JPET Fast Forward. Published on May 9, 2014 as DOI: 10.1124/jpet.114.213306

JPET #213306

Role of microRNAs in resveratrol-mediated mitigation of colitis-associated tumorigenesis in *Apc*$^{Min/+}$ mice

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JPET Fast Forward. Published on May 9, 2014 as DOI: 10.1124/jpet.114.213306
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Running Title: microRNAs in resveratrol mitigated tumorigenesis

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Text Pages: 11

Tables: 0

Figures: 7

References: 50

Words in Abstract: 247

Words in Introduction:748

Words in Discussion: 1478

Nonstandard Abbreviations:

AOM, azoxymethane

COX-2, cyclooxygenase-2

DSS, dextran sodium sulphate

H&E, hematoxylin and eosin

MDSCs, myeloid derived suppressor cells

miRNA, microRNA

Min, multiple intestinal neoplasia

NKT, natural killer T

NSAIDs, non-steroidal anti-inflammatory drugs

Res, resveratrol

UTR, untranslated region

I/P, intraperitoneally (I/P

BrdU, 50-bromo-20-deoxyuridine

MLNs, mesenteric lymph nodes

FBS, fetal bovine serum

TNF, tumor necrosis factor

Recommended Section Assignment: Inflammation Immunopharmacology, and Asthma

Abstract

The pleiotropic effects of resveratrol include anti-inflammatory, antioxidant and anti-cancer activities, and thus unique possibilities exist to explore mechanistic pathways of chemoprevention. The aim of this study was to investigate the role of miRNA alterations induced by resveratrol in the context of chemopreventive mechanisms against dextran sodium sulphate (DSS) induced colitis-associated tumorigenesis in the *Apc*$^{Min/+}$ mouse. To that end, *Apc*$^{Min/+}$ mice were exposed to 2% DSS to enhance intestinal inflammation and polyp development. Concurrently, mice received either vehicle or resveratrol treatment via oral gavage for five weeks. Interestingly, treatment of DSS-exposed mice with resveratrol resulted in decreased number and size of polyps, fewer histological signs of cell damage and decreased proliferating epithelial cells in intestinal mucosa compared to vehicle. Resveratrol treatment dramatically reversed the effects of DSS on the numbers of specific inflammatory CD4$^+$ T cells, CD8$^+$ T cells, B cells, natural killer T (NKT) cells and myeloid derived suppressor cells (MDSCs) in mesenteric lymph nodes. Resveratrol treatment also decreased IL-6 and TNF-α protein levels and reduced IL-6 and COX-2 mRNA expression. Microarray analysis revealed 104 microRNAs exhibiting greater than 1.5-fold differences in expression in the intestinal tissue of resveratrol treated mice. Among them, two microRNAs with anti-inflammatory properties, miRNA-101b and miRNA-455, were validated to be upregulated with resveratrol treatment by RT-PCR. Pathway analysis revealed that numerous differentially regulated miRNAs targeted mRNAs associated with inflammatory processes with known roles in intestinal tumorigenesis. These results suggest that resveratrol mediates anti-inflammatory properties and suppresses intestinal tumorigenesis through miRNA modulation.

Introduction

Bioactive dietary components offer numerous possibilities for chemoprevention, given their potential to target factors linked to the development and progression of cancer (Kim et al., 2009). Further, fewer side effects are associated with consumption of these components than prescription drugs. Resveratrol (Res, trans-3, 5, 40-trihydroxystilbene) is a naturally occurring stilbenoid abundant in plants and plant products such as grapes, peanuts, and red wine. Resveratrol has diverse biological properties including anti-carcinogenic, anti-oxidant, anti-inflammatory, anti-mutagenic, pro-apoptotic, and immunoregulatory activities (Szekeres et al., 2011), indicating promise as a chemoprevention strategy. Recent literature supports a beneficial effect of resveratrol in various mouse models of intestinal cancer. Oral administration of resveratrol decreases polyps in the small intestine and completely suppresses polyp formation in the colon of Apc/Min (multiple intestinal neoplasia) mice (Schneider et al., 2001). Resveratrol also reduces severity of DSS-induced ulcerative colitis and attenuates chronic colonic inflammation in mice (Yao et al., 2010). Similarly, resveratrol inhibits colon carcinogenesis in an azoxymethane (AOM)/dextran sodium sulfate (DSS) murine model, reduced the multiplicity of colon neoplasms, inhibited colon cancer cell proliferation, and promoted apoptosis (Marshall and Kerkvliet). While animal studies provide promising evidence of a benefit of resveratrol on colon cancer, the mechanisms for these effects are unknown.

Among all cancers, the evidence for a relationship between inflammation and colon cancer risk is the strongest (Fantini and Pallone, 2008). Thus, it is no surprise that anti-inflammatory strategies are known to decrease colon cancer risk. Incidentally, resveratrol has well documented anti-inflammatory mechanisms. For example, resveratrol inhibits activated immune cells and downregulates iNOS and COX-2 via its inhibitory effects on NF-□β (Das and Das, 2007). While the anti-inflammatory effects of resveratrol have been characterized in a variety of disease models including staphylococcal enterotoxin B-induced lung injury (Rieder et al., 2012), exposure to environmental toxins (Singh et al., 2011b), diabetes (Singh et al., 2011a), allergic encephalitis (Singh et al., 2007), melanoma (Guan et al., 2012), and ulcerative colitis (Singh et al., 2012a; Singh et al., 2012b), there are relatively few reports of its anti-inflammatory effects in rodent models of intestinal cancer .

The *Apc*$^{Min/+}$ mouse model is the most widely used genetic mouse model for cancer studies that involve the gastro-intestinal tract. *Apc*$^{Min/+}$ mice are heterozygous for a nonsense mutation at position

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850 in *APC* (adenomatous polyposis coli). Apc$^{/Min+}$ mice are predisposed to developing intestinal tumors, as are humans carrying mutations in *APC*. Additionally, approximately 80% of human colorectal tumors harbor mutations in *APC* (Miyoshi et al., 1992). Given the rapid development of polyps, along with its similarities to familial adenomatous polyposis and inherited colon cancer syndrome in humans, the *Apc*$^{Min/+}$ mouse is considered a highly relevant colon cancer model (Corpet and Pierre, 2003). One drawback of this model is that tumors occur predominately in the small intestine and not the colon, whereas humans with germline mutations in *APC* predominantly develop tumors in the colon. However, administration of DSS in the drinking water enhances colon polyp development and promotes inflammation in *Apc*$^{Min/+}$ mice. This mouse model is widely used in studies of anti-inflammatory agents, including both dietary supplements as well as non-steroidal anti-inflammatory drugs (NSAIDs) (Corpet and Pierre, 2003). However, there are few studies that have examined the anti-inflammatory effects of resveratrol in the *Apc*$^{Min/+}$ mouse model of intestinal tumorigenesis (Huderson et al., 2013), and to our knowledge none have examined its effects in the context of DSS-induced inflammation.

MicroRNAs (miRNAs) are 20-25 base-pair single stranded molecules capable of base pairing with complementary messenger RNA (mRNA) transcripts, typically within the 3' untranslated region (UTR). These miRNA/mRNA pairs are subsequently degraded and translation of the targeted mRNA is thereby inhibited. Dysregulation of miRNAs are associated with development of numerous cancers including cancers of the colon (Hutchison et al., 2013). In addition to miRNAs that have cell growth and proliferation targets, new evidence suggests that dysregulation of miRNAs with inflammatory or anti-inflammatory targets also play a mechanistic role in the pathogenesis of inflammatory bowel diseases and colon cancer development. For example, miRNAs such as miR-21 and miR-126 are differentially expressed in active versus inactive ulcerative colitis (Feng et al., 2012). Some of these altered miRNAs have targets that are associated with the downstream regulation of NF-κB and cyclooxygenase-2 (COX-2), both relevant factors in inflammation and tumorigenesis of the colon (Strillacci et al., 2009). Importantly, the as yet poorly understood mechanisms of the pleiotropic activities of resveratrol may act through modulation of miRNAs with targets involved in inflammation.

Thus, the purpose of this study was to examine impacts of resveratrol on inflammation and immune regulation in the *Apc*$^{Min/+}$ mouse model of intestinal tumorigenesis. Furthermore, a goal of this study was to explore polyp characteristics and determine the molecular mechanisms underlying the

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beneficial effects of resveratrol by microRNA (miRNA) microarray analysis. We hypothesized that resveratrol would decrease polyp number and size, decrease inflammation, suppress the immune response, and alter miRNAs known to regulate immunomodulatory pathways.

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Materials and methods

Animals

All animal care and experimental procedures were approved by the University of South Carolina's Institutional Animal Care and Use Committee and performed in accordance with the Guide for the Care and Use of Laboratory Animals. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council. Male *Apc*$^{Min/+}$ mice in a C57BL/6 background (Jackson Laboratories) were bred with female C57BL/ 6 mice at the University of South Carolina's Center for Colon Cancer Research. Offspring were genotyped as heterozygotes by RT-PCR for *APC* by taking tail snips at weaning. The primer sequences were sense: 5'-TGAGAAAGACAGAAGTTA-3'; and antisense: 5'-TTCCACTTTGGCATAAGGC-3'. Female *Apc*$^{Min/+}$ offspring were randomly assigned to either vehicle (DSS + V) or resveratrol (DSS + R) treatment groups at 4 weeks of age. An additional *Apc*$^{Min/+}$ group was euthanized at 4 weeks of age as a naïve control group (Control). Mice were maintained on a 12:12 h light-dark cycle in a low-stress environment (22°C, 50% humidity and low noise) and provided food and water *ad libitum*.

Chemopreventive effect of resveratrol

At 4 weeks of age, *Apc*$^{Min/+}$ mice (DSS + V and DSS + R) were exposed to 2% DSS in their drinking water for one week to induce colitis and enhance polyp development. Beginning on the first day of DSS administration, mice received either resveratrol (100 mg per kg mouse body weight) or vehicle by oral gavage in a volume of 0.2 mL every other day for 5 weeks. The resveratrol was purchased from Supelco (Bellefonte, PA), resuspended in water. Mice (DSS and D+R) were euthanized at 9 weeks of age. An additional *Apc*$^{Min/+}$ group that did not receive DSS was included as a control group (Cont) and was euthanized at 4 weeks of age.

Tissue collection

Fifteen minutes prior to euthanasia, cells synthesizing DNA were labeled by injecting mice intraperitoneally (I/P) with 50-bromo-20-deoxyuridine (BrdU, 120 mg/kg) (BD Biosciences, San Diego,

CA), which was dissolved in normal saline solution containing 30% dimethyl sulfoxide. Mice were euthanized via isoflurane overdose and mesenteric lymph nodes (MLNs) were harvested and mechanically dissociated followed by lysis of red blood cells (Sigma-Aldrich). Cell suspensions were stored on ice in media containing 2% fetal bovine serum (FBS) until analysis for inflammatory cell surface markers using flow cytometry.

The small intestine was carefully dissected distally to the stomach and proximal to the cecum. The large intestine (section 5) was removed from the distal end of the cecum to the anus. The small intestine was divided into 4 equal segments (sections 1–4). All intestinal sections were flushed with PBS, opened longitudinally and flattened with a cotton swab. Sections 1 and 4 of the small intestine and the large intestine (section 5) were fixed flat in 10% buffered formalin at room temperature (RT) for 24 hours for analysis of polyp counts, histopathological evaluation and immunohistochemistry. Sections 2 and 3 were divided into two equal parts and mucosal scrapings were performed in iscoves medium (Invitrogen) [containing 5% FBS and a cocktail enzyme inhibitor (10 mM EDTA, 5 mM benzamidine HCl, and 0.2 mM phenyl methyl sulfonyl fluoride)] and QIAzol reagent (Qiagen) for protein and gene expression analysis, respectively. Samples were stored at -80°C until analysis of inflammatory mediators.

Although DSS exposure in this model allows for the promotion of more polyps in the large intestine, ApcMin/+ mice primarily develop small intestinal polyps. Previous reported findings from our group have shown that an elevation in inflammatory cytokines in the small intestine is positively correlated with the abundance of large polyps as well as overall polyp number (McClellan et al., 2012). In order to detect any potential anti-inflammatory effects of resveratrol in this model, we chose to perform the inflammatory outcome analysis on the small intestine.

Polyp counts

Formalin-fixed intestinal segments from all animals were rinsed in deionized water, briefly stained in 0.1% methylene blue and polyps were counted under a dissecting microscope. Polyps were categorized by size (>2 mm, 1–2 mm and <1 mm).

Histological analyses

Tissue samples from the large intestine (section 5) were fixed in 10% formalin, dehydrated in a graded ethanol series and Swiss rolled in paraffin embedded sections. Samples were cut into 5 µm thick sections using a microtome and stained with hematoxylin and eosin (H&E). Histological alterations including mucosal ulceration, dysplasia and carcinoma were documented by a trained histopathologist.

Antigens were unmasked using proteinase K (Millipore, Billerica, MA) and peroxidase activity was inhibited using BLOXALL (Vector Laboratories, Bulingame, CA) for 10 min. For Proliferating Cell Nuclear Antigen (PCNA) staining, sections were incubated with rabbit polyclonal PCNA antibody (1:200; Abcam, UK) for 1 h at RT. The HRP-DAB Cell and Tissue staining kit (R&D Systems, Minneapolis, MN) was used according to the manufacturer's instructions. Detection was visualized by exposing sections to 3,3'-diaminobenzidine (DAB). PCNA positive cells were visualized using the DAKO Chromavision Systems ACIS 3 system.

Flow cytometric analysis

Cells from the MLN were isolated as described above for analysis of T cells (CD4, & CD8), B cells (CD19), MDSCs and NKT cells. Briefly, for staining of cell surface antigens, cells were washed with a FACS staining buffer (PBS with 2% FBS) and then stained with a fluorescein labelled antibody (isothiocyanate, phycoerythrin, or allophycocyanin (Biolegend, CA)) for 30 min at 4°C. Cells were then washed, thoroughly resuspended in FACS staining buffer, and analyzed using flow cytometry (CXP FC500, Beckman Coulter).

Analysis of IL-6 and TNF-α

Mucosal tissue scrapings were homogenized and samples were centrifuged at 10,000 rpm at 4°C for 10 min. The supernatants were removed and analyzed for IL-6 and TNF-α using an ELISA (Biolegend, San Diego, CA) according to the manufacturer's instructions. Total soluble protein cytokine levels are expressed as pg/100 µl total protein.

Expression of inflammatory markers

RNA was reverse transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad, USA). The cDNA was amplified using forward and reverse primers. The PCR products were identified using 2% agarose gel electrophoresis. The band intensity of the PCR products was quantified using the Bio-Rad Chemi Doc densitometry image analysis system (Bio-Rad, USA).

Immunohistochemical analysis

Intestinal tissues were fixed in 10% formalin, dehydrated through graded concentrations of ethanol, embedded in paraffin and sectioned. Sections (5 µm thick) were mounted on slides, cleared, deparaffinized and rehydrated. Slides were then treated with a buffered blocking solution (3% H2O2 in PBS) for 10 min. Antigen retrieval was performed in a water bath at 89°C and for 10 minutes using an antigen retrieval solution. Following cooling at RT, sections were co-incubated with primary antibodies for BrdU for 1 hour in a humidified chamber at RT. Sections were washed with PBS and incubated with conjugated secondary antibody (streptavidin-HRP) for 30 minutes at RT. Thereafter, sections were washed with PBS and co-incubated with a 3, 30-diaminobenzidine (DAB) solution in the dark at RT for 10 min. Sections were rinsed in PBS, counterstained with hematoxylin and observed under a microscope. Cells that had incorporated BrdU were visualized by counting the proportion of BrdU-labeled cells in 50 typical crypts and/or villi as previously described (Mochida et al., 2003).

Microarray analysis of miRNA

Total RNA including miRNA from intestinal scrapings was isolated using a miRNeasy kit following the manufacturer's instructions (Qiagen, Valencia, CA). The RNA was hybridized on an Affymetricx GeneChip high-throughput miR array containing 609 murine probes. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number INSERT. The data generated from the array was analyzed using hierarchical clustering. Using Ingenuity Pathway Analysis (IPA) software (http:// http://www.ingenuity.com), the results from the miRNA microarray were analyzed to identify molecular pathways potentially altered by single or multiple miRNA target genes. Briefly, this analysis compares each set of miRNAs to all available pathways in the database and assigns priority scores based on the predicted strength of the miRNA interaction with components

of the target pathway. Additional analysis was performed by input of these targets into Cytoscape using the ClueGo plugin (Bindea et al., 2009).

Validation of Microarray Data with RT-PCR

To validate the results of the microarray analysis, two representative miRNAs (miRNA-455 and miRNA-101b), significantly changed based on microarray results and of relevance to intestinal inflammation, were subjected to RT-PCR analysis. miRNAs were reverse-transcribed (miScript II, Qiagen) to make cDNA and RT-PCR was carried out with primers (5' GCAGUCCACGGGCAUAUACAC and 5' UACAGUACUGUGAUAGCUGAA for miRNAs 455 and 101b, respectively). Reactions were performed with SYBR Green Master Mix (Qiagen). Initiation was performed for 15 min at 95°C. The samples were then run for 40 cycles each at 94°C for 5 sec, 55°C for 30 sec and 70°C for 30 sec. All samples were run in triplicate.

Statistical analysis

All statistical analyses were conducted in Microsoft Excel and R (www.r-project.org). The two-tailed Student's t test was used to investigate the difference between the DSS+R and DSS+V groups and a p value < 0.05 was considered statistically significant. The mean ± SEM were reported. Additionally, the heat map and principle component analysis were applied to describe the correlation of miRNA expression levels among these two groups.

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Results

Effect of resveratrol on polyp number and size and histological changes in the colon

In a model for colitis-associated tumorigenesis, exposure of *Apc*$^{Min/+}$ mice to 2% DSS in drinking water for one week induces formation of numerous polyps in the colon. Following DSS-exposure, administration of resveratrol (100 mg/kg.bw) by oral gavage for five weeks reduced the overall number of polyps, although not significantly(32.6 ± 3.2 versus 42.5 ± 4.4; $p<0.09$) and significantly reduced the number of large polyps (5.1 ± 1.1 versus 13.4 ± 1.5; $p<0.01$) (Figure 1A). Additionally, resveratrol treatment reduced the overall polyp number within the colon compared to mice treated with vehicle (6.67 ± 1.63 versus 16.70 ± 2.13; $p < 0.002$ (Figure 1B). Although resveratrol also reduced the overall polyp burden within the small intestine, this did not reach statistical significance (data not shown). Concurrently, resveratrol treatment also reduced polyp size (Representative images in Figure 1C).

Of note, weight loss was observed in both groups, with resveratrol treated mice losing a maximum average of 7% body weight by day 8 and vehicle treated mice losing up to 11% body weight by day 10. This initial weight loss was likely due to the DSS exposure. Weights of both groups thereafter steadily increased, with both gaining approximately 8% body weight by the end of the study (data not shown).

Histopathological analysis was carried out following H&E staining of paraffin embedded Swiss rolled colon samples from DSS-exposed *Apc*$^{Min/+}$ mice treated with resveratrol or vehicle. In general, mice in the vehicle group presented with high grade dysplasia and goblet cell (mucin) depletion. Glandular epithelial cells exhibited 'rounding up' of cigar-shaped nuclei (Figure 1D & E). In contrast, resveratrol treatment dramatically reduced histological signs of cell damage. Histopathology of samples from the resveratrol treatment group ranged from low grade epithelial dysplasia comprising less than 1% of the mucosal surface area to insignificant pathological changes (Figure 1F & G).

Resveratrol treatment decreases the number of proliferating cells

We used BrdU as a marker of DNA synthesis to examine the number of proliferating cells in the colon of DSS-exposed *Apc*$^{Min/+}$ mice treated with resveratrol or vehicle. Briefly, BrdU was injected 15

minutes prior to euthanasia, formalin-fixed colon sections were stained using a BrdU staining kit and proliferation was determined by counting the proportion of BrdU-labeled cells in 50 typical crypts and/or villi (Mochida et al., 2003). A statistically significant (approximately 50%) decrease in the quantification of BrdU positive colonic epithelial cells was evident following resveratrol treatment compared to vehicle ($P< 0.01$) (Figure 2A-C). To support these data, PCNA staining of formalin fixed colon sections was also performed (Figure 2 D-F). Specimens from resveratrol treated mice exposed to DSS showed reduced PCNA staining relative to those treated with vehicle.

Resveratrol decreases inflammatory markers associated with colitis

The protein concentration of pro-inflammatory cytokines (IL-6 and TNF-α), and gene expression of inflammatory mediators (COX-2 and IL-6) in regions 2 and 3 of the small intestine of DSS-exposed *Apc*$^{Min/+}$ mice with and without resveratrol treatment were determined. Regions 2 and 3 were chosen to represent sections of the small intestine with low and high polyp incidence, respectively. Our findings indicated that resveratrol significantly blocked increases in IL-6 and TNF-α protein concentration ($p < 0.03$ & $p < 0.01$, respectively) in DSS-exposed *Apc*$^{Min/+}$ mice (Figure 3A & B). Similarly, resveratrol treatment offset the increase in COX-2 and IL-6 gene expression ($P < 0.02$ & $P < 0.002$, respectively), characteristic in this model (Figure 3C-F).

Resveratrol modulates immune cell populations

Using flow cytometry, we examined changes in the percentage and absolute number of T cells ($CD4^+$ and $CD8^+$), B cells ($CD19^+$), MDSCs and NKT cells in the MLN of DSS-exposed *Apc*$^{Min/+}$ mice with vehicle or resveratrol treatment. While the percentage of these cells were either decreased or slightly altered, enumeration of absolute numbers of these cells revealed that resveratrol treatment reversed the DSS-induced increase in the total number of $CD4^+$, $CD8^+$, $CD19^+$, NKT and MDSCs ($p< 0.001$, $p< 0.004$, $p< 0.004$, $p< 0.01$ and $p< 0.003$, respectively) (Figure 4).

Effect of resveratrol on miRNA expression patterns by microarray assay, validation by qRT-PCR and IPA analysis

To gain an understanding of the differences in miRNA profiles in DSS-exposed *Apc*$^{Min/+}$ mice treated with resveratrol compared to vehicle, miRNA array analysis on intestinal tissue was performed. A total of 104 miRNAs showing a > 1.5-fold change between groups was identified (Figure 5). The heat map (Figure 5A) of miRNA array performed on cell extracts from intestinal mucosal scrapings from two groups, DSS+V and DSS+R showed that miRNAs are differentially expressed. Eleven miRNAs with an increase or decrease in expression by a magnitude greater than 2.0-fold following resveratrol treatment were plotted by principal component analysis (Figure 5B). These altered miRNAs included 56 miRNAs that were upregulated and 48 miRNAs that were downregulated (Figure 5C). Figure 5D depicts all assayed miRNAs within the genome and the magnitude of the fold change, with twelve miRNAs of interest labelled.

Two miRNAs (-455 and -101b) upregulated with resveratrol treatment of DSS exposed *Apc*$^{Min/+}$ mice by microarray analysis that have known roles in inflammatory processes were validated using qRT-PCR. Consistent with the microarray results, we found a significant upregulation of miRNA-455 and miRNA-101b ($p<0.04$ and $p<0.02$ respectively), following resveratrol treatment compared to vehicle. In fact, quantitative estimates of the fold-change from RT-PCR and microarrays were very similar (Figure 6).

miRNAs of interest were further analyzed using IPA software. Of the miRNAs that were up- or downregulated by more than 1.5–fold upon resveratrol treatment, 15 had direct associations with numerous immune modulation targets (Figure 7A). Targets that have known direct, experimentally demonstrated associations with the miRNAs of interest are schematically mapped. These miRNAs had direct affinity to mRNAs with central roles in inflammatory pathways, including IL-6, IL-6R, TNF, TNFRSF1B (tumor necrosis factor receptor superfamily, member 1B) and CD4 among numerous targets relevant to immunomodulation. Molecules with known associations to the miRNAs of interest include transmembrane receptors (ICAM1, IL6R, IL10RA, CD4 and numerous others), cytokines (including IL6, IL7, IL25, IL13,IL1A, IL10, and TNF), ligand dependent nuclear receptors (RORC, AHR, PPARG, NR5A2, and ESR2), transcriptional regulators (including SMAD3, TBX21, DDIT3, and CEBPE), and kinases (MAPK9, IKBKB, and TGFBR2). The disease pathways most prevalent based on the miRNAs with greatest up- or downregulation include cancer, inflammatory disease and gastrointestinal diseases (Figure 7B).

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The targets identified by IPA as having direct interactions with differentially expressed miRNAs were analyzed with ClueGo in Cytoscape to determine the relevant inflammatory pathways altered by resveratrol treatment (Supplementary Figure 1) (Bindea et al., 2009). This analysis revealed that the resveratrol treatment of DSS exposed *Apc*$^{Min/+}$ mice predominantly targets expression of components involved in leukocyte differentiation and Toll like receptor pathways. The most relevant miRNAs modulated by resveratrol treatment that potentially could play a role in modulation of the immune response include: miR-343, miR-130a-3p, let-7a-5p, miR-3909, miR-708-5p, miR-221-3p, miR-504, miR-874-3p, and miR-16-5. The microRNA targets with the greatest involvement in these pathways include: ICAM1, IL12A, CD4, S100A8, IL10, AHR, CD3E, IL6, IL6St, TNF, PPARG, F2RL1, DDX58 (also called “RIP1”), CCR2, and TNFAIP2 (also called “A20”). T cell activation, regulation of leukocyte activation and regulation of the immune response are also significantly linked to targets of differentially expressed miRNAs. Importantly, TNF, IL6 and CD4 had numerous connections to involved pathways. AhR, IL-10, IL-12 and ICAM1 were also centrally featured targets.

Discussion

One bioactive property of resveratrol is its ability to reduce inflammation, a hallmark linked to every step of tumorigenesis (Wood et al., 2010). However, there are few studies examining resveratrol benefits in rodent colon cancer models (Huderson et al., 2013) and even fewer exploring the role of microRNA in resveratrol-mediated anti-inflammatory effects. Therefore, the purpose of this investigation was to examine the effects of resveratrol on inflammation, immune regulation, polyp characteristics and miRNA expression in the *Apc*$^{Min/+}$ mouse model of intestinal tumorigenesis.

Animal studies reporting beneficial effects of resveratrol in cancer have recently been published (Athar et al., 2007). Resveratrol reduces colon adenomas`, limits dysplasia`, increases apoptosis and decreases cell proliferation in ApcMin/+ mice following benzo(a)pyrene induced colon carcinogenesis (Huderson et al.). Resveratrol also reduces aberrant crypt foci in a chemically-induced AOM/DSS model of colon cancer (Boddicker et al., 2011). Likewise, we demonstrated the chemopreventive effects of resveratrol in the AOM/DSS model; resveratrol reduced tumor incidence from 2.4 tumors to 0.2 per mouse (Cui et al., 2010).

In the current study, we found that oral administration of resveratrol decreased the overall polyp burden in the colon of *Apc*$^{Min/+}$ mice. When stratified by size resveratrol specifically reduced the number of large polyps and the percentage of large polyps within the colon as well as histological signs of cell damage. We interpret this to mean that resveratrol can affect both development and growth of polyps.

The cell cycle machinery is a recognized target of resveratrol action (Ahmadi et al., 2009). Thus, we examined the effects of resveratrol on cell proliferation. As expected, BrdU and PCNA immunohistochemical staining of the colonic mucosal epithelium indicated decreased proliferating cells following resveratrol treatment. This confirms the reported antiproliferative properties of resveratrol on several cancer cell lines and decreases in cell proliferation with resveratrol in *Apc*$^{Min/+}$ mice following benzo(a)pyrene induced colon carcinogenesis (Huderson et al., 2013).

Our results show that resveratrol treatment significantly represses the intestinal mucosal protein concentration of IL-6 and TNF-α, and mRNA expression of COX-2 and IL-6. These are pro-inflammatory mediators with a well-documented role in promoting colon cancer and known to be modulated by resveratrol (Oshima and Oshima, 2012). The literature supports a positive relationship

between IL-6 and increasing tumor stage and size, metastasis and decreased survival in colon cancer (Knupfer and Preiss). Additionally, resveratrol reduced circulating IL-6 in an AOM/DSS model of colon cancer (Boddicker et al., 2011).

TNF-α is known to promote colon cancer (Flores et al.) and is associated with human irritable bowel disease and colitis (Natsui et al., 1997). Resveratrol is reported to reduce TNF-α in colon cancer cell lines (Paul et al., 2009). Additionally, resveratrol inhibits COX-2, which is known to be over expressed in gastric tumors, in the *Apc*$^{Min/+}$ mouse model (Sale et al., 2005). In fact, resveratrol may directly inhibit COX-2 expression as resveratrol suppressed growth of COX-2 positive colon cancer cells, whereas there was no response in COX-2 deficient cells (Zykova et al., 2008). Our findings of a reduction in select inflammatory mediators (IL-6, TNF-α, and COX-2) following resveratrol treatment thus offer a plausible explanation for the reported benefits on polyp number and size.

CD4$^+$ T cells were examined as they are reported to play a major role in the induction of irritable bowel disease and colitis-associated colon cancer. In fact, most of the intestinal damage caused by this disease is a result of CD4$^+$ T cell-mediated injury (Elson et al., 1996). Further, RAG1- deficient mice which lack B and T cells do not develop tumors following AOM/DSS exposure implicating a role for lymphocytes in the promotion of colon cancer (Becker et al., 2004). Our findings showed increases in the number of CD4$^+$ T cells in the MLN, which was mitigated by resveratrol treatment. Importantly, the documented increase in the number of CD4$^+$ T cells in this model was accompanied by marked elevation in inflammatory mediators, implicating a link between this cell population and inflammatory responses, which was reduced by resveratrol. Similarly, resveratrol treatment offset the increase in the number of CD8$^+$ T cells and CD19$^+$ B cells within the MLN.

We also measured MDSCs as they are known to downregulate immune surveillance and antitumor immunity, thereby facilitating tumor growth (Ostrand-Rosenberg and Sinha, 2009). In the present study, we found an increase in MDSCs in the MLNs. However, resveratrol treatment resulted in a significant downregulation in the absolute number of MDSCs. As MDSCs exert suppressive activity on cytotoxic T cells along with other immune cells, a reduction in this cell population with resveratrol would presumably result in a more favourable anti-tumor immune response.

Interestingly NKT cells are implicated as both enhancers and suppressors of anti-tumor activity. We found that NKT cells were significantly increased in the MLNs in this model and that resveratrol significantly offset this effect. However, given the dual role of NKT cells, these results should be

interpreted with caution; type I NKT cells enhance tumor immunity by IFN-γ expression and NK cell activation (Yang et al., 2000) and are positively correlated with disease-free survival, whereas, activation of type II NKT cells are associated with pro-tumoral responses. Further investigation is needed to fully elucidate the effects of resveratrol on subsets of NKT cells in this model. Nonetheless, to our knowledge this is the first study to report a beneficial effect of resveratrol on immune regulation in a mouse model of intestinal tumorigenesis.

Resveratrol decreases the expression of several pro-inflammatory and/or oncogenic miRNAs and conversely, upregulates miRNAs with anti-inflammatory and/or antitumor potential (Sonkoly and Pivarcsi, 2009). We found that 104 out of 609 miRNAs exhibited > 1.5-fold change in expression with resveratrol treatment, with 56 miRNAs upregulated and 48 downregulated. We identified the putative targets of several of these miRNAs to be related to inflammatory processes. These include miRNA-455 and miRNA-101b, both upregulated following resveratrol treatment. In particular, miRNA-101b has a high affinity binding site with the mRNA of Ptgs2 (COX-2) (Strillacci et al., 2009). Upregulation of these miRNAs by resveratrol is a potential molecular mechanism for the decrease in TNF-α and COX-2 observed in this investigation.

IPA indicated that the miRNAs altered by resveratrol have important roles in cancer, inflammatory disease, and gastrointestinal disease. In fact, when these miRNAs and their target genes are graphically annotated using the IPA software and Cytoscape, we found direct associations between certain miRNAs with altered expression upon resveratrol treatment and numerous genes with immunomodulatory functions and known associations with colon cancer, including: CD4, IL-6, TNF, ICAM1 (Intercellular Adhesion Molecule 1), PPARG (Peroxisome proliferator-activated receptor gamma) and CCR2 (C-C chemokine receptor type 2) (Supplementary Figure 2).

CD4 is a target of miR-3909, which is upregulated in resveratrol treated mice. CD4+ effector T cells, which can release IL-6, are known to promote chronic inflammation in irritable bowel disease, leading to tumor initiation, promotion and progression (Podolsky, 2002). As such, studies have linked IL-6 expression to sporadic and inflammation-associated colon cancer (Waldner et al., 2012). In the current study, miRNAs targeting IL-6 (let-7a-5p), IL-6R (miR-504 and let-7a-5p), and IL-6ST (miR-130a-3p) were upregulated in resveratrol treated mice, thereby suggesting decreased induction of these inflammatory markers.

TNF-α is also associated with colorectal carcinogenesis, and blocking the expression of TNF-α in a murine model of colon cancer reduces tumor burden (Popivanova et al., 2008). Here, we show that resveratrol reduces TNF-α levels in DSS-exposed *Apc*$^{Min/+}$ mice and that this may be achieved by upregulation of miR-3909 and miR-130a-3p. TNF-α signaling activates NF-κB which in turn induces the expression of COX-2, IL-6, IL-8 and TNF-α (Karin and Greten, 2005). Therefore, inhibition of TNF signaling by miRNA upregulation is a possible mechanism by which resveratrol may reduce intestinal inflammation and tumorigenesis.

Additionally, pathway analysis revealed that several miRNAs upregulated with resveratrol treatment were strongly associated with toll-like receptor (TLR) -3 and -4 signaling pathways. The TLR4 pathway is required for COX-2 induction in DSS treated mice, AOM/DSS treatment induced colon tumorigenesis and development of colitis-associated colon cancer in bone marrow chimera experiments (Fukata et al., 2009).

While animal models allow for examination of stage-specific responses to various dietary strategies, it is important to note that our study design considers only the chemoprevention effects of resveratrol. This is consistent with the majority of animal studies on resveratrol's effects on colon cancer. Thus the chemotherapeutic effects of resveratrol are less clear. Given that polyp number generally does not increase after ~12 weeks of age in the *Apc*$^{Min/+}$ mouse, we would expect that any therapeutic effect of resveratrol in this model would be reflected by a reduction in polyp size but not number.

In summary, we show that dietary resveratrol was associated with decreased cell proliferation, reduced inflammation and suppression of the immune response. Consistent with these findings, miRNA analysis revealed alterations in pathways relevant to inflammation, cancer and gastrointestinal disease with resveratrol treatment. Taken together, these data support further development of resveratrol as a potential chemopreventive strategy for patients at risk for development of colon cancer.

Authorship Contributions

Participated in research design: P.S. Nagarkatti, M. Nagarkatti

Conducted experiments: Altamemi, Murphy, Catroppo, McClellan, Singh

Contributed reagents or analytic tools: P.S. Nagarkatti, M. Nagarkatti, Zhang

Performed data analysis: Altamemi, Murphy, JFC, Zumbrun, Zhang

Wrote or contributed to the writing of the manuscript: Altamemi, Murphy, Zumbrun, P.S. Nagarkatti, M. Nagarkatti

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Footnotes

Funding was provided by The National Institutes of Health [grants P01AT003961, R01AT006888, R01ES019313, R01MH094755, and P20RR032684] and The Veteran's Association [Merit Award BX001357].

JPET Fast Forward. Published on May 9, 2014 as DOI: 10.1124/jpet.114.213306
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Figure legends

Fig. 1. Resveratrol reduces polyp number, polyp size and histopathology in *Apc*$^{Min/+}$ mice exposed to DSS. *Apc*$^{Min/+}$ mice were exposed to DSS in drinking water and treated with vehicle (V) or Resveratrol (R), and compared to naive control group (Control). Resveratrol reversed the effect of DSS on **(A)** number of large polyps in small and large intestine ($p<0.01$), **(B)** total polyp number in colon ($p<0.02$). No large polyps or polyps in the colon were observed in control mice and **(C)** representative images of colon containing intestinal polyps from mice exposed to DSS and treated with vehicle (V) or resveratrol (R). **(D & E)** *Apc*$^{Min/+}$ mice given 2% DSS show H & E stained polyps with high grade epithelial dysplasia. **(F & G)** show minute foci of low-grade epithelial dysplasia comprising less than 1% of the surface area of the roll of colon (D+R group). Magnification power was 4x with a 5 mm field of view **(D)**, 10x with a 2 mm field of view **(E)**, 2x with a 10 mm field of view **(F)**, and 4x with a 5 mm field of view **(G)**.

Fig. 2. BrdU-labeling index of colon crypt cells and adenomas in *Apc*$^{Min/+}$ mice exposed to DSS. *Apc*$^{Min/+}$ mice were exposed to DSS in drinking water and treated with vehicle (V) or Resveratrol (R), and compared naive control group (Control). BrdU (125 mg/kg *i.p.*) was administered 15 minutes before euthanasia to DSS-exposed *Apc*$^{Min/+}$ mice treated with resveratrol (R) or vehicle (V), and the labeling indices in colon crypts were assessed. BrdU-staining is shown in histological sections of intestinal mucosa from *Apc*$^{Min/+}$ mice that received 2% DSS and treated with **(A)** vehicle, or **(B)** resveratrol (100 mg/kg.bw). **(C)** The fraction of cells that are labeled with BrdU was quantified for both intestinal crypts and adenomas, and plotted. Values are averages ± SEM. Asterisks indicates significant difference from DSS + V group, $P< 0.01$. DSS +V (n=10), DSS +R (n=9) and control (n=2). Magnification was 10x with a 2 mm field of view **(A)** and 4x with a 5 mm field of view **(B)**. Intestinal mucousa from naïve **(D)**, DSS +V **(E)**, and DSS + R **(F)** mice were stained for PCNA which appears as dark punctate foci on each of the images.

Fig. 3. Resveratrol reduces the concentration of pro-inflammatory cytokines in the small intestine. The effects of resveratrol (R) compared to vehicle (V) on the concentration of **(A)** IL-6, and **(B)** TNF-α, in pooled sections 2 and 3 of the small intestine of DSS-exposed *Apc*$^{Min/+}$ mice were analyzed by ELISA. Values are averages ± SEM. Asterisks indicate significant difference from DSS + V group, $P< 0.05$. Resveratrol reduces **(C)** COX-2 and **(D)** IL-6 mRNA expression in mucosal scrapings from *Apc*$^{Min/+}$ mouse small intestines. The corresponding densitometry is shown below each PCR for COX-2 **(E)** and IL-6 **(F)**. Data are expressed as mean ± SEM and statistical significance calculated using the Student's *t* test. Asterisks indicate significant difference from DSS group $p< 0.05$.

Fig. 4. Resveratrol diminishes impact of DSS-exposure on immune cell modulation in mesenteric lymph nodes. **(A)** Flow cytometry analysis of mesenteric lymph node cells from three groups of *ApcMin/+* mice (control, DSS + vehicle (V), and DSS + resveratrol (R)). Cells were stained with anti-

CD4 (CD4 T cells), anti-CD8 (CD8 T cells), anti-CD19 (B cells), anti-CD3 and anti-NK1.1 (NKT cells), or anti-Gr1 and anti-CD11b (MDSCs) antibodies. Results show that the treatment with100 mg/kg.bw of resveratrol reversed the impact of DSS on percentage of each of the cell subsets measured. **(B)** Graphs depict the absolute number of these cells. Values are expressed as the average ± SEM. Asterisks indicate significant difference from DSS + vehicle group ($P < 0.05$).

Fig. 5. Differences in miRNA expression profiles of DSS-exposed *Apc*$^{Min/+}$ mice treated with vehicle or resveratrol. **(A)** Heat map of miRNA array performed on cell extracts from intestinal mucosal scrapings from two groups of DSS-exposed *Apc*$^{Min/+}$ mice (DSS + vehicle (V) & DSS + resveratrol (R)). Analysis demonstrated that a cluster of miRNAs are differentially expressed in mice treated with resveratrol. Red represents a positive change, green a represents negative change, and black represents no change. **(B)** Pie chart showing proportion of genes with less than 1.5 fold change, 1.5-2.0 fold up- or downreguation, and greater than 2 fold up- or downregulation. **(C)** The bar graph shows absolute fold-change in expression of 609 miRNAs tested by Affymetrix microarray analysis of DSS-exposed *Apc*$^{Min/+}$ mice treated with resveratrol compared to vehicle. **(D)** miRNAs that were more than 2-fold up- or downregulated are highlighted as well as miR-101b and -455 which were chosen for verification by RT-PCR. A PCA plot was constructed with fold change >2 defined as upregulated and fold change <-2 defined as downregulated.

Fig. 6. qRT-PCR verifying upregulation of miRNA-455 and -101b expression in DSS-exposed *Apc*$^{Min/+}$ mice treated with resveratrol. qRT-PCR shows, resveratrol treatment upregulates relative expression of miRNA-455 and -101b in mucosal tissue. Data are expressed as mean ± SEM and statistical significance was calculated using the Student's *t* test. Asterisks indicate significantly different from DSS + vehicle group $p < 0.05$.

Fig. 7. (A) Network wiring diagrams for miRNAs differentially regulated in DSS-exposed *Apc*$^{Min/+}$ mice treated with resveratrol. Relationships for miRNAs with more than 1.5-fold increase or decreased expression with resveratrol treatment, for which mapping information was available and interaction experimentally demonstrated, were plotted. Relationships known to exist for immunomodulatory mRNAs interacting with the miRNAs of interest are shown. miRNAs in red are upregulated and those in blue are downregulated. **(B)** Differential expression of pathway-specific microRNA following treatment of DSS-exposed *Apc*$^{Min/+}$ mice with resveratrol. Canonical pathway of gene targets of upregulated or down-regulated miRNAs. Pathways are sorted by score (-log *p* value). A higher score indicates that the pathway is more significantly associated with miRNAs of interest. The horizontal line represents statistically significant threshold limit.

Figure 1

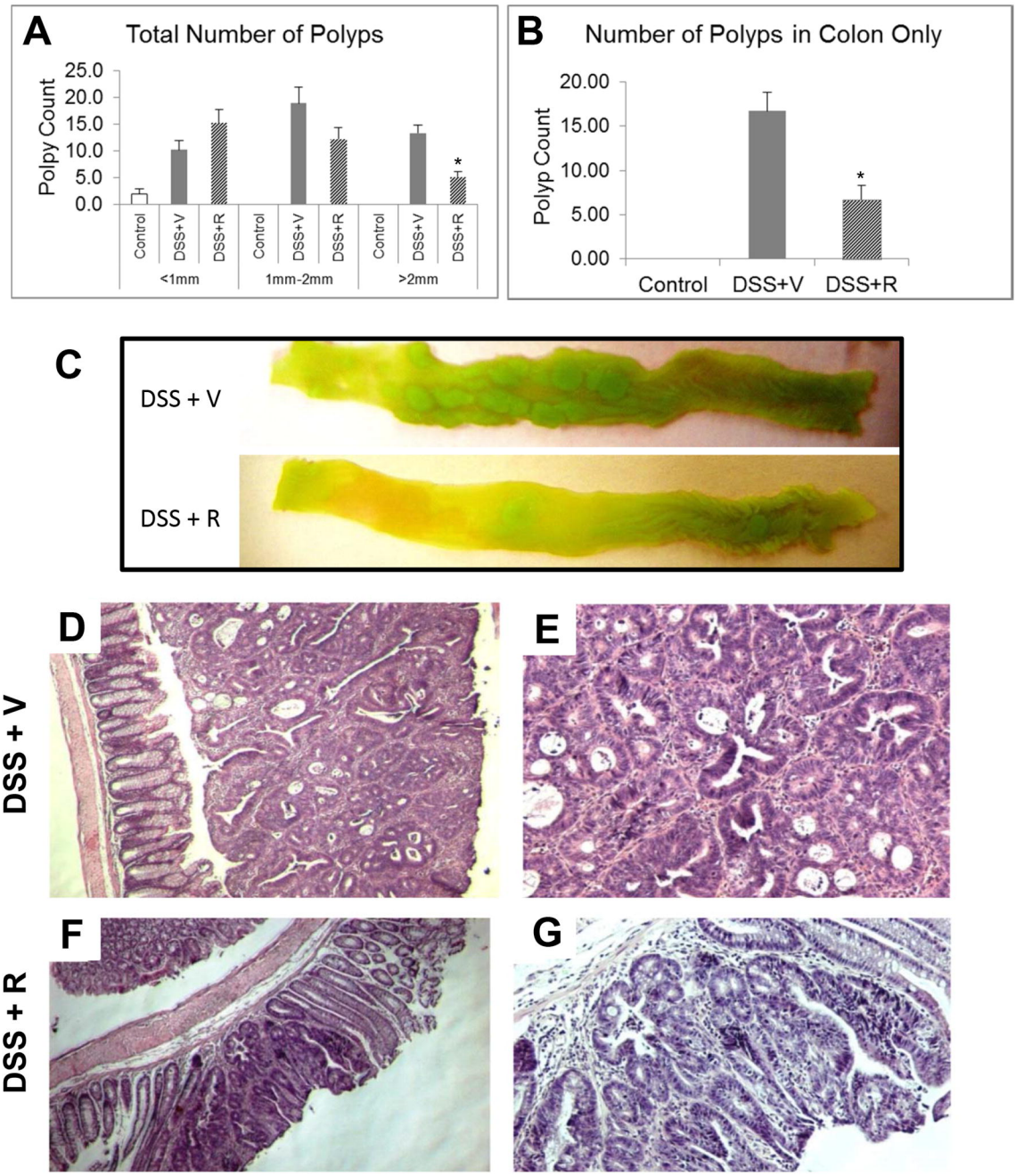

A
Total Number of Polyps
Polpy Count
25.0
20.0
15.0
10.0
5.0
0.0
Control
DSS+V
DSS+R
<1mm
1mm-2mm
>2mm
*
B
Number of Polyps in Colon Only
Polyp Count
20.00
15.00
10.00
5.00
0.00
Control
DSS+V
DSS+R
*
C
DSS + V
DSS + R
D
E
F
G
DSS + V
DSS + R

Figure 2

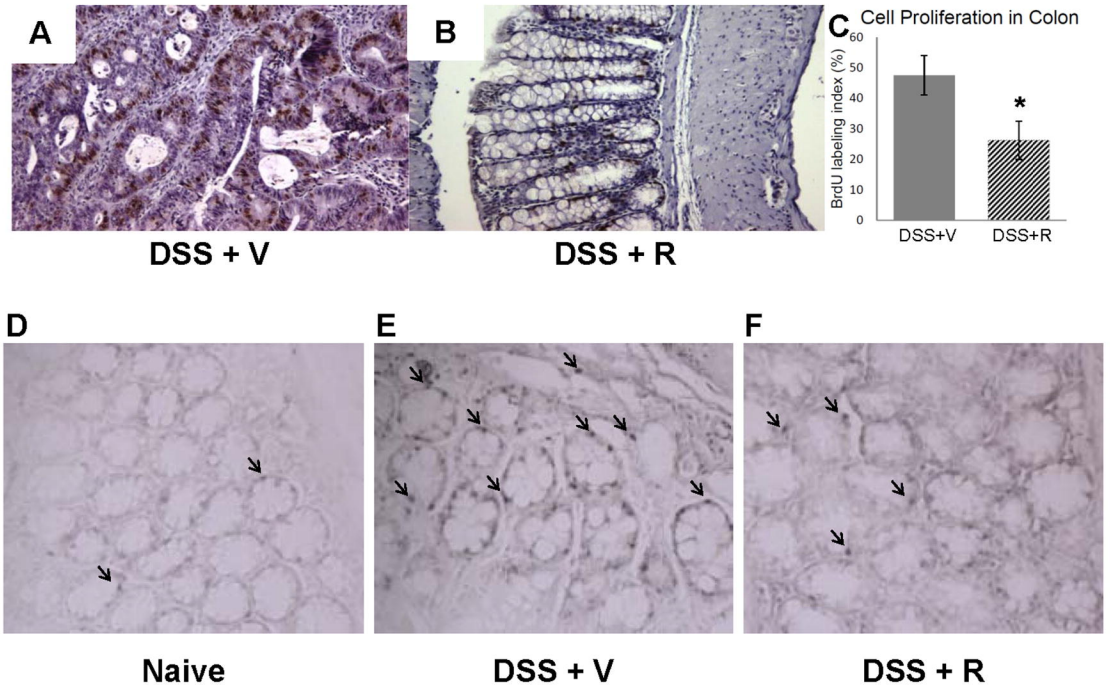

A
B
C
Cell Proliferation in Colon
BrdU labeling index (%)
60
50
40
30
20
10
0
*
DSS+V
DSS+R
DSS + V
DSS + R
D
E
F
Naive
DSS + V
DSS + R

Figure 3

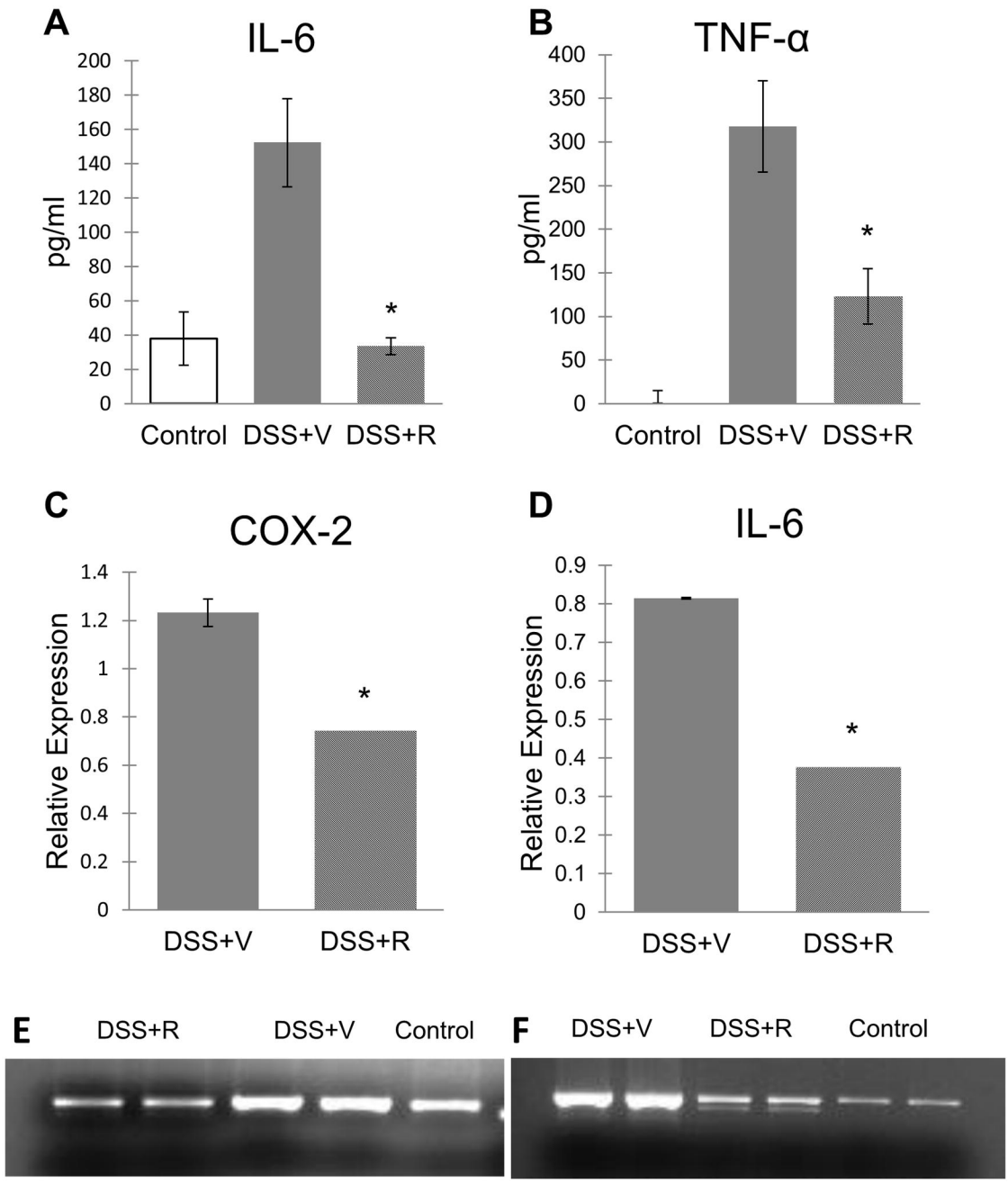
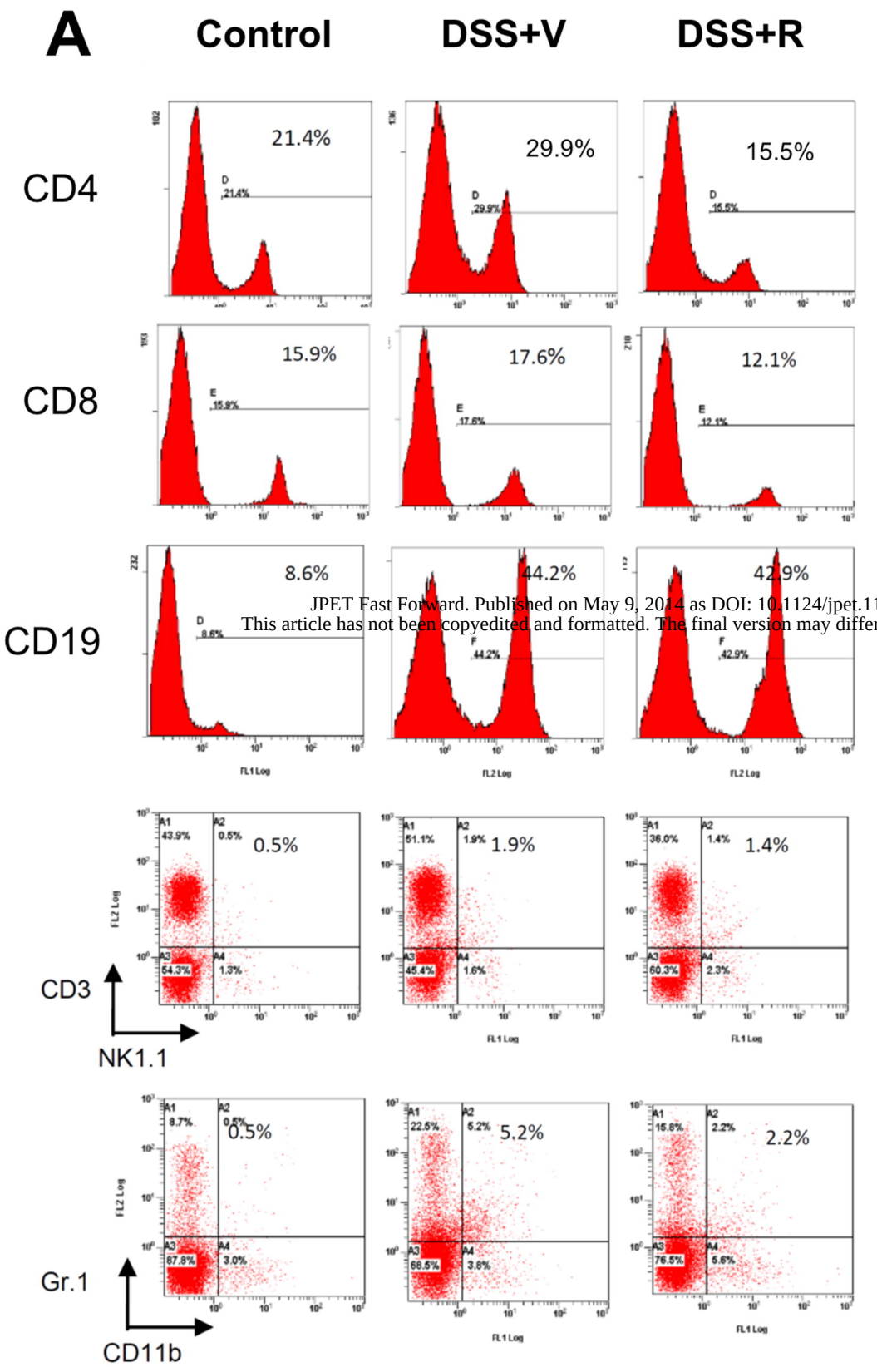
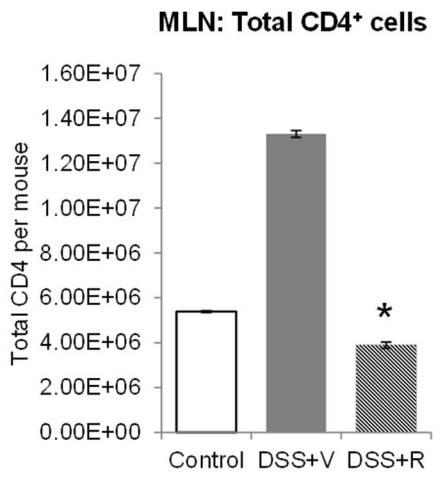
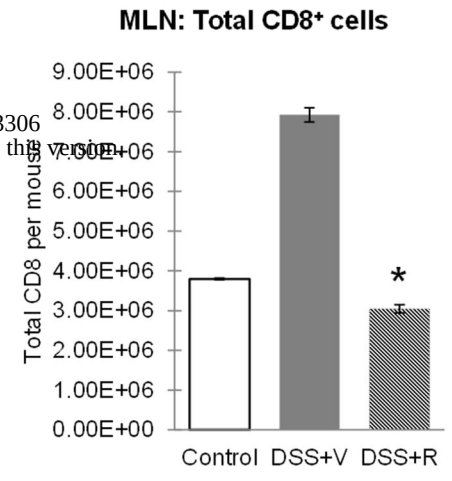
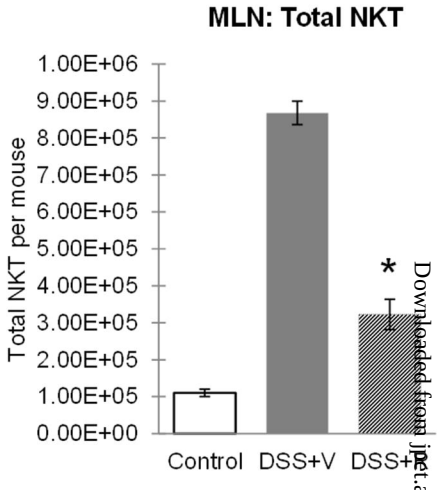

A
IL-6
pg/ml
200
180
160
140
120
100
80
60
40
20
0
Control
DSS+V
DSS+R
*
B
TNF-α
pg/ml
400
350
300
250
200
150
100
50
0
Control
DSS+V
DSS+R
*
C
COX-2
Relative Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
DSS+V
DSS+R
*
D
IL-6
Relative Expression
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
DSS+V
DSS+R
*
E
DSS+R
DSS+V
Control
F
DSS+V
DSS+R
Control

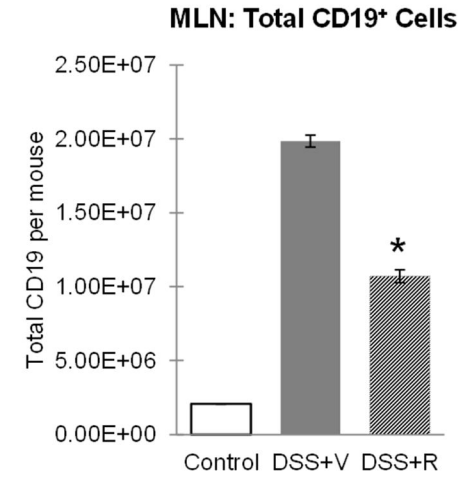
Figure 4

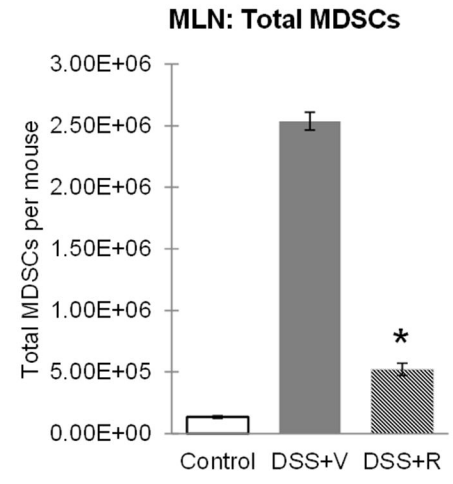












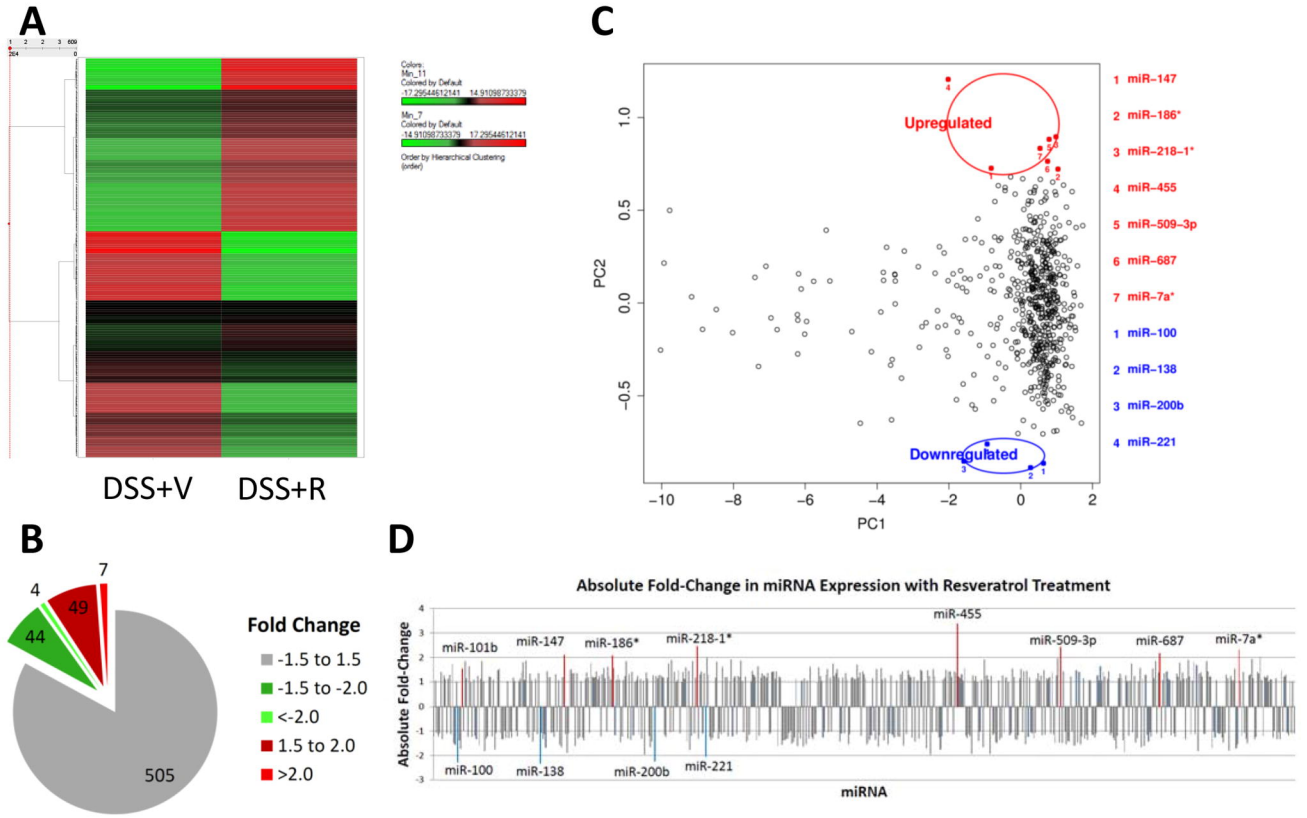

A
DSS+V
DSS+R
B
7
4
49
44
505
Fold Change
-1.5 to 1.5
-1.5 to -2.0
<-2.0
1.5 to 2.0
>2.0
C
Upregulated
Downregulated
PC1
PC2
1 miR-147
2 miR-186*
3 miR-218-1*
4 miR-455
5 miR-509-3p
6 miR-687
7 miR-7a*
1 miR-100
2 miR-138
3 miR-200b
4 miR-221
D
Absolute Fold-Change in miRNA Expression with Resveratrol Treatment
Absolute Fold-Change
miRNA
miR-101b
miR-147
miR-186*
miR-218-1*
miR-455
miR-509-3p
miR-687
miR-7a*
miR-100
miR-138
miR-200b
miR-221

Figure 5

Figure 6

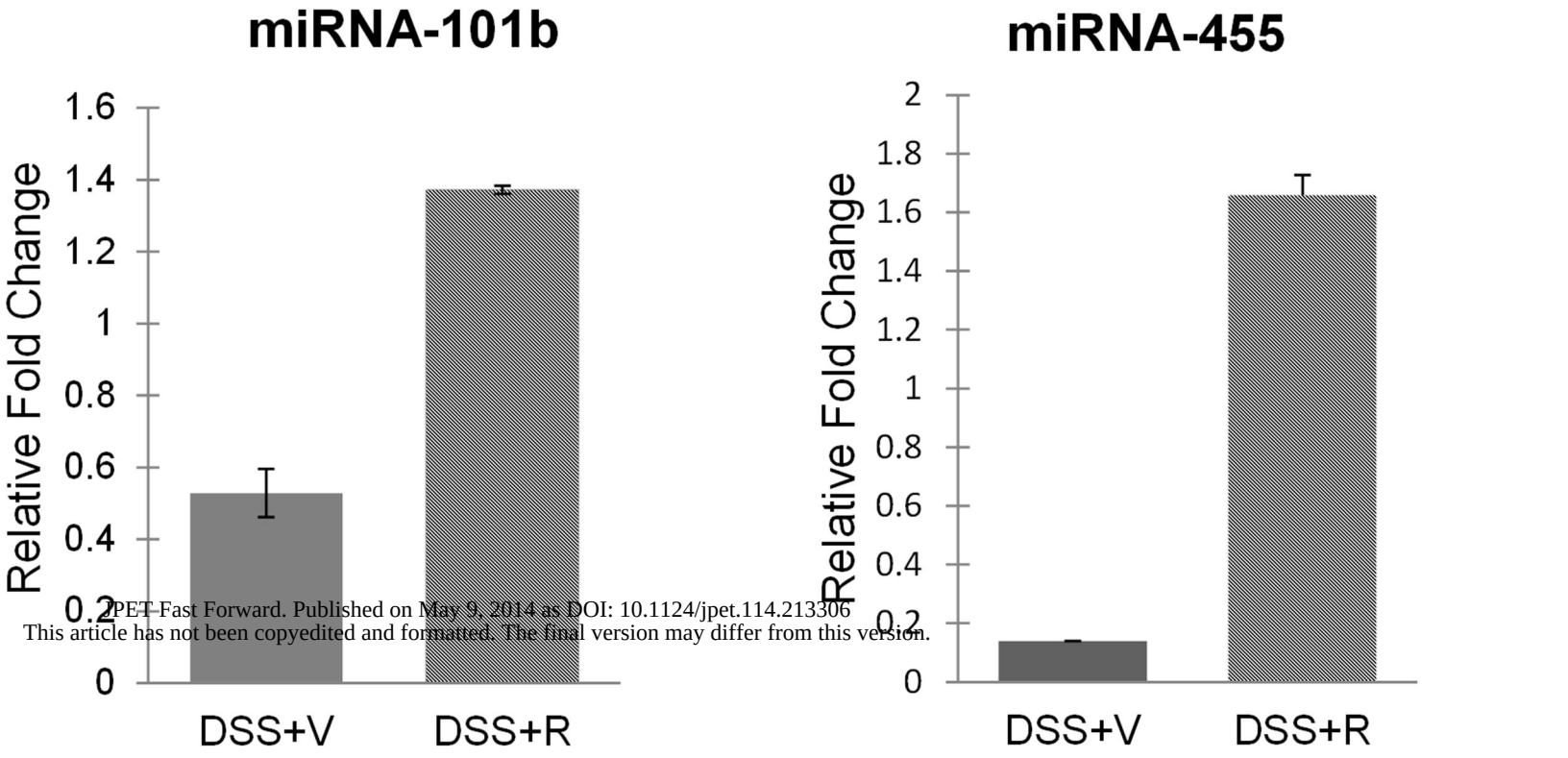
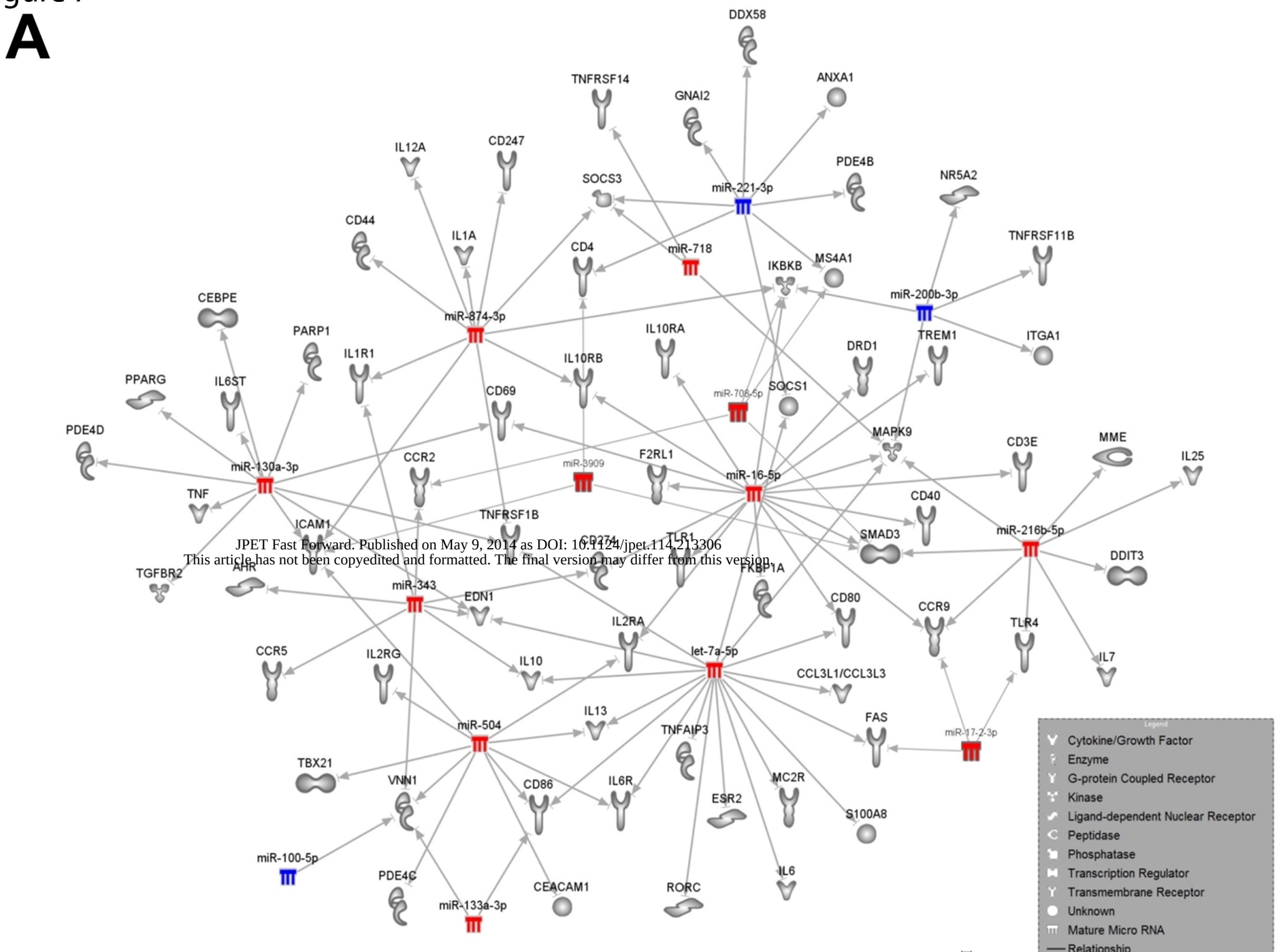


Figure 7

A



JPET Fast Forward. Published on May 9, 2014 as DOI: 10.1124/jpet.114.213306
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