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Epithelial-to-mesenchymal transition contributes to the downregulation of progesterone receptor expression in endometriosis lesions

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ABSTRACT

Endometriosis is a common, estrogen-dependent disease, in which endometrial tissue grows in the peritoneal cavity. These lesions often express low levels of progesterone receptors (PR), which potentially play an important role in the insufficient response to progestin treatment. Here, we uncover an interconnection between the downregulated PR expression and the epithelial-to-mesenchymal transition (EMT) in endometriotic lesions. The majority of ectopic epithelial glands (93.1 %, n = 67/72) display heterogeneous states of EMT by immunohistochemistry staining. Interestingly, low PR expression associated with high N-cadherin expression, a hallmark of EMT. In order to gain mechanistic insights, we performed *in vitro* functional assays with the endometriotic epithelial cell lines EM'osis and 12Z. TGF-β-induced EMT, marked by elevations of CDH2 and SNAI1/2, led to a significant downregulation of PR gene expression in both cell lines. In contrast, silencing of SNAI1 in EM'osis and of SNAI1 plus SNAI2 in 12Z elevated PR gene expression significantly. We found that not only *in vitro*, but also in the epithelial component of endometriotic lesions strong expression of SNAI1/2 concurred with weak expression of PR. In summary, these results suggested the negative correlation association of the heterogeneous states of EMT and suppressed PR expression in endometriotic lesions. Our functional assays indicate that EMT contributes to the downregulation of PR expression *via* the upregulation of EMT-TFs, like SNAI1 and SNAI2, which may ultimately lead to progesterone resistance.

1. Introduction

Endometriosis is a common gynecological disease affecting around 6–10 % of women of reproductive age, causing pelvic pain and infertility [1]. Characterized by the presence of endometrial epithelial and stroma cells outside the uterine cavity, the endometriosis lesion still depends on estrogen to grow [2]. Consequently, the basic rationale of the current medical therapies for endometriosis is to reduce estrogen level.

Progestins, known for their anti-estrogenic effects resulting in endometrial decidualization, and inhibit the surges of gonadotropin-releasing hormone / luteinizing hormone [3,4] and thereby block the ovulation. They also locally reduce estrogenic effects by stimulating the transcription of the enzyme 17β-HSD2 [5], which catalyzes the conversion of the highly active estrogen estradiol (E2) to less potent estrone. Thus, progestins can suppress the endometrial proliferation and induce apoptosis, consequently, impeding the proliferation of the endometriosis lesions. However, the treatment failure for endometriosis patients under progestin-based therapy is increasingly noticed, potentially involving an attenuated response to progestin treatments termed progesterone resistance [6,7]. Progestins exert their effects by binding to progesterone receptors (PR). Suppressed PR expression has been preferentially reported in ectopic lesions [8,9]. Several reports indicated that PR reduction could impede the progestin therapy response [6,10,11]. However, the mechanisms leading to the reduced PR expression in endometriosis remain largely unknown. Comprehensive studies and reviews suggested that the downregulation of PR may be a consequence of the exposure to an altered peritoneal cavity microenvironment [7,12,13]. It is important to understand the specific mechanisms which may

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cause loss of PR expression and whether it contributes to the resistance to progestin therapy.

Epithelial-mesenchymal transition (EMT) is a biological process during which epithelial cells lose the epithelial features and instead gain properties of mesenchymal cells. The hallmark of EMT is the so-called "Cadherin switch", marked by downregulation of E-cadherin and upregulation of N-cadherin [14–16]. E-cadherin is the epithelial marker, which supports columnar morphology, bud outgrowth and luminal structure [15,16]. N-cadherin is the mesenchymal marker, which was reported to promote aggregation and collective cell migration that facilitates invasion and metastasis [14]. A number of studies reported EMT-inducing transcription factors (EMT-TFs) to promote the EMT process through various signaling cascades [17–19]. Examples for such EMT-TFs are Snail family transcriptional repressor 1 (SNAI1), Snail family transcriptional repressor 2 (SNAI2, also known as slug), and Zinc Finger E-Box Binding Homeobox 1 (ZEB1) [19,20]. New insights encourage the re-investigation of these tissue-specific EMT-TFs and their pleiotropic roles in physiological and pathological conditions [18,21]. Particularly in endometriosis, EMT related processes were significantly upregulated in ectopic endometrial lesions compared to eutopic endometrium [22,23]. Therefore, EMT might participate in the pathogenesis of endometriosis [24]. EMT can be induced by various growth and differentiation factors. Among those, TGF-β is recognized as a major inducer of EMT, which increases the transcription factors SNAI1/2 and ZEB1/2 through a well-orchestrated transcription program [25,26]. Interestingly, an increased TGF-β concentrations have been frequently observed in endometriosis patients in peritoneum and peritoneal fluid surrounding endometriosis tissues [27,28].

To our knowledge, there are no studies that investigated EMT as the potential cause for the downregulation of progesterone receptors in endometriosis lesions. Hence, the purpose of this study was to investigate the association between PR expression and EMT in endometriosis and to explore the potential mechanisms of downregulation of PR in ectopic endometrial cells.

2. Materials and methods

2.1. Patient tissue handling

All endometriosis ectopic lesions have been collected following a protocol approved by the local ethical committee. The lesions were categorized as superficial peritoneal endometriosis (SUP), endometrioma (OMA) or deeply infiltrating endometriosis (DIE). The diagnosis of endometriosis was confirmed by pathologists. Menstrual cycle, potential hormonal treatment, endometriosis subtypes and number of identified glands were summarized in the supplementary Table 2. Samples fixed with formalin were stored in 70 % ethanol at 4 °C before proceeding to paraffin embedding.

2.2. Immunohistochemistry (IHC) and immunofluorescence (IF)

Paraffin embedded tissues serially dissected into 4 μm sections. The antibodies are listed in supplementary Table 1.

IHC was performed as previously described by Flores et al. [11]. After antigen retrieval at 95 °C in citrate buffer (pH = 6) for 8 min, paraffin sections were cooled to room temperature (RT) and washed 3 times in Tris-buffered saline (TBS). Each section was incubated in hydrogen peroxide solution (3 %) for 10 min. After washing 3 times in TBS-0.25 %Tween (TBST), blocking buffer (X0909, DAKO, Denmark) was applied to sections for 1 h. Tissue sections were incubated with primary antibodies for N-cadherin, E-cadherin, and progesterone receptors (PR) overnight at 4 °C. Sections were subsequently incubated with biotinylated secondary antibodies at RT for 1 h the next day. The sections were incubated in diaminobenzidine (DAB, D3939, Sigma-Aldrich, USA) for 1−4 min following 30 min streptavidin-horseradish peroxidase (ab7403, Abcam, USA) incubation and removal. Hematoxylin was applied as counterstaining.

For IF, the tissue sections were at 95 °C in citrate buffer (pH = 6) for 15 min for antigen retrieval. Then they were incubated with the primary antibodies overnight at 4 °C and the fluorophore-conjugated secondary antibodies were incubated at RT for 1 h. The cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, D9564, Sigma-Aldrich, USA).

As the negative controls, ectopic tissue samples were incubated with blocking buffer with 3 % goat serum instead of primary antibodies.

Panoramic scanner (250 Flash II, 3DHISTECH) was used for capturing the pictures from IF and IHC. QuPath (version 2.2) was used for detecting the positive and negative cells according to the uniformed thresholds, and for capturing the fluorescence intensities. All of the ectopic epithelial glands were identified and the percentage of positive cells per gland was calculated accordingly.

2.3. Cell culture and treatment

12Z and EM'osis cell lines were both obtained in January of 2013 as the generous gifts from collaborating labs [29,30]. Both cell lines were passaged in IMDM supplemented with 10 % FCS. 12Z at passage 50–51 of and EM'osis at passage 14–15 were used in our experiments. These cells were negative for mycoplasma infection (MycoAlert, Lonza, Switzerland). Transforming growth factor beta1 (TGF-β1, AF-100-21C, Peprotech) was prepared in PBS and SB431542 (1614, TOCRIS), a selective inhibitor of TGF-β1 receptor in DMSO. Cells were cultured in 6-well plate in phenol red-free IMDM supplemented with 5 % charcoal-stripped FBS (A33821-1, Gibco) at the density of 2×10^5 cells per well for 12Z and EM'osis until 70–80 % confluence. To determine the IC_{50} of TGF-β1, cells were treated in triplicates with doses ranging from 0.125 to 1 ng/mL. The involvement of TGF-β1 receptor was assessed with a co-treatment of TGF-β1 (0.5−1 ng/mL) and SB431542 (100 nM). Control groups were treated with an equivalent volume of vehicles. The cells were harvested after 24 h treatment by trypsinization and RNA isolated as described below. All cell cultures were maintained at 37 °C in a 5 % CO_2 atmosphere.

2.4. Quantitative real-time-polymerase chain reaction (qRT-PCR)

RNA extraction was performed with the ReliaPrep™ RNA Miniprep Systems (Z6011, Promega, USA) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed in a final volume of 20 μl with RNase inhibitor (N261A, Promega, USA), reverse transcriptase enzyme (M1708, Promega, USA), nucleotide mix (U1518, Promega, USA), and random primers (C118A, Promega, USA). The resulting cDNA was diluted 1:10. Real-time quantitative polymerase chain reaction (qRT-PCR) using Taqman Fast advanced Master Mix (4444556 Thermofisher, USA) and TaqMan® gene expression arrays for PGR (gene encoding PR, Hs01556702 m1), CDH2 (gene encoding N-cadherin, Hs00983056 m1), SNAI1 (Hs00195591 m1), SNAI2 (Hs0000161904 m1), and ZEB1 (Hs00232783 m1). The qRT-PCR was performed on an Applied Biosystems 7500 fast instrument (Thermofisher, USA), with the following conditions: 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 seconds and 60 °C for 10 s. Multiple reference genes [18S rRNA (Hs99999901_s1), GAPDH (Hs00266705_g1), beta-actin(Hs01060665_g1), YWHAZ (Hs 03044281_g1), UBC (Hs00824723_m1)] were determined the M value of geNorm by Qbase plus software (Biogazelle, Belgium) to compare their stability across the cell lines with/without TGF-β1 treatments. Considering the expression level, YWHAZ, one of the most stable reference genes was employed for normalization among the employed cell types and conditions (supplementary Fig. 1). The comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method was used to assess changes in gene expression.

2.5. Knockdown with small interfering RNA against SNAI1/2

EM'osis was transfected with 20 nM of siRNA against SNAI1 (s13185, Silencer Select siRNA, Ambion Inc., USA) or scrambled control siRNA (4390844, Silencer Select Negative Control #1 siRNA, Ambion Inc., USA). 12Z cells were transfected with 20 nM of a pool of equal proportion of 3 siRNA against SNAI1 (SR304489, Human siRNA Oligo Duplex, Locus ID 6615, Origene, USA), as well as SNAI2 (Silencer siRNA ID 106954, Ambion Inc., USA) or scrambled control from the same set of si-SNAI1 (SR304489, Human siRNA Oligo Duplex, Locus ID 6615, Origene USA). One µl lipofectamine RNAiMAX as transfection reagent (13778075, Thermofisher, USA) was used for one well of 24-well plate under 80 % confluent cells, according to vendor's instructions. RNA was isolated 48 h after transfection.

2.6. Statistical analysis

All statistical analyses were performed in Graphpad Prism version 8.0. Mann-Whitney test was used when the data were not normally distributed, i. e. to compare PR expression between N-cadherin positive/negative groups. One-way ANOVA was used to identify the difference of gene expression between different treatment groups in the *in vitro* models. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Various EMT states can be detected within ectopic lesions

The protein expressions of E-cadherin and N-cadherin in ectopic epithelial cells was quantified by IHC in three subtypes of endometriosis: endometrioma (n = 4), superficial endometriosis (n = 3) and deeply infiltrating endometriosis (n = 4). From these 11 cases a total of 72 epithelial glands were characterized and their profiles are summarized in supplementary Table 2. Interestingly, most of the analyzed ectopic glands displayed various states of EMT, characterized by either reduced E-cadherin and/or increased N-cadherin expression (Fig. 1). Only a small fraction of the glands (5/72, 6.9 %) contained exclusively differentiated epithelial cells (100 % E-cadherin and 0 % N-cadherin positive cells) (Fig. 1B).

3.2. High N-cadherin at protein expression is concurrent with low PR protein expression at in ectopic lesions

IHC staining of serial sections revealed a rather low and heterogeneous protein expression of PR with a median of 10.5 % PR positive epithelial cells per gland. Due to the impact of hormone on PR expression, only the cases without hormonal treatment were considered in further analysis. Within this population (non-hormone users, n = 6; glands, n = 50, Supplementary Table 2), we observed that PR expression was significantly lower in N-cadherin positive epithelial glands compared to that in N-cadherin negative epithelial glands (median 0.00 *vs* 12.09, $P = 0.028$, Fig. 2A). This negative association is illustrated in the representative immunostaining shown in Fig. 2B and C, where PR expression was stronger in the epithelial cells with lower N-cadherin expression. Immunofluorescence staining of ectopic lesions confirms that the expression of N-cadherin and PR is distributed in a mutually exclusive manner in ectopic glands (Fig. 2D–G).

3.3. EMT inducing transcription factors inhibit PGR gene expression in vitro

We next investigated how the induced EMT alters the PGR gene expression. The TGF-β pathway plays a crucial role in the induction of EMT in endometriosis and other model systems [24,25]. When applied to ectopic epithelial derived cell lines (12Z and EM'osis), TGF-β1 indeed increased the expression of CDH2 and SNAI1, both hallmarks for EMT, in a dose depend manner (Fig. 3A and B). In EM'osis cells, CDH2 expression was only minimally altered, most likely due to its mesenchymal status characterized by a high expression level of CDH2 and its typical cell morphology. In line with the inverse correlation reported in ectopic lesion, PGR gene expression is decreased following TGF-β1 induced EMT. The downregulation of PGR under 24 h TGF-β1 treatment is statistically significant in EM'osis and 12Z cells ($P < 0.05$). As expected, TGF-β1 receptor inhibitor abrogated the TGF-β1 induced

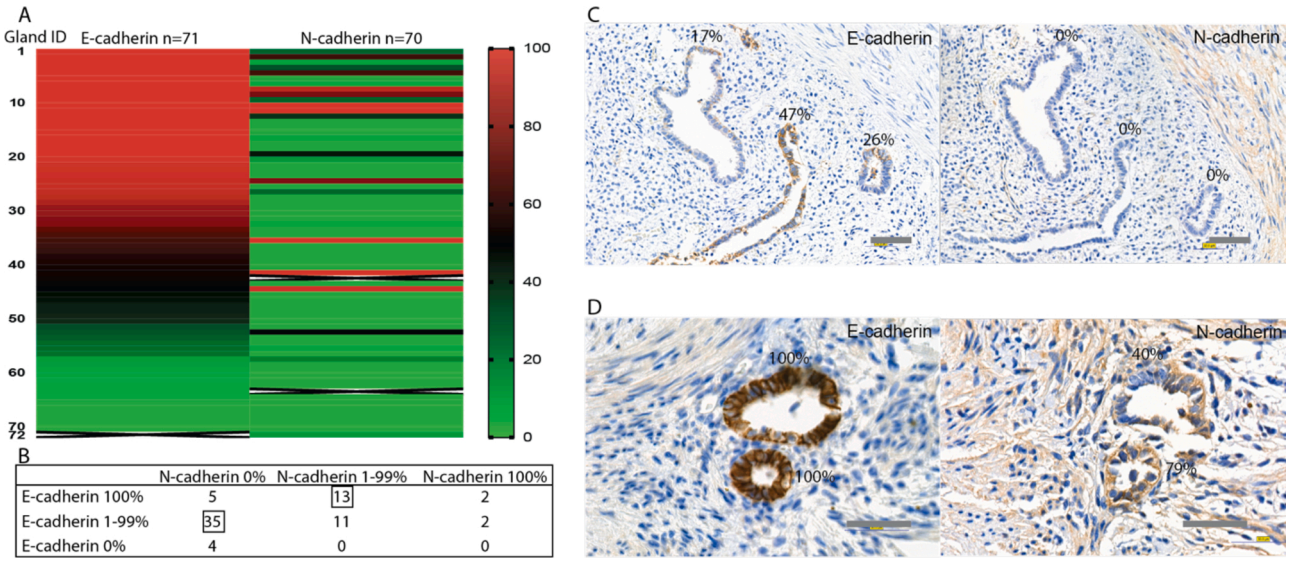

Fig. 1. Ectopic lesions reveal heterogeneous EMT states (A) A heatmap representing the gradual reduction of E-cadherin (left column) in ectopic glands from non-hormone users (Patients, n = 6; SUP glands, n = 3, OMA glands, n = 9 and DIE glands, n = 38) and hormone users (Patients, n = 5; SUP glands, n = 1, OMA glands, n = 2 and DIE glands, n = 19) and concurrently with, or without, N-cadherin expression (right column). The percentage of cells positive for E-cadherin or N-cadherin per glands is illustrated by the color code shown on the right side. The numbers in the left panel represent the identification for each single gland (n = 72). (B) E-cadherin and N-cadherin expression per ectopic gland as unit were classified into 100 %, 1–99 %, and 0 %. Seventy-two glands were categorized accordingly. (C, D) Immunohistochemical stains for E-cadherin and N-cadherin in serial sections of ectopic tissues representing the two main subgroups highlighted in B. Percentages of positive cells per single gland were marked respectively (magnification 600x for C, 1200x for D). The grey scale bars represent 50 µm.

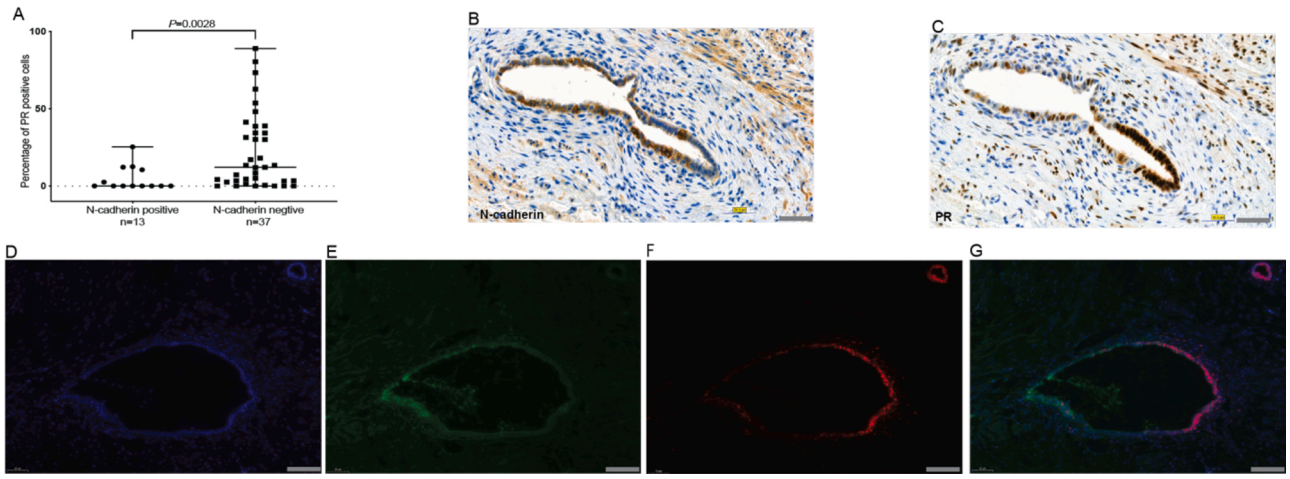

Fig. 2. N-cadherin expression negatively correlates with PR expression (A) PR protein expression level was lower in N-cadherin positive glands than in N-cadherin negative glands amongst the non-hormone users (Patients n = 6; SUP glands n = 3; OMA glands n = 9; and DIE glands n = 38) ($P = 0.0028$, by Mann-Whitney test, data are presented as median with range). (B, C) Representative immunochemistry staining illustrated the mutually exclusive N-cadherin and PR expression in ectopic glands (magnification 600x). (D–G) Immunofluorescent double staining illustrated the differential expression of N-cadherin (in green) and PR (in red) in ectopic glands (magnification 400x). (B-G) The grey scale bars represent 50 μm.

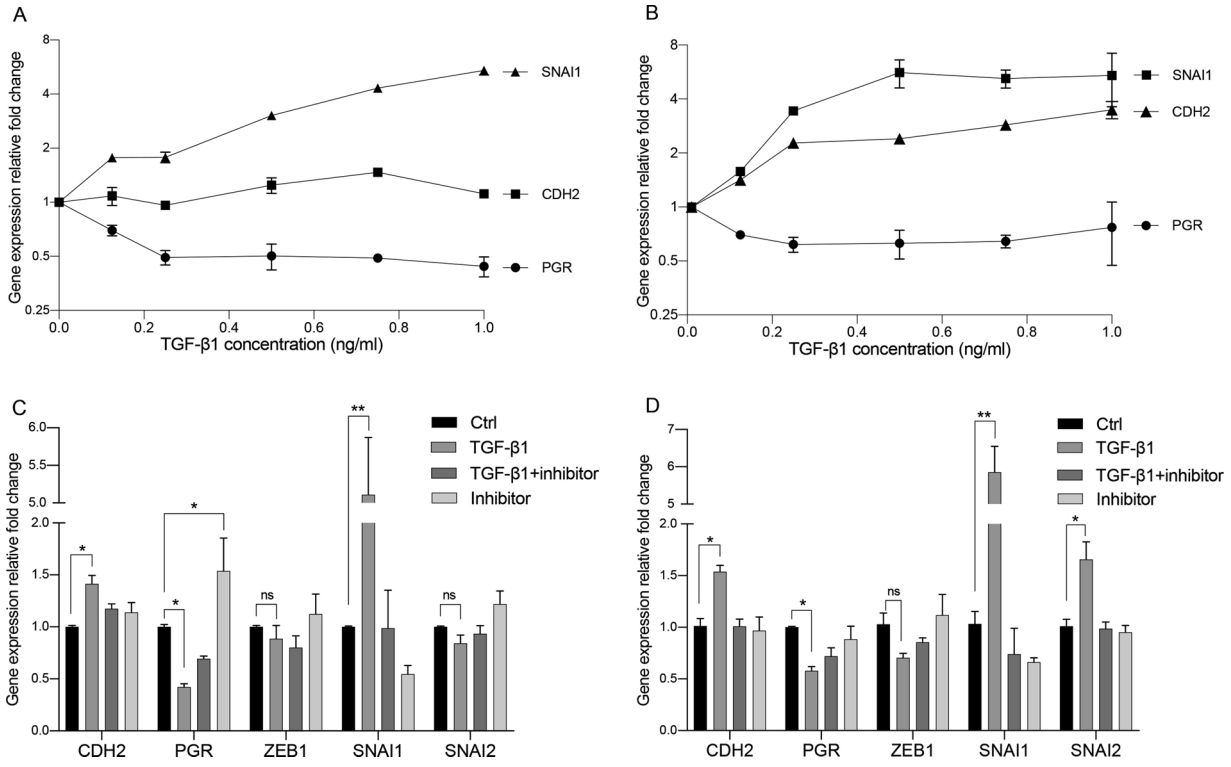

Fig. 3. TGF-β1 dose-dependent changes of gene expression in endometriotic epithelial cell lines (A) EM'osis cells and (B) 12Z cells were treated in triplicates with TGF-β1 (0.125, 0.25, 0.5, 0.75, 1 ng/mL) for 24 h. Values are presented by mean ± SEM of one representative experiment. (C) Expression of CDH2, PGR, and EMT related TFs (ZEB1, SNAI1 and SNAI2) in EM'osis cells treated in four independent experiments with TGF-β1 (1 ng/mL) and TGF-β1 inhibitor (100 nM) for 24 h. Control group were under the equivalent volume of vehicles. (D) Expression of CDH2, PGR, and EMT related TFs (ZEB1, SNAI1 and SNAI2) in 12Z cells treated in four independent experiments with TGF-β1 (0.5 ng/mL) and TGF-β1 inhibitor (100 nM) for 24 h. Values are presented as mean ± SEM and P values were calculated by one-way ANOVA test. * $P < 0.05$, ** $P < 0.001$, "ns" stands for non-significant.

downregulation of PGR, and even upregulated PGR significantly in EM'osis cells ($P < 0.001$) (Fig. 3C and D). SNAI1 was increased by TGF-β1 treatment significantly in both cell lines ($P < 0.001$). In addition, SNAI2 upregulation under TGF-β1 treatment was only observed in the 12Z cell line ($P < 0.05$). ZEB1, another core EMT promoting TF, did not increase under TGF-β1 treatment in either cell line (Fig. 3C and D).

3.4. EMT related PGR repression is SNAI1/2 dependent

12Z and EM'osis cells were transfected with siRNA targeting SNAI1 and SNAI2 to evaluate the role of these TFs in the EMT dependent suppression of PGR. In EM'osis cells, knockdown of SNAI1 reaching 58.7 % reduction at 48 h after transfection, resulted in a significant increase of PGR expression (1.26 fold, $P = 0.028$, Fig. 4A). In 12Z cells, a

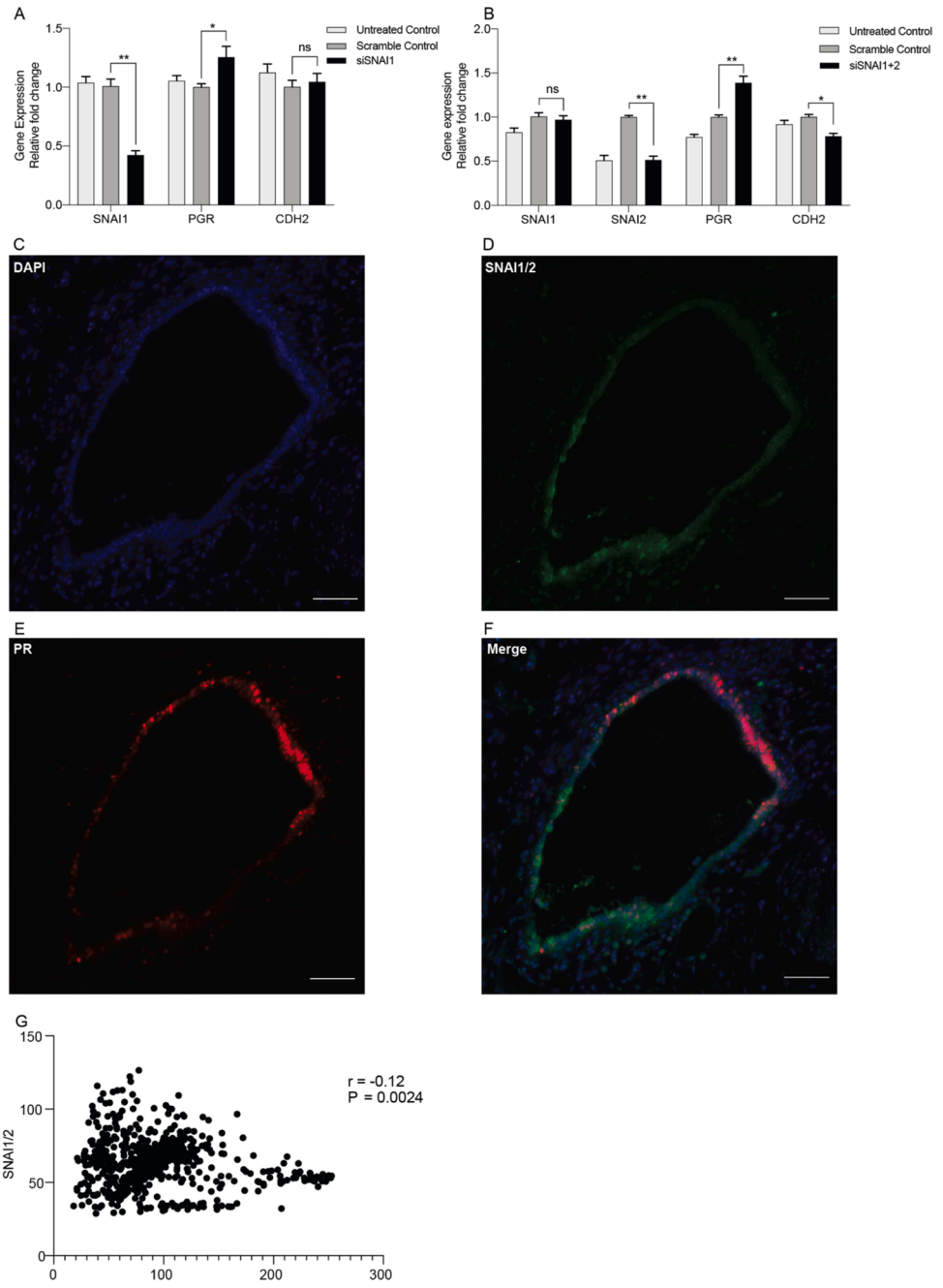

Fig. 4. EMT related PR repression is SNAI-dependent (A) Silencing SNAI1 in EM'osis cells was done by transfection of siRNA against SNAI1 (20 nM) in triplicates in two independent experiments. Transfections with the same amount of scrambled siRNA, and untreated cells were used as controls. (B) Silencing SNAI1/2 in 12Z cells was performed in triplicates in two independent experiments, using the pooled SNAI1/2 with 2:1 ratio of si-SNAI1 and si-SNAI2. Controls were the same as for EM'osis cells. (A, B) Values are presented as mean ± SEM and *P* values were calculated by one-way ANOVA test. * $P < 0.05$, ** $P < 0.001$, "ns" stands for non-significant. (C–F) Representative staining of SNAI1/2 (green) was shown in part of epithelial component, and the representative staining of PR (red) in ectopic epithelial glands (magnification 400x). The grey scale bars represent 50 μm. (G) Correlation analysis on the intensity of fluorescence for SNAI1/2 and PR in epithelial cells. DIE lesions were co-stained with PR and SNAI1/2, and the intensities of fluorescence were quantified by Qupath in each epithelial cells. P value was calculated by Pearson r.

combined knockdown of SNAI1 and SNAI2 was used, since both factors increased after TGF-β1 treatment (Fig. 4B). The use of this siRNA mixture (siRNA against SNAI1 and siRNA against SNAI2) was also supported by the fact that a compensatory mechanism apparently takes place when SNAI1 or 2 are downregulated independently (supplementary Fig. 3). Indeed, SNAI2 downregulation led surprisingly to an increase of SNAI1 mRNA expression (and *vice versa*) in 12Z (supplementary Fig. 3). Hence, the siRNA mixture represented the most efficient way to keep both SNAI1 and 2 at low levels. In fact, in 12Z, the combined knockdown decreased by 48.6 % the mRNA level of SNAI2 while preventing the expected increase of SNAI1 (siSNAI1 + 2, Fig. 4B). All in all, SNAI silencing in 12Z led to a significant elevation of PGR expression (1.39 fold, $P = 0.0002$, Fig. 4B), as found in EM'osis cells.

Next, we performed an *in silico* analysis of the PGR to identify a possible cause for our observed downregulation of the PGR at mRNA level. This analysis revealed 8 potential cis–acting elements (CANNTG, E-box) for both SNAI1 and SNAI2, listed in supplementary Table 3. Altogether, these *in vitro* experiments demonstrated that TGF-β1 induced EMT, characterized by an increase of mesenchymal markers (CDH2 and SNAI1/2), importantly, is accompanied by SNAI dependent PGR downregulation.

3.5. *SNAI1/2 and PR expressions are negatively correlated in DIE ectopic lesions*

We further verified the protein expression level of SNAI1/2 in ectopic lesions by IF (Fig. 4C–F). We found that the high protein expression of SNAI1/2 in the ectopic epithelial cells associates with the low protein expression of PR, while the low protein expression of SNAI1/2 was concurrent with the high protein expression of PR.

And we further used Qupath to detect the intensity of immunofluorescence signals of SNAI1/2 and PR from 647 epithelial cells from 6

ectopic glands from 3 DIE lesions. The negative correlation of protein expressions of SNAI1/2 and PR was indicated by the correlation analysis ($r = -0.12$, $P = 0.0024$). Hence, our study confirmed SNAI1/2 play an important role to regulate PR not only limited *in vitro* but also *in vivo* in patients with DIE.

4. Discussion

Our study revealed a potential negative association between PR and N-cadherin at the protein level. By employing endometriotic cell lines, we verified that TGF-β1-induced EMT downregulated PGR gene expression. Furthermore, we found that SNAI1/2, two core EMT-TFs, play the important roles in regulation of PGR. Most importantly, the results were supported *in situ*, showing a negative correlation between SNAI1/2 and PR protein expression in endometriosis lesions.

We observed that PR was absent in the individual endometriotic epithelial cells, which was widely mentioned previously by multiple studies [8,9,11,31]. Using the open-source software QuPath that automatically quantified the staining intensity of individual cells from the immunohistochemistry, we calculated the percentage of positive cells per epithelial gland. A representative example of the QuPath analysis is shown in supplementary Fig. 2. Interestingly and importantly, the expression of PR is heterogeneous intrasubject. This finding is consistent with a recent report [11]. Due to the limited sample size, we refrained from performing a subgroup analysis on PR expression across different endometriosis subtypes.

Meanwhile, immunohistochemistry revealed that most of the ectopic glands lose E-cadherin expression (n = 48/72) and partially expressing N-cadherin (n = 18/72). In agreement with a previous report, these partial EMT states are present in most ectopic epithelial glands, being likely triggered by the altered pelvic microenvironment [32]. We further found out the PR expression was significantly lower in N-cadherin positive epithelial glands compared to that in N-cadherin negative epithelial glands (Fig. 2A). The low PR expression is concurrent with high N-cadherin expression, illustrated in immunofluorescence co-staining of PR and N-cadherin (Fig. 2D–G). This is in line with a previous report from Liu et al., who found that PR-B, a dominant isoform of PR in endometrium, negatively correlated with EMT markers in adenomyosis lesions [33].

Our hypothesis that EMT contributes to PR downregulation in endometriosis was inspired by a report on endometrial cancer, which explored the reversion of progesterone resistance by suppressing EMT in Ishikawa cells [34]. However, the alterations of PR expression under EMT and reversed EMT were not reported in that study. Hence, to the best of our knowledge, this is the first study reporting a contribution of EMT in the regulation of PR expression in endometriosis. Our study not only revealed this regulation in another system, but also identifies underlying mechanisms.

We verified the regulation of PGR *via* a TGF-β1 induced EMT in endometriosis derived cell lines. In the endometriotic epithelial cells, 12Z and EM'osis, TGF-β1-induced EMT altered PGR expression in a dose dependent manner. The same effect was also observed in Ishikawa cells (data not shown). The downregulation of PGR by TGF-β1 treatment was previously reported in two independent studies [35,36]. These studies were limited to stromal cells from endometriosis. And importantly, the link between TGF-β1 and EMT as well as EMT related pathways has not been suggested in either study.

TGF-β1-induced EMT was characterized by the increase of the mesenchymal marker CDH2 and the EMT-TFs SNAI1/2 in 12Z and EM'osis and ZEB1 in Ishikawa cells (data not shown). We identified SNAI1 and SNAI2 as the most affected TFs during TGF-β1-induced EMT in endometriotic cells *in vitro*. One previous study [37] reported that both TGF-β1 and SNAI1 were expressed strongly in ectopic epithelial cells compared to eutopic endometrium, supporting our results from the *in vitro* model. Strikingly, we noticed that different TFs were activated in a cell-specific manner in our *in vitro* model of TGF-β1-induced EMT, which is consistent with prior knowledge of the tissue-specific activities of these TFs [18,38]. Interestingly, in 12Z cells SNAI1 and SNAI2 compensated each other, which means knockdown SNAI2 leads to a sharp increase of SNAI1, and *vice versa* as shown in supplementary Fig. 3. This reciprocal expression of SNAI1 and SNAI2 was previously found in oral cancer cells [39]. Furthermore, SNAI1 and SNAI2 recognized as transcriptional repressors, share a common organization, and specifically both contain SNAG which is important for the repressor capacity of SNAI factors [19]. Therefore, even though SNAI1 is not downregulated in knock-down experiment in 12Z cells, the efficient downregulation of SNAI2 still leads to an elevation of PGR.

In line with other reports [40–42], knockdown of SNAI1 by siRNA in EM'osis cell lines was only moderately efficient, but nevertheless resulted in a consistent upregulation of PGR. These results suggest that SNA1/2 mediate EMT, which induces PGR repression either by acting directly on the promoter or through an indirect mechanism. SNAI1/2 was previously reported to repress gene expression by binding to its motif constituting a subset of E-box [43]. Interestingly, each PR promoter contains four E-box motifs. Thus, it is plausible to postulate that SNAI1/2 suppresses PGR transcription by binding to the gene promoters.

As the difficulty of setting up culture condition for primary epithelial cells, we employed immortalized endometriotic epithelial cell lines, 12Z and EM'osis. However, we observed both cell lines have lost their epithelial phenotype in culture, especially EM'osis cells. They showed a typical mesenchymal phenotype under the microscope, recognizable by a spindle like shape. The protein expression of E-cadherin was not detectable, instead N-cadherin was strongly expressed as previously reported for 12Z [30]. PR expressions at protein level in both cell lines are also not detectable, which are possibly natural results due to the mesenchymal status. In another hand, our study conclusions have been therefore restricted to the transcriptomic level. However, the negative correlation between SNAI1/2 and PR could be confirmed by immunofluorescent double staining in patient samples. This is in line with the strong protein expression of SNAI in ectopic lesions [37,44,45].

5. Conclusions and outlook

Our study indicates that the clinically relevant, but so far unrevealed downregulation of PR could be the consequence of the heterogeneous EMT states in endometriosis lesions. Since the altered PR expression in ectopic lesions might indicate the attenuated response to progestin-based therapy, we propose that EMT participates in progestin treatment resistance and/or failure. In our study, SNAI1/2 silencing concurrently reversed EMT and increased PGR expression. This highlights the potential role of EMT related factors in the downregulation of PGR. Ultimately, it will be interesting to explore the effect of relevant compounds, such as SNAI inhibitors, to reverse EMT in endometriosis. This might lead to the establishment of a novel adjuvant therapy for endometriosis patients with progesterone resistance.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2021.105943.

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Further reading

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