouse Core Facility. miRNA-155-/- offspring were genotyped as null homozygotes by RT-PCR for the miRNA-155 gene by taking tail snips at weaning. The primer sequences for miRNA-155 were as follows: miRNA-155-/- 5'-GTG CTG CAA ACC AGG AAG G-3', miRNA-155+/- 5'- CTG GTT GAA TCA TTG AAG ATG G-3', and wild-type 5'- CGG CAA ACG ACT GTC CTG GCC G-3'. Mice were maintained on a 12:12h light-dark cycle in a low-stress environment (22oC, 50% humidity and low noise) and provided food (AIN-76A) and water ad libitum. All animal experimentation was approved by the University of South Carolina's Institutional Animal Care and Use Committee.

AOM/DSS protocol. An azoxymethane (AOM) + dextran sodium sulfate (DSS) chemically induced model of colorectal cancer was used [12, 16, 17]. C57BL/6 wild-type mice and miRNA-155-/- mice (n=7-13 group) began their AOM/DSS treatment at 10 weeks of age. Briefly, mice were given a single injection of AOM (10mg/Kg) followed by 3 cycles of DSS (2% in the water for 1 week followed by 2 weeks of plain water) as previously described [12, 16, 17]. A C57BL/6 wild-type group that did not receive AOM/DSS treatment was also included as a comparison (n=5).

Body Weight and Food & Fluid Intake. Mice were weighed twice a week throughout the 10 week colorectal cancer protocol. Food and fluid intake were monitored once a week.

Body Composition. Body composition was assessed at twenty weeks (following the 10 week colorectal cancer protocol and prior to sacrifice). For this procedure, mice were placed under brief anesthesia (isoflurane inhalation) and were assessed for lean mass, fat mass, and body fat percentage via dual-energy x-ray absorptiometry (DEXA) (Lunar PIXImus, Madison, WI) [5].

Symptom Scoring. Symptoms were scored twice weekly throughout the 10 week protocol using a standard scoring system that has previously been used in this model. Briefly, scores were based on diarrhea, blood in stools and weight loss (see Table 1). Diarrhea symptoms were evaluated based on visualization of the fecal matter. Blood in the stools was determined using a Hemoccult Fecal occult blood test. In addition, weight loss was based on the percent change in weight compared to baseline levels. Mice were given scores of 0, 2 or 4 depending on the severity of the symptoms.

Tissue Collection. At 20 weeks of age, mice were sacrificed for tissue collection. Epididymal, mesentery, and retroperitoneal fat pads as well as the spleen were removed, weighed, and immediately snap-frozen in liquid nitrogen and stored at -80°C or fixed in 10% formalin. The large intestine was removed from the distal end of the cecum to the anus and mesentery tissue was removed with tweezers. It was flushed with PBS, opened longitudinally, and flattened with a cotton swab. All large intestines were fixed in 10% buffered formalin (Fisher Scientific, Pittsburg, PA) for 24 h.

Tumor Characteristics. Formalin-fixed large intestines were rinsed in deionized water, briefly stained in 0.1% methylene blue, and counted by the same investigator who was blinded to the treatments. Tumors were counted under a dissecting microscope, using tweezers to pick through the intestinal villi and identify tumors. Tumors were categorized by size (>2 mm, 1–2 mm, and <1 mm). Colon lengths were measured as an indicator of inflammation; a shorter colon length is indicative of greater inflammation. Similarly, colon weight was determined as an increase in colon weight has been associated with elevated inflammation.

CHAPTER 3

RESULTS

Body Weight. Body weight was monitored twice weekly throughout the 10 week AOM/DSS protocol. Body weight over time is expressed as a percent of baseline (i.e. normalized to measured body weight prior to the initiation of the AOM/DSS protocol) (Figure 1A). In general, the disease-free control group gained weight at a greater rate than both of the AOM/DSS groups; this was significant at most of the time-points measured. When comparing between the AOM/DSS groups, the miRNA-155-/- mice had a greater body weight gain than the wild-type mice at 15.5, 18.5, 19.0 and 19.5 weeks ($P<0.05$). We also measured body weight at sacrifice (Figure 2A). As expected, the wild-type AOM/DSS mice had a decrease in body weight compared to the disease-free control wild-type mice (25.47 g versus 31.30 g) ($P<0.05$) and miRNA-155-/- offset this effect; the miRNA-155-/- mice were very similar in weight at end point to the disease-free wild-type control mice (Figure 2A).

Symptom Severity. Symptoms, including diarrhea, blood in stools and weight loss, were scored twice weekly throughout the 10 week AOM/DSS protocol. In general, miRNA-155-/- mice exhibited a lower symptom severity score than wild-type mice over the course of the experiment (Figure 1B); this reached statistical significance at 12.5, 14.5, 15, 15.5, 18, 18.5, 19.5 and 20 weeks of age ($P<0.05$).

Spleen Weight. Spleens were harvested and weighed at sacrifice. Spleen weight, expressed as a percentage of body weight was increased in the wild-type AOM/DSS mice compared to disease-free control mice and this effect was offset in the miRNA-155-/- AOM/DSS mice (Figure 2B). However, these effects did not reach statistical significance.

Body Composition. Prior to sacrifice at 20 weeks of age, mice underwent a DEXA scan to determine body composition (body fat percentage, fat mass and lean mass) (Figure 3). This was performed as cachexia has been associated with severity of disease in colorectal cancer. As expected, the wild-type AOM/DSS mice had a decrease in fat mass compared to the disease-free control mice (4.03 ± 0.3 gm vs. 7.16 ± 0.9 gm) ($P<0.05$) and miRNA-155-/- offset this effect; the miRNA-155-/- mice had a very similar fat mass (6.48 gm) to the disease-free control mice (Figure 3A). Similar effects were observed for body fat percentage. The wild-type AOM/DSS mice had a decrease in body fat percentage compared to the disease free controls (17.45 ± 0.8% vs. 24.98 ± 2.3%) ($P<0.05$) and this effect was negated in the miRNA-155-/- mice (22.81 ± 1.9%) (Figure 3B). There were no significant group differences detected for lean mass at any of the time points (Figure 3C).

Visceral Fat Weights. At sacrifice, epididymal, mesenteric, and retroperitoneal fat pads were collected and weighed. This was done to monitor the degree of fat loss, which has been associated with poor outcome in colorectal cancer. As expected, epididymal fat mass was decreased in the wild-type AOM/DSS mice compared to the disease-free control group ($P<0.05$) and this effect was offset in the miRNA-155-/- mice (Figure 4A). In fact, there was no difference between the disease-free control mice and

the miRNA-155-/- mice that underwent the AOM/DSS protocol. Similar effects were found for mesenteric (Figure 4B) and kidney fat (Figure 4C); in both cases, the fat mass was reduced in wild-type AOM/DSS mice versus the disease free control mice ($P<0.05$) and deficiency of miRNA-155 completely offset this effect.

Tumor Characteristics. At 20 weeks of age, mice were sacrificed and the large intestine (colon) was harvested and tumors were counted on formalin-fixed, methylene blue-stained sections. miRNA-155-/- mice had significantly fewer tumors than wild-type mice (0.17 versus 2.14) ($P<0.05$) (Figure 5A). Tumors were also stratified according to size (Figure 5B); we classified tumors as being large (> 2mm in diameter), medium (<2 > 1 mm in diameter) or small (< 1 mm in diameter). A significant percentage of the tumors found in the wild-type AOM/DSS mice were considered to be large and the only tumors present in the miRNA-155-/- mice were medium size (Figure 5B). After the tumors were counted, the colons were then weighed and measured according to length and width (mm). The colon weight (g) was measured as an indication of the amount of inflammation present. The wild-type AOM/DSS mice had a significantly ($P<0.05$) heavier colon weight than the disease-free control mice (Figure 5C) but there was no effect of genotype; there was no difference between the wild-type AOM/DSS mice and the miRNA-155-/- AOM/DSS mice. Then a colon length: width ratio was calculated as it has been documented that the colon length decreases with inflammation. Both of the AOM/DSS groups showed an increase in colon length: width ratio compared to the disease free controls (Figure 5D).

CHAPTER 4

DISCUSSION

Dysregulated miRNA levels are associated with several types of malignancies including colorectal cancer [3, 21]. Several recent reports have indicated that miRNA-155 is correlated with higher tumor grade, advanced tumor state and lymph node metastasis in breast cancer. Although there is accumulating evidence to suggest a role for miRNA-155 in the development of breast cancer, there are relatively fewer studies that report a link between miRNA-155 and colorectal cancer. Therefore, we examined the role of miRNA-155 on body weight, body composition, symptom severity and tumorigenesis using a well characterized chemically-induced mouse model of colorectal cancer [12, 16, 17]. Our data indicates that miRNA-155 plays a significant role in tumorigenesis as well as the symptoms associated with colorectal cancer; mice deficient for miRNA-155 had a significant reduction in the number of tumors in the colon and this was associated with decreased sickness symptoms and prevention of loss of body weight and fat mass that can accompany the disease.

Knockout mice provide a tool to examine the effects of various genes on tumorigenesis. We used miRNA-155 knockout mice to examine the role of this miRNA on colorectal cancer using a well-characterized chemically-induced mouse model (i.e. the AOM/DSS model) [12, 16, 17]. The miRNA-155-/- mouse is available in a C57BL/6 background, a strain that shows high incidence and multiplicity of colonic adenocarcinoma following AOM/DSS administration. The AOM/DSS model is a two-

stage mouse colorectal carcinogenesis model initiated with AOM and promoted by DSS that was developed to obtain a better understanding of the pathogenesis of inflammatory bowel-related colorectal cancer. In this model, mice initiated with a low dose of a colonic carcinogen, AOM (10 mg/kg body wt), develop tumors after a relatively short-term DSS exposure. The subsequent dysplasia and neoplasms show positive staining for β-catenin, COX - 2 and inducible nitric oxide synthase. Our data indicates that mice deficient for miRNA-155 have fewer overall tumors than wild-type mice. In fact, they were approximately 90% lower than those of wild-type mice. When tumors were stratified according to size, miRNA-155-/- mice had significantly lower numbers of smaller tumors as well as larger tumors and although medium tumor number was also lower in miRNA-155-/- mice, this did not reach statistical significance. While we did observe an increase in colon weight and an increase in the colon length: width ratio in the AOM/DSS treated mice compared to the disease free control mice, we did not see an effect of genotype within the AOM/DSS groups. This was surprising given that an increase in these outcomes is indicative of inflammation and has been associated with increased tumor number. This may be explained by the overall low incidence of tumors in these mice; perhaps a greater number of tumors are needed to clearly observe differences in inflammatory outcomes between these groups. Even so, to our knowledge this is the first report of a benefit of miRNA-155 deficiency in a controlled experimental mouse model of colorectal cancer.

We also examined symptom severity during the course of the AOM/DSS protocol. Symptoms were recorded twice weekly and included scoring for diarrhea, blood in stools and weight loss. The presence of these symptoms is not unusual in

patients with colorectal cancer. In fact, diarrhea, blood in stools and weight loss are a direct result from colorectal cancer. The existence of tumors as well as the reduction of villi in the colon results in a reduced ability to absorb nutrients during digestion, which can lead to diarrhea and weight loss. Also large tumors can cause bleeding during digestion; when the fecal matter is passing through the colon it can catch on the larger tumors causing the presence of blood in the stool especially if there is an absence of villi in the colon. Greater symptom scores indicate increased severity of the cancer. In the wild-type mice, the severity of these symptoms was by far greater than that of the miRNA-155-/- mice following the AOM/DSS protocol. These results imply that miRNA-155 may serve as an important therapeutic target in colorectal cancer; blocking miRNA-155 may reduce the severity of symptoms in colorectal cancer patients.

Body composition was also examined as weight loss, including fat loss, has been associated with colorectal cancer. Cancer cachexia is a wasting disorder in which patients have a significantly decreased appetite without actively trying to lose weight [1, 2, 10]. In fact, patients with cancer cachexia lose a significant amount of weight quickly even when an increased caloric intake is administered. One of the determining factors of cachexia is the loss of lean fat mass while consuming a high calorie diet. Although in the current experiment we did not observe a significant decrease in lean mass in the wild-type AOM/DSS mice compared to the disease-free control group there was a trend towards a decrease. Whereas the miRNA-155-/- had a very similar lean mass to the disease-free control mice. There was however, a significant decrease in fat mass as well as body fat percentage observed in the wild-type mice that were not seen in the miRNA-155-/- mice. In addition, epididymal, mesenteric and kidney fat weight were all reduced

at sacrifice in the wild-type group and miRNA-155-/- offset this effect. In general, the miRNA-155 knockout mice had a more similar body composition to the disease-free control group than the wild-type group of mice. This suggests that targeting miRNA-155 in colorectal cancer patients could possibly reduce the occurrence of cancer cachexia allowing patients a greater chance of maintaining a stable body composition.

While we did not explore specific mechanisms of action that may be contributing to benefits of miRNA-155 deficiency in this model there are several possible explanations. Firstly, miRNA-155 may be promoting angiogenesis [9]. For example, it has been reported that miRNA-155 can downregulate von Hippel-Lindau (VHL) tumor suppressor in breast cancer [9]. VHL acts through stimulating hypoxia-inducible factor (HIFα) family proteins to inhibit the stimulation of vascular endothelial growth factor (VEGF), an important promoter of angiogenesis [9]. Secondly, it has been shown to down-regulate the tumor suppressor CDC73 in various cancer models [11]. For instance, expression of miRNA-155 in a squamous cell carcinoma cell line dramatically reduced CDC73 levels, enhanced cell viability, and decreased apoptosis. Conversely, the delivery of a miRNA-155 antagonist (antagomir-155) to cells overexpressing miRNA-155 resulted in increased CDC73 levels, decreased cell viability, increased apoptosis, and marked regression of xenografts in nude mice [11]. Thirdly, miRNA-155 has been reported to be a positive regulator of inflammatory processes [8, 19]. One study reported that both miRNA-155 overexpression and an inflammatory environment increased the frequency of mutations and down-regulated a kinase that blocks cell-cycle progression indicating that miRNA-155 may be a key player in the treatment of inflammation-related cancers [19]. Similarly, a recent study reported that miRNA-155 expression is inversely correlated with

expression of suppressor of cytokine signaling (SOCS1), a negative regulator of inflammation, in breast cancer cell lines as well as in a subset of primary breast tumors [8].

While our data shows promising effects for a benefit of miRNA-155 deficiency on colorectal cancer and associated symptoms there are several limitations of this study. Firstly, we did not investigate potential targets of miRNA-155 or mechanisms of action of miRNA-155 in this model; this study was limited to examination of tumor number and symptom severity. Secondly, the mice developed relatively few tumors, which may have precluded observation of the true effects of miRNA-155 deficiency. Therefore this study should be repeated with higher doses of AOM and/or DSS to enhance tumorigenesis. Further, pathways and mechanisms whereby miRNA-155 may have mediated its effects on colorectal cancer should be explored. Lastly, anytime a global knockout mouse model is used there is always some speculation as to the clinical translation; having absolutely no presence of miRNA-155 may not fully show what the effects of possible therapeutics would provide in a human.

In summary, our data indicates that miRNA-155 plays a significant role in tumorigenesis as well as the symptoms associated with colorectal cancer; mice deficient for miRNA-155 had a significant reduction in the number of tumors in the colon and this was associated with decreased sickness symptoms and prevention of loss of body weight and fat mass that accompany the disease. Future research should be performed to confirm these findings along with the examination of the mechanisms of action of miRNA-155. If these findings can be clinically translated miRNA-155 may lead to an effective clinical biomarker and/or therapeutic target in colorectal cancer.

TABLE 1. SYMPTOM SCORING

Symptom Scoring			
	0	2	4
Diarrhea	Well-formed pellet	Pasty and semi-formed stools that do not adhere to the anus	Liquid stools that adhere anus
Blood in stools	No blood	Positive hemoccult	Gross bleeding
Weight Loss	0-5% weight loss	11-15% weight loss	>20% weight loss

FIGURE 1.

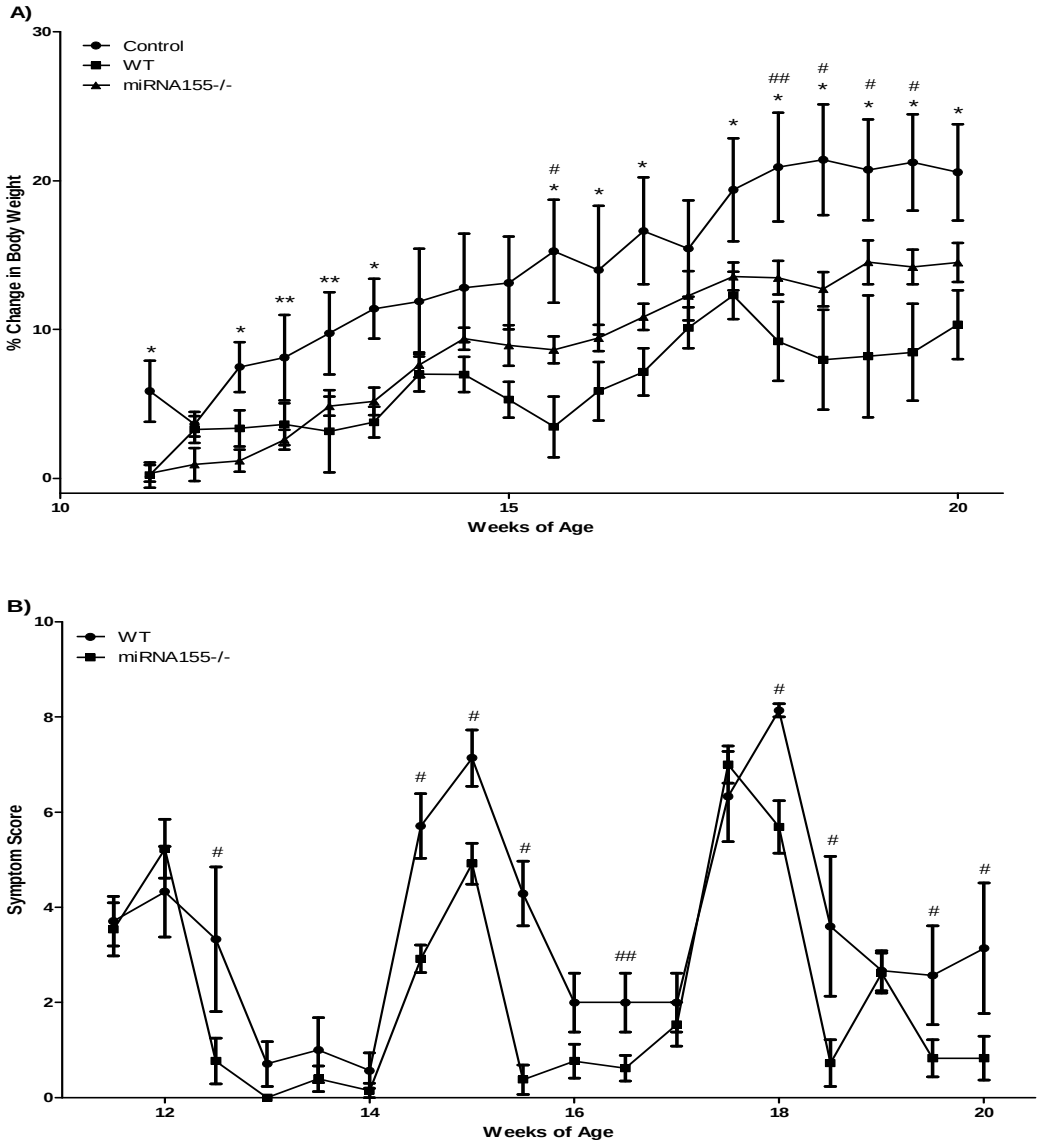

A)
Control
WT
miRNA155-/-
% Change in Body Weight
Weeks of Age
B)
WT
miRNA155-/-
Symptom Score
Weeks of Age

FIGURE 2.

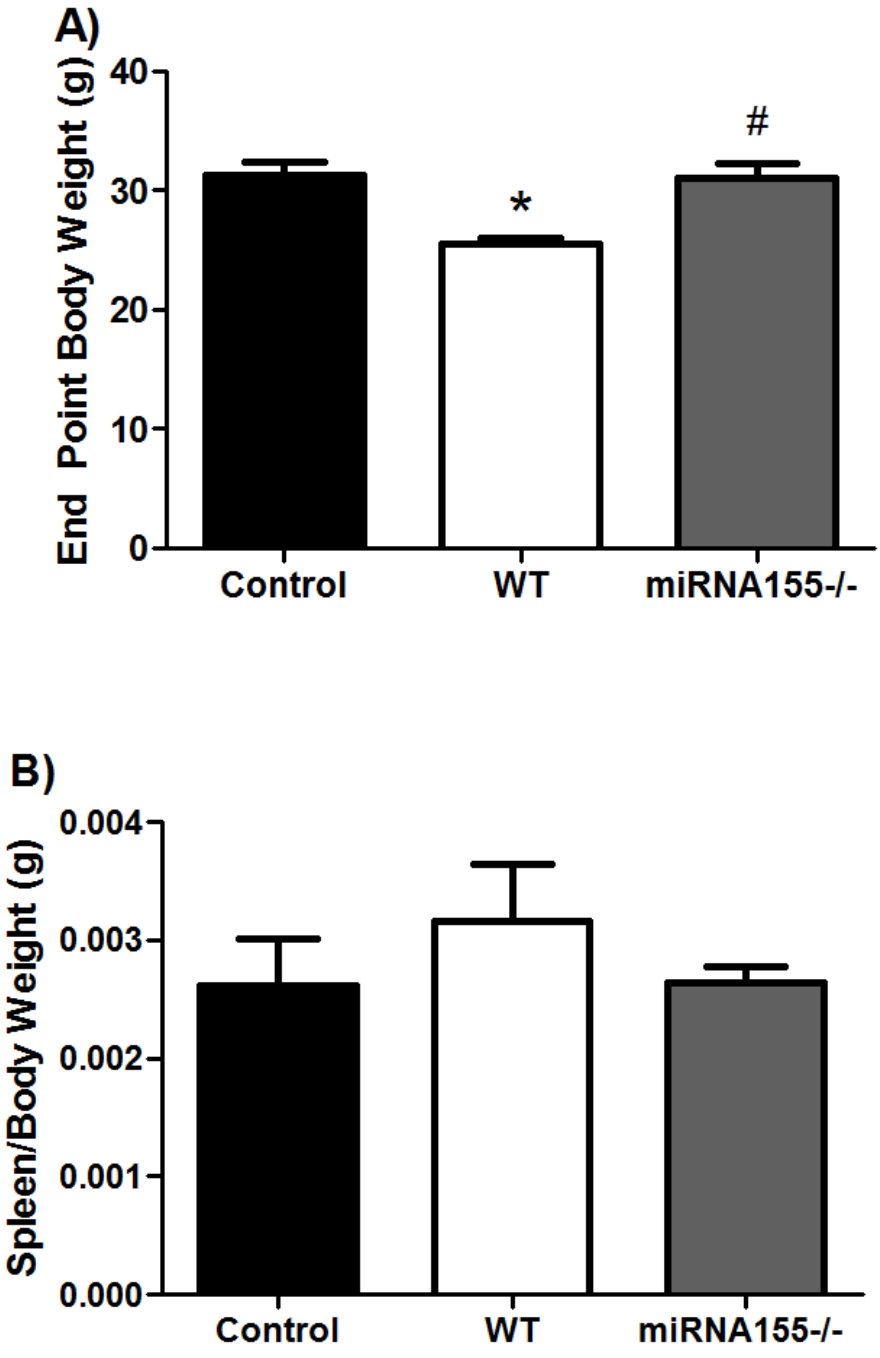

A)
End Point Body Weight (g)
40
30
20
10
0
*
#
Control
WT
miRNA155-/-
B)
Spleen/Body Weight (g)
0.004
0.003
0.002
0.001
0.000
Control
WT
miRNA155-/-

FIGURE 3.

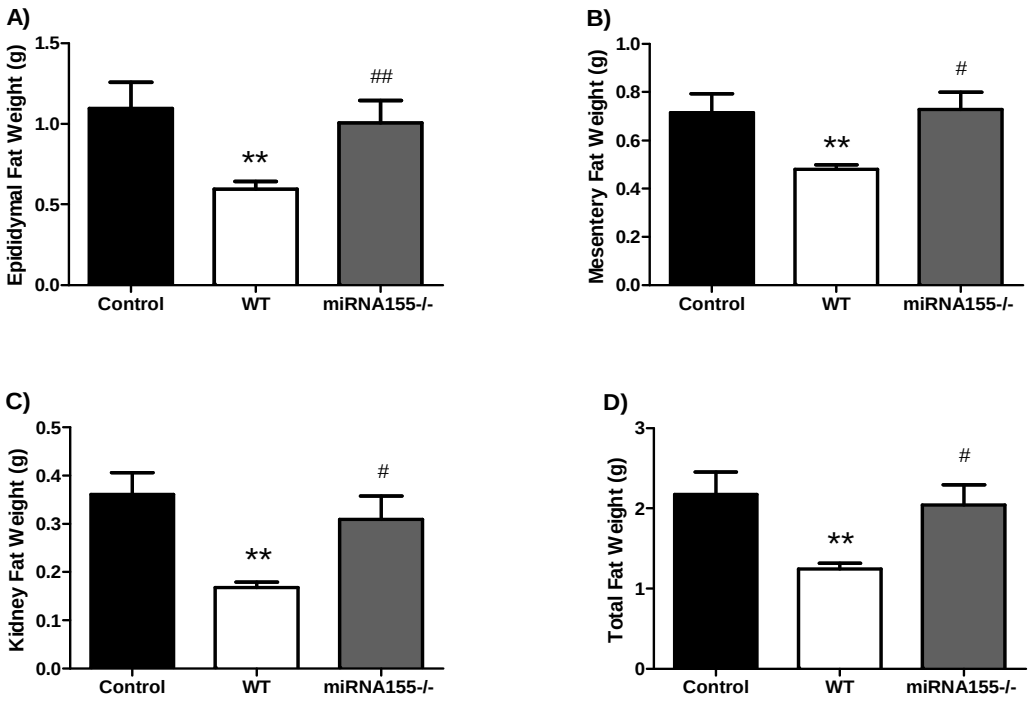

A)
Epididymal Fat Weight (g)
1.5
1.0
0.5
0.0
**
##
Control
WT
miRNA155-/-
B)
Mesentery Fat Weight (g)
1.0
0.8
0.6
0.4
0.2
0.0
**
#
Control
WT
miRNA155-/-
C)
Kidney Fat Weight (g)
0.5
0.4
0.3
0.2
0.1
0.0
**
#
Control
WT
miRNA155-/-
D)
Total Fat Weight (g)
3
2
1
0
**
#
Control
WT
miRNA155-/-

FIGURE 4.

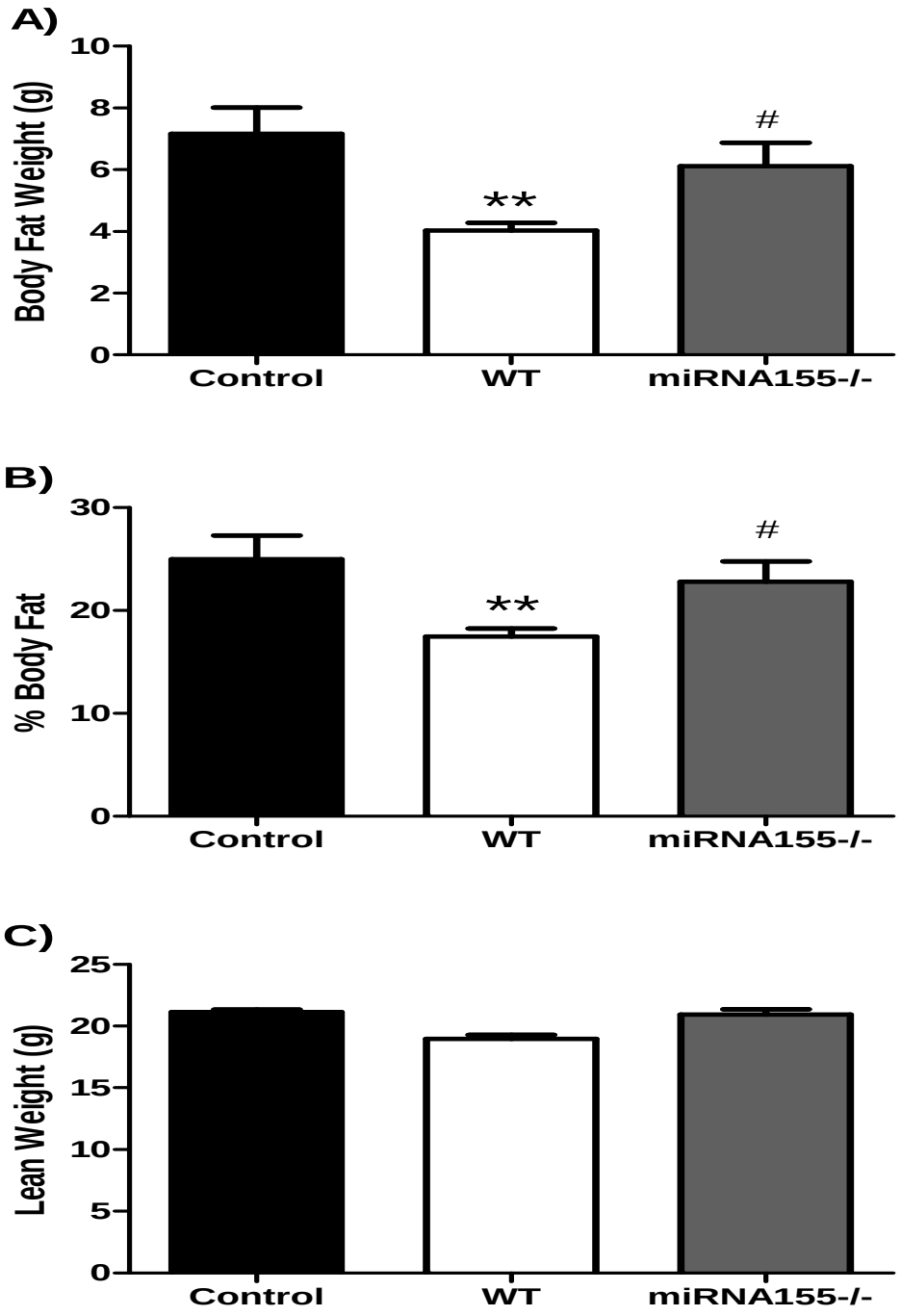
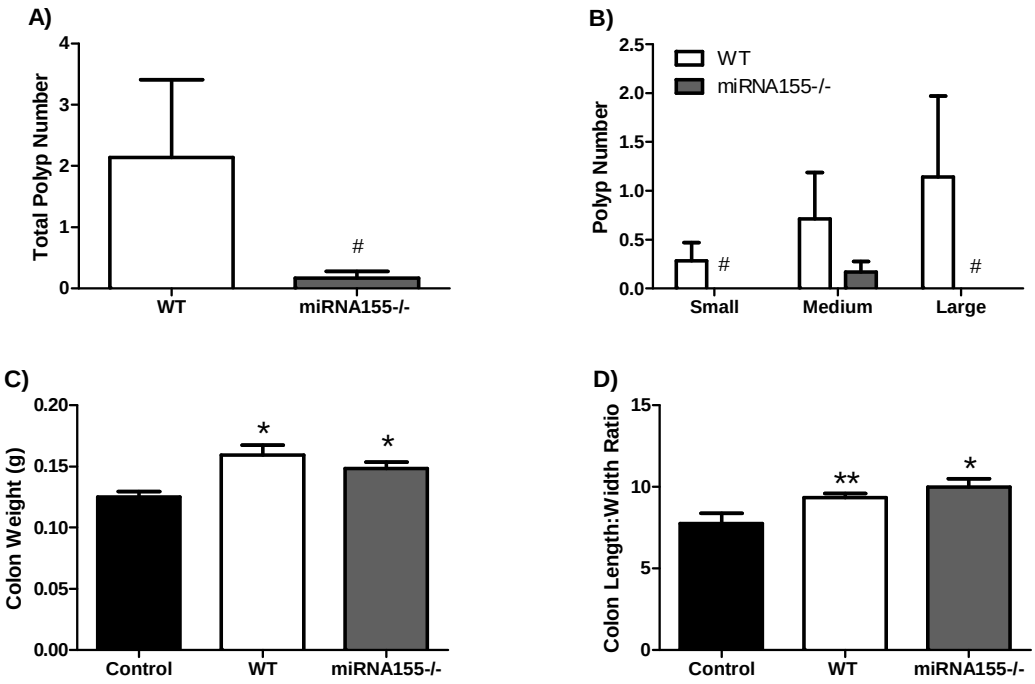

A)
Body Fat Weight (g)
10
8
6
4
2
0
**
#
Control
WT
miRNA155-/-
B)
% Body Fat
30
20
10
0
**
#
Control
WT
miRNA155-/-
C)
Lean Weight (g)
25
20
15
10
5
0
Control
WT
miRNA155-/-

FIGURE 5.


A)
Total Polyp Number
4
3
2
1
0
#
WT
miRNA155-/-
B)
Polyp Number
2.5
2.0
1.5
1.0
0.5
0.0
WT
miRNA155-/-
#
#
Small
Medium
Large
C)
Colon Weight (g)
0.20
0.15
0.10
0.05
0.00
*
*
Control
WT
miRNA155-/-
D)
Colon Length:Width Ratio
15
10
5
0
**
*
Control
WT
miRNA155-/-

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