Human endometriotic lesion expression of the miR-144-3p/miR-451a cluster, its correlation with markers of cell survival and origin of lesion content

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Endometriosis is an inflammatory condition in which endometrial tissue grows in ectopic locations. Survival and growth of these ectopic lesions is associated with pain and infertility. MicroRNAs (miRNAs) have been postulated to play a role in the pathophysiology of the disease and we have previously demonstrated expression of miR-451 in human endometriotic lesion tissue. Here we report elevated expression of the miR-144-3p/miR-451a cluster in human endometriotic lesion tissue. Use of an endometriotic epithelial cell line (12Z) in which the miRNA processing enzyme, DROSHA, was knocked down resulted in an enrichment in the primary (pri) form of miR-144-3p but not that of pri-miR-451a. Using an experimental mouse model of endometriosis in which ectopic endometriotic lesions were deficient for both of these miRNAs revealed that miR-451a, but not miR-144-3p may be derived from exogenous sources such as the circulation/erythrocytes. Together, these data suggest that the miR-144-3p/miR-451a cluster is expressed in human endometriotic lesion tissue, the level of expression correlates with survival status of the lesion tissue and that miR-451a, but not miR-144-3p may be derived from exogenous sources such as erythrocytes.

Endometriosis is a debilitating disease in which endometrial stroma and glands grow in ectopic locations. The disease is characterized by pelvic pain, dysmenorrhea and infertility1, as well as having a profound impact on an individual’s psychological and social functioning2–4. The most well-supported mechanism by which endometriosis is proposed to develop is via reverse menstruation of viable endometrial tissue into the peritoneal cavity. However, because almost all women of reproductive age exhibit some degree of retrograde menstruation5,6, it is postulated that additional factors must contribute to the development and progression of the disease. Potential factors such as alterations in the immune system7, tissue remodeling factors8, inflammatory mediators9,10, stem cells11 and altered cell survival/apoptosis12 have all been proposed, attesting to the complexity of the disease.

MicroRNAs (miRNAs) have been proposed to play a role in the pathogenesis of endometriosis and have garnered considerable attention within the past decade13. miRNAs are small non-coding regulatory RNAs that regulate gene expression post-transcriptionally14. These regulatory small RNAs have been implicated to play essential roles in many cellular events which are conducive to endometriosis development such as cellular proliferation, invasion and apoptosis15. miRNA expression profiles have been established for endometriosis in both the disease tissue and eutopic endometrium as well as eutopic endometrium from control patients16–20.

miRNA-451 (miR-451; now referred to as miR-451a) is one of the most studied miRNAs in endometriosis pathophysiology. Based upon bioinformatic programs such as TargetScan11 and mirDIP22 which predict miRNA

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binding target transcripts, both macrophage migration inhibitory (MIF)23 and tyrosine 3-monooxygenase/tryp- 
tophan 5-monooxygenase activation protein zeta (YWHAZ) 24 have been proposed targets of 
miR-451a which have been examined with respect to endometriosis pathophysiology. However, as 
miR-451a is expressed as part of the 
miR-144-3p/miR-451a cluster25, the expression of both miRNAs products has not been evaluated. This may 
be important as 
miR-144-3p is predicted to target several factors relevant to the pathophysiology of endometriosis 
including PTGS2/COX226–28, TNF-

α29,30, IL-1

β29,30, and IL-630,31. To define the potential role of 
miR-144-3p in the pathophysiology of endometriosis and evaluate the mechanisms for expression of the 
miR-144-3p/miR-451a cluster, we performed the following series of experiments.

Results
Pri- and mature miR-144-3p expression in human endometriotic lesion tissue. Compared to 
matched eutopic endometrial expression, endometriotic lesion, pri-
mir-144-3p (Fig. 1A) as well as mature 
mir-144-3p (Fig. 1B) overall expression was significantly greater. Assessment of individual lesion expression of 
both pri- and mature mir-144-3p revealed not only heterogeneity in the level of expression but also the ratio of 
pri- to mature form (Fig. 1C). Regardless, there was a positive correlation (R = 0.6667; P < 0.0001) between pri-
mir-144-3p and mature mir-144-3p in endometriotic lesion tissue (Fig. 2).

Similarly, both pri-mir-451a (Fig. 3A) and mature mir-451a (Fig. 3B) expression was significantly greater in 
endometriotic lesion tissue compared to matched eutopic endometrium. Much like mir-144-3p, there was a great 
deal of variation within and among lesions with respect to expression levels of both pri-mir-451a and mature 
mir-451a (Fig. 3C). In contrast to mir-144-3p, we detected a significant, negative correlation (R = −0.2906; 
P < 0.05) between pri-mir-451a expression and that of mature mir-451a expression (Fig. 4). In comparing 
pri-forms of both mir-144-3p and mir-451a (Fig. 5A,B), we found that pri-mir-144-3 was approximately more 
than 225-fold more abundant in endometriotic lesion tissue compared to pri-mir-451a. In contrast, mature mir-
451a was approximately 90-fold more abundant in lesion tissue compare to mature mir-144-3p (Fig. 5C,D).

Origin of mature mir-144-3p and mir-451a in endometriotic cells and lesions. To begin to exam-

ine the mechanisms for the disparity between miRNA levels of expression, we transfected endometriotic epithel-
ial 12Z cells with siRNA to DROSHA. As depicted in Fig. 6, transfection of 12Z cells with DROSHA siRNA, but 
not NT siRNA, resulted in significant reduction in transcript (Fig. 6A) and protein levels (Fig. 6B) at both 24- and 
48-hours post transfection. This reduction in DROSHA expression was associated with a significant enrichment
(approximate 55% increase) in pri-miR-144-3p expression suggesting that active processing of miR-144-3p from the genome occurred in 12Z cells. In contrast, knockdown of DROSHA had no effect on levels of pri-miR-451a at either 24 or 48-hours post transfection (data not shown).

These in vitro studies suggested that miR-144-3p may be transcribed from the genome, while the inability of DROSHA inhibition to enrich pri-miR-451a might suggest rapid processing of the pri-form of this miRNA or that lesion content may be derived from exogenous sources. Considering that miR-451a is a major miRNA found in the circulation and erythrocytes and that there is a rich vascularization of endometriotic lesion tissue, we examined the contribution of exogenous miR-144-3p and miR-451a to total lesion expression of these miRNAs.
Figure 7 depicts expression levels of mature miR-144-3p and miR-451a in endometrial fragments from miR-144-3p/miR-451a deficient donor mice which were used to establish endometriotic-like lesions in our experimental animal model. As expected, endometrial tissue from miR-144/miR-451 deficient mice did not express either miRNA. Assessment of miR-144-3p and miR-451a expression in this same miR-144-3p/miR-451a deficient tissue after various timepoints post-induction revealed a significant increase in miR-451a, but not miR-144-3p expression (Fig. 7A). To further confirm that the elevated expression of lesion miR-451a elicited a biological effect on lesion tissue, we evaluated Mif mRNA expression in this same mouse tissue. Associated with increased miR-451a expression was a significant reduction in the miR-451a target Mif (Fig. 7B). This observation is similar to that reported previously by us in human endometriotic lesion tissue and also validates our experimental mouse model as a model which can replicate aspects of the human disease.
Discussion

This is the first study to examine expression of the miR-144-3p/miR-451a cluster in human endometrium and endometriotic lesion tissue as well as explore mechanisms for its expression using a novel animal model. Endometriosis is a complex disease in which inflammation is proposed to play a central role in its pathophysiology. Both members of the cluster (miR-144-3p and miR-451a) have been respectively proposed to target inflammatory factors associated with the pathogenesis of endometriosis including PTGS2/COX2, TNF-α, IL-1β, IL-6, MIF, and YWHAZ, but the majority of emphasis has been placed upon miR-451a. The present findings suggest that miR-144-3p is expressed in both eutopic and ectopic endometriotic lesion tissue, but a role for this miRNA in the pathophysiology of endometriosis has yet to be examined. miR-144-3p has been reported to regulate mediators of inflammation such as TNF-α, IL-6 and IL-1β as well as PTGS2/COX2. Thus, it may be of interest to further evaluate the regulation of these inflammatory mediators by miR-144-3p in the context of endometriosis pathophysiology.

We observed that expression of both miR-451a and miR-144-3p was heterogeneous among lesions, even among similar types (red peritoneal lesions). This heterogeneity of expression cannot be attributed to enrichment/reduction of epithelium or stroma among lesions as we have examined cytokeratin (epithelial cell marker) and vimentin (stromal cell marker) expression in these samples (unpublished observation in this study as well as in a previous study23). Presentation of data from individual lesions (Figs 1 and 3) emphasizes the necessity to evaluate patterns of expression as opposed to simply reporting overall fold-change (increase or decrease from eutopic endometrium). An emerging “working” hypothesis of our research program is that as early endometriotic lesions (red lesions) progress towards becoming less active, miR-451a levels increase and functionally lead to reduced survival of the lesion. We are confident that the heterogeneity in the level of miR-451a expression represents physiological changes within the lesion and is not attributed to differences in lesion content of epithelium. This statement is based upon our observations that the levels of the epithelial markers, cytokerin-18 and cyokertin-19 do not correlate with miR-451a lesion content23.

Within the endometrium and endometriotic lesion tissue, miR-451a localizes predominantly to glandular epithelium. Using the endometriotic epithelial cell line, 12Z as a model, we detected low levels of both the pri- and mature forms of miR-451a and relative levels of the former could not be enriched by knockdown of DROSHA expression. Although transcribed from the same locus, processing of miR-144-3p and miR-451a to the mature forms undergoing differential processing with miR-451a processing occurring via DICER independent mechanisms. However, the initial step for both pri-miRNAs relies on DROSHA activity. As such, we postulated that reduction of DROSHA expression would result in an enrichment of pri-miR-144-3p and pri-miR-451a. While we did observe such an enrichment for miR-144-3p in 12Z cells in which DROSHA expression was...
reduced by siRNA, we did not observe this for miR-451a. These data were interpreted to suggest that miR-451a may be derived from sources other than the cellular genome of endometriotic lesion tissue. This concept was supported by low levels of 12Z cell expression of miR-451a. This postulate was also supported by our observation that expression of pri-miR-451a was exceptionally low in human endometriotic lesion tissue, while expression levels of mature miR-451a were rather robust.

Using our novel mouse model in which wild-type mice (which expressed both miR-144-3p and miR-451a) harbored lesion tissue derived from miR-144-3p/miR-451a deficient donor mice, we confirmed that “lesion” content consists of exogenous sources of miR-451a, as the gene for this miRNA cannot be expressed by this tissue and therefore must be derived from the host tissue/environment. We do not believe that miR-451a, although expressed by peritoneal mesothelial cells (unpublished observation), contributed to the lesion content of this miRNA. We base this statement on two facts. First, in our mouse model, endometriotic lesion tissue does not penetrate the underlying peritoneum and can be easily separate from that tissue. Thus, if there was any contribution from this tissue, it would be minimal. Second, as the levels of miR-451a (and MIF) changed over the time course of this study, the amount of potentially contaminating underlying peritoneum would not change, which supports the notion that the underlying peritoneum was not a major contributor to the level or pattern of miR-451a expression. Based upon these observations, we interpreted these results to suggest that endometriotic lesion miR-144-3p content may be derived from the cellular genome. In contrast, we believe that a significant proportion of miR-451a content may be derived from exogenous sources such as infiltrating erythrocytes and/or exosome crosstalk between cells within the circulation and endometriotic lesion.

Further proof of principal for a role of miR-451a in not only the pathophysiology of endometriosis, but perhaps also the therapeutic utility of miR-451a may be evaluated in non-human primate models. Joshi and colleagues demonstrated that miR-451a was mis-expressed in endometriotic lesion tissue compared to eutopic endometrium but only evaluating a single time point. Time course studies post induction may offer insight as to whether or not a similar pattern of expression might occur in this model as was observed in our mouse model.

While the current study has many strengths, we are aware that it also has limitations. First, we focused on red peritoneal lesions and ovarian endometriomas and did not assess additional lesions such as deep-infiltrating lesions. It would be of interest in future studies to evaluate if deep-infiltrating lesions also express a similar pattern of miR-144-3p/miR-451a expression as well as further explore their pathophysiology. Second, we limited the study to only those women who did not receive hormonal therapy within a minimal 3-month period prior to surgery. It may be of interest to see of hormonal therapies have any impact on miR-144-3p/miR-451a expression in cases of failed treatment to learn more about the regulation and role of these miRNAs in lesion survival and symptoms of the disease. These shortcomings, as well as the potential

Figure 7. Mature miR-144-3p, miR-451a, and Mif transcript expression in endometriotic-like lesions from mice with experimentally induced endometriosis. Endometriosis was induced in wild-type mice using donor tissue from miR-144-3p/miR-451a null mice as described under “Materials and Methods” and mice were sacrificed at 1, 2 and 4 weeks post-induction (week 0 = time of induction). Endometriotic lesion-like structures were removed from each mouse, carefully minimizing underlying peritoneum contamination and total RNA was isolated. Murine miR-144-3p and miR-451a (A) were evaluated by qRT-PCR. miR-451a confirmed target transcript Mif was also evaluated by qRT-PCR in the same lesion tissues at each of the time points (B). Data are displayed as the mean ± SEM. Different letters indicate statistically significant differences among the means as determined by one-way ANOVA followed by post-hoc analysis (N = 6 per group; P < 0.05). Time 0 represents assessments made on tissue fragments (eventual lesions) that were harvested from donors but not transferred to recipient mice and served as the baseline for expression of the indicated miRNAs and their indicated targets.
therapeutic utility of miR-451a in endometriosis treatment may be evaluated in future studies. In summary, we report for the first-time elevated expression of the miR-144-3p/miR-451a cluster in human endometriotic lesion tissue which correlates with survival status of the lesion and that miR-451a, but not miR-144-3p, may be derived from infiltrating or invading sources.

Materials and Methods

Ethical approval and study subjects. The study was approved by the institutional review boards of both the University of Kansas Medical Center and Cleveland Clinic and all experiments were performed in accordance with relevant guidelines and regulations of those institutions. Written informed consent was obtained prior to surgical removal of endometriotic lesion tissue and endometrial biopsies. Following similar approaches to those previously reported, samples were obtained from a total of 48 women (N = 48) between the ages of 21 and 45. Women with endometriosis who presented with pelvic pain due to failed previous endometriosis treatment and were undergoing surgical removal of endometriotic lesion tissue for failed endometriosis treatment were enrolled. A total of 48 subjects were enrolled (N = 18 in the proliferative stage of the menstrual cycle, N = 30 in the secretory stage of the menstrual cycle and included women with stage I/II (N = 15) and stage III/IV (N = 33) endometriosis (Table 1). No subjects had taken GnRH analogs or hormonal therapy within 3 months prior to surgery. A total of 48 endometrial biopsies (eutopic endometrium) and 68 matched (same patient) endometriotic lesions (N = 58 red peritoneal lesions and N = 10 ovarian endometriomas) were collected at the time of surgical removal of endometriosis of all types including deep infiltrating lesions (which were not used for research purposes). All specimens were collected by the same surgeon (TF, RF) at Cleveland Clinic and (KS) at the University of Kansas Medical Center with emphasis on minimizing sample contamination from underlying/surrounding non-endometriotic lesion tissue. To do so, endometriotic lesions were excised and sent to pathology for confirmation of endometriosis, which was defined as the presence of endometrial glands and stroma. Tissue was excised using sharp scissors with no energy. During the excision the underlying tissue was separated from the lesion tissue. Research samples from Cleveland Clinic were immediately snap-frozen, stored at -80 °C and then shipped to the University of Kansas Medical Center, while samples obtained from the University of Kansas Medical Center were transported on ice to the research laboratory where they were snap-frozen upon arrival. Stage of the menstrual cycle was determined from the patient’s medical records with day 1 defined as the onset of menses. Samples were subjected to RNA extraction followed by quantitative real-time (qRT)-PCR analysis as described below. As no difference in any of the pri- or mature forms of the miRNAs assessed was noted among stages of the menstrual cycle, stages of endometriosis, type of lesion (peritoneal or ovarian endometrioma) or influenced by medications, data were collapsed and analyzed as ectopic versus eutopic tissue for each endpoint.

RNA isolation and qRT-PCR of miRNAs and mRNAs. RNA was isolated from tissue using Trizol (Life Technologies) at 1.0 mL of Trizol/100 mg of tissue following the protocols provided by the manufacturer. Total RNA (1 µg in 20 µl) was reverse transcribed using reverse transcription (RT) kits (Applied Biosystems; Foster City, CA) following the manufacturer’s protocol specific for each pri- and mature miRNA. Total RNA (250 ng in 5 µl) was reverse transcribed using RT kits (Applied Biosystems) following the manufacturer’s protocol with the following modifications. Briefly, miRNAs were reverse transcribed in a single reaction using 2 µl of each miRNA specific 5X RT primers. Resulting material was then used for independent qRT-PCR for each miRNA. To normalize for starting material, a reverse snRNA U58 was included in the miRNA RT reactions and qRT-PCR of U58 was performed using validated primers from Applied Biosystems. mRNA was reversed transcribed using 1 µg of total RNA using reverse transcription (RT) kits (Applied Biosystems; Foster City, CA) following the manufacturer’s protocol. Primers for DROSHA were designed using Primer-Blast and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Sequences for the human DROSHA (NM_013325) primers were: forward, 5′-CTGGCAAGGGCATTCCACAT-3′ and reverse, 5′-TGATTGTGGCGCTAGGGTCAGA-3′. DROSHA expression levels were normalized to 18S rRNA using primers from Applied Biosystems). All qRT-PCR reactions were completed on a QuantStudio7 Flex Real-Time PCR System (Applied Biosystems). All samples were run in triplicate and the average value used in subsequent calculations. The 2-delta-delta CT method was used to calculate the fold-change values among samples as previously described by our group. qRT-PCR intra- and inter-assay coefficients of variation were both less than 5%.

| Study group/age range | Diagnosis/stage of menstrual cycle | Lesion type (N) |
|-----------------------|------------------------------------|----------------|
| Endometriosis (N = 48) | Proliferative (N = 6)              | P (7), O (2)   |
|                       | Secretory (N = 12)                 | P (15), O (2)  |
|                       | Stage III/IV endometriosis (N = 30)|                |
|                       | Proliferative (N = 9)              | P (12), O (2)  |
|                       | Secretory (N = 21)                 | P (24), O (4)  |

Table 1. Patient Demographics. N indicates the number of each lesion type within group. Abbreviations: P = peritoneal biopsy; red lesion, O = ovarian endometrioma.
Cell culture of endometriotic epithelial 12Z cells and siRNA transfection. The endometriotic epithelial cell line, 12Z (originally described by Zeitvogel et al.\textsuperscript{[5]}) was obtained from Dr. Linda Griffith (Massachusetts Institute of Technology, Cambridge, MA). Cell culture was conducted following the general approach as previously described\textsuperscript{[5]}. Briefly, cells were cultured in phenol red-free Dulbecco’s Minimum essential medium (DMEM)/Ham’s F12 (Fisher Scientific, Pittsburgh, PA) + 10% charcoal stripped FBS (Atlanta Biologicals, Atlanta, GA) + Pen-Strep (Life Technologies, Carlsbad, CA) in T75 flasks and seeded at 1 × 10^5 cells/ml of media until approximately 90% confluency. Cells were then passed and plated in 6-well plates at a density of 1 × 10^5 cells/ml in DMEM/Ham’s F12 media lacking FBS and Pen-Strep. The next day, cells were transfected as described below for each specific experiment.

To assess the impact of DROSHA on processing of pri-miR-144-3p and pri-miR-451a, 12Z cells were transfected with DROSHA siRNA (Dharmacon RNAi Technologies, Lafayette, CO) or a non-targeting (NT) siRNA (50 nM final concentration for each). Briefly, 12Z cells were cultured in phenol red-free DMEM:F12 supplemented with 10% charcoal-stripped FBS, penicillin, and streptomycin. Cells were transfected at 50% confluence using Lipofectamine-2000 transfection agent according to recommendations of the manufacturer (Applied Biosystems) using siRNAs specific for human DROSHA transcripts (Dharmacon RNAi Technologies, Lafayette, CO) or control, non-targeting sequences (Dharmacon). Twenty-four to forty-eight hours after transfection, DROSHA levels were assessed by Western blotting and qRT-PCR.

Western blotting. Western blot analysis was conducted as previously reported\textsuperscript{[27]}. Briefly, total protein was extracted from cell culture samples using RIPA buffer (1X RIPA, Catalog #9806, Cell Signaling Technologies, Danvers, MA). Protein concentration in each sample was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). The same amount of protein (30 µg) was subjected to 12% Bis (2-hydroxyethyl) amino-tris (hydroxymethyl) methane (w/v) gel electrophoresis and electroblotted onto nitrocellulose membranes (GE Healthcare, Pittsburgh, PA). Rabbit anti-DROSHA (ab183732; 1:10,000; Abcam Inc., Cambridge, MA) and goat anti-rabbit secondary antibody (1:5000; GE Healthcare) were used. Stripping and re-probing for beta-actin (ab8227; Abcam; 1:10,000 dilution) was conducted to normalize DROSHA protein expression levels. Immunodetection was carried out using an enhanced chemiluminescence (ECL) kit (Thermo Scientific, Waltham, MA).

Mouse model of endometriosis. All animal experiments were conducted at the University of Kansas Medical Center under the guidance of Dr. Nothnich following the relevant guidelines and regulations. Experimental procedures incorporating animals were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee (IACUC). Experimental endometriosis was induced as previously described with modifications\textsuperscript{[39]}. Briefly, 22- to 24-day old C57BL/6 female mice deficient for DROSHA levels were assessed by Western blotting and qRT-PCR.

Statistical analysis. Pri- and mature miRNA levels were first separately assessed within stage of endometriosis (stage I/II vs. stage III/IV in endometriosis subjects) and among stage of menstrual cycle. As no significant differences among pri- or mature miRNAs expression could be attributed to stage of endometriosis, type of endometriotic lesion (peritoneal or ovarian endometrioma) or stage of menstrual cycle, data were pooled and analyzed as euotopic endometrial tissue compared to endometriotic lesion tissue. All data were first assessed for normal (Gaussian) distribution. Data which displayed normality of distribution were analyzed by paired t-tests or one-way ANOVA where appropriate with Bonferroni post-hoc analysis. Data which failed to display normality of distribution were analyzed by non-parametric tests as specified below. Differences in the delta Ct values among subject groups or among time points (baboon sera across different times post endometriosis induction) were analyzed using Kruskal-Wallis test followed by post-hoc analysis using Dunn’s multiple comparison test. To examine the correlation among pri- and mature forms of each miRNA, Spearman correlation coefficients were calculated. All analysis was conducted using GraphPad Prism\textsuperscript{6} (GraphPad Software, La Jolla, CA). Significance was set at P < 0.05 for all analyses.

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Author Contributions
Conceived the study: W.B.N.; designed the study: W.B.N., T.F., R.F. and K.S.; involved in clinical sample collection and preparation: T.F., R.F. and K.S.; performed the experiments and analyzed the data: A.G. and W.B.N.; drafted the manuscript: W.B.N.; prepared and approved final manuscript: W.B.N., A.G., T.F., R.F. and K.S.
Additional Information

Competing Interests: The authors declare no competing interests.

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