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The Estrogen-Induced miR-19 Downregulates Secretory Leucoprotease Inhibitor Expression in Monocytes

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Abstract
Compared to females, males are more susceptible to acute viral and other respiratory tract infections that display greater severity and higher mortality. In contrast, females tend to fare worse with chronic inflammatory diseases. Circulating 17β-estradiol (E2) is a female-specific factor that may influence the progression of human lung diseases. Here we hypothesize that E2 modulates the inflammatory response of monocytes through microRNA (miRNA)-based modulation of secretory leucoprotease inhibitor (SLPI), an antiprotease with immunomodulatory effects. Monocytic cells were treated ± E2, and differentially expressed miRNAs were identified using PCR profiling. Cells were transfected with miRNA mimics or antimiRs and *SLPI* mRNA and protein levels were quantified. Luciferase activity assay using wildtype and ΔmiR-19a/b-SLPI3′UTR reporter constructs and chromatin immunoprecipitation on E2-treated monocytes were performed. E2 downregulated SLPI and upregulated miR-19 expression in monocytes. Transfection with premiR-19b reduced SLPI mRNA and protein levels and this effect was abrogated using antimiRs against miR-19b. miR-19b directly binds the SLPI 3′UTR. The mechanism responsible for E2-mediated upregulation of miR-19 occurs via increased MIR17HG promoter activity mediated by c-MYC. Overall E2 decreases SLPI expression in human monocytic cells, via changes in miRNA expression and highlights the potential for estrogen to modulate the innate immune system.

Introduction

All species have sex-related differences in physical characteristics and biological activities [1]. Females on average live longer than men, and as people age, the proportions of women at a particular age increases compared to men [2]. It is now well recognised that females have a lower burden of viral, bacterial and parasitic infections, particularly during reproductive years, and yet experience a higher prevalence of autoimmune diseases [3]. With focus on respiratory illnesses, males outnumber females in the majority of acute inflammatory diseases and have poorer prognoses [4]. In contrast, females fare worse

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with chronic inflammatory diseases, and it has been hypothesised that a higher inflammatory response in females is protective where acute conditions are concerned, but chronic inflammation may cause deleterious tissue damage [5]. Male versus female dichotomy is well recognised in chronic inflammatory lung diseases such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) and asthma. Female sex has been recognised as a negative prognostic factor in CF [6, 7]. In 1996, Corey and Farewell analysed almost 4000 CF patients from the Canadian registry between 1970 and 1989 and detected a diminished survival of females of >5 years compared to males over this period [8]. Poorer survival for females with CF was also confirmed in a UK study [9], and despite significant improvements in CF survival over the last few decades, females have continued to display a lower median survival age compared to males [6, 10–12]. Females with CF also have poorer lung function, higher mortality, earlier colonisation with *Pseudomonas aeruginosa*, deteriorate with *Burkholderia* earlier than males [11, 13–15], require more intensified antibiotic treatments, greater hospitalisation rates and have a higher risk of *P. aeruginosa* and *Burkholderia* colonisation of the lung than that of males [15–17]. Furthermore, females with CF exhibit earlier *P. aeruginosa* mucoid conversion than that of males, leading to worse clinical outcomes [17]. However, some studies report no male versus female differences in children [18, 19]. Therefore, recently, the focus has turned to the role of sex steroid hormones to potentially explain the CF gender gap. Our group has shown that estrogen induces the conversion of *P. aeruginosa* to mucoidy in vitro and is associated with increased exacerbations and mucoid conversion in vivo [20].

Sex differences also exist in other chronic inflammatory lung diseases. The prevalence of asthma is lower in females than in males before adolescence; however, this trend is reversed post-puberty [21–23]. In the United States and elsewhere, >60% of all adult patients with asthma are women, and females asthmatics are more likely to experience hospitalisations and more likely to die from asthma [24]. A similar gender gap is emerging in COPD possibly due in part to the increase in female smokers. Females have now surpassed males with respect to COPD mortality [25]. In oxygen-dependent patients with severe COPD, women have a 50% higher risk of mortality compared with men [26]. Sex hormones, including estrogen, may contribute to the progression of these inflammatory lung diseases in women.

Of the 3 major forms circulatory forms of estrogen in females, 17β-estradiol (E2) is the most potent and is the predominant estrogen in non-pregnant pre-menopausal females. Our group has demonstrated that E2 induces Toll-like receptor hypo-responsiveness in CF bronchial epithelial cells to a range of bacterial agonists [5]. This manifests as an inhibition of interleukin-8 release and was found to be the result of an estrogen receptor (ER) β-mediated upregulation of the expression of secretory leucoprotease inhibitor (SLPI), an important anti-protease widely expressed in the lung, mucosal secretions and the skin. SLPI has been found to be a multifunctional protein; it is a serine protease inhibitor that can protect tissue from degradation by a number of proteases such as neutrophil elastase, cathepsin G and trypsin, and studies in myeloid cells have demonstrated its ability to block NF-κB activation and pro-inflammatory signalling [27–29]. Estrogen has been shown to increase the expression of SLPI in other cell types, including uterine epithelial cells [30, 31]. SLPI, however, is also expressed in non-epithelial cells such as monocytes. What remains unknown is whether estrogen regulates SLPI expression in these key immune cells.

SLPI expression is potentially regulated by microRNAs (miRNA). These are small RNAs involved in the regulation of gene expression at the translational level. A single gene may be targeted by multiple miRNAs and one miRNA targets many (typically over 100) mRNAs. Given that 30–80% of human genes are predicted to be influenced by miRNA, it is likely that miRNAs regulate SLPI expression. SLPI mRNA appears not to be alternatively spliced, with no known transcript variants or alternative 3′UTRs, lending itself as an ideal target for miRNA regulation. Estrogen has been shown to regulate the expression of a wide variety of miRNAs, in a variety of cell types [32–36] and it is unknown yet whether any estrogen-regulated miRNAs target SLPI.

SLPI is especially present at mucosal surfaces. Thus, it is important to elucidate the mechanisms that regulate macrophage-derived SLPI expression in specific contexts, particularly in the lung. As a starting point to address whether E2 affects SLPI expression in monocytes – the precursors of tissue and lung macrophages – we explored the effect of E2 on SLPI in monocytic cell lines. Here we also describe the effects of E2 on SLPI expression in primary human monocytes. We profile global miRNA expression in THP-1 cells in response to E2. To this end, 2 of the upregulated miRNAs are explored further in a second monocytic cell line and in primary monocytes; miR-19a and miR-19b, which are encoded as part of an important 6-miRNA miR-17-92 cluster on chromosome 13 [37]. Predicted to target the 3′UTR of human

Table 1. Primer pairs used in this study

mRNA/gDNA	Primers (5′–3′)	Annealing temperature (° C)
GAPDH	(F)–CATGAGAAGTATGACAACAGCCT (R)–AGTCCTTCCACGATACCAAAGT	57
SLPI	(F)–AATGCCTGGATCCTGTTGAC (R)–AAAGGACCTGGACCACACAG	57
MIR17HG	(F)–GCCCAATCAAACTGTCCTGT (R)–ACCGATCCCAACCTGTGTAG	57
MIR17HG Ebox1 (ChIP)	(F)–AAAGGCAGGCTCGTCGTTG (R)–CGGGATAAAGAGTTGTTTCTCCAA	55

SLPI, secretory leucoprotease inhibitor; ChIP, chromatin immunoprecipitation.

SLPI, the effect of modulation of the levels of miR-19a and miR-19b on SLPI expression is described and their ability to bind directly to the 3′UTR of *SLPI* mRNA is also evaluated. Finally, the mechanism of E2-induced upregulation of the miR-17-92 cluster is investigated.

Methods

Cell Culture and Treatments

All cell lines were maintained at 37 °C in a humidified CO_2 incubator in appropriate media. THP-1 (human acute monocytic leukaemia cell line), U937 (Human monocytic [histiocytic lymphoma] cell line) and HEK293 (human embryonic kidney cell line) cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). THP-1 and U937 cells were cultured in RPMI 1640 and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA). All culture media contained 10% foetal calf serum (Gibco, Billings, MT) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Before treatment, cells were serum starved for at least 2 h. Cell culture grade 17β-E2 (Sigma-Aldrich) was added cells at a final concentration of 10^{-8} M. Equivalent ethanol controls were 0.00028%.

Isolation and Treatment of Primary Human Monocytes

Following informed consent under a protocol approved by Beaumont Hospital Ethics Committee peripheral blood monocytes were isolated from heparinised venous peripheral blood obtained from 6 healthy male individuals. About 15 mL of blood was mixed with an equal volume of 0.9% NaCl and the diluted blood was carefully layered over Lymphoprep (Axis Shield, Dundee, Scotland) in a fresh 50 mL tube, avoiding mixing of blood and Lymphoprep before centrifugation. Ficoll-Paque Plus density gradient centrifugation was carried out at 800 × *g* for 10 min at room temperature with the brake off. The mononuclear cell band was aspirated, washed in Hank's Balanced Salt Solution (Lonza, Basel, Switzerland) and centrifuged at 500 × *g* for 5 min. These mononuclear cells were then resuspended in 1 mL recommended medium (1 mM EDTA, 2% foetal calf serum in Dulbecco's phosphate-buffered saline). Monocytes were purified from this mononuclear cell population using the EasySep® Human CD14 Selection Cocktail (StemCell Technologies, Grenoble, France) as per the manufacturer's protocol.

miRNA Expression Profiling

Total RNA was isolated using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. miRNA profiling was carried out by TaqMan Low Density arrays (TLDA). Prior to miRNA expression profiling, RNA from triplicates treatments was pooled, and miRNA expression profiling was performed by a stem-loop real-time PCR-based miRNA expression profiling method using the TaqMan miRNA Arrays version 3.0 (4444913) from Applied Biosystems (Austin, TX, USA). Two array cards (A and B) for each sample were run on the Applied Biosystems 7900HT fast real-time PCR system, which measured expression levels of 754 different human miRNA in each sample and 4 endogenous controls. RNA (20 ng) from pooled samples was reverse transcribed with the Megaplex™ primer pool allowing simultaneous reverse transcription of 754 miRNA and 4 endogenous controls across 2 RT pools on a Thermal cycler. A pre-amplification step was performed on the Megaplex™ RT product (5 μL) using TaqMan® PreAmp Master Mix (2×) and PreAmp Primer Mix (10×) in a 25 μL reaction on the 7900HT fast real-time PCR system The PreAmp primer pool contained forward primers specific for each miRNA and a universal reverse primer. Specific Megaplex™ primer pools and PreAmp Primer Mix were used for A and B cards. PCR for TLDA was carried out with a sample specific cDNA pool, made up to 100 μL (25 μL of PreAmp reaction and 75 μL of H_2O) and combined with TaqMan® 2× Universal PCR Master Mix. One hundred microliter of each sample was added to the 8 fill ports on each card to populate individual wells. The cards were sealed, centrifuged and then run on the 7900HT.

Quantitative Reverse Transcription-PCR for mRNA and miRNA Levels

For quantification of mRNA, equal quantities of RNA were reverse transcribed into cDNA using the Quantitect Reverse Transcription kit (Qiagen, Valencia, CA, USA). The resulting cDNA was used as template for quantitative real-time PCR. Oligonucleotide primers were synthesized (MWG Biotech, Ebersberg, Ger-

many) and quantitative PCR reactions performed in 20 μL containing 2 μL of template cDNA, SYBR Green MasterMix (Roche, Basel, Switzerland) and 10 pmol of each primer (Table 1).

Individual miRNA levels were measured using Taqman miRNA assays (Applied Biosystems, Austin, TX, USA) according to the manufacturer's instructions. Amplification for both mRNA and miRNA was performed on the Roche LC480 Lightcycler in triplicate samples, including no-template controls. Relative expression of transcripts and miRNAs relative to GAPDH and U6 snRNA, respectively, were determined using the $2^{-\Delta\Delta Ct}$ method, where $2^{-\Delta\Delta Ct} = 2^{-(\text{experimental condition } \Delta CT - \text{control } \Delta CT)}$.

Transfection of Pre-miRs, anti-miRs and Reporter Plasmids

U937 cells (1×10^5 in triplicate) were left non-transfected or transiently transfected (48 h) with indicated concentrations of a negative control pre-miR, synthetic pre-miRs or anti-miRs (Ambion, Life Technologies) using Ribojuice (Novagen, Madison, WI, USA) in OptiMEM-reduced serum media (Life Technologies, Carlsbad, CA, USA). RNA was isolated for quantitative reverse transcription-PCR (qRT-PCR) and Taqman miRNA assays, whereas supernatants were recovered for ELISA.

The SLPI 3′UTR was cloned immediately downstream of the coding sequence of Firefly luciferase in the pMIR-REPORT (Origene, Rockville, MD, USA) reporter plasmid, to create pMIR-SLPI-3′UTR. A mutant of this construct lacking the miR-19a/b-3p binding site in the SLPI 3′UTR was generated by inverse PCR using primers flanking the 7 bp predicted miRNA recognition element (MRE) for miR-19. This approach utilises a pair of back-to-back 5′phosphorylated primers, facing outward, each binding to one of the DNA strands of a circular plasmid. The entire plasmid is PCR amplified, using these primers. For this study, these primers were designed to create a 7 base pair deletion in the miR-19a/b binding site in the SLPI 3′UTR (base 170–177), and the following primers were used which flank each side of this sequence: SLPI-MUT-F 5′-AAAGCTTAATAAAGGATCTTTTATTTTCATTGG-3′ and SLPI-MUT-R 5′-GAGAAATAGGCTCGTTTATTTATTC-3′. The absence of the miRNA binding site in this mutant construct, pMIR-SLPI-3′UTR-*Δ19a/b*, was verified by DNA sequencing. HEK293 cells (1×10^5 in triplicate) were transiently transfected with 250 ng pMIR-SLPI-3′UTR or pMIR-SLPI-3′UTR-*Δ19a/b* and 100 ng of the reference *Renilla* luciferase reporter plasmid pRLSV40 (Promega, Madison, WI, USA) with Genejuice (Novagen). Lysates were prepared and assayed for both Firefly and *Renilla* luciferase using the Luciferase assay system (Promega) and coelenterazine (Marker Gene Technologies, Eugene, OR, USA). Firefly luciferase activity was normalized to the *Renilla* luciferase activity.

SLPI ELISA

SLPI protein concentrations in cell supernatants were determined by sandwich ELISA using specific antibodies to SLPI (R&D Systems, Minneapolis, MN, USA).

Chromatin Immunoprecipitation Analysis

Chromatin immunoprecipitation (ChIP) analysis was carried using the High Cell Number protein A ChIP kit following the manufacturer's instructions (Diagenode, Ougrée, Belgium). In brief, cells were cultured and treated as described above. Following stimulation the cells were formalin-fixed. The cells were sonicated to shear DNA (12 × 15 s at 40% amplitude) to approximately 200–1,000 bp in length. ERα and c-MYC were immunoprecipitated using 1 μg of antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After reversal of cross-linking, the DNA was purified and quantitative PCR was carried out as described above. The amount of DNA recovered by ChIP was normalized to an input control. IgG isotype antibodies (provided by Diagenode) were used as a negative control for the ChIP assay.

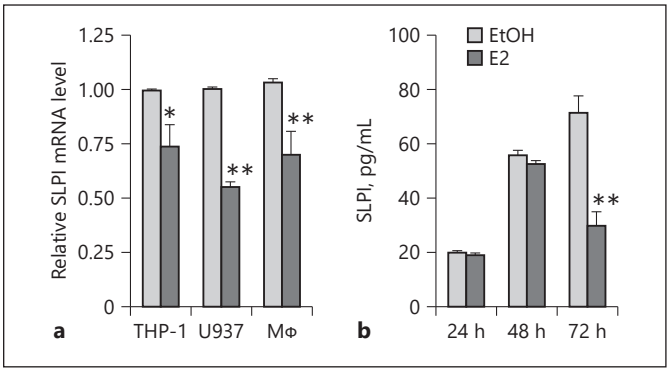

Fig. 1. SLPI expression in monocytes in response to estrogen treatment. Cells were treated, in triplicate, with E2 (10^{-8} M) or with EtOH. **a** Relative levels of SLPI expression in THP-1 ($n = 4$) and U937 ($n = 3$) monocytic cell lines and peripheral blood monocytes ($n = 6$) in response to E2 treatment after 6 h, as determined by qRT-PCR. Results (mean ± SEM) are normalised to *GAPDH* expression. **b** SLPI protein levels in U937 monocytic cell supernatants in response to E2 treatment, as determined by ELISA. * $p \leq 0.05$, ** $p \leq 0.01$; one-tailed *t* test. SLPI, secretory leucoprotease inhibitor; EtOH, ethanol vehicle control; E2, estradiol.

Statistical Analysis

All analyses were performed using GraphPad Prism 4.0 software package (San Diego, CA, USA). Results are expressed as the mean ± SEM and were compared by Student *t* test (non-parametric, 1 tailed) or 2-way ANOVA, as appropriate. Differences were considered significant at *p* values of ≤0.05.

Results

Estrogen Downregulates SLPI in Monocytic Cell Lines and in Primary Monocytes

17β-E2 has previously been shown to increase the expression of SLPI in bronchial epithelial cells [5]. To examine the effect of E2 on the expression of SLPI in monocytes, 2 monocytic cell lines were treated with either 10^{-8} M E2 or ethanol vehicle control (EtOH). E2 significantly downregulated *SLPI* mRNA abundance in both cell lines examined (THP-1; $p < 0.05$; $n = 4$ and U937; $p < 0.01$; $n = 3$), as determined by qRT-PCR (Fig. 1a). E2 also significantly downregulated the expression of *SLPI* mRNA in primary monocytes obtained from the peripheral blood

Table 2. miRNAs downregulated in THP-1 monocytic cells in response to estrogen treatment

miRNA	Fold change (down)
hsa-miR-519b-3p	–5.81E+05
hsa-miR-135a	–2.37E+04
hsa-miR-29c	–10.04
hsa-miR-523	–9.39
hsa-miR-378	–7.57
hsa-miR-203	–7.41
hsa-miR-646	–7.06
hsa-miR-454	–4.38
hsa-miR-205	–4.06
hsa-miR-377	–3.98
hsa-miR-324-3p	–3.96
hsa-miR-628-3p	–3.23
hsa-miR-544	–3.10
hsa-let-7e	–3.06
hsa-miR-1208	–2.86
hsa-miR-302b	–2.76
hsa-miR-335*	–2.53
hsa-miR-571	–2.52
hsa-miR-639	–2.48
hsa-miR-1260	–2.02
hsa-miR-200a	–1.85
hsa-miR-628-5p	–1.80
hsa-let-7d	–1.78
hsa-miR-575	–1.76
hsa-miR-320B	–1.68

RNA from triplicate E2 (10^{-8} M) or EtOH 6 h treatments carried out in THP-1 cells was pooled and profiled by TaqMan® Low Density Array. Relative quantification was determined using the $2^{-\Delta\Delta Ct}$ method.

miRNA, microRNA. miR* (i.e., miR-star) is the old nomenclature for a passenger, rather than a guide strand.

($p < 0.01$; $n = 6$; $p < 0.01$; $n = 6$). SLPI protein expression from U937 cells increased over time as determined by ELISA (Fig. 1b) A similar effect was observed in THP-1 cells where SLPI protein levels increased from 90 ± 12 pg/mL at 6 h to 180 ± 5 pg/mL at 24 h (data not shown). E2 significantly decreased SLPI protein secretion in U937 monocytes at later time points (T = 72 h, $p < 0.01$; Fig. 1b).

Putative SLPI-Targeting miRNAs miR-19a and miR-19b Are Increased in Monocytic Cells in Response to E2

To determine the effect of E2 on global miRNA expression in monocytic cells, miRNA profiling was carried out on THP-1 cells treated ± E2 using TLDA. Of 768 miRNAs profiled, 25 miRNAs were identified as downregulated (relative quantification, RQ, of <0.6; Table 2) and 56 were upregulated (RQ >2.0; Table 3) in response to E2 treatment, when normalised to vehicle control (EtOH) treatment.

Given that E2 downregulates SLPI expression and that it also upregulates the expression of a range of miRNAs in THP-1 cells, the possibility that E2-induced miRNA regulation of SLPI occurs was examined. A selection of target prediction databases (TargetScanHuman 6.2, miRanda and PITA) was interrogated to identify miRNAs predicted to regulate SLPI (NM_003064.2). In total, 211 MREs were predicted in the 3′UTR of the *SLPI* mRNA. Two of the miRNAs that were identified as potentially regulating SLPI across these 3 prediction databases, hsa-miR-19a and hsa-miR-19b (Fig. 2a), were upregulated by E2 treatment in THP-1 cells, as determined by miRNA profiling. Both of these miRNAs had good mirSVR scores of –1.1873 as determined by the miRanda algorithm (microRNA.org) indicating a high probability of targeting SLPI.

Next, in order to determine that this effect of E2 on was not unique to THP-1 cells, qRT-PCR assays were performed for hsa-miR-19a and hsa-miR-19b in another monocytic cell line, U937 (Fig. 2b). Basal miR-19b levels were significantly higher than miR-19a in both U937 ($p = 0.0060$). This effect was also evident in THP-1 ($p = 0.0049$, data not shown). Cells were treated with either 10^{-8} M E2 or EtOH. The effect of E2 on mature miR-19a and miR-19b levels was examined by qRT-PCR. An increase in the expression of both miRNAs post-E2 treatment was observed in U937 cells with respect to time. This increase due to E2 treatment was statistically significant for both miRNAs after 6 h (miR-19a: $p = 0.0124$, miR-19b: $p = 0.0016$).

miRNA Modulation Affects SLPI Expression in Monocytic Cells

As bioinformatic analysis revealed that the miRNAs hsa-miR-19a and hsa-miR-19b are predicted to regulate SLPI, the effect of modulation of these miRNA levels on SLPI expression was investigated. Synthetic pre-miRs for miR-19a and miR-19b or scrambled non-targeting controls were transfected into U937 cells. Transfection efficiency was evaluated by analysis of fluorescent microscope images of cells transfected with fluorescent miRNA (Dharmacon Miridian miRNA-Dy547). These assessments repeatedly showed at least 70% efficiency. Forty-eight hours post-transfection, cells were lysed and RNA was isolated. qRT-PCR analysis was carried out for SLPI expression (Fig. 3). Significant knockdown (63%, $p < 0.01$) of *SLPI* mRNA occurred with transfection of pre-

Table 3. miRNAs up-regulated in THP-1 monocytic cells in response to estrogen treatment

miRNA	Fold change (up)	miRNA	Fold change (up)
hsa-miR-302c	2.01	hsa-miR-135b*	5.40
hsa-miR-872	2.01	hsa-miR-19b	5.96
hsa-miR-24	2.42	hsa-miR-518d-3p	6.32
hsa-miR-618	2.45	hsa-miR-484	6.35
hsa-miR-548a-3p	2.54	hsa-miR-92a	6.91
hsa-miR-662	2.57	hsa-miR-505	7.30
hsa-miR-19b-1*	2.64	hsa-miR-645	7.38
hsa-miR-197	2.80	hsa-miR-9	8.73
has-miR-1305	2.82	hsa-miR-548c-3p	9.07
hsa-miR-191	2.88	hsa-miR-19a	10.41
hsa-miR-1267	2.95	hsa-miR-190b	11.68
hsa-miR-549	2.99	hsa-miR-376a	12.31
hsa-miR-374b	3.00	hsa-miR-342-3p	13.02
hsa-miR-1275	3.07	hsa-miR-1298	17.30
hsa-miR-432	3.22	hsa-miR-521	17.36
hsa-miR-30d	3.24	hsa-miR-299-5p	18.07
hsa-miR-25	3.45	hsa-miR-29b	20.04
has-miR-155	3.55	hsa-miR-625*	23.55
hsa-miR-30b	3.62	hsa-miR-451	25.34
hsa-miR-146b-5p	3.65	hsa-miR-151-3p	58.23
hsa-miR-200a*	3.71	hsa-miR-21	62.26
hsa-miR-302d	3.79	hsa-miR-378	83.69
hsa-miR-106a	3.80	hsa-miR-1243	118.04
hsa-miR-17	3.85	hsa-miR-148b*	1.35E+08
hsa-miR-142-3p	3.89		
hsa-miR-15b	3.91		
hsa-miR-224	4.06		
hsa-miR-720	4.44		
hsa-miR-320	4.70		
hsa-let-7b	4.93		
hsa-miR-551b	5.19		
hsa-miR-16	5.31		

RNA from triplicate E2 (10^{-8} M) or EtOH 6 h treatments carried out in THP-1 cells was pooled and profiled by TaqMan® Low Density Array. Highlighted miRNAs are predicted by in silico bioinformatic analysis to regulate SLPI by binding to its 3′UTR. Relative quantification was determined using the $2^{-\Delta\Delta Ct}$ method.

miRNA, microRNA. miR* (i.e., miR-star) is the old nomenclature for a passenger, rather than a guide strand.

miR-19b, to a greater extent than pre-miR-19a, as compared to scrambled control (Scr; Fig. 3a). This result was mirrored at the protein level, as determined by ELISA, although protein knockdown was statistically significant using both pre-miRs (Fig. 3b).

Conversely, transfection of these cells with synthetic antimiRs for inhibition of miR-19a and miR-19b resulted in an increase in *SLPI* mRNA in U937 cells. This increase was only statistically significant in anti-miR-19b transfected cells, which resulted in almost 6-fold higher *SLPI* mRNA levels ($p = 0.045$) as compared to Scr (Fig. 3c). An effect on SLPI protein production was only seen when miR-19b levels were modulated (by antimiR-19b), resulting in an increase in SLPI at the protein level (Fig. 3d).

miR-19 Directly Binds to the SLPI 3′UTR

In order to confirm whether SLPI is a molecular target of miR-19b, pMIR-SLPI-3′UTR or pMIR-SLPI-3′UTR-Δ19a/b was transiently transfected into HEK293 cells (Fig. 4) and co-transfected with either pre-miR-19a, pre-miR-19b or Scr. Co-transfection with pre-miR-19a or pre-miR-19b resulted in a significant decrease in luciferase gene expression from the reporter vector containing the wild-type pMIR-SLPI-3′UTR when compared with a Scr (Fig. 4a). There was no decrease in gene expression from the reporter vector containing the deleted miR-19-binding site pMIR-SLPI-3′UTR-*Δ19a/b*, when compared with a Scr, demonstrating direct targeting by miR-19 (miR-19a: $p < 0.01$; miR-19b: $p < 0.001$).

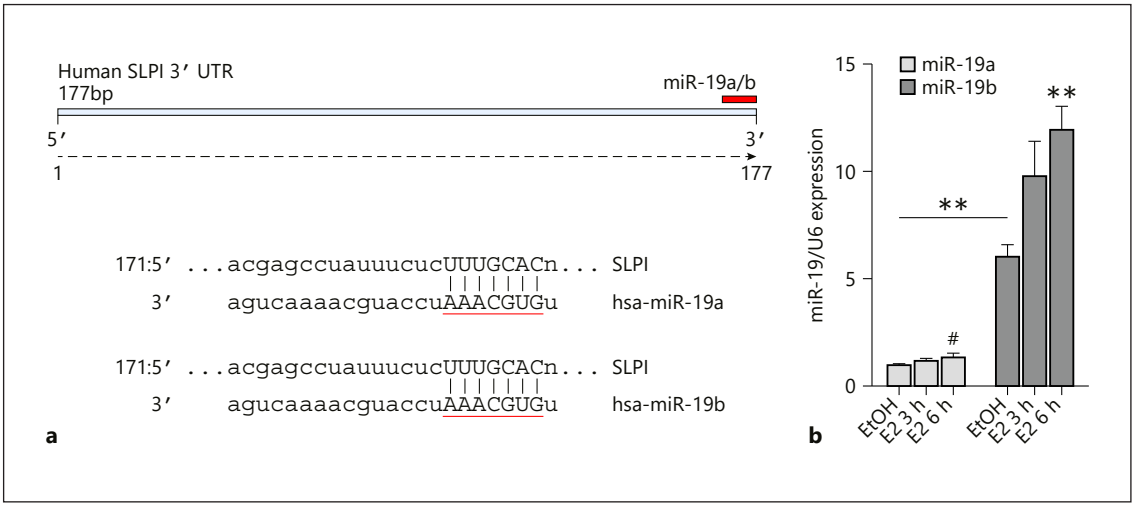

Fig. 2. miR-19a and miR-19b are increased in monocytic cells in response to E2 and are predicted to target the 3′UTR of SLPI. **a** miR-19a/b is predicted by TargetScanHuman (version 6.2) to target a site at the distal end of the 3′UTR of human SLPI. Alignment of miRNAs 19a and 19b with their predicted target SLPI mRNA, as illustrated by TargetScanHuman (version 6.2) and microRNA.org, The 7mer-m8 (exact match to positions 2–7 of the miRNA followed by an exact match at position 8) seed region is underlined. **b** Validation of E2-mediated induction of miR-19 in U937 cells. U937s cells (1×10^5/mL, in triplicate) were treated with EtOH or E_2 (10^{-8} M) for 3 or 6 h. miR-19a and miR-19b levels were measured by qRT-PCR. Results (mean ± SEM, $n = 3$) are normalised to U6 snRNA expression. # $p \leq 0.05$, ** $p \leq 0.01$ versus EtOH or miR-19a versus miR-19b as indicated; one-tailed Student *t* test. SLPI, secretory leucoprotease inhibitor; EtOH, ethanol vehicle control; E2, estradiol.

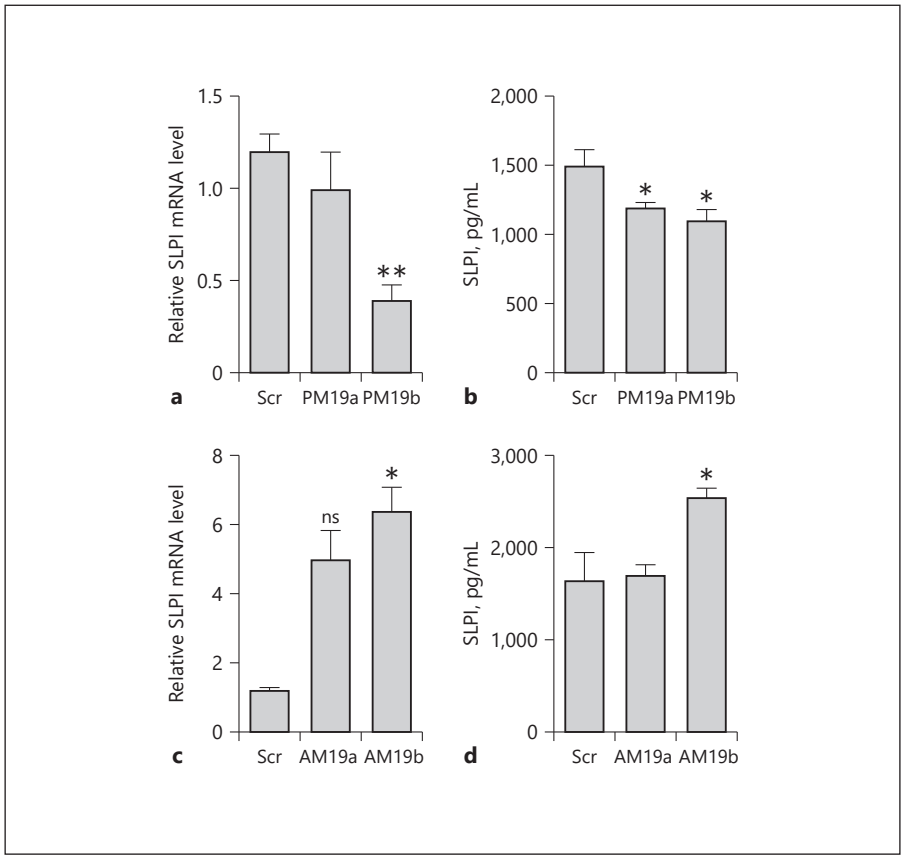

Fig. 3. Modulation of miR-19a/b and SLPI in U937 cells using pre-miRNA mimics. U937 cells (1×10^5/mL, in triplicate) were transfected with a Scr, and either pre-miR (PM) mimics to or antimiR (AM) inhibitors against miR-19a and miR-19b for 48 h. **a**, **b** *SLPI* mRNA and SLPI protein (pg/mL) in cell supernatants, respectively, post transfection with pre-miR mimics. **c**, **d** *SLPI* mRNA and SLPI protein in cell supernatants, respectively, post-transfection with antimiR inhibitors. mRNA and protein levels were measured by qRT-PCR (normalised to *GAPDH* mRNA) or ELISA, as appropriate. Results shown are representative of experiments carried out $n = 3$. * $p \leq 0.05$, ** $p \leq 0.01$; ns vs. Scr, one-tailed *t* test. SLPI, secretory leucoprotease inhibitor; Scr, scrambled control; ns, not significant.

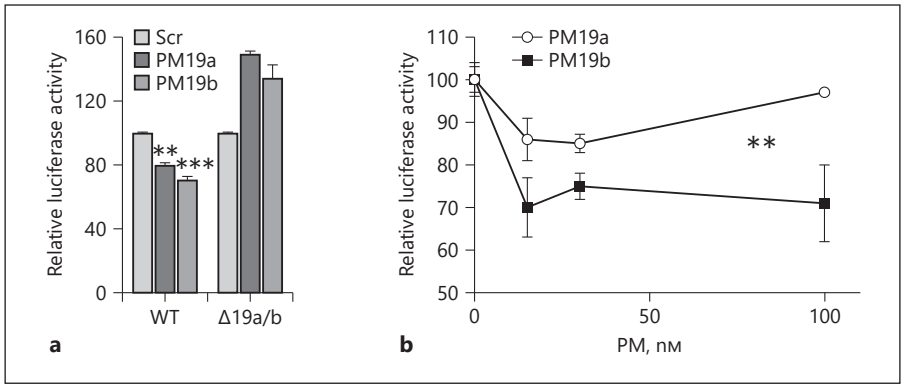

Fig. 4. miR-19 directly targets SLPI. HEK293 cells (1×10^5/mL, in triplicate) were transiently transfected with pRLSV40 (encoding *Renilla* luciferase, *r-luc*), pMIR-SLPI-3′UTR (WT) or pMIR-SLPI-3′UTR-Δ*19a/b* (MUT; both encoding Firefly luciferase, *f-luc*), and co-transfected with either pre-miR-19a (PM19a), pre-miR-19b (PM19b) or Scr pre-miR (Scr). **a** The effects of pre-miR-19a and pre-miR-19b on luciferase activity 48 h post-transfection of HEK293 cells with pMIR-SLPI-3′UTR or pMIR-SLPI-3′UTR-Δ*19a/b*. **b** The effects of pre-miR concentration on luciferase activity 48 h post-transfection of HEK293 cells co-transfected with pMIR-SLPI-3′UTR. *f-luc* activity were normalized to *r-luc* activity. Data are represented as mean ± SEM. Data from (**a**) were compared by one-tailed *t* test; ** $p \leq 0.01$; *** $p \leq 0.001$ ($n = 3$). Data from (**b**) were compared by 2-way ANOVA; ** $p \leq 0.01$ ($n \geq 2$).

Table 4. Summary of observed effects of E2 on SLPI in monocytic cells

	THP1	U937	1° monocytes
SLPI mRNA	↓	↓	↓
SLPI protein	↓	↓	nd
miR-19a/b	↑ (profiling)	↑ (miR assay)	nd
miR-17HG	↑	↑	nd
miR-17HG promoter occupancy	nd	c-MYc and ERα	nd

1°, primary; nd, not determined; SLPI, secretory leucoprotease inhibitor.

To distinguish the effect of miR-19a and miR-19b on SLPI expression increasing concentrations of pre-miR-19a and pre-miR-19b were co-transfected with the SLPI 3′UTR reporter plasmids into HEK293 cells. Luciferase gene expression was reduced by both pre-miRs (Fig. 4b). However, pre-miR-19b had a more potent inhibitory effect than pre-miR-19a transfection at all concentrations examined ($p < 0.01$), indicating that miR-19b is a stronger regulator of SLPI than miR-19a.

Estrogen-Mediated Upregulation of the miR-17-92 Cluster Host Gene Is Mediated by c-MYC and ERα

The miRNAs miR-19a and miR-19b-1 are encoded by the miR-17-92 cluster host gene (MIR17HG). This gene is a non-protein coding gene located on chromosome 13 and encodes a polycistronic primary transcript that yields at least 6 mature miRNAs including miR-17, miR-18, miR-19a, miR-19b-1, miR-20 and miR-92 (Fig. 5a). The effect of E2 treatment on the expression of this transcript in monocytes was examined. THP-1 and U937 cells were treated in triplicate with either 10^{-8} M E2 or EtOH control. qRT-PCR was performed to determine mRNA levels. There was no difference for time points 3, 48 and 72 for U937 (data not shown). However, E2 significantly upregulated the expression of MIR17HG at 6- and 24-h post-E2 treatment in both cell lines examined (Fig. 5b, c).

In addition to chromosome 13, miR-19b is also encoded on the X chromosome (as miR-19b-2) from the paralogous miR-106a–363 cluster. The expression of this locus in response to estrogen in U937s was also examined, but this transcript was not expressed in these cells and E2 did not stimulate its expression (data not shown). Although E2 has been shown to increase the expression of the miR17-92 cluster via transactivation by the transcrip-

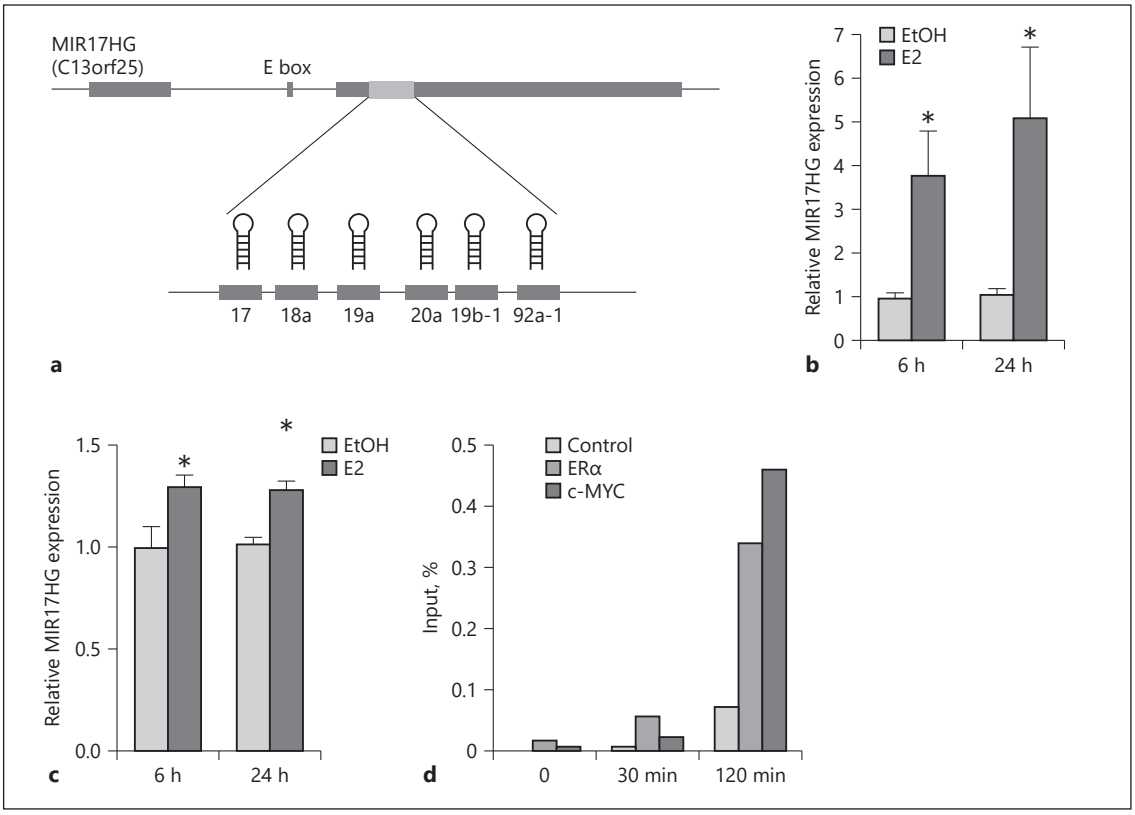

Fig. 5. MIR17HG encodes miR-19 and is controlled by ER and c-MYC in response to E2 in monocytes. **a** Schematic of the MIR17HG transcript. MIR17HG is the host gene for the miR-17-92 cluster of 6 miRNAs, including miR-19a and miR-19b-1. Also depicted is a conserved E box sequence in the promoter region for this gene. THP-1 cells (**b**) or U937 cells (**c**; 1×10^5/mL, in triplicate) were treated with vehicle control (EtOH) or E_2 (10^{-8} M) for 6 or 24 h. MIR17HG levels were measured by qRT-PCR. Results (mean ± SEM, $n = 3$) are normalised to *GAPDH* expression. THP-1 and U937 data are representative of 2 and 3 independent experiments, respectively. **d** U937 cells (1×10^7) were treated with EtOH or E_2 (10^{-8} M) for various time points. ChIP was carried out to determine MIR17HG promoter occupancy. Cells were treated with 1% formaldehyde and subjected to ChIP analysis using c-MYC IgG, ERα IgG or non-immune IgG, as a control. The immunoprecipitated complexes were quantified by PCR using specific primers designed to amplify putative response elements. The ChIP data were normalized to input data. Data are representative of 3 independent experiments. * $p \leq 0.05$; one-tailed Student *t* test. EtOH, ethanol vehicle control; E2, estradiol.

tion factor c-MYC in breast cancer cells, little is known about the E2-specific molecular interactions at this locus in monocytic cells [38]. We hypothesised that E2-mediated upregulation of miR-19 expression occurs *via* binding of c-MYC and ERα to a regulatory region in the *MIR17HG*. ChIP assays showed similar enrichment of E2-induced c-MYC and ERα DNA binding within the MIR17HG promoter (Fig. 5d).

Discussion

It is believed that sex differences in susceptibility to and progression of certain chronic inflammatory diseases are linked in part to steroid sex hormones such as estrogen. Our group has previously reported that E2 increases the expression of SLPI in bronchial epithelial cells leading to a state of TLR hypo-responsiveness in these cells [5]. Here we have examined the relationship between E2 and SLPI expression in monocytes, key cells that regulate the immune response to invading pathogens (Table 4). Figure 6 summarises the mechanism underlying the findings presented here. Unexpectedly, SLPI expression was determined to be downregulated in response to E2 in monocytes. This was the case not only in the monocytic cell lines U937 and THP-1 but also in primary human monocytes isolated from peripheral blood.

We hypothesised that E2-induced miRNAs play a role in regulating SLPI expression in monocytes. To this end, we carried out miRNA profiling to determine the global

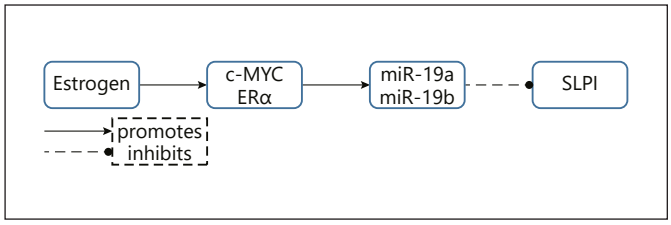

Fig. 6. Mechanism by which estrogen downregulates SLPI in monocytic cells. Estrogen promotes binding of the transcription factors c-MYC, and to a lesser extent ERα, to the promoter of the miR-17 host gene thereby leading to increased expression of miR-19a and, in particular, miR-19b. Both miRNAs can bind to the single miR-19a/b site in the SLPI 3′UTR; however; miR-19b has a stronger effect than miR-19a on inhibition of SLPI expression. SLPI, secretory leucoprotease inhibitor.

miRNA expression in response to E2 in these cells. Of the 768 miRNAs examined, a total of 81 were differentially expressed in E2-treated monocytic cells compared to control. E2 is well-known to regulate the expression of a wide variety of miRNAs, and our results sit well with other studies which have found similar widespread E2-controlled changes in miRNA expression [32–36]. Most of these studies have been carried out in breast cancer cells, and to our knowledge, this is the first study to examine E2-regulated miRNA in monocytes.

The results outlined here confirm that E2 upregulates miR-19, as seen in other studies using the MCF-7 (human breast adenocarcinoma) cell line [32, 39]. The E2-mediated induction of MIR17HG (the pri-mir-17-92 transcript that encodes 6 miRNAs including miR19a and miR-19b-1 as part of the miR-17-92 cluster) observed here in monocytic cells has also been observed in other cells, including breast cancer cells [34, 39]. The miR-17-92 cluster (also known as OncomiR-1) was originally identified as being overexpressed in B cell lymphoma due to chromosome 13q31.3 amplification [37]. miR-17-92-deficient mice display neonatal lethality with developmental defects in lung hypoplasia and ventricular septal defects [40]. These and other studies suggest that this cluster has an anti-apoptotic role. miR-19, and specifically miR-19b, has also been shown to positively regulate NF-κB signalling, at least in primary fibroblast-like synoviocytes, by acting on repressors of NF-κB signalling, such as members of the A20/Tnfaip3-ubiquitin editing complex [41]. Interestingly, we determined that miR-19b expression in U937 cells is produced from this cluster on chromosome 13 and not its paralogous cluster; miR-106a–363 on the X chromosome. Unfortunately, it was not possible at this time to determine whether the latter is expressed in primary monocytes or induced in response to E2. Regarding miR-19a, its upregulation in asthma has been shown to influence T cells and in particular to modulate Th2 cytokine production in the lung [42]. Furthermore, miR-19a participates in allergic lung inflammation by promoting IL-13 and IL-5 production from type 2 innate lymphoid cells [43].

The individual miRNAs encoded by the miR-17-92 polycistron have both pro- and anti-inflammatory validated targets. For example, miR-17 and miR-20a target signal-regulatory protein α, a negative regulator of inflammation, thus increased MIR17HG potentially increases TNF-α, IL-6 and nitric oxide [44]. In contrast, miR-92 suppresses inflammatory responses via targeting MKK4 kinase, which would be expected to reduce TNF-α and IL-6 production [45]. Therefore, the overall consequence of E2 on the balance between the individual effects of the miR-17-92 miRNAs is likely to be complex and, as we have reported for other miRNAs, to be highly cell- and context specific [46].

Bioinformatic analysis revealed a binding site for both miR-19a and miR-19b at the end of the SLPI 3′UTR. miRNA binding sites located toward the distal end of 3′UTRs have been shown to be more effective in target repression and this is potentially due to increased site accessibility [47]. Indeed both miR-19a and miR-19b were found to have good mirSVR scores, predicted by the Miranda tool [48], indicating a high probability of targeting SLPI. Both were subsequently confirmed to be upregulated by E2 in monocytic cells in additional samples and modulation of these by using pre-miRs and anti-miRs evidently modulated SLPI expression. Direct binding of both miRNAs to a predicted site at the end of the SLPI 3′UTR was confirmed by luciferase assay using a full-length SLPI 3′UTR inserted immediately downstream of the firefly luciferase coding sequence. A full-length 3′UTR was utilised to more closely replicate the in vivo accessibility of MREs to miRNAs, including all potential RNA secondary structures and folding, that may be altered with shorter 3′UTRs [49].

The results presented here demonstrate that miR-19b is more effective in targeting SLPI than miR19a. This is to a certain extent unexpected, as the sequence difference between these miRNAs is only a single nucleotide (U > C) at position 11. Being 3 nucleotides away from the seed region, no difference in *SLPI* mRNA binding is predicted using well-established target prediction databases such as miRanda and TargetScan. In a study by Philippe et al. [50], both miRNAs have been experimentally validated to target TLR2 mRNA, with apparent equal efficiency. It is

unknown why there is such a difference in *SLPI* mRNA binding and knockdown between these miRNAs in monocytic cells. The single nucleotide difference may potentially result in greater affinity or more favourable binding to *SLPI* mRNA. It may also be explained by the basal levels of both of these miRNAs in the cells used, with miR-19b levels over 5 times higher than miR-19a levels. Although the levels of these miRs in HEK293 cells were not determined, published sequencing data suggest that the basal expression of miR-19b is also higher than miR-19a in these cells [51]. Interestingly, this study is not the only one to show a variance between these 2 miRNAs in function. Gantier et al. [41] have also shown that the effect of miR-19a and miR-19b is not fully redundant with miR-19b being the more effective of the 2 at suppressing negative regulators of NF-κB.

Here we also investigated the molecular mechanism by which E2 increases the expression of miR-19a and miR-19b in monocytic cells. It has been previously demonstrated that some E2 responsive genes contain both c-MYC and ER-binding sites located within close proximity in their promoters [52]. It has also been postulated that E2 can increase the expression of the miR 17-92 cluster via transactivation by c-MYC [32, 38]. Indeed, Castellano et al. [32] identified a conserved ERE half site and a c-MYC consensus site (E-box) in close proximity within the miR-17-92 promoter region and showed promoter occupancy by c-MYC and not ER, in response to E2, at least in breast cancer cells. In an approach to determine whether the E2-induced upregulation of miR-19 in monocytes occurs through either altered DNA methylation or increased c-MYC expression, we determined that neither the levels of the DNMT1 nor c-MYC are significantly altered by E2 treatment in monocytic cells (data not shown). However, our ChIP experiments determined that c-MYC, most likely in combination with ERα, actively binds to the miR-17-92 cluster upon E2 treatment.

The difference between the effects of E2 on SLPI expression in the lung epithelium [5] versus monocytes may be explained by the difference in basal levels of miR-19 between these cells. Basal levels of both miR-19a and -19b are significantly higher (30- to 50-fold, respectively) in monocytes compared to bronchial epithelial cells or bronchial brushings (data not shown), implying that miRNA-based regulation by miR-19 may be more significant in monocytes.

Having determined that E2 downregulates SLPI in monocytes via miRNA activity, it is unclear as to the clinical significance of this observation. E2 has been shown to regulate SLPI expression in many tissues such as mucosal epithelia. Most studies indicate a positive correlation between E2 and SLPI levels [30–32, 53] as opposed to the negative correlation we report in monocytes. SLPI is a multifunctional protein, with anti-protease, immunemodulatory and antimicrobial roles. It can antagonise activation of NF-κB and inhibit subsequent pro-inflammatory cytokine production [27–29]. Thus, high E2 concentrations that reduce SLPI expression may aid in priming cells to be ready to produce pro-inflammatory mediators in response to microbial stimuli, such as LPS. This may help to explain why females generally fare better with sepsis [54–56], although studies such as these are beyond the scope of the current work and will be addressed in the future. In contrast to monocytes, which are specialised innate immune cells, the primary functions of bronchial and other epithelial cells are to act as a barrier and mediate ion transport, therefore mechanisms to rapidly modulate anti-inflammatory protein expression such as we propose here would be less important in these cells.

Limitations of this study include that pooled samples were used for miRNA profiling; however, validation of the selected miRNAs was confirmed in additional samples. It was not possible to replicate all of the cell line studies using peripheral blood monocytes or macrophages, nor was it feasible to demonstrate the effect of E2 on SLPI in the presence of miR-19b antagonism, which one would expect to be inhibited.

In summary, we have shown that 17β-E2, the primary estrogen in circulation in non-pregnant females, downregulates the expression of SLPI in human monocytes. We demonstrate a widespread change in the miRNA profile of monocytes in response to E2 and describe for the first time that the expression of SLPI is controlled through the interaction of targeting miRNA. In particular, we describe the mechanism of E2-mediated induction of miR-19 in monocytes, and its ability to bind directly to the 3′UTR of SLPI, with the capacity to reduce its expression. Elsewhere we have demonstrated the effect of PLGA nanoparticles encapsulating a miR-19b mimic on SLPI expression in PMA-differentiated U937 cells [57]. This proof-of-concept study demonstrated that miR-19b-targetting nanoparticles can effectively alter target gene expression in macrophages. Given the importance of monocyte-derived macrophages in lung inflammatory processes, it will be interesting to explore the expression and function of miR-19b in alveolar macrophages in future studies and to evaluate the therapeutic potential of an anti-miR-19b approach to increase SLPI expression in those cells.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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