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ORIGINAL RESEARCH

60S acidic ribosomal protein P1 (RPLP1) is elevated in human endometriotic tissue and in a murine model of endometriosis and is essential for endometriotic epithelial cell survival *in vitro*

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ABSTRACT: Endometriosis is a female disease which is defined as the presence of ectopic endometrial tissue and is dependent on estrogen for its survival in these ectopic locations. Expression of the ribosomal protein large P1 (RPLP1) is associated with cell proliferation and invasion in several pathologies, but a role in the pathophysiology of endometriosis has not been explored. In this study, we aimed to evaluate the expression and function of RPLP1 with respect to endometriosis pathophysiology. RPLP1 protein was localised by immunohistochemistry (IHC) in eutopic and ectopic tissue from 28 subjects with confirmed endometriosis and from 20 women without signs or symptoms of the disease, while transcript levels were evaluated by qRT-PCR in 77 endometriotic lesions and 55 matched eutopic endometrial biopsies, and protein expression was evaluated using western blotting in 20 of these matched samples. To evaluate the mechanism for enhanced lesion expression of RPLP1, an experimental murine model of endometriosis was used and RPLP1 expression was localized using IHC. *In vitro* studies using an endometriosis cell line coupled with shRNA knockdown was used to demonstrate its role in cell survival. Expression of RPLP1 mRNA and protein were significantly higher in ectopic lesion tissue compared to paired eutopic endometrium and immunohistochemical localisation revealed predominant localisation to epithelial cells. This pattern of lesion RPLP1 was recapitulated in mice with experimentally induced endometriosis. Stable knockdown of RPLP1 protein resulted in a significant decrease in cell survival *in vitro*. These studies reveal that RPLP1 is associated with cell proliferation and/or survival and may play a role in the pathophysiology of endometriosis.

Key words: endometriosis / RPLP1 / c-Myc / proliferation / 12Z cell line

Introduction

Endometriosis is a chronic inflammatory disease, characterised by the development of ectopic endometrial tissue which establishes primarily in the pelvic cavity. The primary symptoms of the disease are pelvic pain and infertility (Burney and Giudice, 2012). Endometriosis is thought to develop through 'seeding' of the pelvic cavity via retrograde menstruation of shed endometrial tissue during menses (Sampson, 1927). However, essentially all women exhibit some degree of reverse menstruation, suggesting that additional factors must contribute to the survival of these ectopic lesions. It is well established that endometriosis is an estrogen-dependent disease, and in accord, the majority of endometriosis treatments are based upon inhibition of estrogen production and/or estrogen action.

Estrogen mediates its affects through two cognate nuclear receptors: estrogen receptor-1 (ESR1 also known as estrogen receptor-α) and ESR2 (also known as estrogen receptor-β). ESR2 expression, as well as that of several estrogen-regulated genes including growth-regulating estrogen receptor binding-1 (GREB1), cyclin D1 (CCND1) and c-Myc, are increased in human peritoneal endometriotic lesions (Pellegrini *et al*., 2012). ESR2 further interacts with inflammatory factors to inhibit cell apoptosis and promote endometriotic lesion invasiveness, as demonstrated using an experimental mouse model of the disease (Han *et al*., 2015). Additional estrogen signalling factors, including fibroblast growth factor 9 (FGF-9; Wing *et al*., 2003) and steroid receptor coactivator-1 (SRC1; Han *et al*., 2012), are also over-expressed in endometriotic lesion tissue. Clearly, endometriosis development and progression is estrogen-dependent, but our understanding on the complexity of this environment is far from complete.

RPLP1 (ribosomal protein large P1) is part of the 60S ribosomal subunit and functions in regulating elongation translation factors, and its expression is regulated by the estrogen target gene, c-Myc (Guo *et al*., 2000). RPLP1 has two alternatively spliced transcript variants that encode different proteins, with variant 1 coding for a protein of ~12-kDa molecular weight and variant 2 coding for an ~8.4-kDa protein, and the variant 1-transcribed protein appears as the major isoform. RPLP1 is proposed to play a role in diseases associated with cell proliferation and cell survival (Chen *et al*., 2002; Martinez-Azorin *et al*., 2008). For example, RPLP1 induces cell immortalisation of primary cells and transforms previously immortalised cells (Artero–Castro *et al*., 2009). Interestingly, RPLP1 was initially identified as one of only 17 mRNAs upregulated in tumour tissue in a global gene expression profiling study. Subsequent studies have confirmed its over-expression in gynecologic cancers, including endometrial cancer (Artero-Castro *et al*., 2011). Considering the similarities between endometriosis and endometrial cancer, including their dependency on estrogen, we wished to determine if RPLP1 may be mis-expressed in endometriosis where it may play a role in the pathophysiology of the disease.

For the current study, we focused on evaluating RPLP1 mRNA and protein expression, examining its function in human endometriotic lesion tissue and cells and validating its expression and association with lesion survival and pelvic pain in an experimental mouse model for endometriosis.

Materials and Methods

Human tissue acquisition

All studies on humans were approved by the Institutional Review Boards (IRB) at the University of Kansas Medical Center and Cleveland Clinic, and all patients signed consent forms for the procured tissue samples. Samples were obtained through the University of Kansas Medical Center, Department of Obstetrics and Gynecology and the Department of Pathology and Laboratory Medicine as well as the Department of Obstetrics and Gynecology at the Cleveland Clinic. Samples included those obtained from the operating room by TF, RF and KS as well as paraffin-embedded tissue sections for immunohistochemistry from the Department of Pathology and Laboratory Medicine.

Samples were obtained from subjects between the ages of 21 and 45 who presented with pelvic pain due to failed previous endometriosis treatment and were undergoing surgical removal of endometriotic lesion tissue. A total of 55 subjects were enrolled ($N = 20$ in the proliferative stage of the menstrual cycle, $N = 35$ in the secretory stage of the menstrual cycle), and these included women with stage I/II ($N = 20$) and stage III/IV ($N = 35$) endometriosis (Table I). No subjects had taken GnRH analogues or hormonal therapy within 3 months prior to surgery. A total of 55 endometrial biopsies (eutopic endometrium) and 77 matched (same patient) endometriotic lesions were collected. All specimens were collected by the same surgeons (TF and RF) at the Cleveland Clinic or University of Kansas Medical Center (KS) with emphasis on minimising 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. A portion of the same sample lesion which was sent for endometriosis confirmation by a pathologist was utilised for research. Research samples were immediately snap-frozen, stored at −80°C and then shipped to the University of Kansas Medical Center. Stage of the menstrual cycle was determined from the patient's medical records with Day 1 defined as the onset of menses.

Tissue samples used as non-endometriosis controls were obtained from the University of Kansas Medical Center Department of Pathology and Laboratory Medicine. Endometrial tissue in the control groups were from women with uterine leiomyomas ($N = 15$) or endometrial polyps ($N = 5$) and included 10 samples obtained during the proliferative stage of the menstrual cycle and 10 were from the secretory stage of the menstrual cycle (Table I).

The eutopic and matched ectopic endometriotic lesion tissues were obtained from women with endometriosis during the proliferative ($N = 16$; $N = 3$ stage I/II and $N = 13$ stage III/IV) and secretory ($N = 12$; $N = 3$ stage I/II and $N = 9$ stage III/IV) stage of the menstrual cycle (Table I). As generally no difference in RPLP1 (mRNA and/or protein) expression was noted among stages of the menstrual cycle or stages of endometriosis, data were collapsed and analysed as ectopic versus eutopic tissue unless otherwise noted.

Experimental endometriosis mouse model

All animal experiments were conducted at the University of Kansas Medical Center under the guidance of Dr Nothnick 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 (Nothnick *et al*., 2014). Immature (22- to 24-day old C57BL/6 female mice) were injected s.c. with pregnant mare serum gonadotropin (PMSG; 2 IU; Sigma Chemical Company, St. Louis, MO) to stimulate endogenous estrogen production and subsequent estrogenic response within the uterus. Uteri were then harvested from these donors 42–44 h after PMSG injection. Uterine stroma and epithelium (endometrium) were separated from the myometrium with the aid of a dissecting microscope. Endometrial tissue (which contained stromal as well as glandular and luminal epithelium) was cut into 10 fragments

Table 1 Patient demographics.

Study group/age range	Diagnosis/stage of menstrual cycle	Lesion type (*N*)[1]
Control (*N* = 20)	Leiomyomas	
24–48 years of age	Proliferative (*N* = 7)	
	Proliferative (*N* = 7)	
	Endometrial polyps	
	Proliferative (*N* = 3) Secretory (*N* = 2)	
Endometriosis (*N* = 79)	**qRT-PCR studies (*N* = 55)**	**Lesion type (*N* = 79)**
21–45 years of age		
	Stage I/II endometriosis (*N* = 20	
	Proliferative (*N* = 9)	P (10), O (2), F (1)
	Secretory (*N* = 11)	P (12), O (2), F (1), CDS (1)
	Stage III/IV endometriosis (*N* = 35)	
	Proliferative (*N* = 11)	P (13), F (1)
	Secretory (*N* = 24)	P (25), O (7), F (2), CDS (2)
	IHC studies (*N* = 28)	
	Stage I/II endometriosis	
	Proliferative (*N* = 3)	P (2), O (1)
	Secretory (*N* = 3)	P (2), O (1)
	Stage III/IV endometriosis	
	Proliferative (*N* = 13)	P (2), O (1)
	Secretory (*N* = 9)	P (6), O (3)

[1]*N* indicates the number of each lesion type within group. Abbreviations: P = peritoneal biopsy, O = ovarian endometrioma, F = fibrotic lesion, CDS = cul-de sac lesion.

of equal size (1 mm^3). Uterine fragments were suspended in 0.4 mL of sterile saline and prepared for transfer to recipients. Recipient mice consisted of mature (2–4 months old) C57BL/6 immunocompetent, reproductively intact females. For the transfer procedure, recipients were anesthetised with ketamine/xylazine and an antibiotic ointment was placed over the corneas to avoid corneal abrasions. The area over the right rib cage was prepared for surgery and a small incision (~0.5 cm) was made exposing the peritoneal cavity. Tissue fragments were injected into the peritoneal cavity through the incision, and the incision was then closed with wound clips. Carprofen analgesic was given post-operatively at the conclusion of the surgery and again 24 h later. A sham group consisted of recipient mice which received a fat pad (10 equal 1 mm^3). Mice then underwent assessment of pain/visceromotor reflex as described below and were then sacrificed for tissue harvest. This experiment utilised seven sham (*N* = 7) and nine endometriosis mice (*N* = 9).

Assessment of pain/visceromotor reflex

Pain/referred hypersensitivity was assessed by measuring the visceromotor response (VMR), which assess a visceromotor reflex, during vaginal balloon distension (VBD), as previously described (Pierce *et al.*, 2014). Two weeks after induction of endometriosis, mice were anesthetised with inhaled isoflurane (4% induction, 2% maintenance) and two stainless steel electrode wires were acutely implanted into the right and left lateral abdominal musculature using a 26-gauge needle. A custom-made latex balloon (1 cm in length) was inserted into the vagina and secured to the tail. Mice were placed into a Broome-style restraint (Kent Scientific, Torrington, CT), the free ends of the electrodes were attached to a differential amplifier (Model 1700, A-M Systems, Sequim, WA), and the mice were allowed to recover from anaesthesia for 30 min. Electromyographic (EMG) electrode activity was amplified, filtered and recorded using Spike2 software (Cambridge Electronic Design, Cambridge, UK) during triplicate balloon distensions to 40, 60, 80, 100 and 120 mmHg (20-s distension with 4-min inter-trial interval) and analysed off-line. VMR was quantified by measuring the area under the curve for the entire distension period and was expressed as a percent of baseline activity (the 10 s immediately prior to distension). As indicated above, these mice were fully awake and not anesthetized during the VBD recordings.

Cell culture, transfection and lentiviral infection

For *in vitro* studies, we used the well-characterized endometriotic epithelial cell line, 12Z (Banu *et al.*, 2008), which were obtained from Dr Linda Griffith, (Massachusetts Institute of Technology, Cambridge, MA) via a Materials Transfer Agreement (MTA). 12Z cells were seeded

at density of 1×10^6 cells/ml of media in a T-75 flask using complete culture media, phenol red-free Dulbecco's minimum essential medium (DMEM)/Ham's F12 (Sigma Chemical Co., St. Louis, MO) containing 10% charcoal stripped FBS (Atlanta Biologicals, Atlanta, GA), and Pen-Strep (Sigma Chemical Co.) was used for maintenance (referred to as complete media). Once the cells reached ~90% confluency, the cells were passed and plated into six-well plates at a density of 1×10^5 cells/mL in phenol red-free Dulbecco's minimum essential medium (DMEM)/Ham's F12 (Sigma Chemical Co.), with no FBS and Pen-Strep added, and incubated at 37°C under 5% CO_2.

For RPLP1 transient knockdown studies, siRNA for RPLP1 (small interfering RNA; ON-TARGETplus smart pool, Dharmacon Inc., Lafayette, CO) was used. To prepare the cells for transfection, 12Z cells were plated in complete media. As a control, non-targeting NT siRNA cDNA was used. For RPLP1 knockdown, cells were transfected using siPORT^{TM} NeoFX^{TM} transfection agent (Life Technologies/Ambion, Inc.) following the recommendations of the manufacturer. Briefly, transfection reagent was mixed with 50 nM siRNA cDNA for RPLP1 and added to 6-well plates (0.2 mL volume). Then, the 12 Z cells (1×10^5 cells in 1.8 mL of complete media) was added to the plates and cultured for 24 or 48 h. All assessments were performed in duplicate, and this experiment was repeated a minimum of three times using different passages of the 12Z cells.

For Myc knockdown, cells were transfected as described above using 40 nM siRNA for Myc or a non-targeting siRNA (negative control) added to six-well plates. Cells harvested 24 h after transfection were transfected once, but cells harvested at 48 h were transfected twice, once at the initial plating and again at 24 h. This double transfection for c-Myc was required to continue protein knockdown to the 48-h time point. At 24 and 48 h post-transfection, protein was isolated from the 12Z cells and western blot analysis was performed for c-Myc, RPLP1 and β-actin. For each treatment, cells were cultured in duplicates using four different cell passages ($N = 4$).

To develop stable RPLP1-deficient cells, 12Z cells were plated at 1×10^5 cells/well of six-well plates in DMEM:F12 with Pen-Strep and 5% FBS for 24h. Cells were transduced with either lentiviral particles expressing a shRNA for RPLP1 or with a non-targeting shRNA (negative control) per the manufacturer's instructions (GeneCopoeia, Inc., Rockville, MD). Cells were then maintained in the same media with puromycin (2 µg/mL) to select positively infected cells. Negative controls consisted of cells in the same media devoid of puromycin to verify infection. Viral infection was confirmed by expression of eGFP protein. To assess cell survival, cells were removed from culture plates at 96 h post-puromycin treatment and counted manually by Trypan blue exclusion. Briefly, cells were trypsinised from plates and a 100-µL aliquot was diluted 1:5 with PBS and Trypan blue (10% v/v). Then, 10 µL of cell suspension was loaded into each chamber (two total) of a hemocytometer for cell counting. Average cell counts from four sections per hemocytomer were assessed in duplicate (eight total fields), and the number of cells were calculated.

RNA isolation and qPCR

For assessment of mRNA, total RNA was isolated using the Tri-reagent (Sigma Chemical Co., St. Louis, MO, USA). Then, total RNA (1 µg in 20 µL) was used for reverse transcription using RT kits (Applied Biosystems; Foster City, CA, USA), according to the manufacturer's instructions. Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used for primer design. Sequences for the primers used in this study were RPLP1 V1: forward 5′-TGACAGTCACGGAGGATAAGA-3′, reverse 5′-CCAGGCCAAAAAGGCTCAAC-3′. RPLP1 V2: forward 5'-CTCACTTCATCCGGCGACTA-3', reverse 5′-GCCAGGGCCGTGACTGT-3′. CCNE1: forward 5′-CAGGGAGCGGGATGCG-3′, reverse 5′-GGTCACGTTTGCCTTCCTCT-3′. The primers were synthesised by Integrated DNA Technology (IDT, Coralville, IA). mRNA expression was determined by qRT-PCR on a QuantStudio 7 Flex System (Thermo Fisher). For analysis of human tissues, human 18S primers were used (Thermo Fisher) and the fold changes were normalised to the eutopic endometrium and values were expressed as fold change as indicated. For analysis of 12Z human endometriotic cells, human 18S primers were analysed, and the fold changes were normalised to the non-targeting (negative control). 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 (Graham et al., 2015).

Protein isolation and western blot

Tissues were homogenised in RIPA Buffer (Cell Signaling Technology, Danvers, MA) with PMSF (Cell Signaling Technology), and total protein concentration was measured using Bio-Rad Protein Assays (Bio-Rad Laboratories, Richmond, CA, USA). To isolate the protein from the 12Z human endometriotic epithelial cell line, the protein was isolated by using lysis buffer (Cell Signaling Technology) with PMSF (Cell Signaling Technology), and then the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA, USA) was used to determine the total protein.

The same amount of protein (50 µg) was electrophoresed through 12% bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (w/v) gels and electro-blotted onto nitrocellulose membrane (Invitrogen). Primary antibody RPLP1 (1:300; Proteintech, Rosemont, IL, #216363-1-AP), primary antibody c-Myc (Protein Tech; # 10828-1-AP) and goat anti-rabbit secondary antibody (1:5000; GE Healthcare/Fisher Scientific, Pittsburgh, PA, USA) were used. To normalise the protein expression levels, the membranes were stripped and re-probed for β-actin (ab8227; Abcam, Cambridge, MA, USA). The immuno-detection was performed by a using enhanced chemiluminescence (ECL) kit (Thermo Scientific, Waltham, MA, USA).

Immunohistochemistry staining and quantitation

The tissues were fixed with 10% neutral buffered formalin and subjected to immunohistochemical (IHC) localisation using RPLP1 (Anti-RPLP1 antibody; Abcam 121190) at a dilution of 1:300. IHC was performed following the recommendations of the manufacturer using VectaStain ABC system (Vector Laboratories, Inc., Burlingame, CA). Protein localisation was identified as dark brown colouring on the tissue slides.

To semi-quantitatively assess immunohistochemically detected RPLP1 protein expression, we used the H-Score system in which the level of protein was quantitated in each cell type (epithelial, stroma, etc.) indicated as regions of interest (ROI). We selected several sections as ROI within each endometrial biopsy, and due

to the limited amount of tissue in endometriotic lesions compared to eutopic endometrial biopsies, we quantitated the entire lesion cross section as the ROI. The reaction product signal intensity was then scored as 0 (absent), 1 (weak), 2 (moderate) or 3 (strong), and the percent of cells expressing these levels of intensity were calculated in each ROI. The H-Score was then obtained from this information (average level of intensity multiplied by the percent of cells at that intensity) for semi-quantitative measurement of the positive staining.

Statistical analysis

Data were analysed using GraphPad Instat 3 (GraphPad Software, Inc, La Jolla, California). All data from human eutopic endometrium and endometriotic lesion samples were expressed as fold change from matched eutopic endometrial controls (ectopic/eutopic). Matched eutopic control values were expressed as the mean $\pm$ SEM, which was determined by averaging baseline expression of each eutopic endometrial sample. Once the mean values were calculated for eutopic and ectopic end points, data were then subjected to normality tests, and those which were not considered normally distributed were analysed using nonparametric tests. Specific data analysis methods are provided in each figure legend for lesion and eutopic endometrium (unpaired *t* test, Mann–Whitney *U* test, one-way ANOVA). When an *F* test indicated statistical significance, post hoc analysis was made using Bonferroni testing or SNK procedure. For correlation studies, Pearson's correlation was used. For comparison studies of the *in vitro* 12Z cells treatment groups, one-way ANOVA was used followed by post-analysis using Bonferroni testing. Visceromotor reflex data were analysed using two-way ANOVA (with repeated measures) followed by Bonferroni's test. All data are displayed as the mean $\pm$ standard error of the mean (SEM) and significance was set at alpha <0.05.

Results

RPLP1 expression is increased in endometriotic lesions, and it localises to glandular epithelium

As RPLP1 had not been described in human endometriotic tissue, we first confirmed its expression and localisation. To do so, we examined endometriotic lesion tissue and matched eutopic endometrial specimens as well as control eutopic endometrium from women without signs or symptoms of endometriosis (Table I). Samples were prepared for immunohistochemical localisation of RPLP1 as described in 'Materials and Methods'. As depicted in Figure 1A, RPLP1 was localised primarily to the glandular epithelium of ectopic endometriotic lesions (indicated by black arrows) with lower levels of staining in eutopic endometrium from both subjects with (Eutopic-endometriosis) or without (Eutopic-controls) endometriosis. (Higher magnification is provided in Supplementary Figure S1.) Assessment of H-Scores (Fig. 1B) among the three groups within stages of the menstrual cycle revealed that the level of RPLP1 expression (staining) was significantly greater in lesion tissue compared to either eutopic endometrium (endometriosis or control), although eutopic endometrial tissues from both groups expressed low, but consistent, levels of RPLP1 protein and this level of expression did not differ between the study groups or by stage of menstrual cycle. RPLP1 expression in ectopic lesions from women in proliferative stage of the menstrual cycle was significantly higher compared to that in ectopic lesions from women in the secretary stage of the menstrual cycle (Fig. 1B indicated by the asterisk).

RPLP1 mRNA and protein is expressed in human ectopic endometriotic lesions and correlates with a lesion proliferation marker

To further evaluate the expression and potential function of RPLP1 in human endometriotic lesion tissue, we utilised portions from human tissues (Table I) and assessed whole tissue transcript and protein expression. We first analysed RPLP1 (variant 1 and variant 2) mRNA expression, by qRT-PCR, in matched eutopic and ectopic tissue from women with endometriosis. Stage of endometriosis (I/II versus III/IV) did not influence expression of either variant of RPLP1, so the data were collapsed and analysed as eutopic versus ectopic endometrial tissue. As depicted in Figure 2A, the fold change of expression (ratio of lesion/eutopic) of RPLP1v1 was significantly higher (3.72-fold increase; $P < 0.01$) in ectopic endometriosis lesion tissue compared to that in matched eutopic endometrium. Similarly, the fold change of expression of RPLP1v2 was greater (2.56-fold increase; $P < 0.05$) in ectopic endometriosis lesion tissue compared to matched eutopic endometrium. There was a high degree of correlation in RPLP1v1 and v2 mRNA expression within lesion tissue (Pearson $r = 0.8542$; $P < 0.0001$; data not shown). We next performed western blot analysis on a portion of the matched eutopic and ectopic lesion samples to examine the expression of RPLP1 at the protein level. As displayed in Figure 2B, RPLP1 protein expression was greater in ectopic lesion tissues compared to that in matched eutopic endometrium. (An image of the full x-ray film is provided as Supplementary Figure S2.)

To examine the relationship between RPLP1 and a known marker of proliferation in endometriotic lesion tissue (Graham *et al*., 2015), we examined the correlation between cyclin E1 (CCNE1) and RPLP1 in the same lesions. There was a significant, positive correlation between CCNE1 and both variants of RPLP1 (Fig. 3), supporting the notion that RPLP1 expression is associated with cell proliferation *in vivo*.

RPLP1 is necessary for endometriotic epithelial 12Z cell survival *in vitro*

These studies confirmed the up-regulation of RPLP1 transcript and protein levels in human endometriotic lesion tissue which is associated with cell/tissue survival. As RPLP1 has been previously identified as a survival and proliferation promoter in tumours, we next determined if this protein plays a similar role in endometriosis. To do so, an endometriotic epithelial cell line, 12Z cells, were infected with GFP-lentiviral pseudoparticles containing a non-targeting cDNA or shRNA to RPLP1 ($N = 3$). As displayed in Figure 4, endogenous depletion of RPLP1 resulted in a highly significant reduction in the number of viable cells (Fig. 4A). Very few cells remained attached to the cell culture plate (circled cell) while most cells were rounded up and free floating (white arrows; Fig. 4B). These *in vitro* studies further support a potential role of RPLP1 in cellular proliferation and/or survival in endometriotic epithelial 12Z cells.

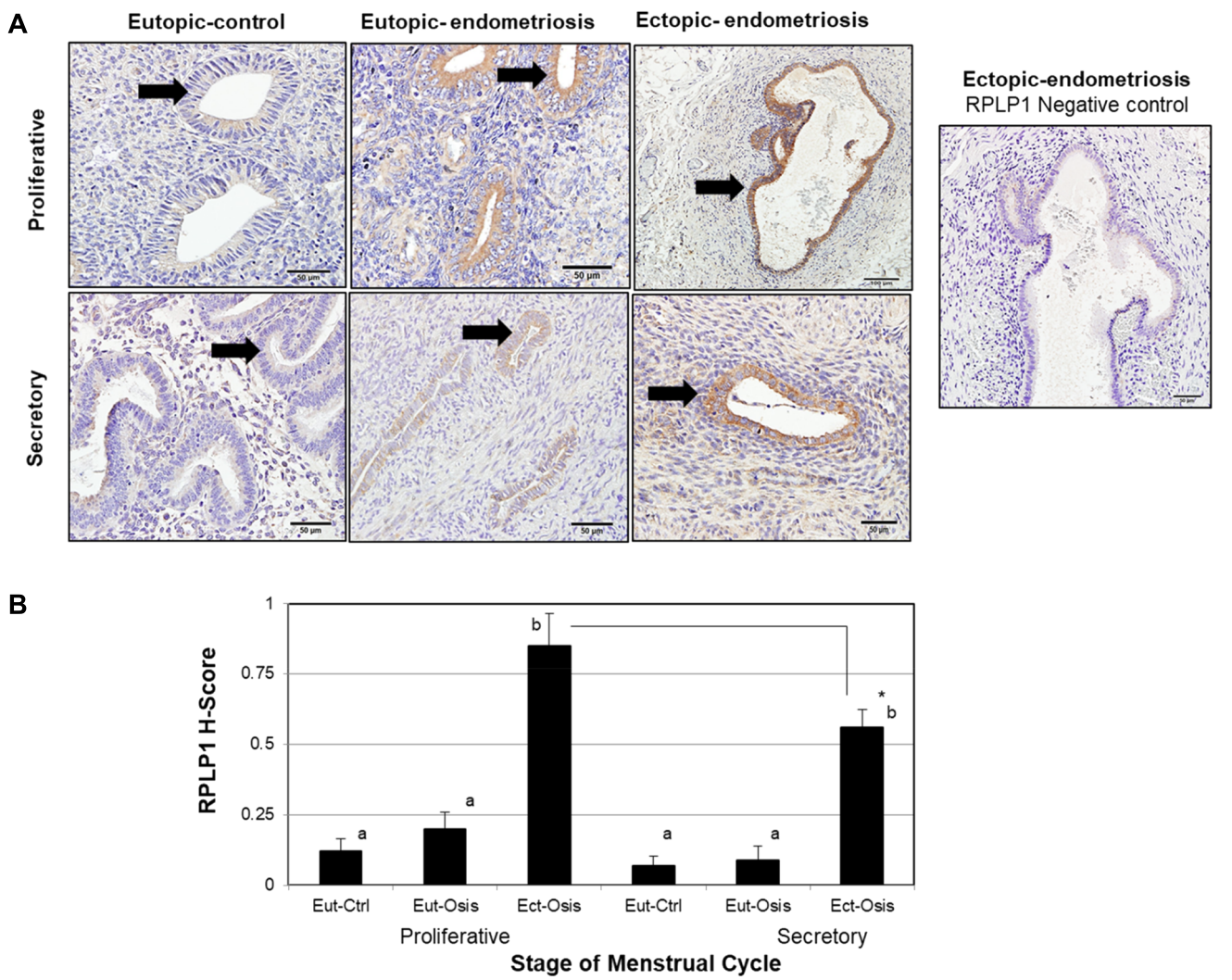

Figure 1 RPLP1 expression is elevated in human ectopic lesion tissue and localised in the glandular epithelium. (**A**) RPLP1 was immunohistochemically localised in ectopic endometriotic tissue and matched eutopic tissues from women with confirmed endometriosis, and eutopic control from women without endometriosis during the proliferative (upper panel) and secretory (lower panel) stages of the menstrual cycle. The arrow illustrates the location of RPLP1 protein in the glandular epithelium of the ectopic tissues (where the brown colour indicates positive staining). All magnifications were ×20 (scale bar indicates 50 μm), except for proliferative ectopic endometriosis which was ×10 (scale bar indicates 100 μm). Negative, isotype-matched control is depicted in the far-right panel at ×20 magnification (scale bar indicates 50 μm). (**B**) Immunohistochemical histological score (H-Score) of RPLP1 in endometrial tissues (ectopic and eutopic) in both stages of menstrual cycle. Data are presented as the mean ± SEM and were analysed by one-way ANOVA followed by Bonferroni post hoc analysis. Different letters indicate statistically significant different mean values, and the asterisk indicates significantly lower expression in secretory ectopic lesion tissue (Ect-Osis) compared to the proliferative ectopic lesion tissue. Data are from a total of 48 specimens, 20 control subjects ($N = 10$ in the proliferative and $N = 10$ in the secretory stage of the menstrual cycle) and 28 endometriosis subjects ($N = 16$ in the proliferative stage and $N = 12$ in the secretory stage of the menstrual cycle).

Myc deletion reduces the expression of RPLP1 in 12Z endometriotic epithelial cell line

To confirm that the expression of RPLP1 is regulated by c-Myc, we knocked down c-Myc expression using siRNA technology. As shown in Figure 5, the expression levels of c-Myc protein detected by western blotting were reduced at both 24 and 48 h compared to the negative control (Fig. 5A). Reduction of c-Myc protein expression was associated with a significant downregulation of RPLP1 protein levels (Fig. 5B), supporting the notion that RPLP1 may be regulated by c-Myc.

RPLP1 is over-expressed in endometriotic lesion tissue of mice with experimentally induced endometriosis

Our observations to this point strongly suggest that RPLP1 is necessary for endometriotic epithelial 12Z cell survival *in vitro* and its expression is positively correlated with cell proliferation in human tissue *in vivo*. To begin to evaluate the necessity of endometriotic lesion RPLP1 in endometriotic lesion survival *in vivo* as well as to determine if expression by the lesion is a result of its ectopic location (as opposed to an inherent upregulation in the eutopic endometrium),

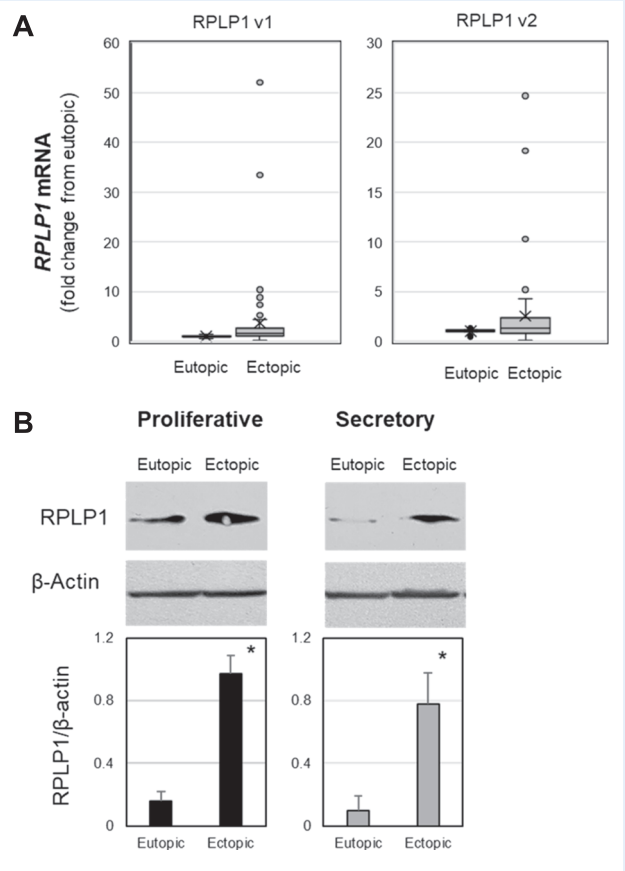

Figure 2 RPLP1 mRNA and protein are elevated in endometriotic lesions. Endometriotic lesions and matched eutopic tissue were assessed for mRNA and protein expression of RPLP1v1 and RPLP1v2. (**A**) Average fold change from matched eutopic endometrium was calculated for both RPLP1v1 and v2, normalising to eutopic endometrium values. Box and whisker plots display data comparing RPLP1 mRNA variant 1 (v1) and variant 2 (v2) between eutopic and ectopic endometrial samples. Data were not normally distributed and were therefore analysed using non-parametric Mann–Whitney *U* tests. Both RPLP1 v1 and RPLP1 v2 transcript levels were significantly higher ($P < 0.01$) in ectopic lesion tissue (mean value is indicated by the X in each whisker and box plot). (**B**) Western blot analysis confirms expression of greater levels of RPLP1 protein expression in lesion tissue compared to matched eutopic tissue. Western blot data are representative from a total 20 matched eutopic and ectopic (lesion) samples for 8 stage I/II disease ($N = 4$ proliferative, $N = 4$ secretory stage) and 12 stage III/IV disease ($N = 7$ proliferative, $N = 5$ secretory stage). Bar graph depicts RPLP1 / β-actin levels comparing eutopic and ectopic levels of expression. $*P < 0.001$ by unpaired *t* test, ectopic compared to eutopic within stage of menstrual cycle.

we utilised an experimental mouse model for endometriosis. All mice in which experimental endometriosis was induced developed ectopic lesions, with an average of two lesions per mouse (data not shown). Compared to eutopic uterine tissue from both sham controls as well as from mice with experimentally induced endometriosis, ectopic lesion tissue expressed robust levels of RPLP1 protein expression which localised predominantly to the epithelium (Fig. 6), similar to that detected in human lesion tissue (Fig. 2). Associated with lesion viability and RPLP1 expression, there was an increase in vaginal sensitivity, which has been validated as a measure of endometriosis-induced pain in rats (Berkley *et al.*, 2001; McAllister *et al.*, 2009). Compared to sham controls, mice with experimentally induced endometriosis exhibited a significantly higher ($P < 0.05$) visceromotor response to vaginal balloon distension across all applied pressures (Fig. 7). Neither mice with induced endometriosis or sham surgeries developed adhesions at 2 weeks post-surgery. Collectively, these results suggest that experimentally induced endometriotic lesions express robust levels of RPLP1 once established in the pelvic cavity, and there may be an association with lesion viability and 'pelvic pain'.

Discussion

Endometriosis is well known as an estrogen-dependent disease, and there is a strong association between estrogen and the inflammatory environment contributing to the disease. However, the complex downstream mediators which impact the pathophysiology of endometriosis are only partially understood. ESR2 is one of the two nuclear receptors that mediate estrogen action, and in the context of endometriosis, ESR2 is expressed at a significantly higher level (over 100-fold) in endometriotic lesions compared with eutopic endometrium (Brandenberger *et al.*, 1999; Fujimoto *et al.*, 1999; Pellegrini *et al.*, 2012). Overexpression of ESR2 leads to a decrease in ESR1 expression (Trukhacheva *et al.*, 2009), which is associated with elevated expression of cyclooxygenase-2 (Bulun *et al.*, 2012), while activation of ESR2 induces expression of several factors associated with endometriosis pathophysiology, including macrophage migration inhibitory factor (MIF; Lin *et al.*, 2010; Veillat *et al.*, 2012), GREB1, CCND1 and c-Myc (Pellegrini *et al.*, 2012).

Endometriosis shares many similarities with endometrial cancer, including an estrogen-driven pathophysiology. Specifically, ESR2 and CCND1 polymorphisms, as well as up-regulation of MIF and c-Myc, are associated with both endometrial cancer and endometriosis (Lin *et al.*, 2010; Pellegrini *et al.*, 2012). A recent systematic literature review and meta-analysis suggests an increased risk of endometrial cancer in women with endometriosis. These observations along with a report that RPLP1 is over-expressed in endometrial cancer led to our assessment of RPLP1 in the pathophysiology of endometriosis.

RPLP1 is a ribosomal protein that forms the ribosomal stalk in the 60S subunit and aids in translation (Martinez-Azorin *et al.*, 2008). However, deletion of RPLP1 in fibroblasts did not affect total protein synthesis, but did influence the expression pattern of some proteins, primarily those involved in protein folding and unfolding, apoptosis and cell signalling (Artero-Castro *et al.*, 2015). In this same study, Artero-Castro and colleagues reported the importance of RPLP1 in cell survival, a trait which is essential in the pathophysiology of the ectopic endometriotic lesion as well as cancer. RPLP1 expression is elevated in gynaecological tumours (Artero-Castro *et al.*, 2011) and associated with increased cellular proliferation. More recently, He *et al.* (2018) reported that RPLP1 promotes tumour metastasis, is associated with poor prognosis in triple-negative breast cancer patients and affects epithelial-mesenchymal transition (EMT). Emerging data indicate that the EMT may play a role in the pathophysiology of endometriosis. Most recently, reported that genes linked to endometriosis are integral

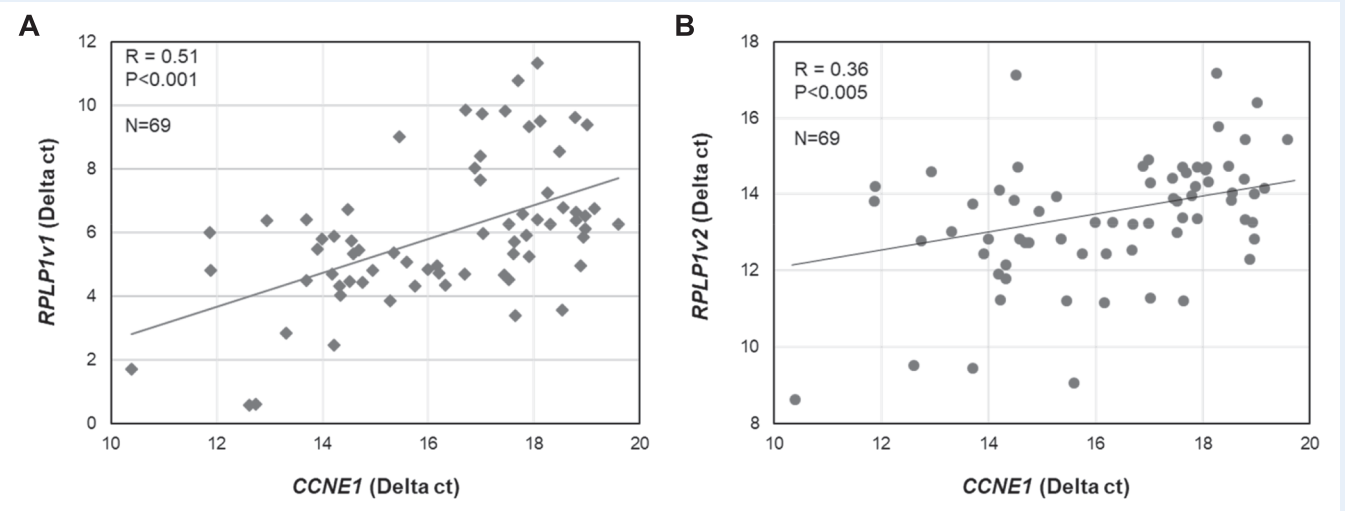

Figure 3 Endometriotic lesion RPLP1v1 and v2 mRNA levels positively correlate with CCNE1 mRNA. mRNA expression was quantitated by qRT-PCR for each transcript. Delta ct values (target—18S) were calculated for each sample and the correlation between delta ct values was determined. (**A**) RPLP1v1, (**B**) RPLP1v2 and CCNE1 values are from a total of 69 lesions.

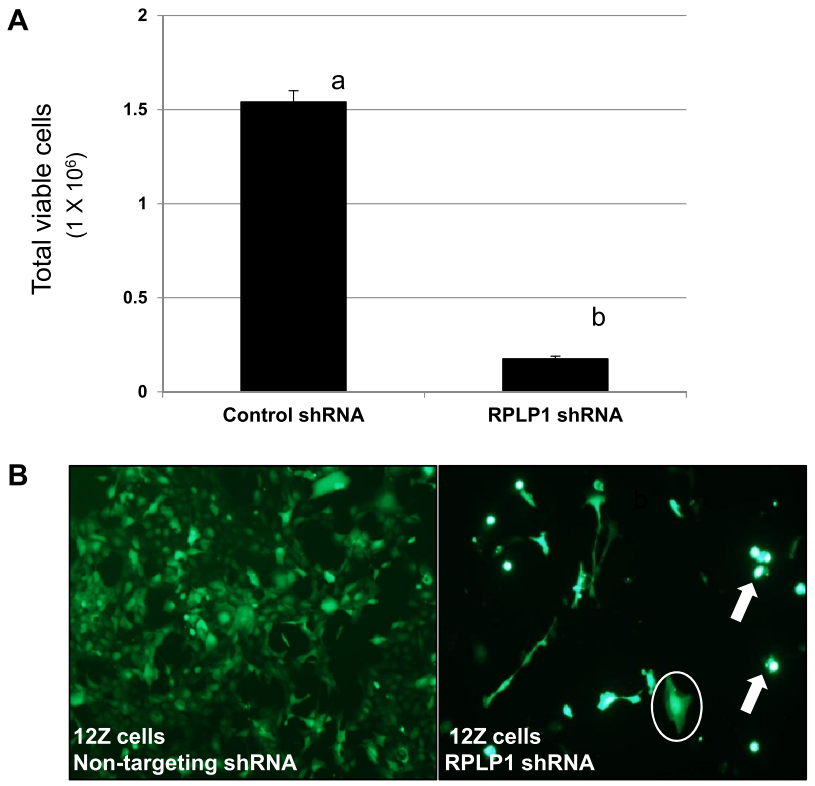

Figure 4 Stable RPLP1 knockdown by lentiviral pseudoparticles results in reduction in cell survival. (**A**) Endometriotic epithelial cells (12Z) were infected with GFP-lentiviral pseudoparticles containing a non-targeting (control) cDNA or shRNA for RPLP1, and the cells were cultured and treated with puromycin after 48 h for positive cell selection. The cells then were then assessed for cell viability at 72 h post transfection. The total number of viable cells was assessed by manual counting of GFP-expressing cells as described in the Materials and Methods. Data are representative of three separate experiments ($N = 3$) which resulted from three different infections. (**B**) Data are displayed as the mean $\pm$ SEM. Different letters indicate statistical significance ($P < 0.001$) between the means using unpaired t test. B depicts a representative culture of 12Z cells infected with non-targeting control shRNA and RPLP1 shRNA. The arrows depict dead/floating cells and the circle depicts a cell attached to culture plate.

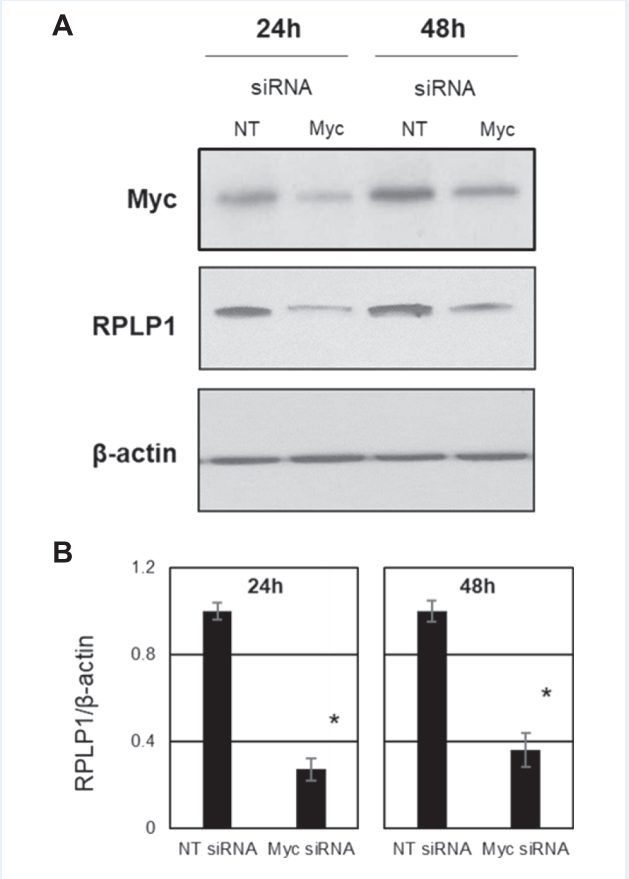

Figure 5 Suppression of c-Myc expression is associated with reduced levels of RPLP1 protein in endometriotic epithelial 12Z cells. (**A**) 12Z cells were transfected with either siRNA to c-Myc or a non-targeting (NT, negative control) siRNA and protein levels of c-Myc and RPLP1 were determined at 24 and 48 h post-transfection by western blot analysis. (**B**) RPLP1 expression levels were normalized to β-actin and are expressed as a fold change from levels detected in NT-siRNA transfected cells. Data are displayed as the mean $\pm$ SEM and are representative of four separate experiments ($N = 4$) using different cell passages for each experiment. $*$ indicates statistical significance ($P < 0.001$) between NT and c-Myc siRNA transfected cells at each time point, by unpaired *t* tests.

to cytoskeleton regulation, implying that mesothelial barrier homeostasis may be a factor in the pathogenesis of endometriosis. This notion is supported by early studies which reported alteration in the expression of EMT markers in endometriotic lesion tissue (Matsuzaki and Darcha, 2012; Proestling *et al*., 2015). Thus, currently available data on RPLP1 support the notion that this protein is essential for cellular growth and proliferation and associated with the EMT. Based upon the current findings reported in our study as well as in data from the literature, we hypothesised that RPLP1 expression may play a role in the survival and/or proliferation of early/newly established ectopic lesions. Further, we postulate that RPLP1 up-regulation occurs once the endometrial tissue is established ectopically, as lower levels of RPLP1 protein were expressed in eutopic endometrial tissue prior to their transplantation and ectopic establishment. Selective targeting of the pathway(s) which contribute to RPLP1 up-regulation or the targeting of RPLP1 itself may hold promise as a future therapeutic target, but much work remains to be conducted to fully understand the role and regulation of RPLP1 in endometrial and endometriotic tissue.

While our current observations suggest that RPLP1 may play an essential role in cell/tissue survival of endometriosis, the mechanism by which it may do so, as well as the mechanisms which lead to its over-expression, remain unclear. The observation the RPLP1 immunolocalisation was greater in tissue from the proliferative stage of the menstrual cycle may suggest an effect of estrogen on RPLP1 expression, but to the best of our knowledge, there is no information on steroidal regulation of RPLP1 in any tissue (outside of the association data presented here and within references). Whole tissue RPLP1 protein levels (as assessed by western blot analysis) did not differ between the menstrual cycle stages and this may be due to the small sample size compared to that for IHC studies.

RPLP1 is a c-Myc responsive gene (Guo *et al*., 2000), and c-Myc is proposed to regulate the expression of 15% of genes in the human genome (Gearhart *et al*., 2007). Among those, c-Myc controls expression of several genes responsible for proliferation, tumourigenesis and apoptosis (Armelin *et al*., 1984; Baudino *et al*., 2002; Beer *et al*., 2004; Dang, 2012). Altered expression of Myc is defined as a 'hallmark' of cancer (Gabay *et al*., 2014), and it is detected in several types of cancer including breast (Palaskas *et al*., 2011), lung (Beroukhim *et al*., 2010), ovarian (Ross *et al*., 2013) and prostate (Gurel *et al*., 2008) cancer. In endometriosis, the expression of Myc was found to be highly expressed in the epithelial cells of ectopic lesion tissue compared to eutopic tissues, and its expression was associated with proliferation (Proestling *et al*., 2015). Previous studies have indicated the up-regulation of Myc in endometriotic lesions compared to eutopic endometrium from women with or without endometriosis (Schenken *et al*., 1991; Schneider *et al*., 1998; Zamarripa *et al*., 2000; Meola *et al*., 2010; Kobayashi *et al*., 2014; Ping *et al*., 2016). However, most of these were descriptive studies to show the differentially expressed protein profile and they have not specifically investigated the proliferative mechanism of Myc in endometriotic lesions. As reported in our current study, RPLP1 expression is significantly reduced when Myc expression is reduced *in vitro*. These observations would suggest that elevated levels of c-Myc may in turn induce the expression of RPLP1 which is essential for endometriotic epithelial (12Z) cells, at least *in vitro*.

As RPLP1 appears to be essential for cell proliferation/survival, we were surprised at the low levels of protein expression in eutopic endometrium. However, despite the lower levels of protein, RPLP1 transcripts (variant 1 being greater than variant 2) appear to be quite abundant and consistently expressed among eutopic endometrial samples (ct values in the range of 19–21 by qRT-PCR (compared to 18S values which range from 12- 14 on average). Due to its abundant levels, RPLP1 has been proposed by several investigators as a reference gene for qRT-PCR in Atlantic cod (Aursnes *et al*., 2011), rodent (Marques *et al*., 2013) and human (Barragán *et al*., 2015) studies. It should be noted, however, that the protein levels were not assessed in any of these studies. Clearly, our current study suggests that RPLP1 transcript expression is robust in eutopic endometrial tissue and endometriotic lesion tissue, but only the later have significantly elevated levels of protein expression. As both our current data

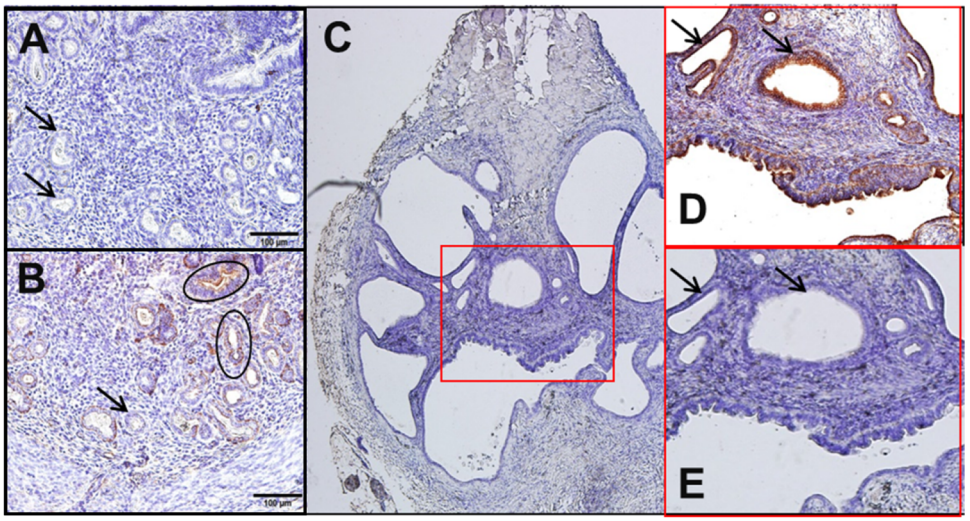

Figure 6 Immunohistochemical localisation (IHC) of RPLP1 in mouse endometrium and matched endometriotic lesion tissue. IHC was performed on eutopic uterine tissue from sham (**A**) and mice with experimentally-induced endometriosis (**B**) as well as endometriotic lesion tissue (**D**). All tissues were obtained during the estrogen-dominate stages (proestrus or oestrus) of the estrous cycle 2 weeks after endometriosis was induced. Arrows indicate glandular epithelium and the black ovals in B indicate glands in eutopic endometrium of mice with endometriosis which show elevated RPLP1 localisation. A and B are eutopic endometrium, C represents the entire lesion (H&E staining only) and the red box indicates the sections depicted in D and E, which represent RPLP1 antibody (1:300) staining and a negative control (isotype-matched antibody), respectively. Observations are representative of data obtained from four animals per group ($N = 4$). Scale bar = 100 μm in A and B.

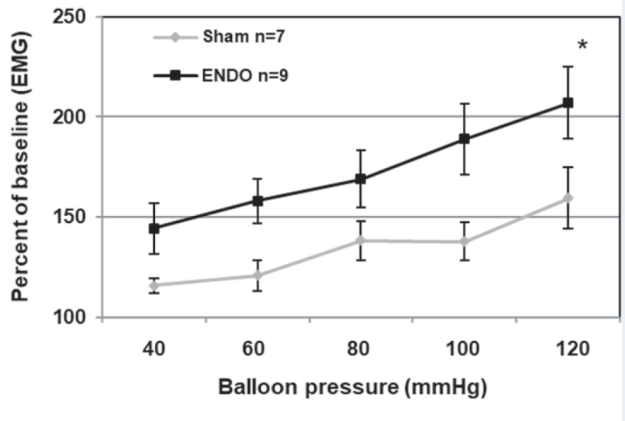

Figure 7 Endometriotic-like lesions significantly increases vaginal sensitivity in an experimental mouse model. The visceromotor response (VMR) was measured by recording the EMG activity of the abdominal musculature during graded balloon distension of the vagina (VBD). Mice with experimentally-induced endometriosis (ENDO) had a significantly higher VMR during VBD than sham-surgery mice (Sham) over the entire distension series ($P < 0.05$), as well as at 100 and 120 mm Hg intra-balloon pressures. $*P < 0.05$ vs Sham; two-way repeated-measures ANOVA and Bonferroni's post hoc test. Data are displayed as mean ± SEM.

and that of the literature clearly indicate an association between RPLP1 over-expression and pathologic levels of cell proliferation, a mechanism to tightly control protein expression seems appropriate. The observations of robust transcript expression, but low protein expression levels in eutopic cells may suggest post-transcriptional regulation of RPLP1. However, to the best of our knowledge, potential mechanisms of post-transcriptional regulation for RPLP1 expression remain to be determined, although microRNAs (miRNAs) may be one plausible mechanism for post-transcriptional regulation. MIR196A2 genetic variation promotes endometriosis development and progression through up-regulation of ribosome biogenesis (Chang *et al*., 2016), but a role for miR-196a2 in the specific regulation of RPLP1 remains to be determined. Future efforts will be dedicated to examining the role of miRNAs in post-transcriptional regulation of RPLP1 as well as developing an experimental mouse model of endometriosis in which we can selectively induce and inhibit RPLP1 expression to evaluate the necessity of this protein for lesion survival *in vivo*.

In summary, the ribosomal protein, RPLP1 is over-expressed in human endometriotic lesion epithelium where it is associated with cell survival/proliferation and this pattern of expression can be recapitulated in an experimental mouse model of endometriosis. *In vitro* studies demonstrate an essential role for RPLP1 in cell survival and the expression of RPLP1 may be mediated through c-Myc. Furthering our understanding of the mechanisms for RPLP1 over-expression, and its function in endometriotic lesion tissue may lead to the development of novel therapeutics for endometriosis treatment.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

Authors' roles

Z.A. performed and designed the experiments, analysed the data, performed the statistical analysis and wrote and edited the manuscript. A.G. performed the experiments and edited the manuscript. T.F, R.F and K.S. oversaw collection of and provided human specimens and wrote and edited the manuscript. W.C. identified and provided pathology specimens and edited the manuscript. X.Y. and J.A.C. performed the vaginal balloon distention and edited the manuscript. W.B.N. conceived the ideas, designed the experiments, performed the experiments, performed the statistical analysis and edited the manuscript.

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Conflict of interest

None declared.

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