Molecular Pharmacology Fast Forward. Published on March 9, 2015 as DOI: 10.1124/mol.114.096743
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MOLPHARM/2014/096743

Exposure to diethylstilbestrol during pregnancy modulates microRNAs expression profile in mothers and fetuses reflecting oncogenic and immunological changes

Narendra P. Singh, Ikbal K. Abbas, Martin Menard, Udai P. Singh, Jiajia Zhang, Prakash Nagarkatti, and Mitzi Nagarkatti

Department of Pathology, Microbiology & Immunology, University of South Carolina School of Medicine, Columbia, SC 29208 (NPS, IKA, MM, UPS, PN, and MN); and Department of Epidemiology & Biostatistics, Arnold School of Public Health, University of South Carolina, Columbia, SC 29208 (JZ)

Molecular Pharmacology Fast Forward. Published on March 9, 2015 as DOI: 10.1124/mol.114.096743
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MOLPHARM/2014/096743

Running Title: microRNA profile in thymus of prenatal mice post DES exposure

Address for Correspondence:

Mitzi Nagarkatti, Ph. D.
Professor and Chair,
Department of Pathology, Microbiology, and Immunology
University of South Carolina School of Medicine,
Columbia, SC 29208
Tel (off): 803-216-3404
FAX: 803-216-3413.
E-mail address: mnagark@uscmed.sc.edu

Number of Text Pages: 32
Number of Tables: 3
Number of Figures: 10
Number of References: 49
Number of words in Abstract: 233
Number of words in Introduction: 582
Number of words in Discussion: 1366

Abbreviations used in this paper: GD, gestation day; DES, diethylstilbestrol; miR, microRNA; UTR, untranslated region; FasL, Fas ligand;

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ABSTRACT

Prenatal exposure to diethylstilbestrol (DES) is known to cause increased susceptibility to a wide array of clinical disorders in humans. Previous studies from our laboratory demonstrated that prenatal exposure to DES induces thymic atrophy and apoptosis in thymus. In the current study, we investigated if such effects on the thymus result from alterations in the expression of microRNA (miR). To that end, pregnant C57BL/6 mice were exposed to DES and miR profiles in thymocytes of both the mother and fetuses on postnatal day 3 (GD17) were studied. Of the 609 mouse miRs examined, we noted 59 miRs altered that were common for both mothers and fetuses, whereas 107 altered miRs were specific to mothers only, and 101 altered miRs were specific to fetuses only. Upon further analyses in the fetuses, we observed that DES-mediated changes in miRs expression may regulate genes involved in important functions such as apoptosis, autophagy, toxicity, and cancer. Of the miRs that showed decreased expression following DES treatment, miR-18b and miR-23a were found to possess complementary sequences and binding affinity for 3'UTR regions of Fas ligand (FasL) and Fas, respectively. Transfection studies confirmed that DES-mediated down-regulation of miR-18b and miR-23a led to increased FasL and Fas expression. These data demonstrated that prenatal DES exposure can cause alterations in miRs leading to changes in the gene expression, specifically, miR-mediated increased expression in FasL and Fas causing apoptosis and thymic atrophy.

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INTRODUCTION

Diethylstilbestrol (DES; 4,4'-(3E)-hex-3-ene-3,4-diyldiphenol), a synthetic estrogen, was used in the US (from early 1940s to 1970s) to prevent spontaneous abortion (Herbst, 1981; Laitman, 2002). Approximately 5–10 million expectant mothers and developing fetuses were exposed to DES during this period. DES exposure of mothers and fetuses during this time has caused long-term adverse effects. For example, mothers were shown to exhibit increased risk of breast cancer (Goodman et al., 2011; Herbst, 1981) while DES-exposed daughters, showed increased risk of cervicovaginal cancer (Hoover et al., 2011; Palmer et al., 2005). Other abnormalities, such as immune system disorders, psychosexual effects, and reproductive abnormalities in DES daughters and sons, have also been reported (Giusti et al., 1995). There are studies showing prenatal exposure to DES alters immune functions in T cells as well as in other immune cells (Ford et al., 1983; Ways et al., 1987). Increased incidence of autoimmune diseases in adult life following prenatal DES exposure have also been reported (Noller et al., 1988). All these studies suggest that the immunological effects of DES exposure may be far reaching in the offspring (sons and daughters) of exposed mothers.

DES exposure of prenatal mice has been shown to cause various abnormalities including those in thymus, skeletal tissues, female reproductive organs, and muscles (Maier et al., 1985; Okada et al., 2001; Takasugi, 1963). In the thymus, prenatal exposure to DES has been shown to cause thymic atrophy and several other changes such as apoptosis, T cell differentiation, immunotoxicity, and immunosuppression (Brown et al., 2006a; Ford et al., 1983). We have also demonstrated that exposure to DES during pregnancy caused thymic atrophy in mothers as well

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as in the fetuses and new born pups (Brown et al., 2006b). Furthermore, we have also shown DES-induced alteration in positive and negative selection of T cells in the thymus (Brown et al., 2006a). Holladay et al have shown that DES exposure caused postnatal alterations in T-cell and natural killer (NK) cell functions and increased incidence of autoimmune diseases (Holladay et al., 1993). There are studies demonstrating DES effect on the expression profile of several genes in the thymus (Doherty et al., 2010; Geier et al. 2010). In a recent study, Frawley et al have reported DES-induced alteration in gene expression profile in thymic cells (Frawley et al. 2011).

In recent years, microRNAs (miRs) have been investigated extensively for their role in gene expression. miRs are highly conserved noncoding single-stranded small RNA molecules (17–27 nucleotides). They control gene expression by binding the 3' untranslated region (UTR) of target gene mRNA post transcriptionally. The binding of miRs with the target UTR of mRNA results in the degradation of the target mRNA or inhibition of the translation of the mRNA (Bartel, 2004; Iorio and Croce, 2009). miRs account for 1% of the genome, regulate 30% of the genome, and play a critical role in cellular processes such as apoptosis, proliferation, and differentiation (Iorio and Croce, 2009; Lee et al., 1993).

While DES-induced alterations in gene expression have been well studied, the role of miRs in such process remains unclear. In the current study, we therefore investigated if prenatal exposure to DES would alter miRs expression in the thymus, an organ that has been well characterized to be the target of DES-induced toxicity. Our studies demonstrated specific alterations in miRs that alter the apoptotic pathways as well as other pathways such as cancer. These studies demonstrate that DES-induced immunotoxicity may result from alterations in the expression of miR.

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MATERIALS AND METHODS

Mice. Pregnant mice (C57BL/6) were purchased from Jackson Laboratory. The mice were kept in University of South Carolina School of Medicine Animal facility. The mice were cared and maintained according to the guidelines for the care and use of laboratory animals as adopted by Institutional and NIH guidelines.

Cell line. EL4 cells were cultured in complete medium (RPMI 1640 medium, 10% heat-inactivated fetal bovine serum, 10 mM L-Glutamine, 10 mM HEPES, and 100 µg/ml penicillin/streptomycin) at 37°C and 5% CO_2).

Chemicals. We purchased Diethylstilbestrol (DES; 4,4'-(3E)-HEX-3-ENE-3,4-DIYLDIPHENOL) from Sigma (Sigma-Aldrich, St. Louis, MO). DES suspended in DMSO used in in vitro studies and suspended in corn oil was used in *in vivo* studies. The following reagents: RPMI 1640, HEPES, L-Glutamine, Penicillin/streptomycin and fetal bovine serum, Epicentre's PCR premix F and Platinum *Taq* Polymerase kits, and Lipofectamine RNAMAX transfection kit were purchased form Invitrogen (Invitrogen Life Technologies Carlsbad, CA). miRNeasy kit, miScript cDNA synthesis kit, miScript primer assays kit, and miScript SYBR Green PCR kit were purchased from QIAGEN (QIAGEN INC, Valencia, CA). EL4 transfection kits was purchased from Lonza (Lonza Cologne GMBH, Cologne, Germany)

***In vivo* exposure of pregnant mice with DES. We used** a single dose of DES (5 µg/kg) in this study. DES was administered intraperitoneally (ip) into pregnant mice (C57BL/6) on GD 14, as described previously (Singh et al. 2012a). We performed dose-response studies and found that the dose of 5 mg/kg body weight was the optimal dose for our studies (Singh et al. 2012a).

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Also, administration of higher than this dose led to profound thymic atrophy making it difficult to secure enough cells for the proposed experiments. Also, this dose range has been used by others (Ohta et al. 2012). On day 3 (GD 17), thymi were harvested and thymic weight and cellularity were determined. Mice treated with vehicle (VEH; corn oil) were used as control. For each treatment group, at least three pregnant mice were used and from each pregnant mother, we obtained an average of 5-8 pups. We combined the three litters from each treatment group to generate a pool of 18-24 fetuses. Due to low thymic cellularity in the fetus, thymi from 5 fetuses were randomly pooled per sample and ~5 replicate pools were used for statistical analysis.

Determination of thymic cellularity and apoptosis post DES exposure. Thymi from mothers and fetuses were harvested and transferred in complete RPMI-1640 medium. We prepared single cell suspensions of thymi as described earlier (Camacho et al., 2004a; Camacho et al., 2004b). The cell viability was determined using an inverted phase contrast microscope. Thymic cellularity was expressed as total number of thymocytes/ mouse. We pooled 5-6 replicate pools and were compared from each treatment group for statistical analysis and data were depicted as mean ± SEM. Apoptosis in thymic cells post DES exposure was determined as described previously (Singh et al. 2012a). TUNEL assays were performed using TUNEL kit from Roche and following the protocol of the company (Roche Applied Sciences, Indianapolis). Apoptosis in thymocytes was analyzed using flow cytometry (CXP 500; Beckman Coulter).

Determination of Fas and FasL expression in thymocytes. Expression of Fas and FasL in mother's and fetal thymic cells was measured using RT-PCR as described earlier (Fisher et al., 2004). In brief, single cell suspension of thymic cells post VEH- or DES- treatments was prepared as described above. Expression of Fas and FasL was determined by RT-PCR as

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described previously (Singh et al., 2008). For RT-PCR, total RNAs from VEH- or DES-treated groups were isolated using RNeasy Mini Kit and following the protocol of the company (QIAGEN, Maryland). cDNA synthesis was performed in a 20 µl reaction mix containing 1 µg total RNA using iScript Kit and following the protocol of the manufacturer (Bio-Rad). PCR was performed using mouse FasL- or Fas-specific sets of forward and reverse primers as described earlier (Singh et al., 2008). The PCR products, generated from mouse Fas and FasL primer pairs, were normalized against mouse-specific 18S PCR products as described previously (Singh et al., 2008). The intensity of PCR products was determined using ChemiDoc image analysis system (BioRad).

miR array assays. Total RNAs including miRs from the thymi of mothers and fetuses exposed to DES or VEH were isolated using miRNeasy kit from QIAGEN and following the manufacturer's instructions (QIAGEN, Valencia, CA). miRs arrays were performed –using Affymetrix GeneChip miR platform. The data generated from miR arrays were analyzed using hierarchical clustering and pathway network. Induction or repression of miR expression was determined using 2-sample t-test as described previously (Hegde et al., 2013; Singh et al. 2012b) and p-value of <0.01 in the t-test was considered significant. In this study, a fold-change (FC) of more than 1.5-fold was considered positive.

Real-Time PCR to validate the expression of miRs in thymocytes. We selected several downregulated miRs (miR-18b, -23a, -30a, -31, -146a, 155, and -217) and one upregulated miR (miR-320) to validate their expression. Real-Time PCR assays were performed on cDNAs generated from total RNAs including miRs isolated from fetal thymocytes exposed to DES or VEH as described earlier. We used miScript primer assays kit (details in Supplemental

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Table 1) and miScript SYBR Green PCR kit from QIAGEN and following the protocol of the company (QIAGEN, Valencia, CA).

We used StepOnePlus Real-Time PCR system V2.1 (Applied Biosystems, Carlsbad, CA). The following conditions: 40 cycles using the following conditions: 15 min at 95°C (initial activation step), 15 s at 94°C (denaturing temperature), 30 s at 55°C (annealing temperature), and 30 s at 70°C (extension temperature and fluorescence data collection) were used. Normalized expression (NE) of miRs was calculated and the data were normalized to various miRs against internal control miR (Snord96a) and fold change of miRs were calculated against control miR and DES treatment group was compared with VEH group. ANOVA was performed using GraphPad version 4.0 (GraphPad Software, Inc., San Diego, CA) to define significant differences in miR levels in the thymocytes post DES- or VEH-treated groups and differences between treatment groups were considered significant when, $*p < 0.05$.

Analysis of miRs and their association with various pathways. Post generation of heatmap and analysis of miR expression, we selected dysregulated (upregulated/downregulated) miRs (>1.5 fold) in fetal thymi exposed to DES, when compared to those exposed to VEH. Next, the selected miRs were analyzed for their role in expression of various genes and pathways using IPA software and database of the company (Ingenuity INC, California), as described previously (Hegde et al., 2013; Singh et al. 2012b). DES-regulated miRs were also analyzed using Cytoscape 3.0.1 and Cluego software (Cytoscape Consortium) as described previously (Hegde et al., 2013; Singh et al. 2012b).

miR-mRNA target interactions. We identified miR-specific mRNA targets using micro RNA.org, TargetScan mouse 5.2, miRWalk, and miRGEN (version 3) software and their

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database, as described earlier (Hegde et al., 2013; Singh et al. 2012b). The details of some of miRs and UTR region of their target gene (RNA targets) are described in Supplemental Table 2.

Transfection with mature miR-23a and miR-18b and determination of Fas and FasL expression in the presence or absence of DES. EL4 cells ($5x10^6$) were transfected using Lipofectamine RNAMAX transfection kit from Invitrogen and following the protocol (Reverse Transfection) of the company (Invitrogen), as described previously (Hegde et al., 2013; Singh et al. 2012b). Forty eight hrs post transfection, EL4 cells were treated with VEH or DES (10 μM/ml) for 24 hrs and the expression of Fas and FasL in EL4 cells was determined. To this end, total RNAs from EL4 cells transfected with miR-23a or miR-18b and treated with DES or VEH were isolated using RNeasy mini kit from QIAGEN and following the protocol of the company (QIAGEN, Valencia, CA). Fas and FasL expression was determined as described previously (Singh et al. 2012b). The PCR products, generated from mouse Fas and FasL primer pairs, were normalized against PCR products generated from mouse 18S after electrophoresis -. The band intensity of PCR products was determined using ChemiDoc image analysis system (BioRad, Hercules, CA).

Generation of the reporter constructs containing Fas UTR region or FasL UTR region. To this end, we used pmirGLO reporter vector from Promega (Promega Corporation, Madison, WI). Mouse-specific Fas UTR region or FasL UTR region was cloned into pmirGLO vector and these were designated as pmirGLO-Fas UTR or pmirGLO-FasL UTR, as described previously (Singh et al. 2012b).

Generation of miR-23a-specific Fas UTR or miR18b-specific FasL UTR oligonucleotides. First, oligonucleotides pairs containing Sac1 and Xba1 restriction sites

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(forward and reverse) of mouse Fas UTR region containing miR-23a-specific binding sites and mouse FasL UTR region containing miR-18b-specific binding sites were generated by IDT (IDT INC). The details of oligonucleotide pairs for miR-23a-specific Fas UTR region and miR-18b-specific FasL UTR region are as described below.

Oligonucleotide pairs (Fas UTR) of miR-23a
Forward Primer: 5'-AGTC**GAGCTCGTTCCAGGGACTGCTTCTG**-3'
Reverse Primer: 5'-AGTC**TCTAGAGGAAGGTCTTCAATTAACTGCGAG**-3'

Oligonucleotide pairs (FasL UTR) of miR-18b
Forward Primer: 5'-AGTC**GAGCTCAGCTTTGGGCTGCTGTGTG**-3'
Reverse Primer: 5'-AGTC**TCTAGATGGTGCCAATGAGACACTGTC**-3'

In the presence of oligo annealing buffer (46 µl), 2 µl of each oligonucleotide of mouse Fas UTR (specific to miR-23a) or mouse FasL UTR (specific to miR-18b) were annealed at 90°C for 3 minutes and 37°C for 15 minutes. The annealed oligonucleotides of Fas UTR or FasL UTR were used immediately or stored at -20°C.

Ligation and Transformation. Annealed oligonucleotides of Fas UTR or FasL UTR were ligated to pmirGLO vector restricted with Sac1 and Xba1 following the protocol of the company (Promega Corporation, Madison, WI). Ligated pmirGLO-Fas UTR or pmirGLO-FasL UTR was transformed and positive clones were selected for further use after confirming the clones by sequencing.

Transfection of EL4 cells and luciferase assays. Freshly cultured EL4 cells ($5x10^6$) were transfected with 5-10 µg of pmiRGLO plasmid without an insert (MOCK) or pmirGLO-Fas UTR plasmid or pmirGLO-Fas UTR plasmid and mature miR-23a or pmirGLO-FasL UTR plasmid or pmirGLO-FasL UTR plasmid and mature miR-18b using Amaxa Nucleoector

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instrument and EL4 transfection kits from Lonza and as described previously (Singh et al. 2012b). EL4 cells were replated in triplicate in 96-well plate two days post transfection and the cells were treated with VEH or DES (10 μM/ml). The cells were incubated for 24 h at 37 °C, 5% CO_2 post treatments. The cells were harvested, washed twice with PBS, centrifuged, and extracts were prepared by lysing cells in 70 μl of reporter lysis buffer (Promega Corporation, Madison, WI). Luciferase activity was determined using the Dual-Glo® Luciferase Assay System obtained from Promega Corporation (Promega corporation, Madison, WI) using Victor^2 (Perkin Elmer). Normalized firefly luciferase activity (firefly luciferase activity/Renilla luciferase activity) for each construct was compared to that of the pmirGLO Vector without insert (MOCK) and "normalized-fold induction" was determined.

Statistics. In this study, statistical analyses were performed using GraphPad Prism software (San Diego, CA). The significance of analysis of microarrays was performed using Kaplan-Meier method. Student's t-test was used for paired observations if data followed a normal distribution to compare DES-induced apoptosis in T cells, and expression and quantification of Fas and FasL expression in thymocytes. Differential (upregulated or downregulated) expression of miRs was analyzed using 2-sample t-test method. Multiple comparisons were made using ANOVA (one-way analysis of variance) test and Turkey-Kramer Multiple Comparisons Tests. P-value of ≤ 0.05 was considered to be statistically significant.

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Results

DES induces alterations in thymic cellularity in mothers and prenatal fetuses. We have reported previously that prenatal exposure to DES induces thymic atrophy and apoptosis resulting from upregulation of Fas and Fas ligand expression (Brown et al., 2006b; Singh et al. 2012a). In this study, our goal was to investigate the role of microRNA in such immunological changes induced by DES. To this end, we administered a single dose of DES (5 μg/kg) into pregnant C57BL/6 mice on gestation day (GD) 14, as described previously (Brown et al., 2006b; Singh et al. 2012a) and studied the effect on the thymocytes. On day 2 and day 3 post-DES treatment (GD 16 and GD 17), thymic cellularity of mothers and fetuses were determined. There was a significant decrease in thymic cellularity of the mothers on both day 2 (GD 16) and day 3 (GD 17) post-DES exposure (Fig. 1A). In fetuses, however, no significant difference in thymic cellularity of fetuses was observed on day 2 (GD 16) but significant decrease in thymic cellularity of fetuses was observed on day 3 (GD 17) post-DES exposure (Fig. 1B). These data are consistent with previously published results from our laboratory that DES induces a decrease in thymic cellularity both in the pregnant mothers and fetuses (Brown et al., 2006a; Brown et al., 2006b; Camacho et al., 2004a; Camacho et al., 2004b). We also confirmed in these experiments that exposure to DES led to increased apoptosis in thymocytes from both mothers and fetuses and that DES-exposed cells had higher levels of the expression of Fas and FasL (data not shown), consistent with our earlier studies (Brown et al., 2006b; Singh et al.2012a).

Analysis of DES-regulated miRs profile in thymocytes from the mother and fetus: The data obtained from miR arrays of the thymocytes post DES exposure were further analyzed.

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To this end, cluster analyses of 609 miRs (Fig 2A) were performed using Ward's method. Similarity, measure of miRs of the four groups (MV: Mother+VEH, MDES: Mother+DES, FV: Fetus+VEH, and FDES: Fetus+DES) was done using Half Square Euclidean Distance method and ordering function of miRs was done on the basis of Input rank. As shown in Fig 2A, the visualization of cluster analysis of miRs have been presented as a dendrogram and their expression pattern is reflected (Fig 2A). Upon comparison of more than 1-fold dysregulated (upregulated or downregulated) miRs in DES-treated mothers versus VEH-treated mothers or DES-treated fetuses versus VEH-treated fetuses, there were 59 miRs that were common for both mothers and fetuses, whereas there were 107 miRs that were specific to mothers only and 101 miRs that were specific to fetuses only (Fig 2B).

Differential Expression of miRs in fetal thymocytes. For all further studies, we focused on miR profiles seen in fetal thymocytes to investigate if their expression correlated with thymic atrophy. To that end, we analyzed differential (upregulated or downregulated) expression of miRs in fetal thymocytes using 2-sample t-test method. The significance of analysis of microarrays was performed using Kaplan-Meier method. A p-value of <0.01 in the t-test was considered significant. As shown in Fig 3A-B, of the total 609 miRs screened, there were 63 miRs showing more than 1.5 fold change (Fig 3B), of which 34 miRs showing upregulated and 29 miRs showing downregulated expression in DES-exposed fetal thymocuyes when compared to vehicle controls. Upon analysis of upregulated (34) miRs, 15 miRs showed expression from 1.5 to 1.9 fold whereas 19 miRs showed 2 or more than 2-fold expression in DES-exposed thymocytes. Similarly upon examination of downregulatd (29) miRs following DES treatment, there are 19 miRs that showed expression from 1.5 to 1.9 fold and 10 miRs that showed

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expression of 2 or more than 2-fold (Supplemental Table 3). Change in expression of miRs, 1.5-fold or higher, was considered positive and thus, for all further analyses, we used miRs showing such changes.

Validation of miR Expression by Real-Time PCR. Based on the analysis of miR array data, we randomly chose eight miRs (seven downregulated miRs; miR-18b, -23a, -30a, -31, -146a, -155, and -217 and one upregulated miR (miR-320) to verify and validate their expression in fetal thymocytes post-DES or vehicle exposure (Fig 4A). To this end, Real-Time PCR was performed on cDNAs converted from total RNAs including miRs from thymocytes treated with DES or vehicle as described in Methods. Data obtained from Real-Time PCR demonstrated downregulated expression of miR-18b, -23a, -30a, -31, -146a, -155, and -217 and upregulated expression of miR-320 in fetal thymocytes exposed to DES when compared to vehicle-treated thymocytes (Fig 4B). Thus, the Real-Time PCR data validated the expression profile of selected miRs obtained from miR arrays.

DES-regulated miRs play important role in various disease pathways. We next analyzed how DES-induced alterations in the expression of miRs in the fetus would affect various disease pathways. To this end, 63 miRs were analyzed which showed 1.5 or greater fold change, using IPA software and database of the company (Ingenuity Inc). Although, there were as many as 28 pathways that were found to be affected by the altered expression of miRs, there were at least 12 dominant pathways that were significantly affected (Fig 5A). These included: genetic disorder (26 miRs), skeletal and muscular disorder (15 miRs), connective tissue disorder (15 miRs), inflammatory diseases (18 miRs), organismal injury (13 miRs), respiratory diseases (11 miRs), developmental disorder (13 miRs), and cancer (26 miRs) (Fig 5A). Similarly, as

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shown in Fig 5A, there were several miRs involved in other pathways including cellular development (16 miRS), cellular growth and proliferation pathways (16 miRs), cardiovascular diseases (7 miRs), gastrointestinal diseases (15 miRs), hematological disease (10 miRs), endocrine development (4 miRs), organ development (4 miRs), organismal development (4 miRs), tissue development (5 miRs), cell death (13 miRs), and cell cycle (7 miRs). Upon further analysis of DES-regulated miRs (>1.5 fold upregulated or downregulated expression) using Ingenuity IPA software, the following pathways were found to be more significantly affected, including cancer pathway (Fig 5B), genetic disorder pathway (Fig 5C), reproductive diseases pathway (Fig 5D), and cellular differentiation pathways (Fig 5E).

Upon further analysis of DES-regulated miRs and their relationship with various genes using Cytoscape and Cluego softwares (Cytoscape Consortium), there were large numbers of miRs that regulated various cytokine genes (Fig. 6A-B, 7A, 7C) and genes involved in molecular pathways (Fig. 7B). These data together demonstrated that DES-induced alterations in the expression of miRs in the fetus may play a role in affecting various pathways and genes that are important in biological, immunological, and molecular functions of cells.

Analysis of miRs associated with Fas and FasL Expression. Previous studies from our laboratory have reported that DES induces increased expression of Fas and FasL in thymocytes, which plays a critical role in induction of apoptosis (Brown et al., 2006a; Brown et al., 2006b). In this context, we investigated potential miRs involved in the expression of Fas and FasL. We used TargetScanMouse 5.2, miRWalk, and microRNA.org software, and detected significant binding affinity of miR-23a and miR-18b with 3'-UTR region of Fas and FasL genes respectively (Supplemental Table 2).

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To confirm this analysis, we used EL4 T cells not transfected or transfected with mature miR-23a or miR-18b and cultured them in the absence or presence of DES for 24 hrs. The expression of Fas and FasL was determined by performing RT-PCR. EL-4 cells spontaneously expressed Fas and FasL (VEH-treated) and treatment with DES further augmented their expression (Fig 8A-B). Interestingly, expression of Fas in EL4 cells transfected with miR-23a was decreased significantly when compared to VEH-treated EL-4 cells (Fig 8A-B). Moreover, EL4 cells transfected with miR-23a and treated with DES also showed a decrease in Fas expression, when compared to DES-treated cells (Fig 8A-B). Similarly, upregulated expression of FasL was observed in EL4 cells treated with DES, when compared to VEH-treated cells (Fig 8A-B). Upon transfection of EL4 cells with miR-18b, there was significant downregulation of FasL expression but upon DES treatment, downregulation of FasL expression was significantly reversed (Fig 8A-B). Overall, in these studies, we also noted that transfection of vehicle-treated EL-4 cells with miR-23a, decreased the expression of only Fas but not FasL and similarly, transfection with miR-18b decreased the expression of FasL but not Fas. Also, DES reversed the miR transfection-induced inhibition of the expression of Fas and FasL. These data together suggested that DES-induced decrease in the expression of miR23a and miR-18b may indeed be the mechanism through which Fas and FasL, respectively, get up-regulated.

Association of DES-induced downregulates miR-23a and miR-18b and expression of Fas and FasL. To further examine if DES mediated its effect through downregulation of miR-23a and miR-18b, Fas UTR containing miR-23a binding region (Fig 9A) or FasL UTR region containing miR-18b binding region (Fig 9B) was cloned into pmiRGLO luciferase expression vector and the clones were designated as pmirGLO-Fas UTR and pmirGLO-FasL UTR. EL4

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cells transfected with pmiRGLO without insert (MOCK) or pmirGLO-Fas UTR or pmirGLO-Fas UTR + mature miR-23a or pmirGLO-FasL UTR or pmirGLO-FasL UTR + mature miR-18b were next treated with VEH or DES for 24hrs. EL4 cells were also transfected with a vector containing GFP as a positive control for transfection. There was more than 68% transfection of EL4 cells (Fig 10A). Upon analysis of luciferase expression in the presence of Fas or FasL UTR region, there was significantly upregulated expression of luciferase (3-3.5 fold) in EL4 cells transfected with pmirGLO-Fas UTR in the presence of DES when compared to EL4 cells transfected with pmirGLO-Fas UTR but treated with VEH (Fig 10B). Similarly, luciferase expression was upregulated (2-2.5 fold) in EL4 cells transfected with pmirGLO-FasL UTR and treated with DES, when compared to similar cells treated with VEH (Fig 10B). Next, we examined luciferase expression in EL4 cells that were transfected either with pmirGLO-Fas UTR + mature miR-23a or pmirGLO-FasL UTR + mature miR18b in the absence or presence of DES. The expression of luciferase was decreased in EL-4 cells with pmirGLO-Fas UTR + mature miR-23a+DES when compared to cells with pmirGLO-Fas UTR + DES (Fig 10B). Similar results were seen in luciferase expression in EL-4 cells transfected with pmirGLO-FaL UTR + mature miR-18b + DES when compared to cells with pmirGLO-FasL UTR + DES (Fig 10B). These data demonstrated that DES regulates expression of Fas and FasL, at least in part, through down-regulation of miR-23a and miR-18b expression respectively.

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DISCUSSION

Exposure to DES during pregnancy in humans has been shown to trigger a number of clinical disorders both in the mother as well as the developing fetus well into adulthood (Goodman et al., 2011; Herbst, 1981; Hoover et al., 2011; Palmer et al., 2005). Prenatal exposure to DES has also been shown to cause significant alterations in the immune response including altered susceptibility to autoimmune diseases, cancer and infections (Besteman et al., 2005; Brown et al., 2006a; Giusti et al., 1995; Hilakivi-Clarke et al., 2013; Holladay et al., 1993; Laitman, 2002; Maier et al., 1985; Marselos and Tomatis, 1992a; Marselos and Tomatis, 1992b; Singh et al. 2012a). Although in recent years, miRs have been the focus of several studies due to their direct role in gene expression, there is not much information in literature regarding DES-mediated effects on gene expression and function regulated by miRs. Therefore, in the present study, we investigated whether exposure to DES during pregnancy alters the miR profile in the pregnant mother as well as the fetus. We noted that while DES caused similar alterations in the mother and the fetus as 59 miRs showing similar changes, there were significant numbers of miRs that were uniquely altered in these two groups. The differences in miR expression between mother and fetus may result from the differential amounts of DES exposure between the mother and the fetus. This may be because of direct exposure of mother's thymocytes to DES, whereas the fetal thymocytes may be exposed to the amounts that cross the placenta to reach the fetus. In addition, while mother has mature thymus, the fetus, on the other hand, has immature thymus, which may be more susceptible to DES toxicity.

Molecular Pharmacology Fast Forward. Published on March 9, 2015 as DOI: 10.1124/mol.114.096743
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Cluster analysis of miR expression profile showed clustering of a large number of miRs that were differentially expressed in fetal thymi upon exposure to DES. We identified several upregulated or downregulated miRs in the presence of DES. We validated the expression profile of some of the miRs (miR-18b, -23a, -30a, -31, -130b, -146a, -155, -217, -301a, and -320) by performing Real-Time PCR and the data confirmed and corroborated with the miRs expression profile data obtained from miR arrays analysis. Furthermore, we also verified the relationship of selected miRs (miR-23a and miR-18b) and their target gene expression. We selected miR-23a and miR-18b because miR-23a was found to have binding affinity with mouse Fas UTR whereas, miR-18b had binding affinity with mouse FasL UTR and both miRs were downregulated in fetal thymi post-DES exposure (Fig 4B). Moreover, previous studies from our laboratory showed that DES caused increased expression of Fas and FasL expression in thymocytes of pregnant mothers as well as the fetus (Brown et al., 2006a; Brown et al., 2006b). Examination of Fas and FasL expression by RT-PCR, on the other hand, showed increased expression in fetal thymi upon exposure to DES, when compared to VEH (Fig 8A-B).

As negative regulators of gene expression, expression profile of miRs in the thymus is very important. In this study, we considered DES-regulated miR expression to be significant when they were altered more than 1.5 fold, as also reported in other studies (Moffat et al., 2007). The downregulated expression of several miRs (miR-18a, -18b, -23a, -23b, -26b, -30a, -31, -98, -130b, -146a, -155, -204, -217, -298-301a, and -320) in fetal thymi post-DES exposure indicated that the expression of respective genes that they regulate, may be increased. Upon closer analysis of miR-gene relationship, we observed that miR-18a and miR-18b possessed binding affinity with FasL 3'UTR whereas, miR-23a, miR-23b, possessed binding affinity with Fas

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3'UTR. Similarly, miR-146a possessed binding affinity with BRCA1 3'UTR, miR-155 with SHIP1 3'UTR, miR-217 with Foxp3 3'UTR, and miR-298 with BACE1 3'UTR. Thus, downregulated expression of these miRs post-DES exposure suggests that they may be involved in regulation of many genes including apoptosis in fetal thymi.

In the current study, we also noted downregulated expression miR-31, miR-34b, and miR-181a in fetal thymi post-DES exposure. Upon analysis, miR-31 showed binding affinity with 3'UTR region of Foxp3 and CYP1A1 genes. Foxp3 expression is known to be expressed predominantly in regulatory T cells (Biller et al., 2007; Ruan et al., 2009). The computational analysis of miR-34b and miR-181a showed binding affinity with 3'UTR of Notch ligand JAG-1 and large number of zinc finger (ZNFs) genes respectively. Expression of miR-34b has been shown to be regulated by p53, which mediates cell-cycle arrest and promotes apoptosis (Hermeking, 2010; Hermeking, 2007). Also, overexpression of miR-34b has been shown to decrease the expression of a number of cell cycle regulatory proteins including cyclin D1, c-MET and CDK4 (Hermeking, 2010; Hermeking, 2007), and thus hamper cell cycle progression (Hermeking, 2010; Hermeking, 2007). miR-34b has been reported to act as a tumor suppressor in colorectal cancer (Wu et al. 2014), prostate cancer (Geier et al. 2010), and gastric cancer (Wang et al., 2014; Yang et al. 2014). In another recent study, Lee et al have shown the role of miR-34b in expression of ER and ER-mediated growth of breast cancer cells *in vitro* (Lee et al. 2011). These data are consistent with our previous findings that DES alters T cell selection process in the thymus (Brown et al., 2006a). It has also been demonstrated that miR-181a modulates expression of zinc finger family member genes by directly targeting their coding regions (Huang et al. 2010). Together, these data demonstrated that the miRs that are

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downregulated in fetal thymi by DES may control the expression of genes involved in various mechanisms and functions like T cell selection in thymus, thymic atrophy, immunosuppression, and toxicity. This may explain how DES may alter the positive and negative selection of T cells in the thymus as reported by us previously (Brown et al., 2006a).

We also noted DES-induced upregulation of several miRs (miR-21, -101a, -126, -133b, -141, 200a, -200b, -320, -429, -451, -466f, and -466g). These miRs regulate the expression of various genes that control different physiological and biological mechanisms and functions. For example, miR-21 that showed increased expression (>2.3 fold) in fetal thymi post-DES exposure, plays an important role in cancer development 2010). miR-21 expression has been shown to be activated in multiple types of cancers, such as breast, liver, brain, prostate, myometrial cancers (Jazbutyte and Thum 2010). Thus, miR-21 regulates a large number of target proteins that may be involved in cellular survival, apoptosis, and cell invasiveness (Jazbutyte and Thum 2010). Tanaka et al (2009) reported that miR-101a controls mammary gland development in mice, through alterations in the expression of Cox-2 (Tanaka et al., 2009). Several miRs such as MiR-141, miR-200a, and miR-200b were upregulated in fetal thymi post-DES exposure. These miRs play significant role in ovarian tumorigenesis and cancer development. In a recent study, it was shown that miR-141 and miR-200a affect ovarian tumorigenesis by controlling oxidative stress response (Mateescu et al. 2011). Furthermore, there were several miRs that were downregulated (varied from 1.5 to 2.5 fold) post-DES exposure. These miRs were miR-23a, -23b, -18b, -26b, -98, -148b, and -186 and are expressed in various tissues such as breast, cartilage, endothelial cells, and embryonic tissues. These down-regulated miRs have been shown to control genes that are involved in various physiological

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functions in these tissues (Baltimore et al., 2008). Thus, overall, our miR data were consistent with DES-induced toxicity in reproductive organs and ability to act as a carcinogen (Walker et al. 2013).

The current study suggests that prenatal exposure to environmental stressors can have a significant impact on the miR profile. For example, prenatal exposure to arsenic was shown to alter miR expression associated with innate and adaptive immune responses (Rager et al. 2014). Additionally, we have also shown that TCDD altered the expression of miRs in thymuses, when pregnant mothers were exposed to TCDD (Singh et al. 2012b). In summary, we demonstrate for the first time that prenatal exposure to DES can cause a significant effect on the miR profile in thymuses of both the mother and fetus. These upregulated or downregulated miRs may influence the regulation of genes that affect the development of the immune cells and other organ systems. Identification of miRs as targets for DES-induced modulation of gene expression offers novel mechanisms to understand DES-regulated molecular mechanisms and its long-term effects.

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Molecular Pharmacology Fast Forward. Published on March 9, 2015 as DOI: 10.1124/mol.114.096743
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Acknowledgements

We would like to thank Drasti Patel for technical help.

Molecular Pharmacology Fast Forward. Published on March 9, 2015 as DOI: 10.1124/mol.114.096743
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Authorship Contribution

Participated in research design: N. P. Singh, P. Nagarkatti, and M. Nagarkatti.

Conducted experiments: N. P. Singh, Abbas, Menard, and U. P. Singh.

Contributed new reagents or analytic tools: N. P. Singh, P. Nagarkatti, and M. Nagarkatti.

Performed data analysis: N. P. Singh, Abbas, U. P. Singh, Zhang, P. Nagarkatti, and M. Nagarkatti.

Wrote or contributed to the writing of the manuscript: N. P. Singh, P. Nagarkatti, and M. Nagarkatti.

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Molecular Pharmacology Fast Forward. Published on March 9, 2015 as DOI: 10.1124/mol.114.096743
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Footnotes

This work was supported in part by National Institutes of Health Grants: National Center for Complementary and Alternative Medicine [P01AT003961 and R01AT006888], National Institute of Environment and Health Sciences [R01ES019313], National Institute of Mental Health [R01MH094755], and National Institute of General Medical Sciences [P20GM103641]; Veterans Affairs Merit Award [BX001357]; and University of South Carolina ASPIRE 1 Grant [A011].

Molecular Pharmacology Fast Forward. Published on March 9, 2015 as DOI: 10.1124/mol.114.096743
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Legends for Figures

FIGURE 1. DES decreases thymic cellularity in both mothers and fetuses. DES (5 μg/kg body weight) or VEH (control) was administered into pregnant mice (GD 14). The thymi from mothers and their fetuses were harvested on days 2 (GD 16) and 3 (GD 17) post DES or VEH treatment to determine thymic cellularity. A. DES-induced changes in thymic cellularity of mothers. B DES-induced changes in thymic cellularity of fetuses. The bars represent mean +/- SEM from groups of 5 mice ($p>0.05$). Experiment repeated thrice with consistent results.

FIGURE 2. Heat map of miR expression profile in thymi of mothers and fetuses post exposure to DES. Total RNAs including miRs from thymi of mothers and fetuses post DES or VEH exposure were isolated. miR array specific to mouse was performed on Affymetrix GeneChip miR platform. A. Heat map depicting miR expression profile in thymi of mothers and prenatal fetuses exposed to DES or VEH (control). The expression pattern (green to red) represents the spectrum of downregulated to upregulated expression pattern of miRs. B. Venn diagram showing relationship amongst various miRs differentially expressed in thymi of mothers treated with DES when compared to VEH, and miRs differentially expressed in thymi of fetuses treated with DES when compared to VEH.

FIGURE 3. Expression profile of miRs in thymi of fetuses post prenatal exposure to DES. Data obtained from miR arrays were analyzed for the expression of miRs. Dysregulation (fold change) of miR expression in DES-treated thymi was compared to miRs expression in VEH-

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treated thymi. A. Depicts fold change expression profile of miRs post-DES exposure in comparison to VEH. B. A significant number of miRs showed more than 1.5 fold change (upregulated or downregulated) in their expression profile.

FIGURE 4. Validation of expression profile of selected miRs in fetal thymi post prenatal exposure to DES. Based on expression profile obtained from miR arrays, eight miRs (seven downregulated and one upregulated) were selected for validation. Real-Time PCR using mouse-specific miR assays was performed. In panel A, miR expression profile from miR arrays is depicted. B, Expression profile of selected miRs (miR-18b, -23a, 30a, -31, -146a, -155, -217, and -320) in fetal thymi post DES exposure was determined using miR-specific primers and by performing Real-Time PCR. Data are depicted as mean ± SEM of three independent experiments. Asterisk (*) in panel B indicates statistically significant ($p<0.05$) difference between groups compared.

FIGURE 5. DES-regulated miRs and their association with functional networks and various pathways. DES-induced up- or down-regulated (more than 1.5 fold change) miRs were analyzed using IPA software and database of Ingenuity Systems (QIAGEN). The data presented in the graph demonstrate various pathways regulated by DES-induced miRs. Y-axis, –log(p-value) represents significance of function by random chance (IPA software, Ingenuity Systems, QIAGEN). Number over each bar represents number of miRs involved in pathways. DES-regulated miRs as described in panel A were further analyzed using IPA software and the database (Ingenuity Systems, QIAGEN). B, miRs involved in cancer pathway, C, genetic

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disorder pathway, D, reproductive pathway, and E, cellular differentiation pathway. In Figure 5 B-E, thin line empty circles represents mature miRs with various functions, thick line empty circles represents various genes, magenta circles represent upregulated mature miRs, green circles represent downregulated mature miRs, and blue ovals represent various genes involved in the pathways.

FIGURE 6. Potential gene targets of DES-regulated miRs. First, potential gene targets of DES-regulated miRs were analyzed by Ingenuity IPA software online (QIAGEN) and then the relationship between various DES-regulated miRs and genes were analyzed using Cytoscape version 3.0.1. A-B, data showing relationship between various DES-regulated miRs and genes post Cytoscape 3.0.1 software analysis. Various sizes of miR boxes indicate number of possible regulated genes by the particular miR.

FIGURE 7. Mapping of miRs and possible pathways. DES-regulated miRs and selected target genes were analyzed using Cytoscape suite with ClueGo plugins. A, demonstrates Biological pathway, B, demonstrates molecular pathway, and C, demonstrates Immunological pathway regulated by miRs and associated genes.

FIGURE 8. Expression of Fas and FasL in EL4 cells in the presence or absence of miR-23a or miR-18b post DES treatment. Expression of Fas and FasL was determined by performing RT-PCR on cDNAs generated from total RNAs isolated from EL4 cells not transfected or transfected with mature miR-23a or mature miR-18b and treated with VEH or DES. 18S was used as an

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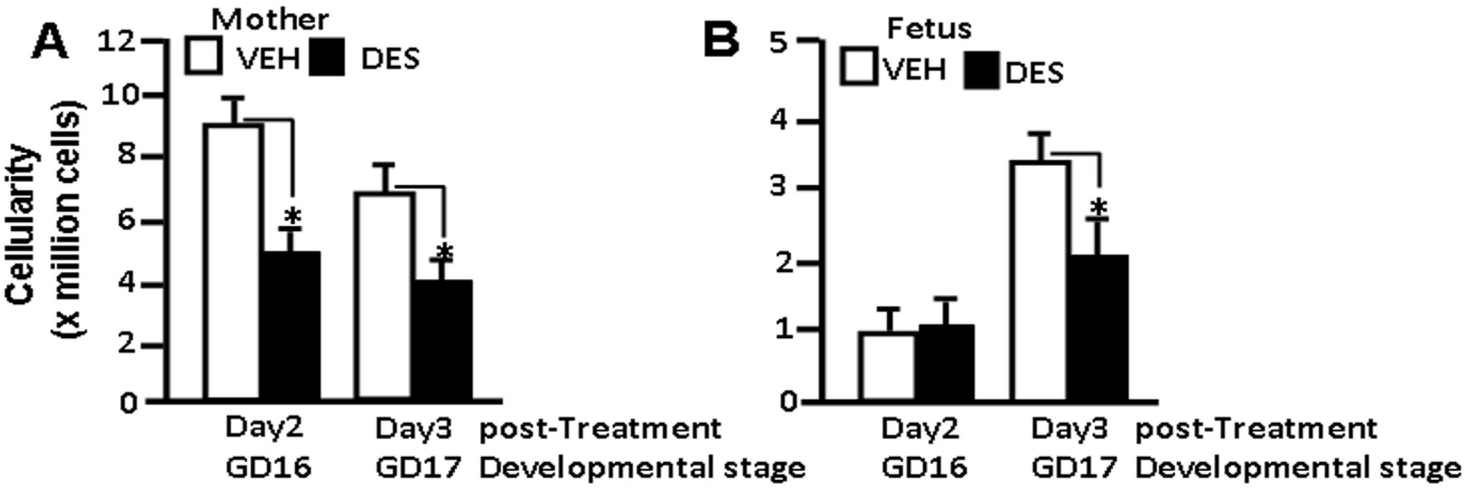
internal control. A, RT-PCR data for Fas and FasL expression are presented. EL4 cells not transfected or transfected with mature miR-23a or mature miR-18b exposed to DES or VEH were analyzed for the expression of Fas and FasL by performing RT-PCR. In panel B, RT-PCR data are presented as percentage of 18S expression with the latter being considered as 100%. Data are depicted as mean ± SEM of three independent experiments. Asterisk (*) in panel B indicates statistically significant ($p<0.05$) difference between groups compared.

FIGURE 9. Physical map of Fas and FasL UTR showing miR-23a and miR-18b binding sites. A: Physical map of Fas UTR region demonstrating binding site of miR-23a and the position of the primer pairs in the Fas UTR. B: Physical map of FasL UTR region demonstrating binding site of miR-18b region and the position of the primer pairs in the FasL UTR.

FIGURE 10. Expression of luciferase in EL4 cells in the presence or absence of Fas UTR containing miR-23a binding site or FasL UTR containing miR-18b binding site post VEH or DES treatment. EL4 cells were transfected with pmiRGLO without an insert (MOCK), pmiRGLO-Fas UTR, pmiRGLO-Fas UTR + mature miR-23a, pmiRGLO-FasL UTR, or pmiRGLO-FasL UTR + mature miR-18b. EL4 cells were also transfected with a vector containing GFP. Forty eight hrs post transfection, cells were examined for GFP expression by flow cytometry for transfection efficiency (A). Luciferase assays were performed to determine luciferase expression in EL4 cells post-transfection. (B). Luciferase data are presented as fold change in expression. Data are depicted as mean ± SEM of three independent experiments. Asterisk (*) in panel B indicates statistically significant ($p<0.05$) difference between groups compared.


A
Mother
VEH
DES
Cellularity
(x million cells)
12
10
8
6
4
2
0
*
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Day2
GD16
Day3
GD17
post-Treatment
Developmental stage
B
Fetus
VEH
DES
5
4
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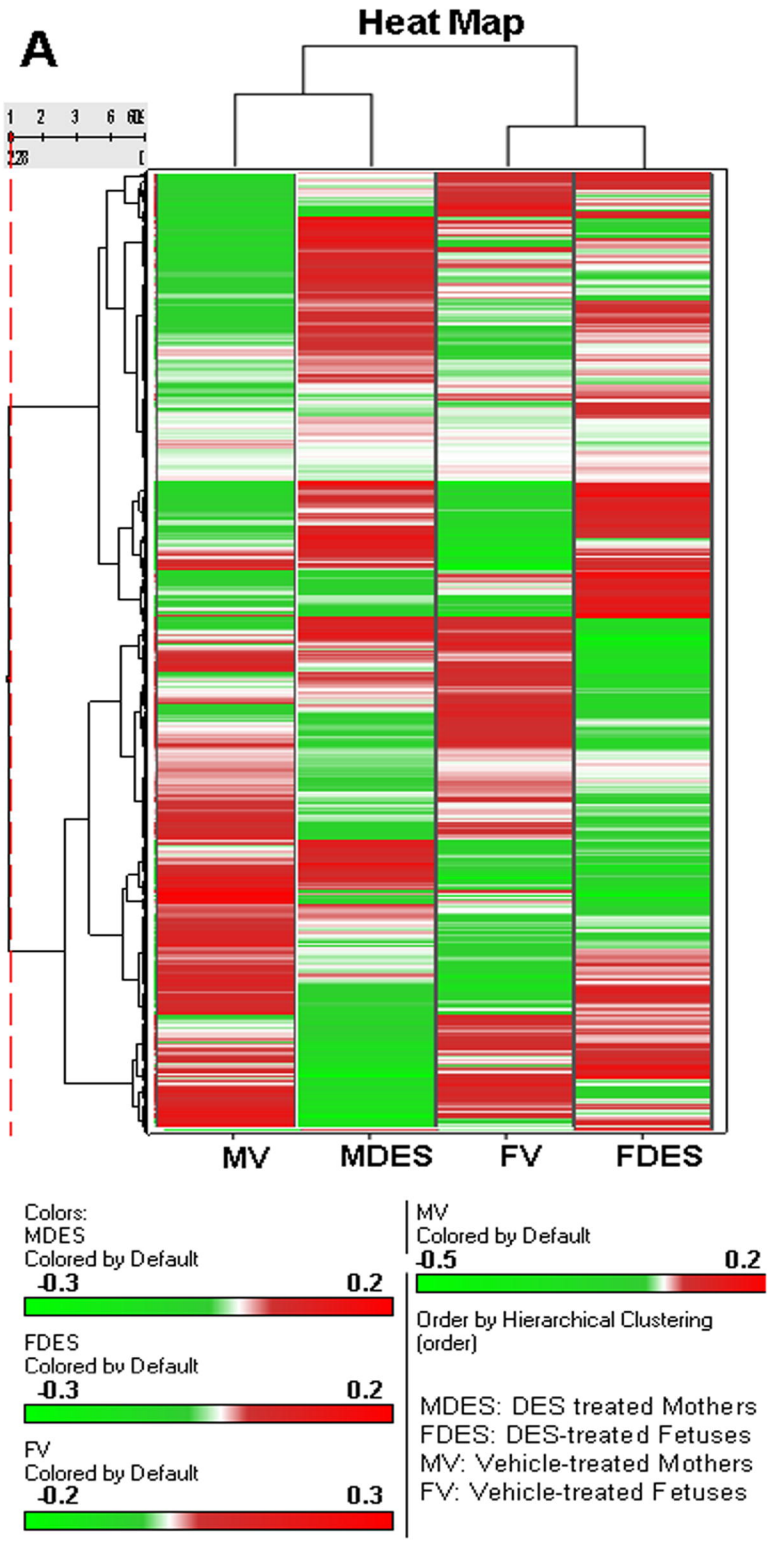
Day2
GD16
Day3
GD17
post-Treatment
Developmental stage

Figure 1


A
Heat Map
MV
MDES
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FDES
Colors:
MDES
Colored by Default
-0.3
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FDES
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-0.3
0.2
FV
Colored by Default
-0.2
0.3
MV
Colored by Default
-0.5
0.2
Order by Hierarchical Clustering (order)
MDES: DES treated Mothers
FDES: DES-treated Fetuses
MV: Vehicle-treated Mothers
FV: Vehicle-treated Fetuses

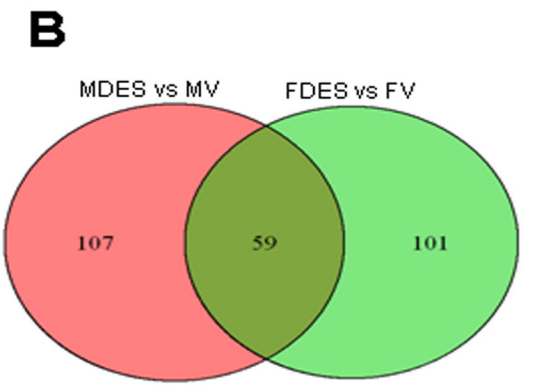

B
MDES vs MV
FDES vs FV
107
59
101

Figure 2

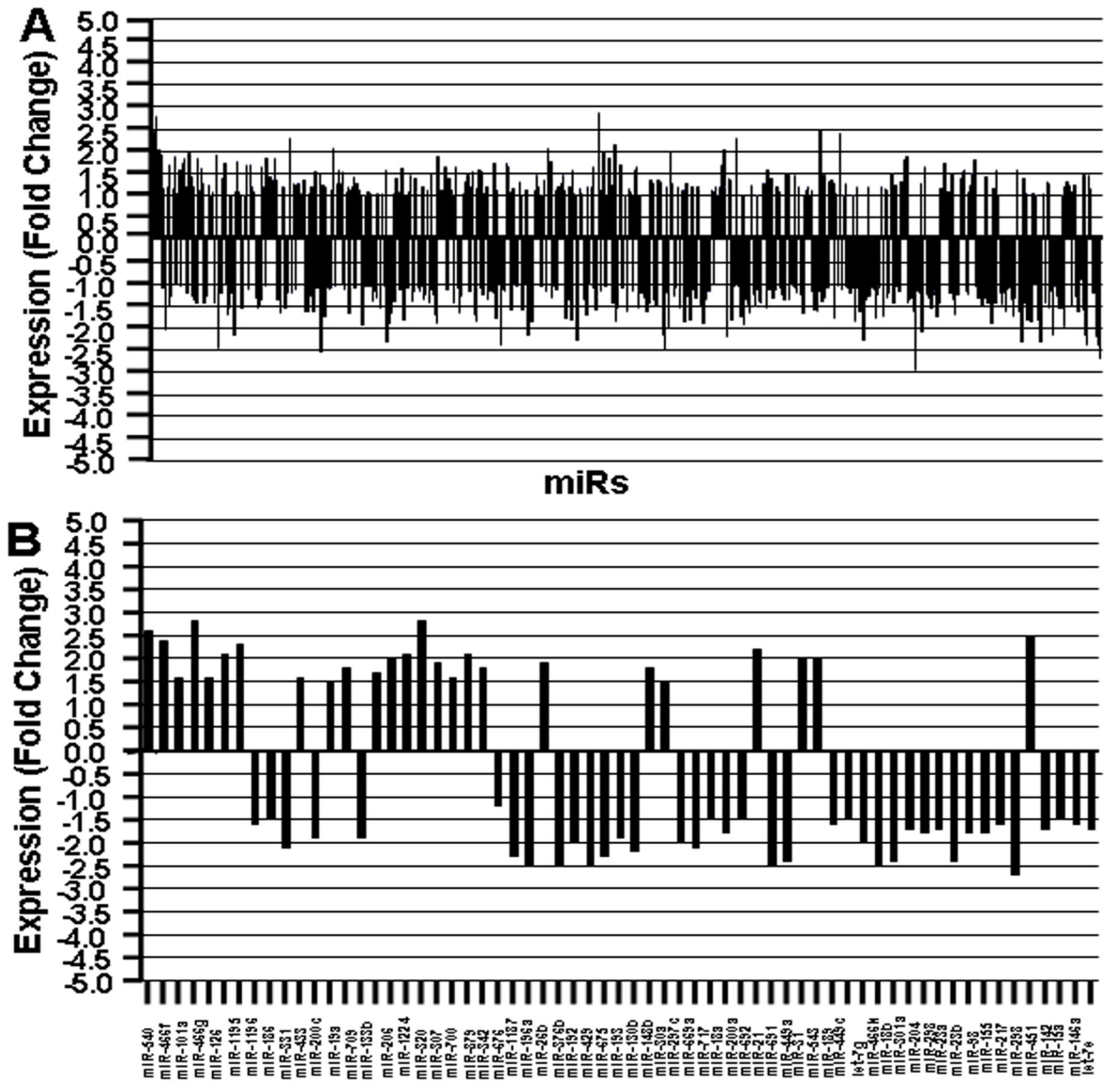

A
Expression (Fold Change)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
miRs
B
Expression (Fold Change)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
miR-340
miR-4661
miR-1013
miR-466g
miR-126
miR-1195
miR-1196
miR-186
miR-331
miR-433
miR-200c
miR-193a
miR-709
miR-133b
miR-206
miR-1224
miR-320
miR-307
miR-7-2
miR-379
miR-342
miR-675
miR-1187
miR-196a
miR-26b
miR-376b
miR-192
miR-429
miR-675
miR-133
miR-130b
miR-148b
miR-30a
miR-297c
miR-669a
miR-7-1
miR-183
miR-200a
miR-492
miR-21
miR-451
miR-449a
miR-31
miR-545
miR-183
miR-448c
let-7g
miR-466m
miR-18b
miR-3011a
miR-204
miR-26a
miR-23b
miR-93
miR-155
miR-217
miR-26b
miR-451
miR-142
miR-183
miR-146a
let-7e

Figure 3

A

miRs	Expression (Fold Change)
18b	(2.5) ⬇
23a	(2.2) ⬇
30a	2.2 ⬇
31	2.4 ⬇
130b	2.3 ⬇
301a	2.4 ⬇
146a	1.7 ⬇
155	1.8 ⬇
217	1.6 ⬇
320	2.0 ⬆

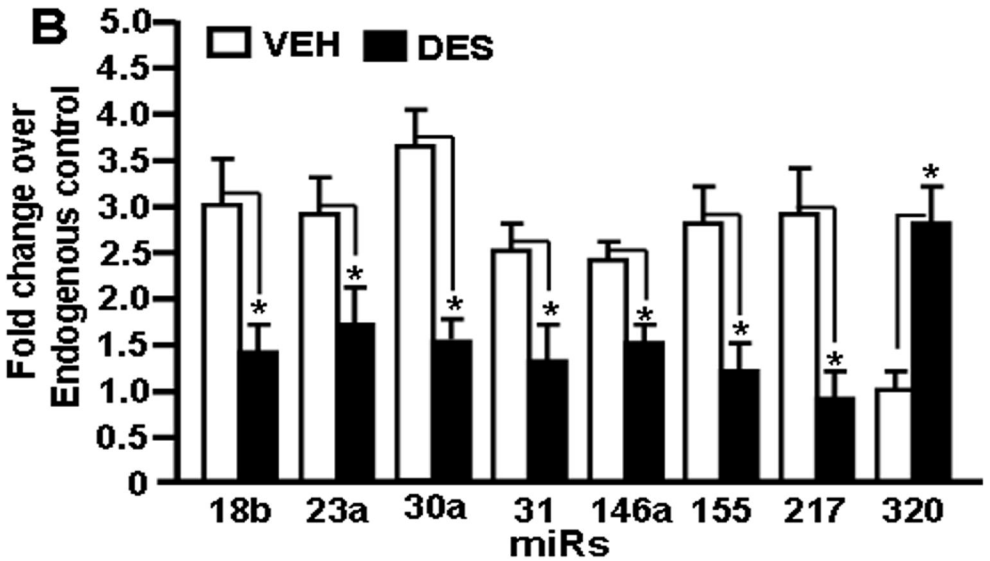
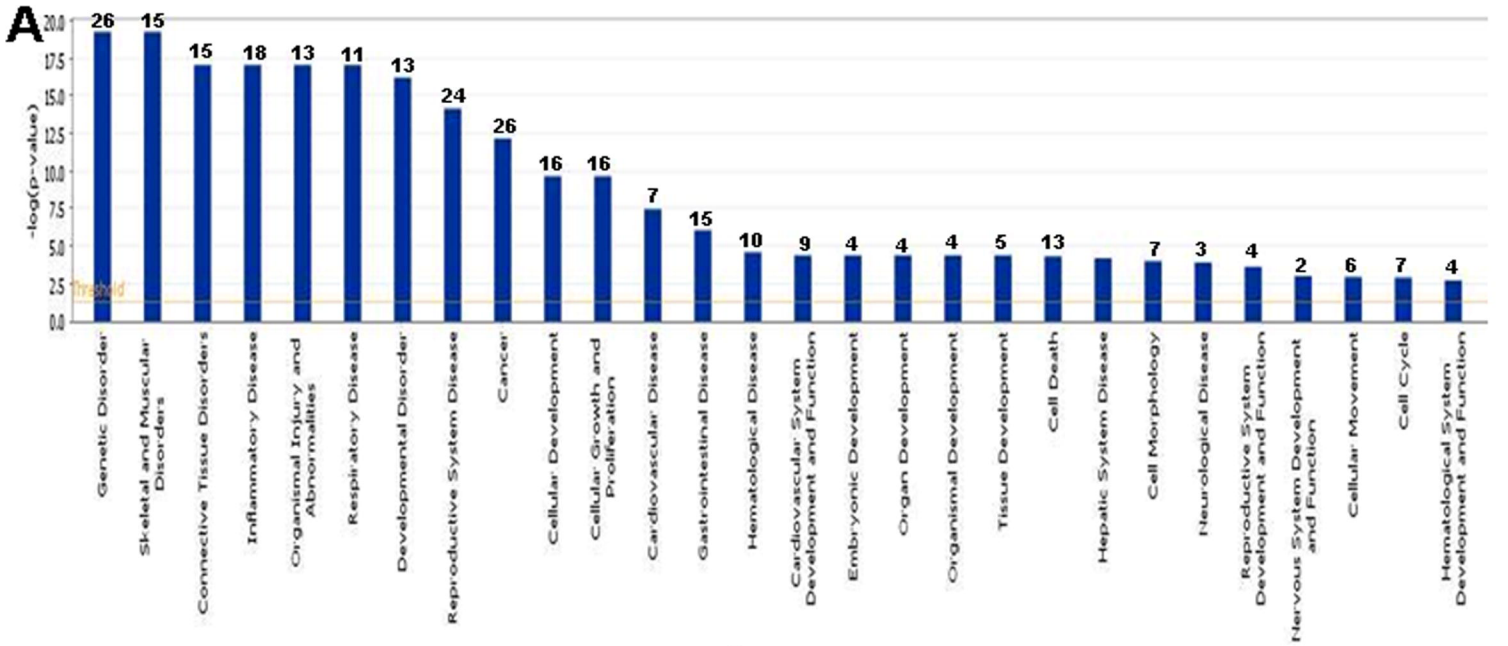
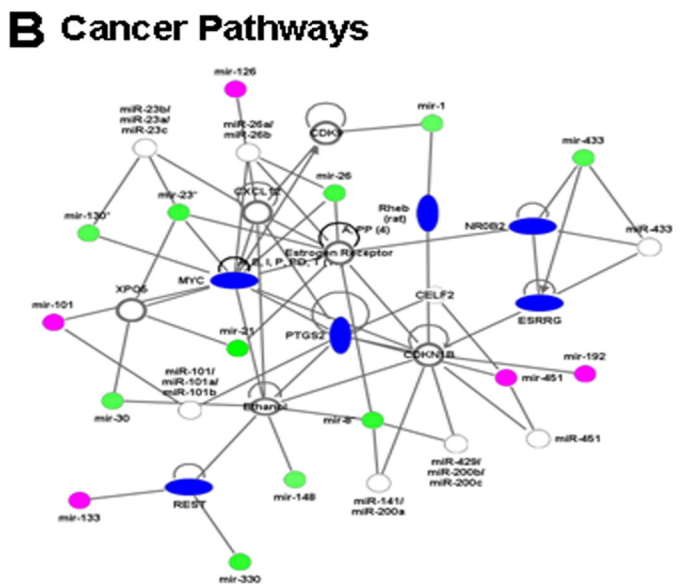
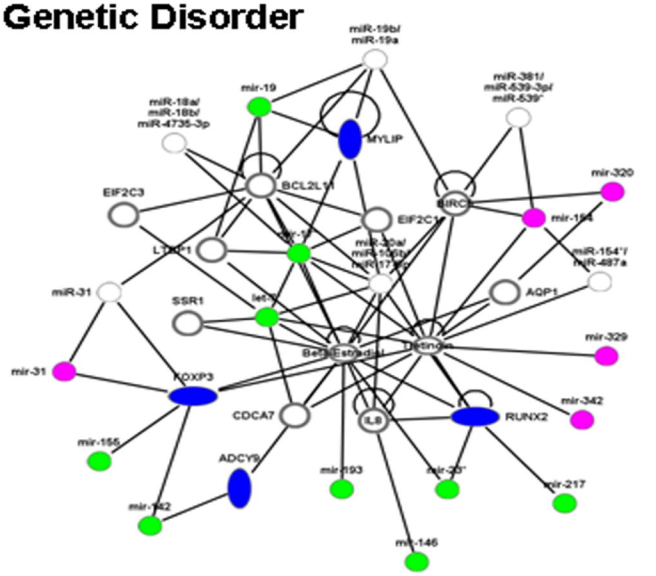
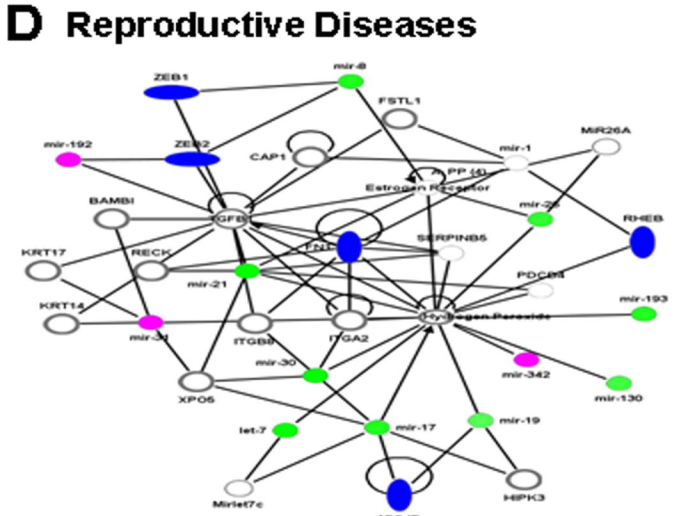


Figure 4


A
-log(p-value)
Threshold
26
15
15
18
13
11
13
24
26
16
16
7
15
10
9
4
4
4
5
13
7
3
4
2
6
7
4
Genetic Disorder
Skeletal and Muscular Disorders
Connective Tissue Disorders
Inflammatory Disease
Organismal Injury and Abnormalities
Respiratory Disease
Developmental Disorder
Reproductive System Disease
Cancer
Cellular Development
Cellular Growth and Proliferation
Cardiovascular Disease
Gastrointestinal Disease
Hematological Disease
Cardiovascular System Development and Function
Embryonic Development
Organ Development
Organismal Development
Tissue Development
Cell Death
Hepatic System Disease
Cell Morphology
Neurological Disease
Reproductive System Development and Function
Nervous System Development and Function
Cellular Movement
Cell Cycle
Hematological System Development and Function


B Cancer Pathways
miR-126
miR-23b/ miR-23a/ miR-23c
miR-26a/ miR-26b
CDK6
mir-1
mir-433
mir-26
CXCL12
mir-23*
mir-130*
Rheb (rat)
NR0B2
miR-433
Estrogen Receptor
MYC
XPO5
CELF2
mir-101
mir-21
PTGS2
ESRRG
CDKN1B
mir-192
mir-451
miR-101/ miR-101a/ miR-101b
mir-30
mir-8
mir-451
miR-429/ miR-200b/ miR-200c
REST
mir-148
miR-141/ miR-200a
mir-133
mir-330


C Genetic Disorder
miR-19b/ miR-19a
miR-19
miR-18a/ miR-18b/ miR-4735-3p
MYLIP
miR-381/ miR-539-3p/ miR-539*
BCL2L11
mir-320
EIF2C3
EIF2C1
mir-154
LTBP1
miR-154*/ miR-487a
miR-31
SSR1
AQP1
mir-329
FOXP3
CDCA7
IL8
RUNX2
mir-342
mir-155
ADCY9
mir-193
mir-217
mir-146


D Reproductive Diseases
ZEB1
mir-8
FSTL1
MIR26A
mir-192
ZEB2
CAP1
mir-1
BAMBI
TGFB1
RHEB
KRT17
RECK
SERPINB5
mir-21
PDCD4
KRT14
mir-193
ITGB8
ITGA2
mir-30
mir-342
mir-130
XPO5
let-7
mir-17
mir-19
Mirlet7c
HIPK3
MYLIP

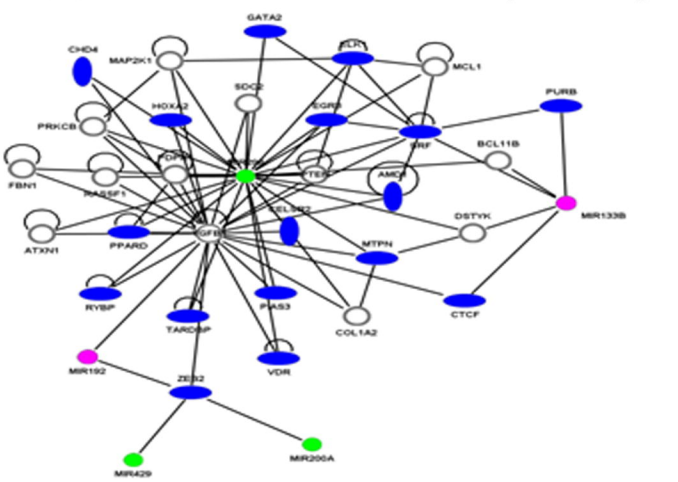

E Cellular Differentiation pathway
GATA2
CHD4
MAP2K1
MCL1
SOX2
HOXA2
PURB
PRKCB
BCL11B
FBN1
MAGI1
AMOT
DSTYK
MIR133B
ATXN1
PPARD
MTPN
RYBP
TARDBP
PIAS3
COL1A2
CTCF
MIR192
ZEB2
VDR
MIR429
MIR200A

Figure 5

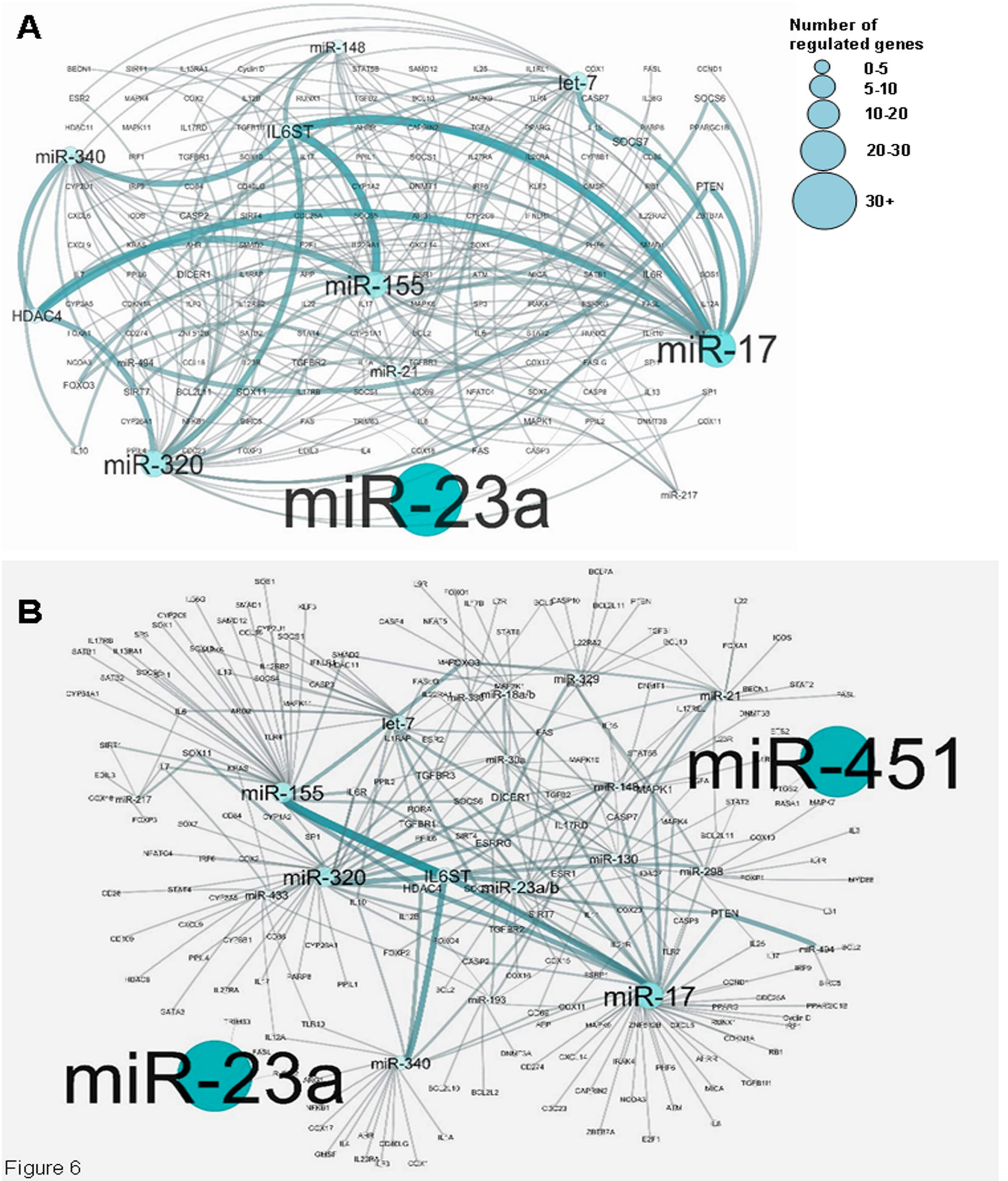

A
Number of regulated genes
0-5
5-10
10-20
20-30
30+
miR-148
let-7
IL6ST
miR-340
SOCS7
SOCS6
PTEN
miR-155
HDAC4
miR-17
miR-21
miR-320
miR-23a
miR-217
B
miR-451
miR-155
miR-320
IL6ST
miR-23a
miR-340
miR-17
let-7
miR-21
miR-329
miR-18a/b
miR-30a
miR-148
miR-130
miR-298
miR-433
miR-193
miR-217
miR-23a/b
PTEN

Figure 6

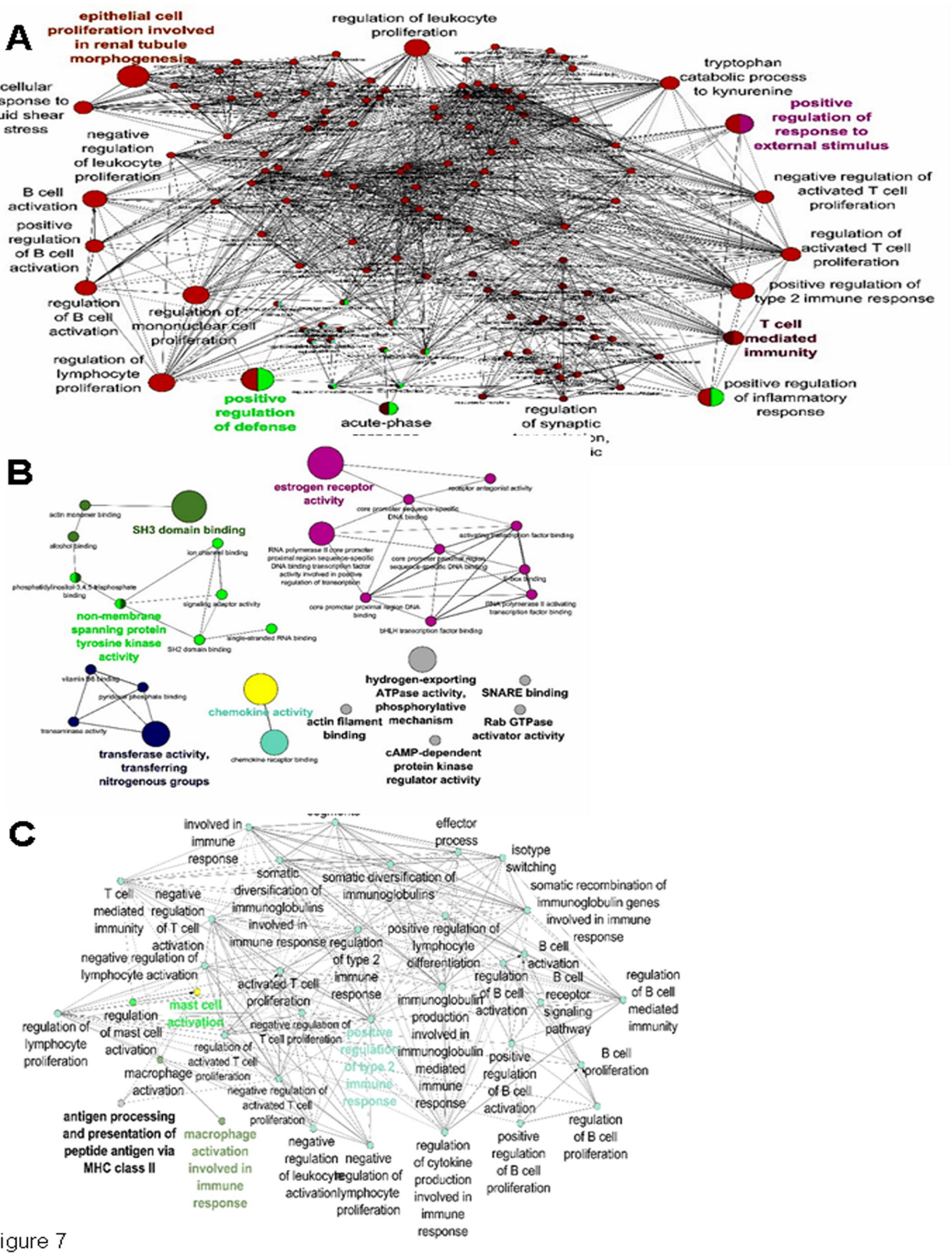

A
epithelial cell proliferation involved in renal tubule morphogenesis
regulation of leukocyte proliferation
cellular response to fluid shear stress
tryptophan catabolic process to kynurenine
positive regulation of response to external stimulus
negative regulation of leukocyte proliferation
B cell activation
negative regulation of activated T cell proliferation
positive regulation of B cell activation
regulation of activated T cell proliferation
regulation of B cell activation
regulation of mononuclear cell proliferation
positive regulation of type 2 immune response
T cell mediated immunity
regulation of lymphocyte proliferation
positive regulation of inflammatory response
positive regulation of defense
acute-phase
regulation of synaptic
B
estrogen receptor activity
SH3 domain binding
non-membrane spanning protein tyrosine kinase activity
chemokine activity
hydrogen-exporting ATPase activity, phosphorylative mechanism
SNARE binding
actin filament binding
Rab GTPase activator activity
cAMP-dependent protein kinase regulator activity
transferase activity, transferring nitrogenous groups
C
involved in immune response
effector process
isotype switching
somatic diversification of immunoglobulins involved in immune response
somatic diversification of immunoglobulins
somatic recombination of immunoglobulin genes involved in immune response
T cell mediated immunity
negative regulation of T cell activation
positive regulation of lymphocyte differentiation
negative regulation of lymphocyte activation
regulation of type 2 immune response
B cell activation
activated T cell proliferation
regulation of B cell activation
B cell receptor signaling pathway
regulation of B cell mediated immunity
mast cell activation
immunoglobulin production involved in immunoglobulin mediated immune response
regulation of lymphocyte proliferation
regulation of mast cell activation
negative regulation of T cell proliferation
positive regulation of type 2 immune response
positive regulation of B cell activation
B cell proliferation
regulation of activated T cell proliferation
macrophage activation
negative regulation of activated T cell proliferation
antigen processing and presentation of peptide antigen via MHC class II
macrophage activation involved in immune response
negative regulation of leukocyte activation
negative regulation of lymphocyte proliferation
regulation of cytokine production involved in immune response
positive regulation of B cell proliferation
regulation of B cell proliferation

Figure 7

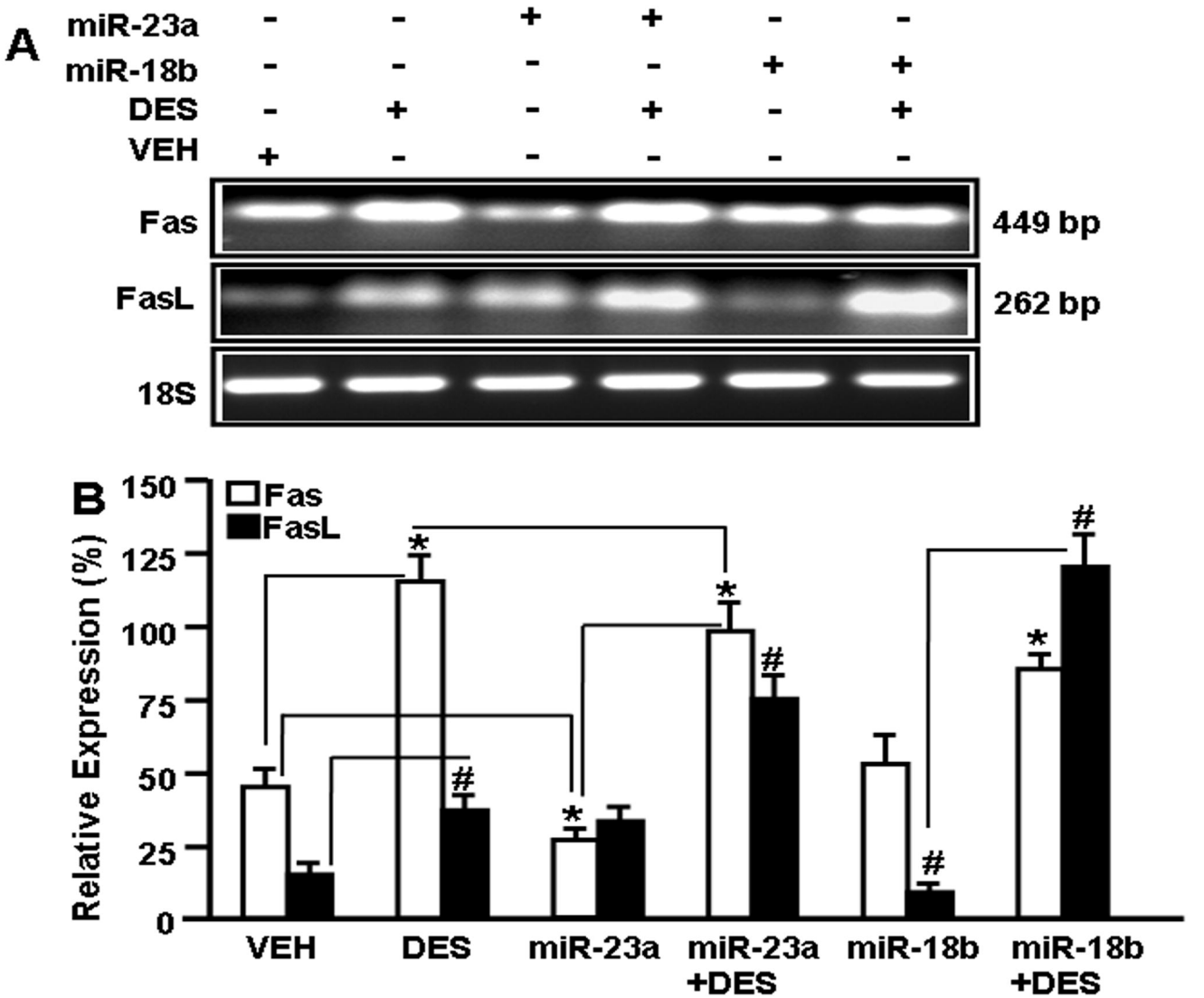

A
miR-23a - - + + - -
miR-18b - - - - + +
DES - + - + - +
VEH + - - - - -
Fas
FasL
18S
449 bp
262 bp
B
Fas
FasL
Relative Expression (%)
150
125
100
75
50
25
0
VEH
DES
miR-23a
miR-23a +DES
miR-18b
miR-18b +DES

Figure 8

A

AAACTACCTCAGTTCCAGCCATGAAGAGAGGAGAGAGCCTGCCACCCATGATGGAA
ACAAAATGAATGCCAACTGTATTGACATTGGCAACTCCTGGTGTGTTCTCTTTGCCA
GCAAATGGTAGTTGATACTCAGTGAGGGTCAAATGACTAGCAG**GTTCCAGGGACTG**
CTTCTGTTATTCTCTGCAGTTGCTGAGATGAACCATTTTCTCTGTCTACTGCAATTTTT

23a/23b Fas UTR Binding site

ACATTCAAATGTCCATG**AAATTTGTATTAAATGTGA**AGTGGAATCTGCAGTGTTTGT
GTTTATATTCATATACTATGAACTGAGGAGAATTTAAACTGAAACAAATACT**CGCAGT**
TAATTGAAGACCTTCCATTGATGGACAGTTCTTTTCCTCTCTATGTGGAAATGTATAA
TAGAAGAAATAATTTTTAAATTAAAGTATCTCTTTTTGCATTTC

B

GACCTCATGACCGCAAGGTCCAACAGGTCAGCTATCCTTCATTTTCTCGAGGTCCA
TGGAGTGGTCCTTAATGCCTGCATCATGAGCCAGATGGAAGGAGGTCTGTGACTG
AGGGACATAA**AGCTTTGGGCTGCTGTGTG**ACAATGCAGAGGCACAGAGAAAGAAC

miR-18b FasL UTR Binding site

TGTCTGA**TGTTAAATGGCCCTTT**ACACTCACTTCCAGGGTGGGTCTACTTACTACCT
CACAGAGGCCGTTTTTGAGACATAGTTGTGGTATGAATATACAAGGGTGAGAAAGG
AGGCTCATTTGACTGATAAGCTAGAGACTGAAAAAAA**GACAGTGTCTCATTGGCAC**
CATCTTTACTGTTACCTAATGTTTTCTGAGCCGACCTTTGATCCTAACGGAGAAGTA
AGAGGGATGTTTGAGGCACAAATCATTCTCTACATAGCATGCATACCTCCAGTGCA
ATGATGTCTGTGTGTTTGTATGTATGAGAGCAAACAGATTCTAAGGAGTCATATAAA
TAAAATATGTACATTATGGAGTACATATTAGAAACCTGTTACATTT

Figure 9

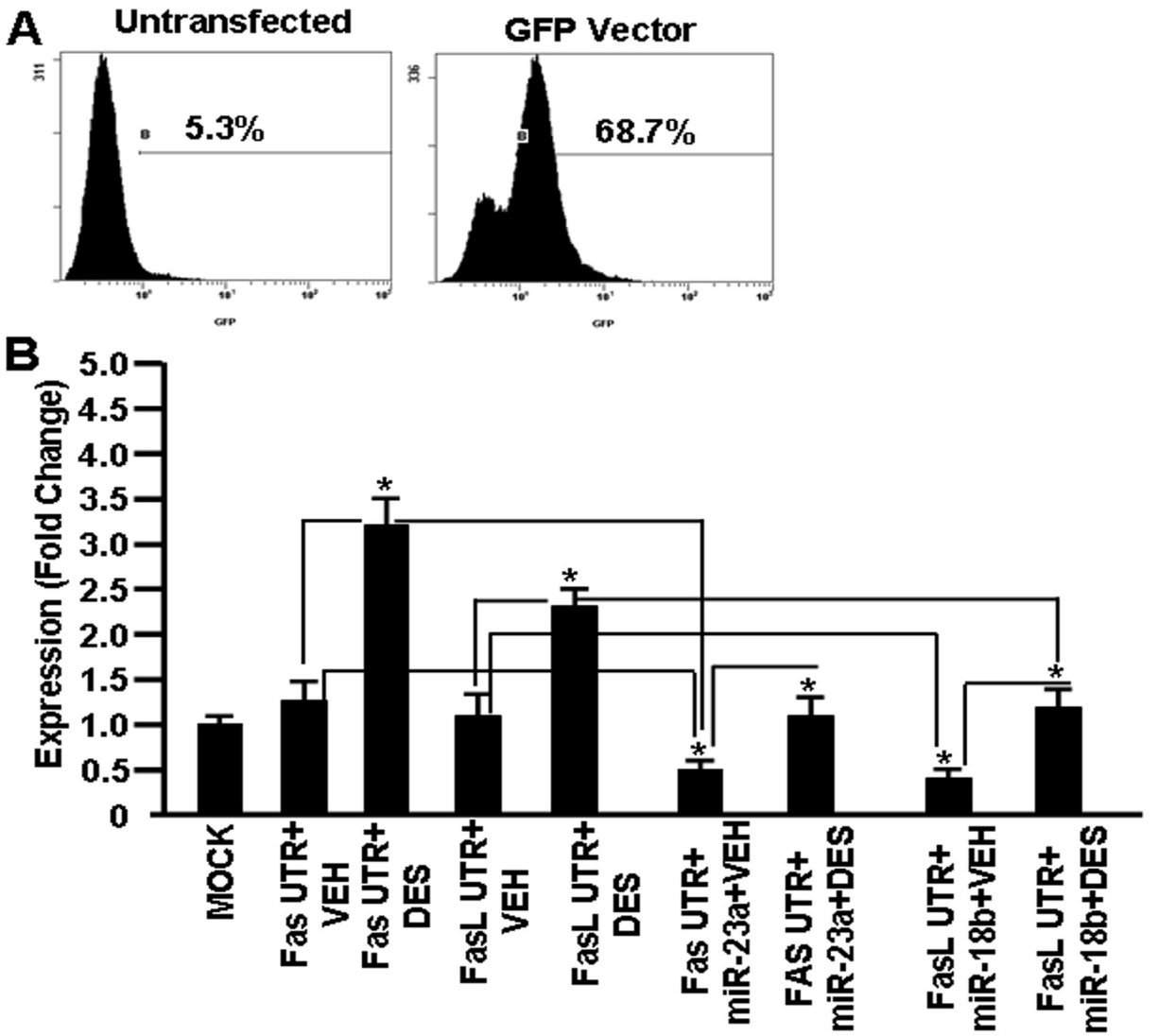

A
Untransfected
GFP Vector
5.3%
68.7%
GFP
B
Expression (Fold Change)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
MOCK
Fas UTR+ VEH
Fas UTR+ DES
FasL UTR+ VEH
FasL UTR+ DES
Fas UTR+ miR-23a+VEH
FAS UTR+ miR-23a+DES
FasL UTR+ miR-18b+VEH
FasL UTR+ miR-18b+DES

Figure 10