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Diethylstilbestrol (DES) induces autophagy in thymocytes by regulating Beclin-1 expression through epigenetic modulation

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Abstract

Diethylstilbestrol (DES) is an endocrine disruptor that was used to prevent adverse effects of pregnancy in women in late 1940s until early 1970s. Its use was banned following significant toxicity and negative effects not only in the mothers but also transgenerationally. Previous studies from our laboratory showed that DES induces thymic atrophy and immunosuppression in mice. In this study, we investigated the molecular mechanisms through which DES triggers thymic atrophy, specifically autophagy. To that end, we treated C57BL/6 mice with DES, and determined expression of two autophagy-related proteins, microtubule-associated protein-1 light chain 3 (LC3) and Beclin-1 (Becn1). We observed that DES-induced thymic atrophy was associated with increased autophagy in thymocytes and significant upregulation in the expression of both Becn1 and LC3. DES also caused downregulation in the expression of miR-30a in thymocytes, and transfection studies revealed that miR-30a targeted Becn1. Upon examination of methylation status of Becn1, we noted hypomethylation of Becn1 in thymocytes of mice exposed to DES. Together, these data demonstrate for the first time that DES induces autophagy in thymocytes potentially through epigenetic changes involving hypomethylation of Becn1 and downregulation of miR-30a expression.

Keywords

Diethylstilbestrol; Thymus; Autophagy; Epigenetics; microRNA; Hypomethylation

1. Introduction

Diethylstilbestrol (DES) is a nonsteroidal estrogen that was first synthesized in 1938 and classified as an endocrine disruptor (Alves and Oliveira, 2013; Gibson and Saunders, 2014; Nohynek et al., 2013). DES exposure to adult mice has been shown to cause various abnormalities including thymic atrophy, skeletal tissue damage, female reproductive organs, and muscles (Maier et al., 1985; Okada et al., 2001). In the thymus, several changes such as

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

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apoptosis in thymic cells, T cell differentiation, immunotoxicity, and immunosuppression have been reported post-DES exposure (Badewa et al., 2002; Brown et al., 2006a). Previous studies have demonstrated that DES caused a decrease in prothymocyte stem cells (Holladay et al., 1993), decrease in double positive CD4⁺ CD8⁺ cells (Smith and Holladay, 1997; Brown et al., 2006a), as well as cell death in thymocyte subsets CD4⁺ CD8⁺, CD4⁺CD8⁻ and CD4⁻CD8⁺ (Calemine et al., 2002; Brown et al., 2006b). DES has also been shown to induce apoptosis in double-negative (CD4⁻CD8⁻) cells in fetal thymic organ culture system (Lai et al., 2000). The studies from our laboratory have also demonstrated DES-mediated thymic atrophy as well as upregulation of Fas and FasL expression leading to apoptosis occurs in both mothers and neonatal mice (Brown et al., 2006b; Shamran et al., 2017).

Autophagy has been in the forefront of regulating cellular physiological processing and is a major degradation system responsible for removing cellular constituents. It is a lysosome-dependent physiological mechanism that degrades and recycles cellular proteins and organelles (Xie and Klionsky, 2007). It has been reported that basal level of autophagy contributes to the maintenance of intracellular homeostasis and is required for cell cleansing and remodeling (Dikic et al., 2010). The thymus is an important organ in which pre-T cells differentiate into mature T cells following positive and negative selection. In addition to apoptosis, autophagy in the thymus has also been shown to shape the T cell repertoire (Nedjic et al., 2008). Moreover, there is cross-talk between autophagy and apoptosis (Kemp, 2017). Beclin-1 (Becn1) and microtubule-associated protein light chain 3 (LC3) are two important players in autophagy.

It is estimated that ~5–10 million Americans received DES during pregnancy or were exposed to DES in utero. Such an exposure to DES is associated with an increased risk for breast cancer in DES mothers and cervicovaginal cancers in DES daughters (Giusti et al., 1995). Based on the transgenerational toxic effects of DES, it has been suggested that DES may trigger epigenetic events (Newbold et al., 2006). However, the precise mechanisms of epigenetic pathways are still unclear. Because DES is well-established to trigger thymic atrophy, in the current study, we decided to test if epigenetic changes can be seen following DES exposure in thymocytes and whether such changes can induce autophagy in thymocytes.

The current study demonstrates that DES induces autophagy in thymocytes, which correlates with increased expression of Becn1 and LC3. Furthermore, we demonstrate that these changes are related to the ability of DES to cause hypomethylation of Becn1 and down-regulation of miR-30a which targets Becn1. To our knowledge, this study is the first to demonstrate DES-induced autophagy in the thymus and it highlights the potential role of epigenetic pathways in the regulation the autophagy.

2. Materials and methods

2.1. Chemicals and reagents

We purchased Diethylstilbestrol (DES) powder and Acridine Orange (AO) solution from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). DES suspended in corn oil was used for in vivo studies and DES suspended in Dimethyl sulfoxide (DMSO) was used for in vitro

studies, as described (Singh et al., 2012, 2015a,b). The culture medium (RPMI1640, Penicillin/ Streptomycin, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), L-glutamine, Fetal Bovine Serum (FBS), and PBS (Phosphate-buffered saline) were purchased from Invitrogen Life Technologies (Carlsbad, CA). Polymerase chain reaction (PCR) reagents, Epicentre's PCR premix F, and Platinum *Taq* Polymerase, were purchased from Invitrogen Life Technologies (Carlsbad, CA). Anti-LC3 (PA5-22990) polyclonal antibody was purchased from Thermo-Fisher Scientific (Rockford, IL), and anti-Becn1 (H-300) polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The following reagents including miRNeasy kit, miScript cDNA synthesis kit, miScript primer assays kit, miScript SYBR Green PCR kit, and EpiTect Bisulfite Kit were purchased from QIAGEN (QIAGEN INC., Valencia, CA).

2.2. Mice

Female C57BL/6 mice (8 weeks old) were purchased from National Cancer Institute (NCI). The mice were housed in the animal facility at the University of South Carolina School of Medicine. All the mice were given ad libitum access to water and normal chow diet and were housed at 23–24 °C with a 12-h/12-h light/dark cycle. Mice were used for the experiments when they were 9 weeks old. The mice were sacrificed at the end of experiments and were ~9.4 weeks old. This AAALAC-accredited animal facility is equipped with a light- and temperature-maintained system. The mice were maintained and cared according to the guidelines for the care and use of laboratory animals as adopted by Institutional IACUC and NIH guidelines.

2.3. Cell line

EL4, a mouse T cell lymphoma cell line, purchased from American Type Culture Collection (ATCC), was maintained in complete RPMI 1640 medium containing 10mM HEPES, 10mM L-Glutamine, 100 µg/ml penicillin/ streptomycin), and 10% heat-inactivated fetal bovine serum (FBS) at 37 °C and 5% CO₂. The medium was changed every other day to maintain healthy growth of EL4 cells. Before changing the medium, EL4 cells in flask were observed to determine the confluency of the cells as well as for viability by observing the shape of EL4 cells under inverted phase contrast microscope. Dead EL4 cells were removed by washing the cells repeatedly using complete medium. After washing, the cells were suspended in fresh complete RPMI medium (1×10^6 /ml) to maintain healthy growth of EL4 cells. EL4 cells were analyzed for STR profile (DDC Medical, Fairfield, OH) and for Mycoplasma as follows: EL4 cells, cultured for two-three days and when the cells were 80–85% confluence, were tested for the presence of Mycoplasma species. PCR using Mycoplasma Detection kit from Applied Biological Materials, Inc (abm, Inc, Richmond BC, Canada) and primers for over 200 Mycoplasma species was performed at the following amplification conditions (Enzyme activation at 95 °C for 5 min, PCR amplification (40 cycles) was performed with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 60 s, and final extension at 72 °C for 10 min. There was no Mycoplasma contamination present in the culture medium of EL4 cells as both EL4 cultures (1 and 2) showed no PCR amplification (500 bp) for Mycoplasma species, whereas, positive control for Mycoplasma showed the amplified band (500 bp) (Fig S1). Also, negative control for Mycoplasma did not show the amplified band.

2.4. Treatment of mice with DES

Mice (C57BL/6) were administered (ip) with a single dose of DES (5 µg/kg), as described previously (Singh et al., 2015a, 2015b). Mice, treated with corn oil, were used as control for vehicle.

2.5. Determination of thymic cellularity and autophagy

On day 2 and 4 following DES treatment, mice were euthanized, thymi from DES- and VEH-treated mice were harvested, thymic cellularity and autophagy was determined. In brief, single cell suspensions of thymi were prepared as described earlier (Camacho et al., 2004a,b). Thymic cell number and viability were determined by staining the cells with trypan blue dye and using Hematocytometer and an inverted phase contrast microscope. The dead and live thymic cells were counted in all the 64 squares of the Hematocytometer. Cells were counted at least 4–5 times with a minimum five mice for each group. The dead and live cells of thymi of both groups were also verified using an automated cell counter TC20 from Bio-Rad (Bio-Rad). Thymic cellularity was expressed as total number of thymocytes/mice. To determine autophagy in thymic cells post-DES exposure, thymic cells were stained with Acridine orange (AO) as it has been used to determine autophagy (Thome et al., 2016). AO crosses into lysosomes (and other acidic compartments) and becomes protonated. The protonated dye stacks and stacked AO emits in the red range. In brief, thymic cells freshly isolated from thymi of mice treated with VEH or DES were stained for 20 min with AO (1 µg/ml) at room temperature as described previously by Thome et al. (2016). AO emission was captured by red laser using FC500 Flow cytometer (Beckman Coulter). At least five mice were used for each treatment group and the experiments were repeated at least three times.

2.6. Histopathology of thymi exposed to DES

The thymi of VEH- and DES-treated mice were fixed by immersion in 4% paraformaldehyde in PBS. Paraffin blocks of fixed thymi were prepared, and microtome sections (5-µm-thick) were generated. The tissues sections were stained using hematoxylin and eosin (H&E) as described by Singh et al. (2009). In brief, the slides were washed in deionized water and stained with Mayer's hematoxylin and eosin (Sigma-Aldrich) for 1–2 minutes. Next, the tissue sections were washed with deionized water and then de-hydrated in 70%, 95%, and absolute alcohol for 5 min each. The slides were then passed three times through xylene for one minute each. Finally, the sections were mounted with histomount mounting solution (ThermoFisher Scientific). The thymi sections post H&E staining were examined for architecture of cortex and medulla of the thymi using a Cytation-5 Imaging Reader (BioTek Instruments, Winooski, VT, USA). Five sections per mouse were analyzed, and a minimum of five mice were included in the study.

2.7. Real-Time PCR (Q-PCR) to determine the expression of Becn1 in thymic cells post DES treatment

Total RNAs from thymic cells of mice treated with VEH or DES were isolated using RNeasy Mini Kit and following the protocol of the company (Qiagen, Maryland). First strand cDNA synthesis was performed in a 20 µl reaction mix containing 1 µg total RNA

using iScript Kit and following the protocol of the company (Bio-Rad). Following first strand synthesis, 2 µl (10% of the reaction volume) was used as a template for Q-PCR amplifications. Real-Time PCR was performed to detect mouse *Becn1* (96 bp) expression using mouse *Becn1*-specific forward (5'-CCA GGAAGTACAGCTCCATTAC-3') and reverse (5'-CTCCTCTC CTGAGT TAGC CTC-3') primer pairs. We used CFX Connect Bio-Rad Real-Time PCR system (Bio-Rad) at the following conditions: 30 s 95 °C (denaturing temperature), 40 s at 58 °C (annealing temperature), and 60 s at 72 °C (extension temperature), with a final incubation at 72 °C for 10 min were used. We used mouse ribosomal 18S as an internal control and mouse 18S-specific forward (5'-GCCCAGCCGCTGGATAC-3') and reverse (5'-CCGGCGGGTCATGGGAATA AC-3') primer pairs were used for amplification. We used Bio-Rad CFX Maestro software to analyze Q-PCR data (Bio-Rad). This software supports the entire process of Q-PCR, including reference gene selection, statistical analysis, and data graphing as detailed at: <http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10000068703.pdf>

The software does not rely completely on ddCt value but uses as starting reference for calculations. In addition, Bio-Rad CFX Maestro software considers Pfaffl equations and MIQE guidelines as reported previously (Bustin et al., 2009, 2013; Huggett et al., 2013; Svec et al., 2015). The data were normalized to *Becn1* against internal control 18S and fold change of *Becn1* was calculated against control 18S and DES treatment group was compared with VEH-treatment group. To define significant differences in *Becn1* expression in the thymi of DES- or VEH-treated groups, ANOVA was performed using GraphPad prism version 6.0 (GraphPad Software, Inc., San Diego, CA). Differences between treatment groups were considered significant when $*p < 0.05$.

2.8. Immunoblot analysis

Immunoblotting was performed to determine the expression of *Becn1*, and LC3 in thymic cells post-DES treatment as described previously (Tanida et al., 2008). Polyclonal antibodies were obtained as follows: *Becn1* (1:200; Santa Cruz Biotechnology), LC3 (1:2000; ThermoFisher Scientific), and β -actin (1:50,000; Sigma-Aldrich). HRP-conjugated secondary Ab was used 1:4000 dilution (Cell Signaling). Lysates from DES-treated thymic cells were prepared by freezing and thawing and the protein concentration was measured using standard Bradford assay (BioRad, Hercules, CA). The proteins were fractionated in SDS-PAGE and transferred onto PVDF membranes using a dry blot apparatus from BioRad following the protocol of the company (Hercules, CA). The membrane was incubated in 5% nonfat dry milk (BioRad, Hercules, CA) in PBS (blocking buffer) at RT for 1 h and then in primary antibody at 4 °C overnight. The membrane was then washed 3 times (10–15 min) with washing buffer (PBS + 0.2% Tween 20) and incubated for 2h in HRP-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA) in blocking buffer. The membranes were then washed several times and incubated in developing solution (equal volume of solution A and B; ECL Western Blotting Detection Reagents, GE Life Sciences, England) and signal was detected using BioRad ChemiDoc System and densitometric analyses of the Western blots were performed using ChemiDoc software (Hercules, CA).

2.9. Reverse transcriptase PCR (RT-PCR) to determine the expression of LC3 in thymic cells post-DES treatment

RT-PCR was performed to detect LC3 (263 bp) expression using mouse LC3-specific forward (5'-AGAAGTTGAACGAGTACCGCCTG-3') and reverse (5'-AGCACCTGACTTTA TGGCTTCCAG-3') primer pairs. We performed PCR for 35 cycles using the following conditions: at 95 °C (denaturing temperature) for 30 s, at 58 °C (annealing temperature) for 50 s, and at 72 °C (extension temperature) for 60 s, with a final incubation at 72 °C for 10 min. The PCR products, generated from mouse LC3 primer pairs, were normalized after electrophoresis on 1.2% agarose gel and visualization with UV light against PCR products generated from mouse 18S forward and reverse primers as described earlier in Material and Methods. The band intensity of PCR products was determined using ChemiDoc image analysis system from Bio-Rad (Bio-Rad, Hercules, CA).

We also performed Q-PCR using CFX Connect Bio-Rad Real-Time PCR System (Bio-Rad) to confirm the expression of LC3 in the thymic cells post-DES treatment. The above mentioned amplification conditions were used. 18S was used as an internal control. Q-PCR data were analyzed using Bio-Rad CFX Maestro software (Bio-Rad).

2.10. Real-Time PCR to determine the expression of miR-30a in thymic cells post-DES treatment

Real-Time PCR assays were performed to determine the expression of miR-30a on cDNAs generated from total RNAs isolated from thymic cells post-DES or VEH exposure. miScript primer assays kit from Qiagen (Catalog No: MS00011704) and miScript SYBR Green PCR kit (Catalog No: 218073) from QIAGEN were used and the protocol of the company (QIAGEN, Valencia, CA) was followed. Real-Time PCR was performed using CFX Connect Bio-Rad Real-Time PCR System (Bio-Rad) and the following conditions were used: 40 cycles using with 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. We used Bio-Rad CFX Maestro software to analyze Real-Time PCR data (Bio-Rad). The data were normalized to miR-30a against internal control miR (Snord96a) and fold change of miR-30a was calculated against control miR (Snord96a) and treatment group (DES) was compared with VEH group. To define significant differences in miR-30a level in the thymi of DES or VEH-treated groups, ANOVA was performed using GraphPad prism version 6.0 (GraphPad Software, INC., San Diego, CA). Differences between treatment groups were considered significant when $p < 0.05$.

2.11. Detection of methylation in *Becn1* promoter

We examined methylation/demethylation of *Becn1* promoter in thymic cells post-DES in vivo exposure. To examine DES-induced regulation *Becn1* expression in thymic cells, total genomic DNAs from thymic cells post DES- or VEH-treatment were isolated using DNeasy Blood & Tissue kit from QIAGEN and following the protocol of the company (QIAGEN, Valencia, CA). The DNA concentration was measured using a spectrophotometer. Bisulfite modification of total DNA was performed using EpiTect Bisulfite kit from QIAGEN and following the protocol of the company (QIAGEN, Valencia, CA). Post bisulfite,

DNA was purified and its concentration was measured. Purified DNA post bisulfite conversion was either used immediately or stored at -20°C for future use.

2.12. Methylated (MSP) PCR

MSP PCR was performed to amplify methylated or demethylated regions of mouse *Becn1* promoter using mouse bisulfite converted genomic DNA and mouse *Becn1*-specific forward (5'-TCGGGTTTGGGT TTAGTTTC-3') and reverse (5'-GCTAACCGCAATTTTC ACG-3') primer pairs to amplify methylated region or forward (5'-TTTTTGGGTTT GGGTTTTAGTTTT-3') and reverse (5'-CCCACTAACCACAATTTTC ACA-3') primer pairs to amplify demethylated region. Primers were generated using Methyl Primer Express version 1.0 software and synthesized from IDT (Coralville, IA, USA). PCR reactions were carried out in a volume of 20 μl containing 1X EpiTect master mix (EpiTect MSP PCR kit, QIAGEN), bisulfite-converted DNA (500 ng), and primer pairs (0.3–0.4 μM). Amplification was performed in a thermal cycler (BioRad) using the following profile: 95 $^{\circ}\text{C}$ for 10 min, three steps cycling: 35 cycles at 94 $^{\circ}\text{C}$ for 30 s, 58 $^{\circ}\text{C}$ for 30 s, and 72 $^{\circ}\text{C}$ for 45 s, and 72 $^{\circ}\text{C}$ for 10 min. *Becn1* PCR products were detected by electrophoresis using 2% agarose gel and visualization with UV light. The band intensity of PCR products was determined using BioRad image analysis system (BioRad).

We also performed Real-Time PCR using the above-described mouse-specific methylated and unmethylated sets of primers. We used CFX Connect Bio-Rad Real-Time PCR system (Bio-Rad) at the following conditions: 95 $^{\circ}\text{C}$ for 30 s, 58 $^{\circ}\text{C}$ for 40 s, and at 70 $^{\circ}\text{C}$ for 60 s, with a final incubation at 70 $^{\circ}\text{C}$ for 10 min. Universal Methylated (100%) Mouse DNA Standard (S8000; Millipore, Billerica, MA, USA) and low (0–2%) methylated mouse DNA (EpigenDX, INC, Hopkinton, MA) were used as a positive and negative controls respectively with all primers. Methylation index $\{[(M)/(M + UM)] \times 100\}$, where M is methylated, and UM is unmethylated, was used to assess DNA methylation.

2.13. Interaction of miR-30a and *Becn1* mRNA

We identified interaction between miR-30a and *Becn1* mRNA target using microRNA.org, TargetScan mouse 5.1, and miRGen V.3 software and their database. Computational algorithms aid this task by examining base-pairing rules between miR and mRNA target sites, location of binding sites within the target's 3'-UTR, and conservation of target binding sequences within related genomes. Computational algorithms and base-pairing rules between miR and mRNA was done by the softwares described above. The details of miR-30a and UTR region of its target gene *Becn1* is described in Fig. 4A.

2.14. Transfection of EL4 cells with mature miR-30a and anti-miR-30a and determination of expression of miR-30a and *Becn1* in the presence of DES or VEH

Freshly cultured EL4 cells (5×10^6) were transfected with mature miR-30a and anti-miR-30a 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., 2015a,b). We also used p-GFP as a positive control for transfection. Forty-eight hrs post transfection, EL4 cells were treated with VEH or DES (10 $\mu\text{M}/\text{ml}$) for 24 h. The expression of miR-30a and *Becn1* in EL4 was determined post-

treatment with DES or VEH. In brief, total RNAs including miRs from EL4 cells transfected with miR-30a, anti-miR-30a, or miR-30a and anti-miR-30a and treated with DES or VEH were isolated using RNeasy mini kit from QIAGEN and following the protocol of the company (QIAGEN, Valencia, CA). First strand cDNA synthesis was performed on total RNA (1 µg) using iScript cDNA Synthesis Kit and following the protocol of the company (Bio-Rad). Expression of miR-30a and Becn1 was determined performing Real-Time PCR. Snord96a primer pairs were used as an internal control for miR-30a and 18S primer pairs were used as an internal control for Becn1 gene. Western blotting was also performed to determine the expression of Becn1 in transfected EL4 cells.

2.15. Statistics

We used GraphPad Prism version 6 software (San Diego, CA) for statistical analyses. Student's *t*-test was used for paired observations if data followed a normal distribution to compare DES-induced autophagy in thymic cells, and expression and quantification of Becn1 and LC3 in thymic cells. Expression of miR-30a was analyzed using 2-sample *t*-test method. Multiple comparisons were made using ANOVA (one-way analysis of variance) test and Tukey-Kramer Multiple Comparisons Tests. P-value of < 0.05 was considered to be statistically significant.

3. Results

3.1. DES triggers autophagy in thymocytes in vivo

Thymi from VEH- and DES-exposed mice were collected on day 2 and day 4 post-treatment and single cells were prepared. Upon examination of thymic cells, we noted significant decrease in their numbers in mice that received DES treatment, when compared to mice-treated with VEH (Fig. 1A). Thymic cells were examined for autophagy by staining with Acridine Orange (AO) dye. Upon analysis of autophagic cells using flow cytometry, there was significantly more autophagy on day 2 and 4 in thymic cells of DES-treated mice (Fig. 1B–C) when compared to vehicle-exposed thymic cells on similar days (Fig. 1B–C). The results from a representative experiment have been shown in Fig. 1B and data from multiple experiments have been plotted in Fig. 1C. Histopathological analyses of thymi following H&E staining showed highly disorganized cortex and medulla in the thymi of DES-treated mice (right panel; Fig. 1D), when compared to thymi of VEH-treated mice (left panel; Fig. 1D). These results demonstrated that DES causes significant autophagy in thymic cells and a reduction in the number of thymocytes.

3.2. DES upregulates expression of Becn1 and LC3 in thymic cells

Becn1 and LC3 have been well characterized for their role in autophagy (Cicchini et al., 2014; Gawriluk and Rucker, 2015). Therefore, we determined the expression of Becn1 and LC3 in thymic cells post VEH or DES exposure by performing Real-Time PCR. We observed significant upregulation of Becn1 (Fig. 2A) and LC3 (Fig. 2B) in thymic cells of DES-treated mice, when compared to thymic cells of VEH-treated mice (Fig. 2A–B). We also performed Real-Time PCR to confirm the expression of LC3 in thymic cells post VEH and DES treatment. There was significant upregulation of LC3 (Fig. 2C) expression in thymic cells post DES treatment, when compared to VEH treatment. We also performed

Western blot to determine the expression of both Bcl-2 and LC3 in thymic cells. There was significantly more expression of Bcl-2 (Fig. 2C–D) and LC3, especially LC3 I and LC3 II (2C and 2E) in DES-treated thymic cells, when compared to VEH-treated thymic cells. Western blot analyses of Bcl-2 (Fig. 2C–D) and LC3 (LC3I and LC3II; 2C and 2E) expression in thymic cells post DES- and VEH-treated mice corroborated with data obtained from the Real-Time PCR and/or data obtained from RT-PCR. These data demonstrated that DES triggers enhanced expression of both Bcl-2 and LC3 in thymic cells in vivo.

3.3. DES-mediated hypomethylation of Bcl-2 promoter and consequent upregulation of its expression

Previous studies have shown that DNA methylation plays an important role in the regulation of genes leading to autophagy (Li et al., 2010; Syed et al., 2013; Yuanchao et al., 2014). In this study, therefore, we examined the effect of DES on the methylation/demethylation of Bcl-2 gene in thymic cells. As shown in Fig. 3A, we identified CpG islands rich in the promoter of Bcl-2 and selected methylated/un-methylated sets of primers to amplify these regions. We performed methylated PCR (MSP) using bisulfite converted genomic DNA from thymic cells, using sets of mouse Bcl-2-specific forward and reverse primers that could amplify methylated or unmethylated region of Bcl-2 promoter (Fig. 3B–C). The data showed lower intensity of DNA amplicon of Bcl-2 in DES-treated thymic cells in comparison to VEH-treated thymic cells, when methylated sets of primers were used. However, the band intensity was significantly higher in DES-treated thymic cells when compared to VEH-treated thymic cells, when unmethylated sets of primers were used (Fig. 3B–C). We also performed Real-Time PCR using 100% methylated mouse DNA (positive control for Methylation) and low (0–2%) methylated mouse DNA (negative control for Methylation) to determine methylation in thymic cells post-DES treatment. Analysis of Methylation Index (as described in Materials and Methods), showed that Bcl-2 promoter had significantly lower Methylation Index (Fig. 3D). These data supported the observation that DES-induced hypomethylation of Bcl-2 promoter may cause increased expression of Bcl-2 in thymic cells in vivo.

3.4. Identification of Bcl-2-specific miR (miR-30a) and determination of miR-30a expression in thymic cells by Real-Time PCR

Previous studies have shown the role of miR-30a in autophagy by regulating Bcl-2 (Chen et al., 2015; Huang et al., 2015; Wang et al., 2003). Upon examination of miR-30a binding affinity with 3' UTR region of mouse Bcl-2 using various analyses tools (microRNA.org, TargetScan, miRWalk etc as described in Materials and Methods), we observed binding affinity of miR-30a with mouse Bcl-2 3'UTR region (Fig. 4A). To that end, Real-Time PCR was performed on cDNAs converted from total RNAs including miRs from thymic cells treated with DES or VEH in vivo. Data obtained from Real-Time PCR demonstrated significant downregulated expression of miR-30a in thymic cells treated with DES, when compared to VEH-treated thymic cells (Fig. 4B). The Real-Time PCR data demonstrated that DES downregulated miR-30a in thymic cells demonstrating a possible role of miR-30a in Bcl-2 expression.

3.5. Analysis of miR-30a-associated Becn1 expression

To directly demonstrate the role of miR-30a in the regulation of Becn1 expression, we first transfected EL4 T cells with mature miR-30a or anti-miR-30a and 48 h post transfection, EL4 cells were cultured in the absence or presence of DES for 18–24 hrs. The expression of miR-30a and Becn1 was determined by performing Real-Time PCR. EL-4 cells not transfected with miR-30a showed moderate miR-30a expression but there was significant downregulation of miR-30a in the presence of DES (Fig. 5A). EL4 cells transfected with miR-30a showed significantly higher expression of miR-30a but the expression was significantly downregulated in the presence of DES (Fig. 5A). Interestingly, EL4 cells transfected with anti-miR-30a, showed significantly downregulated miR-30a expression and DES further decreased its expression (Fig. 5A). EL4 cells transfected with both miR-30a and anti-miR-30a, as suggested by the protocol of the manufacturer, showed significantly downregulated expression of miR-30a and DES treatment further downregulated the expression of miR-30a (Fig. 5A).

Next, we determined the expression of Becn1 in EL4 cells not transfected or transfected with miR-30a or anti-miR-30a or both and treated with VEH or DES. There was a moderate expression of Becn1 in VEH-treated untransfected EL4 cells but in the presence of DES, Becn1 expression was significantly higher in EL4 cells (Fig. 5B). Interestingly, expression of Becn1 in EL4 cells transfected with miR-30a was decreased significantly when compared to VEH-treated untransfected EL-4 cells (Fig. 5B). DES treatment further downregulated the expression of Becn1 in EL4 cells (Fig. 5B). Moreover, EL4 cells transfected with anti-miR-30a treated with VEH showed a significant increase in Becn1 expression, when compared to VEH-treated untransfected EL4 cells (Fig. 5B). DES treatment of anti-miR-30a transfected EL4 cells significantly increased Becn1 expression (Fig. 5B). Upon transfection of EL4 cells with both miR-30a and anti-miR-30a, there was significant downregulation of Becn1 expression but upon DES treatment, downregulation of Becn1 expression was significantly reversed (Fig. 5B). Becn1 expression in EL4 cells in various groups was also confirmed by Western blot analysis of Becn1 (Fig. 5C–D). These data together suggested that DES-mediated decreased expression of miR-30a may indeed be the mechanism through which Becn1 expression is regulated in the thymus.

4. Discussion

DES is a synthetic estrogen and an endocrine disruptor (Alves and Oliveira, 2013; Gibson and Saunders, 2014; Hilakivi-Clarke et al., 2013; Nohynek et al., 2013) that has been linked to a wide range of abnormalities including thymic atrophy, cancer, immune system disorders such as immune suppression, and increased incidence of autoimmunity (Giusti et al., 1995). Studies from our laboratory have previously shown that DES induced thymic atrophy and caused apoptosis in murine thymic cells (Brown et al., 2006a,b). DES-induced apoptosis was related to increased expression of Fas and FasL in fetal thymic cells and T cells involving transcription factors and cis-regulating elements (Shamran et al., 2017). In addition to apoptosis, autophagy in the thymus has also been shown to shape the T cell repertoire (Nedjic et al., 2008). Moreover, there is cross-talk between autophagy and apoptosis (Kemp,

2017). In the present study, therefore, we investigated if DES would also mediate autophagy in thymocytes and if so, whether this is regulated by epigenetic pathways.

Autophagy is a biological and physiological process in which the intracellular contents are engulfed and consumed by vesicles, known as autophagosomes (Bialik et al., 2010; Wang and Klionsky, 2003). The autophagosomes then fuse with lysosomes to form autolysosomes and the components of autolysosomes are degraded by lysosomal hydrolases (Tanida et al., 2008). There are reports that aggresome formation can also be used to cause autophagy, since the aggresome serves as a storage compartment for protein aggregates and can be actively involved in their degradation with the help of autophagic clearing (Zaarur et al., 2008). Despite marked differences between apoptosis and autophagy, recent studies have suggested that there is crosstalk between the autophagic and apoptotic pathways (Thorburn, 2008; Hsieh et al., 2009). Studies indicated that components of the core regulating apoptotic machinery can control autophagy with other studies showing autophagy regulators capable of controlling apoptosis (Thorburn, 2008). It has been found that some connections occur upstream of the autophagic and apoptotic machinery which can be triggered by upstream signals resulting in combined autophagy and apoptosis or the cells switching between the two types of cell death in a mutually exclusive manner (Thorburn, 2008; Maiuri et al., 2009). This illustrates that the apoptotic and autophagic response machineries share common pathways that either link or polarize the cellular responses (Maiuri et al., 2009). In a recent study, a novel estradiol analogue was shown to cause autophagy in esophageal carcinoma cells (Wolmarans et al., 2014). However, there are no studies demonstrating DES-mediated autophagy in animals or humans.

The current study demonstrated that DES not only decreased thymic cellularity in mice but also induced autophagy in thymic cells (Fig. 1). Upon analysis of *Becn1* expression in thymic cells, we noted significant increase in its expression in the thymic cells following DES exposure in comparison to VEH-exposed thymic cells (Fig. 2A, C, and D). Moreover, LC3 expression, especially LC3 II, was significantly upregulated in the thymic cells post-DES exposure, when compared to VEH-exposed thymic cells (Fig. 2B and E). While our studies did not directly test if DES-induced upregulation of *Becn1* was directly responsible for autophagy, based on the well-established role of this molecule in autophagy, our findings suggested that the pro-autophagic proteins *Becn1* and LC3 are upregulated by DES, which in turn may promote autophagy in thymic cells. There are several studies demonstrating the role of *Becn1* and LC3 in autophagy (Martyniszyn et al., 2011; Shrivastava et al., 2012). Beclin-1 binds to class III phosphatidylinositol 3-kinase (PI3KC3) and activates autophagosome formation and maturation (Shrivastava et al., 2012; Levine and Kroemer, 2008). Also, Beclin-1 acts as a tumor suppressor in mammalian systems, and the deletion of Beclin-1 was observed in various cancers, including prostate, ovarian, breast, brain, and lung cancers (Miracco et al., 2007; Maiuri et al., 2009). LC3, on the other hand, is a soluble protein of a molecular mass of ~17kD and is distributed ubiquitously in mammalian tissues and cultured cells. During autophagy, autophagosomes engulf cytoplasmic components, including cytosolic proteins and organelles. Concomitantly, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes. Autophagosomes fuse with lysosomes to form autolysosomes, and intra-autophagosomal components are

degraded by lysosomal hydrolases. At the same time, LC3-II in autolysosomal lumen is degraded. Thus, lysosomal turnover of the autophagosomal marker LC3-II reflects starvation-induced autophagic activity, and detecting LC3 by immunoblotting or immunofluorescence has become a reliable method for monitoring autophagy and autophagy-related processes, including autophagic cell death.

Although the molecular pathways of autophagy have been well characterized, whether autophagy is regulated by epigenetic pathways is not well understood (Baek and Kim, 2017). To address this, we examined methylation status of *Becn1* in thymic cells post-DES exposure *in vivo*. It should be noted that bisulfite conversion does not discriminate between methylcytosine and hydroxymethylcytosine as both modifications protect the cytosine from bisulfite conversion. Nonetheless, there was hypomethylation of *Becn1* in the presence of DES (Fig. 3) demonstrating a DES may induce the expression of *Becn1* by altering methylation status. Because miRs have been shown to regulate gene expression (Bandi and Vassella, 2011; Singh et al., 2015a,b; Baltimore et al., 2008), we also determined the status of miR in thymic cells. In a recent study, we reported that DES affects a large number of miRs in thymic cells of both adult and fetuses (Singh et al., 2015a,b). We had also observed that DES caused downregulation of miR-30a in the thymus (Singh et al., 2015a,b) and miR-30a has been shown to influence autophagy by regulating *Becn1* expression (Yu et al., 2012). It is for these reasons that we further examined the role of miR-30a in regulating the expression of *Becn1*. We used transfection studies to test if miR-30a directly targeted *Becn1* expression. Our data showed that transfection of cells with mature miR-30a led to higher expression of miR-30a and decreased expression of *Becn1* while transfection with anti-miR-30a led to decreased miR-30a expression and enhanced *Becn1*. Furthermore, DES treatment caused relatively additional downregulation of miR-30a and enhanced expression of *Becn1*. These data clearly indicated that miR-30a targets *Becn1* and that DES may modulate the expression of *Becn1* through suppression of miR-30a. Previous studies have shown the regulation of *Becn1* by miR-30a. For example, miR-30a mimic inhibits expression of *Becn1* and ATG5 and downregulates autophagy, whereas antagomir-30a increases their expression and autophagy (Mizushima et al., 1998). It has also been shown that knocking down of *Becn1* and ATG5 inhibits antagomir-30a- induced autophagy, confirming that *Becn1* and ATG5 are downstream effectors of miR-30a-mediated autophagy (Mizushima et al., 1998). In the current study, while DES also caused upregulation of LC3, we found that LC3 did not express miR-30a binding sites. Thus, the induction of LC3 by DES may result as a consequence of *Becn1* upregulation.

The current study demonstrates that DES exposure triggers hypomethylation of *Becn1* and downregulates miR-30a, which targets *Becn1*. Consistent with the current study, we demonstrated in an earlier investigation that pregnant C57BL/6 mice exposed to DES showed changes in miR profiles in thymocytes of both the mother and fetuses (Singh et al., 2015a,b). Of the 609 mouse miRs examined, we noted 59 altered miRs that were common for both mothers and fetuses. Moreover, pathway analyses suggested that these miRs may regulate genes involved in various functions, such as apoptosis, autophagy, toxicity, and cancer (Singh et al., 2015a,b).

In summary, we demonstrate for the first time that exposure to DES in vivo triggers significant decrease in thymic cellularity, through potential induction of epigenetic mechanisms, which cause autophagy. Our study suggests that DES downregulates the expression of miR-30a and triggers hypomethylation of *Becn1* which together increase the expression of *Becn1*, thereby triggering autophagy in thymic cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

DES	diethylstilbestrol
ER	estrogen receptor
miR	microRNA
miR-30a	microRNA-30a
Becn1	Beclin 1
LC3	microtubule-associated protein light chain 3

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Fig. 1. DES causes decrease in thymic cellularity and induces autophagy in thymocytes of adult mice.

Adult mice (C57BL/6) were injected i.p. with DES (5 μ g/kg body weight) or VEH (corn oil; control). On days 2 and 4 post DES- or VEH-treatment, the thymi were harvested and thymic cellularity was determined (A). Data from groups of 5 mice are depicted as mean $\pm$ SEM. Vertical bars in panel A represent mean $\pm$ SEM. Asterisk (*) indicates statistically significant ($p < 0.05$) differences between indicated groups. The thymocytes from mice on day 2 and 4 (GD 18) post-DES treatment were analyzed for autophagy using Acridine Orange (AO). Panel B shows a representative histogram of three independent experiments

and panel C shows data from groups of 5 mice (mean $\pm$ SEM). Asterisk (*) in panel C indicates statistically significant ($p < 0.05$) difference in autophagy between DES and VEH-treated groups. The thymi harvested on day 4 from DES- or VEH-treated mice were stained with H&E. The organization of medulla and cortex of thymi were analyzed using microscope (D). The representative figure for DES and VEH treatments are shown.

Fig. 2. Expression of Becn1 and LC3 in thymocytes following DES exposure.

Expression of Becn1 and LC3 in thymic cells was determined by Real-Time and/or standard RT-PCR post-DES or VEH treatments (2 A–B). Data in panel A represent three independent Real-Time PCR depicting as Mean $\pm$ SEM. Asterisk (*) indicates statistically significant ($p < 0.05$) difference in expression of Becn1 between DES and VEH-treated thymic cells. Data in panel B demonstrates the expression of LC3 in VEH- and DES-treated thymic cells and presented as a representative of three independent assays, which are also depicted respectively as Mean $\pm$ SEM. Asterisk (*) indicates statistically significant ($p < 0.05$)

difference in expression of LC3 between VEH and DES-treated thymic cells, when compared to internal 18S control. Panel C shows LC3 expression and represents data from three independent Real-Time PCR assays depicted as Mean $\pm$ SEM. Asterisk (*) indicates statistically significant ($p < 0.05$) difference in expression of LC3 between VEH- and DES-treated thymic cells. D–F, Expression of Becn1 and LC3 in thymic cells were also determined by Western blot. Data in panel C is a representative of three independent experiments and are plotted in panel D as Mean $\pm$ SEM for Becn1 and in panel E as Mean $\pm$ SEM for LC3 I and LC3 II. Asterisk (*) indicates statistically significant ($p < 0.05$) difference in expression of Becn1 and LC3 I and LC3 II between VEH- and DES-treated thymic cells.

A BISULFITE MODIFICATION OF DNA

TAGAGAGAATCGGGATTTCGACGTAGTGTATTATTGGAGATTAGGTTATT
 TATTTATTTGATTATAAGAAATAGTTGGTTTATTTTAAAGAGAGAAGTA
 AGGTTTGGGATTAGCGTTTAATGGGGTGTTTTTTGTTTAGTTTGTTT
 AATTTTTTGAGTTAGTATAAAAGTAGGAGAGAATTTT**TCGGGTTTGG**
GTTTAGTT**TCGG**TAATATGGTTAATTAGGATT**CG**TTATGGTGTTTTT
 TTT**CG**GAAAAATGGTAAAGAAAATTGAAATTAGTTAGTA**CG**TAAAGT
 ATTTTGTTTTTTTTTGTGGTAAGATTAAAGATGAAGAGT**CGAGT****CGTC**
GGTATTTGGTATTGGGGTTTATG**CGTGAAATTGCGGTTAGC**GGGG
 TTTGGATTTATATTTTATTTTGTAGGTATAGTGAAGTTTGTATTAGA
 AGATTGAAGGAATTGAAAGATTAGTAGAAGTTTTGTCGTTTGAGATT
 TGTTTAGTTTGTAATAAACGGGTATTACGTTAAAAAAAAAAAAAAAAA
 AAAGTAGGAGAGAATTAATTTTATGGAGTTTTTTTTTTGATTTTACGTAT
 ATTATGGTATATATATATGCGCGCGGGCGCGTATATATATATATATATAT
 ATATTATTAGTTTATTATATTGGGTTTGGGTTGTGTATTAAAGGAGTATT
 GAGTTTGGTTTGATGTTGTTTATATTAAATTTAGTTAAATATTAGCG
 TTTAGGGTTTGTTTG

Fig. 3. Effect of DES on methylation of Becn1 promoter in thymic cells.

A: Shows distribution of CpG islands (in red color) and the position of the MSP primer pairs on the mouse Becn1 promoter. B: Shows representative methylated or unmethylated PCR amplicons of Becn1 promoter from three independent experiments. Becn1 promoter region was amplified using methylated or unmethylated sets of mouse-specific primers in thymic cells post VEH or DES treatment. C: Demonstrates PCR data (mean $\pm$ SEM) obtained from three independent experiments for methylation/demethylation status of Becn1 promoters. Asterisks (*) represent statistically significant ($p < 0.05$) difference between VEH- and

DES-treated groups. Panel D represents Methylation Index obtained from Real-Time PCR. This panel shows data from three independent experiments (mean $\pm$ SEM) and asterisks (*) represent statistically significant ($p < 0.05$) difference in methylation index between VEH- and DES-treated groups.

Fig. 4. Expression of miR-30a in thymic cells post DES treatment.

Expression of miR-30a in thymic cells post-DES treatment. A: Showing miR-30a complementary region and binding affinity with 3' UTR region of *Becn1* gene using microRNA.org analysis software (I) and using miRWalk analysis software (II). B: Shows relative expression of miR-30a to internal control SNORD96a (Real-Time PCR data) in thymic cells post DES or VEH treatment. Data in panel B represent mean $\pm$ SEM obtained from three independent experiments and asterisks (*) represent statistically significant ($p < 0.05$) difference in the expression of miR-30a in thymic cells post DES treatment.

Fig. 5. Expression of miR-30a and Becn1 in EL4 cells in the presence or absence of miR-30a post DES treatment.

A: EL4 cells, not transfected or transfected with mature miR-30a or anti-miR-30a and treated with VEH or DES, were analyzed for the expression of miR-30a (A) or Becn1 (B) by performing Real-Time PCR. Real-Time PCR data are presented as fold change in expression relative to internal control (SNORD96a). Data are depicted as mean $\pm$ SEM of at least three independent experiments. Asterisks (* and #) in panel A and B indicate statistically significant ($p < 0.05$) difference between groups compared. Panel C, Western blot analysis of Becn1 expression at the protein level in EL4 cells not transfected or transfected with mature

miR-30a or anti-miR-30a and treated with VEH or DES. Data are depicted as mean $\pm$ SEM of at least three independent experiments in panel D and asterisks (*) in panel D indicate statistically significant ($p < 0.05$) difference between groups compared.