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γ-Secretase inhibition affects viability, apoptosis, and the stem cell phenotype of endometriotic cells

Laura Ramirez Williams[1] | Kathrin Brüggemann[1] | Marina Hubert[1] | Nurjannah Achmad[1] | Ludwig Kiesel[1] | Sebastian D. Schäfer[1] | Burkhard Greve[2] | Martin Götte[1]

[1]Department of Gynecology and Obstetrics, Münster University Hospital, Albert-Schweitzer-Campus 1, Münster, Germany

[2]Department of Radiotherapy-Radiooncology, Münster University Hospital, Albert-Schweitzer-Campus 1, Münster, Germany

Correspondence
Martin Götte, Department of Gynecology and Obstetrics, Münster University Hospital, Albert-Schweitzer-Campus 1, D11, 48149 Münster, Germany.
Email: mgotte@uni-muenster.de

Present address
Nurjannah Achmad, University Muhammadiyah of Jakarta, KH Ahmad Dahlan Ciputat, Cirendeu South Jakarta, 15419, Indonesia.

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Abstract

Introduction: Stem cells mediate cyclic regeneration of the endometrium. The up-regulated expression of receptors and modulators of the notch signaling pathway in endometriosis suggests an involvement in the pathogenetic process. Here, we investigated the effects of notch pathway inhibition by a γ-secretase inhibitor (GSI) on stemness-associated properties of the epithelial endometriotic cell line 12Z and of primary endometriotic stroma cells.

Material and methods: 12Z cells and primary endometriotic stroma cells of 7 patients were treated with or without GSI, and analyzed for changes in gene expression by TaqMan low-density arrays, quantitative PCR, and flow cytometry. The functional impact of GSI treatment was studied by MTT assay, cell cycle analysis, colony formation assay, annexin V apoptosis assay, and aldehyde dehydrogenase activity assays.

Results: In 12Z cells, GSI treatment reduced aldehyde dehydrogenase activity and colony formation, and induced a shift to the G2/M phase of the cell cycle. Cell viability was decreased and apoptosis was increased in both cell models. GSI further induced transcriptional downregulation of the stemness-associated factors leukemia inhibitory factor receptor (LIFR), sex-determining region Y (SRY)- box 2, interferon-induced transmembrane protein 1, and hes-related family bHLH transcription factor with YRPW motif 1, in 12Z cells and in primary cell cultures. Downregulation of LIFR expression by GSI was confirmed at the protein level by flow cytometry.

Conclusions: Our in vitro data suggest that application of GSI may be a worthwhile approach in the treatment of endometriosis that warrants further investigation.

KEYWORDS

endometriosis, interferon-induced transmembrane protein 1, leukemia inhibitory factor receptor, Notch, podocalyxin-like, sex-determining region Y (SRY)- box 2, stem cells, therapy

Abbreviations: ALDH, aldehyde dehydrogenase; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; GSI, γ-secretase inhibitor; HEY1, hes-related family bHLH transcription factor with YRPW motif 1; IDO1, indoleamine 2,3-dioxygenase-1; IFITM1, interferon-induced transmembrane protein 1; LIFR, leukemia inhibitory factor receptor; Msi1, Musashi-1; MTT, methylthiazolyldiphenyltetrazolium bromide; PODXL, podocalyxin-like; SOX2, sex-determining region Y (SRY)- box 2.

Laura Ramirez Williams, Kathrin Brüggemann and Marina Hubert are contributed equally. Burkhard Greve and Martin Götte are joint senior investigators of this study.

1 | INTRODUCTION

Between 6% and 10% of women of reproductive age develop endometriosis, an estrogen-dependent chronic inflammatory disease where endometrial-like tissue resides in ectopic sites.[1] Women with endometriosis suffer from pelvic pain, painful periods, pain at sexual intercourse, and subfertility.[1] Treatment options are limited to repeated surgeries or hormonal intervention, which is often associated with adverse effects similar to a premature menopause.[1,2] Different general etiological concepts of endometriosis include retrograde menstruation, coelomic metaplasia, lymphovascular metastasis, or the embryonic rest theory, but the underlying molecular mechanisms remain unclear, impeding the development of targeted therapies.[1,2]

Mechanisms relating to the pathogenesis of endometriosis include altered inflammation and proteolysis, endocrine alterations, as well as epigenetic modifications such as microRNA dysregulation.[2-5] Notably, all classical concepts of endometriosis fit to an altered endometrial stem cell function.[5-7] For instance, the key role of stem cells during development may contribute to the process of coelomic metaplasia. Retrograde menstruation or lymphovascular trafficking of stem cells may induce the establishment of particularly persistent ectopic lesions, related to specific stem cell properties like unlimited proliferation and high developmental plasticity.[5-7] Notch signaling emerges as one particularly relevant stemness-related pathway in this context, as it is more active in deep infiltrating endometriotic lesions of patients than in controls.[6] Moreover, Musashi-1 (Msi1), a stem cell factor and important modulator of notch function, is significantly upregulated in endometriotic tissue.[7] In line with these findings, glandular notch-1 expression is upregulated in the eutopic endometrium of patients suffering from deep infiltrating endometriosis compared with the endometrium of an endometriosis-free in vitro fertilization collective.[8] However, in contrast to this, a study by the Fazleabas group found decreased notch signaling in the eutopic endometrium of women with endometriosis, resulting in impaired decidualization.[9] Notch activation was linked to progesterone resistance in endometriotic lesions,[10] and to angiogenesis in a mouse model.[11]

The notch family consists of 4 transmembrane proteins, which share distinct sequence similarity with epidermal growth factor-1 receptors. Their intracellular domain is proteolytically released upon binding of the integral membrane ligands of the Delta Serrate Lag-2 family (DLL-1,-3,-4 and Jagged-1 and -2). In association with accessory proteins, this domain can act as a transcription factor driving the expression of stemness-related genes, consisting of the transcription factor Hes-1 and the transcriptional repressor Hey-1.[6-11] Numb antagonizes notch signaling by posttranscriptional regulation of Notch, which itself is regulated by Msi1.[7,8,12] Proteolytic cleavage by γ-secretase is a key step in Notch activation, and γ-secretase inhibitors (GSIs) have already entered clinical trials in the context of neurogenerative and malignant diseases.[13] As a result of the endometriotic stem cell concept and the pivotal role of Notch in this context, the application of GSIs may hold promise in the context of endometriosis. In this study, we evaluated the impact of GSI on functional properties and the stem cell phenotype of endometriotic cells in vitro. Moreover, we aimed to identify stemness-related genes in endometriotic cells that are sensitive to GSI treatment, with the perspective of widening the range of potential therapeutic targets for this disease.

Key message

Aberrantly distributed endometrial stem cells may promote endometriosis because of their unlimited proliferative capacity and high developmental plasticity. This preclinical laboratory study shows that γ-secretase inhibition targets this cell population, and may therefore be a worthwhile future therapeutic approach.

2 | MATERIAL AND METHODS

2.1 | Material

Media, fetal calf serum (FCS), and tissue culture supplies were from Gibco BRL/Thermo Fisher (Waltham MA, USA). Unless stated otherwise, all chemicals were from Sigma (Deisenhofen, Germany).

2.2 | Cell culture

12Z is an immortalized epithelial endometriotic human cell line kindly provided by Prof. Anna Starzinski-Powitz, Frankfurt, Germany.[14] Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 1% glutamine, and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. We obtained primary endometriotic stroma cells from biopsies of 7 women with endometriosis who underwent surgical treatment at the Department of Gynecology and Obstetrics of Münster University Hospital between October 2012 and March 2014. The modified American Society for Reproductive Medicine classification was used to assess endometriosis.[1] Patient characteristics are shown in Table 1. Primary cells were isolated as described previously[15] and cultured in DMEM supplemented with 10% FCS, 1% glutamine, and 1% penicillin-streptomycin in a humidified atmosphere of 7.5% CO_2 at 37°C. Cells were either treated with 0.01-1 μmol/L γ-secretase inhibitor I (benzyloxycarbonyl-leucyl-leucyl-norleucinal; Calbiochem/Millipore, Darmstadt, Germany) dissolved in dimethyl sulfoxide (DMSO), or DMSO vehicle control (<1% final concentration).

2.3 | Cell viability assay and cell cycle analysis

Cell viability was determined after treatment with vehicle or GSI for 24 hours by methylthiazolyldiphenyltetrazolium bromide (MTT) assay as described previously.[5] Cell cycle analysis was done 24 hours after treatment with 1 μmol/L GSI or DMSO vehicle. Cells were trypsinized and 100 μL of cell suspension was adjusted to 1 mL

TABLE 1 Characteristics of endometriotic biopsies

Number and laboratory code	Patient age at biopsy	Endometriosis manifestations and rASRM score	Location of biopsy
#1 - OP 10	19	rASRM score II Periurethral, pelvic wall Septum rectovaginale Vagina	Pelvic wall
#2 - OP 5	35	rASRM score III Septum rectovaginale Deep infiltrating: retrocervix Vagina Uterus Rectum	Vagina
#3 - OP 26	35	rASRM score III Gut Left ovary Septum rectovaginale Pouch of Douglas Vagina Peritoneal (pelvic) Deep infiltrating: lig. sacrouterinae, pelvic wall	Pelvic wall
#4 - OP 18	22	rASRM score III Uterine wall Pelvic wall Rectum Deep infiltrating: plica vesicouterina, ligamentum sacrouterinum	Urinary bladder
#5 - OP 28	39	rASRM score III Disseminated with deep infiltration: peritoneum/urinary bladder Disseminated: pelvic walls close to urethra	Peritoneum
#6 - OP 8	33	rASRM score II Pelvic wall Recessus ovarii links Plica vesicouterina Ligg. sacrouterinae Pouch of Douglas	Pouch of Douglas/ ligamentum sacrouterinum
#7 - OP 23	45	rASRM score IV Urinary bladder peritoneum vagina Pouch of Douglas Deep infiltrating: rectum, adnexe Disseminated: peritoneum	Periurethral

rASRM, revised American Society for Reproductive Medicine.

with 4,6-diamidino-2-phenylindole (DAPI, CyStain; Sysmex/Partec, Görlitz, Germany). After 5 minutes of incubation at room temperature, cells were analyzed by flow cytometry on a CyFlow Space (Sysmex/Partec). Excitation was carried out with a 375-nm UV laser and fluorescence emission was measured at 455 nm in FL4. For cell cycle distribution analysis FLOMAX software was used (Quantum Analysis, Münster, Germany).

2.4 | Colony formation assay

Colony formation was assessed essentially as previously described.[16] Either 1000 (24 hours treatment) or 2000 (10 days treatment) 12Z cells were resuspended in 1 mL of culture medium and plated onto 3.5-cm Petri dishes with a 2.5-mm grid (Nunc, Langenselbold, Germany). Cells were either treated for 24 hours or for 10 days with vehicle (DMSO) or 1 μmol/L GSI and incubated for 10 days in a CO_2 incubator at 37°C. Cell colonies with >50 cells were counted with a microscope (Olympus, Hamburg, Germany).

2.5 | Annexin V assay and flow cytometry

Determination of apoptotic cell death was done following treatment with DMSO vehicle, 0.1 μmol/L GSI, or 0.5 μmol/L GSI. Cells were stained for apoptosis using the annexin V test kit from Becton

Dickinson (Franklin Lakes, NJ, USA) and measured on a flow cytometer (CyFlow Space, Sysmex/Partec). Labeled cells were excited with a 488 nm blue argon laser and signals were collected at 527 nm in FL1 and at 665 nm in FL3. The dotplot histogram of FL1 and FL3 allows differentiation between viable (quadrant Q3) and apoptotic (quadrant Q4) and late apoptotic/necrotic cells (quadrants Q1, Q2).

2.6 | Aldehyde dehydrogenase activity assay

Aldehyde dehydrogenase (ALDH) activity was determined 24 hours after treatment with 1 μmol/L GSI or DMSO vehicle control, exactly as previously described.[12] Flow cytometric analysis on a Cyflow Space cytometer (Sysmex/Partec) was performed using a 488-nm argon laser. Fluorescence emission was measured at 527 nm in FL1. Analysis was confined to living cells by setting a live cell gate in the forward scatter versus side scatter plot. Data are shown as percentage of ALDH-positive cells over the whole cell population.

2.7 | Flow cytometric measurement of LIFR expression

12Z cells treated with 1 μmol/L GSI or DMSO vehicle for 24 hours were harvested by incubation with 2 mmol/L EDTA in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) buffer for <10 minutes at 37°C with gentle agitation. Then, 2×10^6 cells in 100 μL PBS/0.1% bovine serum albumin (PBS/BSA) were incubated with 20 μL mouse anti-human leukemia inhibitory factor receptor (LIFR; AN-E1) PE monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA) or 20 μL Isotype control antibody (BD Pharmingen, BD Biosciences, Frankland Lakes, NJ, USA) for 30 min at room temperature. Subsequently, cells were centrifuged (450 ***g***, 30 seconds) and washed twice with 300 μL PBS/BSA. The cells were resuspended in 1 mL PBS/BSA before analysis on a flow cytometer (CyFlow, Sysmex/Partec). Excitation took place with a 20-mW 488-nm laser and fluorescence emission was measured at 590 nm in FL2.

2.8 | RNA isolation and reverse transcription

The isolation of mRNA from cultured cells was done using the innuPREP RNA Mini Kit (Analytik Jena AG, Jena, Germany) following the manufacturer's instructions. Conversion into complementary DNA (cDNA) was carried out by the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) and addition of random hexamer primers according to the manufacturer's instructions.

2.9 | TaqMan low-density array analysis and quantitative real-time PCR

Expression of stemness-related genes was analyzed in 12Z cells subjected to 1% DMSO (control), or 1 μmol/L GSI or 10 μmol/L GSI, respectively, for 24 hours. Expression was determined using a TaqMan Low-density array (TaqMan® Human Stem Cell Pluripotency Array, ABI/Thermo Fisher, Darmstadt, Germany) according to the manufacturer's instructions. The TaqMan array is based on 384-well microfluidic cards, preloaded with TaqMan gene expression assays. The simultaneous screening of about 100 stemness- and differentiation-related genes including normalizing controls and 6 genes related to undifferentiated cells, 43 genes linked to the maintenance of pluripotency, 33 stemness-related genes, and 50 differentiation markers is possible. Genes of interest from the TaqMan Low-density array data were confirmed in independent biological replicates. Investigation of additional potentially GSI-regulated genes was carried out using predesigned TaqMan assays (all from ABI/Thermo Fisher). Details on intron-exon boundaries, amplicon length, and probe site can be accessed by entering the accession number at https://www.thermofisher.com/order/genome-database/. The following TaqMan assays were used: chorionic gonadotropin subunit β (CGB): Hs00361224_gH, Dead-box helicase 4 (DDX4): Hs00987130_m1, Desmin (DES): Hs00157258_m1, FOXA2: Hs00232764_m1, IFITM 1: Hs00705137_s1, IFITM 2: Hs00829485_s1, IL6ST: Hs00174360_s1, Lefty: Hs00764128_s1, LIFR: Hs00158730_m1, MNX1: Hs00907365_m1; MyoD1: Hs00159528_m1,nanog:Hs02387400_g1,Nestin:Hs00707120_s1, nodal: Hs00415443_m1, Podocalyxin (PODXL): Hs00193638_m1, SOX2: Hs00602736_s1, Sox17: Hs00751752_s1, TERT: Hs00162669_m1, Wnt 1: Hs00240913_m1. Target gene expression was normalized to 18S ribosomal RNA expression (Hs99999901_s1). indoleamine 2,3-dioxygenase-1 (IDO1) expression was evaluated using the primers fwd-5′-CAAAGGTCATGGAGATGTCC-3′ and rev- 5′-CCACCAATAGAGAGACCAGG-3′ at 0.375 μmol/L each with Power SYBR Green PCR mix (Invitrogen/Thermo Fisher, Carlsbad, CA, USA), normalizing to the expression of β-actin (fwd-5′-TCAAGATCATTGCTCCTCCTGAG-3′ and rev-5′-ACATCTGCTGGAAGGTGGACA-3′) (Biolegio BV, Nijmegen, the Netherlands). An ABI PRISM 7300 Sequence Detection System was used for quantitative RT-PCR experiments with default thermal cycling conditions. The evaluation of the TaqMan low-density array was carried out on a 7900 HT fast real-time PCR system (ABI). For relative quantification the comparative cycle threshold method was used.

2.10 | Statistical analyses

Statistical analysis was performed using SigmaStat 3.1 (Systat Software Inc., San Jose, CA, USA) and SPSS 15 software (SPSS, Chicago, IL, USA), using the Mann-Whitney *U*-test. All experiments have been performed on at least 3 biological replicates. A *P*-value of <0.05 was considered statistically significant.

2.11 | Ethical approval

The study was carried out in accordance with the Declaration of Helsinki, and approved by the local ethics commission

(Ethikkommission der Ärztekammer Westfalen-Lippe und der Medizinischen Fakultät der WWU; approval no. 1 IX Greb 1 from 19 September 2001, updated 2012). All participants gave written informed consent.

3 | RESULTS

3.1 | γ-Secretase inhibitor treatment affects viability, apoptosis, and cell cycle progression of endometriotic cells

To study the impact of the notch signaling pathway on endometriotic stem cell properties, we used a GSI. As a model system, we used the immortalized endometriotic cell line 12Z, which was originally isolated from a peritoneal lesion, and has been extensively characterized.[14] Z has been proven to be a useful model for mechanistic studies of cell proliferation, invasive growth, endometrial stem cell properties, and growth of endometrial lesions in vivo.[5,14,17] Quantitative PCR analysis revealed that all relevant components of the notch pathway (Notch 1-4, the transcriptional targets HES1 and HEY1, δ-like ligands and the notch modulators Msi1/2 and numb) were expressed in 12Z cells (see Supplementary material, Figure S1). Treatment of 12Z cells with GSI over a range of 0.01-1 μmol/L GSI resulted in a significant inhibition of cell viability at doses higher than 0.25 μmol/L, as assessed by MTT assay (Figure 1A). We next evaluated the impact of GSI treatment on cell viability in patient-derived primary endometriotic cells. For this purpose, we established stroma cell cultures from ectopic lesions of women with endometriosis (see Table 1 for patient characteristics). Treatment with 0.5 μmol/L GSI significantly reduced primary endometriotic stroma viability by 23% (Figure 1B). We next evaluated the impact of GSI treatment on apoptosis. Annexin V assays revealed a significant doubling of apoptosis rates in 12Z cells compared with basal apoptosis after exposure to 0.5 μmol/L GSI (Figure 1C). Primary endometriotic stroma cells showed substantially higher basal apoptosis rates compared with 12 Z cells (Figure 1D). GSI treatment resulted in a significant 1.4-fold increase in apoptosis rates (Figure 1D). Finally, cell cycle analysis revealed that GSI treatment of 12Z cells had an antiproliferative effect, as demonstrated by a decrease in cells of the S-phase of the cell cycle and a shift to the G2/M phase (Figure 1E).

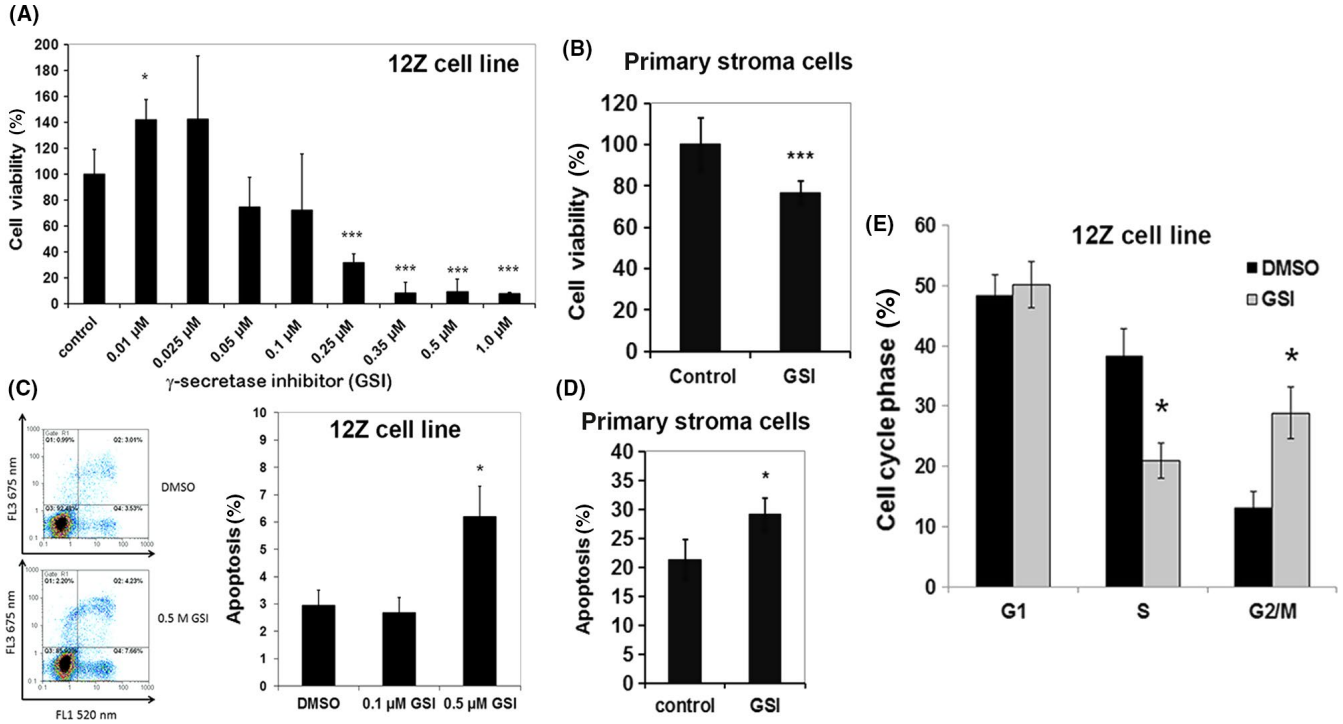

FIGURE 1 γ-Secretase inhibitor (GSI) treatment affects viability, and apoptosis of endometriotic cells (A,B) Viability of the endometriotic cell line 12Z (A) and primary stroma cells isolated from endometriotic lesions (B) is significantly inhibited by GSI treatment (MTT assay). 5000 cells/well in a 96-well plate were cultured for 72 hours in the presence and absence of GSI and subjected to a photometric MTT assay. Error bars = SEM. n ≥ 3. ***P < 0.001, *P < 0.05. (C,D) GSI-treatment induces apoptosis. 12Z cells (C) and primary endometriotic stroma cells (D) were treated with 0.1 μmol/L (12Z) and/or 0.5 μmol/L (12Z and primary cells) GSI for 24 hours and subjected to flow cytometric analysis of apoptosis using fluorescently labeled annexin V. GSI (0.5 μmol/L) significantly increased the percentage of apoptotic cells. Error bars = SEM. n ≥ 3. *P < 0.05. (E) GSI treatment affects the cell cycle in 12Z cells. 12Z cells were subjected to treatment with vehicle (DMSO) or 0.5 μmol/L GSI for 24 hours, and subjected to a flow cytometric analysis of the cell cycle. GSI treatment induced a shift to the G2/M phase and reduced the proportion of cells in the S phase and G1 phase, respectively. Error bars = SEM. n = 4. *P < 0.05 [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | GSI affects expression of the stemness-related factors *HEY1*, *LIFR*, *SOX2* and *IFITM1* in primary endometriotic stroma cells and the 12Z cell line

As GSI treatment affects stemness-associated pathways with relevance to endometriosis,[8-11] we next took an unbiased screening approach for stemness-associated factors potentially linked to the observed phenotypic changes. For this purpose, we subjected 12Z cells that were treated with 1 and 10 μmol/L GSI for 24 hours, respectively, to TaqMan low-density array analysis. The human stem cell panel microfluidic card allowed for a simultaneous quantitative PCR analysis of >90 stemness- and differentiation-related markers (see Supplementary material, Table S1). In 12Z cells, 27 gene products were at the limit of detection with a ΔCt >34. Transcriptional changes were observed for the following genes: *ACTC*, *CD34*, *CD9*, *CDH5*, *CGB*, *COL1A1*, *COL2A1*, *COMM*, *CRABP2*, *CTNNB1*, *DES*, *DNMT3B*, *FOXA2*, *GAL*, *GATA6*, *GCG*, *GFAP*, *GRB7*, *IFITM1*, *IFITM2*, *IL6ST*, *LAMB1*, *LEFTB*, *LEFTY1*, *LIFR*, *LIN28*, *MYOD1*, *Nanog*, *NOG*, *PODXL*, *RAF1*, *REST*, *RUNX2*, *SEMA3A*, *SERPIN A1*, *SOX17*, *SYP*, *TAT*, *TDGF1*, *TERT*, *WT1*, and *ZFP42*. GSI treatment resulted in no change of expression for 32 additional gene products. We next aimed at confirming altered gene expression of the 19 most clearly regulated genes by quantitative PCR analysis of independent biological replicates. This revealed a significant and substantial (>50%) downregulation of the cytokine receptor leukemia inhibiting factor receptor (*LIFR*), of the pluripotency-associated transcription factor sex-determining region Y (SRY)- box 2 (*SOX2*), and of the stemness-related interferon-induced transmembrane protein 1 (*IFITM1*) (Figure 2A). Notably, expression of the transcription factor *HEY1*, a downstream regulatory target and readout of notch signaling, was significantly downregulated by GSI treatment. Moreover, expression of podocalyxin-like (*PODXL*) was significantly reduced by >25% upon GSI treatment of 12Z cells (Figure 2A). Following treatment with 1 μmol/L GSI, a significant downregulation of *LIFR*, *SOX2*, and *IFITM1* compared with DMSO controls was detected in patient-derived primary endometriotic cells by quantitative PCR (Figure 2B). Compared with 12Z cells, the downregulation of *LIFR* was in a similar range of about 50%, whereas downregulation of *SOX2* and *IFITM1*, although still substantial, was not as pronounced in primary endometriotic stroma cells. Moreover, *PODXL* expression was significantly upregulated in these cells, whereas a downregulation was observed in 12Z cells (Figure 2A,B). As GSI affected the cell viability of our cell models, we evaluated a potential GSI-induced dysregulation of *IDO1*, which has been linked to proliferation, invasive growth, and immunomodulatory potential of endometriotic stroma cells.[18,19] Although GSI treatment did not significantly affect *IDO1* mRNA expression in 12Z cells, a trend for an upregulation ($P = 0.077$) was observed in the case of patient-derived endometriotic stroma cells (Figure 2A,B). In contrast to *LIFR*, *SOX2*, and *IFITM1*, we could not independently confirm a significant regulation of *TERT*, *MNX1*, *IFITM2*, *DDX4*, *FOXA2*, *Nestin*, *nanog*, *WNT1*, *DES*, Lefty, nodal, *SOX17*, *MYOD1*, *IL6ST*, and *CGB* in 12Z cells (Figure 3).

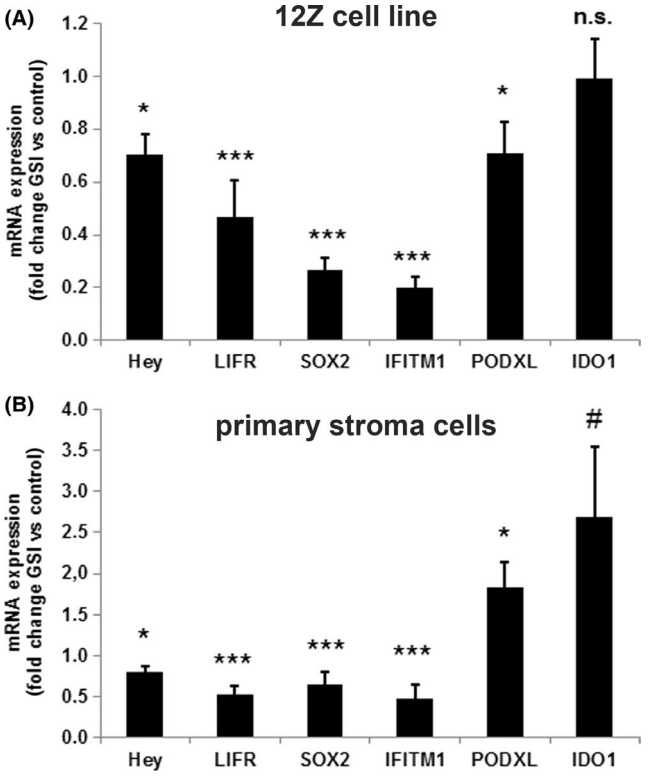

FIGURE 2 The stemness-related gene products Hey, LIFR, SOX2, and IFITM1 are downregulated by γ-secretase inhibitor (GSI) treatment of the endometriotic cell line 12Z and of primary endometriotic stroma cells. (A) Quantitative PCR confirmation of TaqMan Low-density array screening results of GSI-treated 12Z cells. 12Z cells were treated with 1 μmol/L GSI or vehicle (DMSO control) for 24 hours before quantitative PCR analysis. A significant downregulation of Hey, LIFR, SOX2, IFITM1, and PODXL is observed in GSI-treated cells relative to controls. n ≥ 6, error bars = SEM, $^{*}P < 0.05$, $^{***}P < 0.001$. (B) GSI treatment of primary endometriotic stroma cell cultures obtained from lesions of 7 endometriosis patients results in significant downregulation of the stemness-associated factors Hey, LIFR, SOX2, and IFITM1, and an upregulation of PODXL. In contrast, the inflammation-related indoleamine 2,3-dioxygenase-1 (IDO1) was not significantly altered by GSI treatment. Individual cultures of primary endometriotic cells were treated with 1 μmol/L GSI or vehicle (DMSO control) for 24 hours before quantitative PCR analysis. n = 4 to n = 7, error bars = SEM, $^{*}P < 0.05$, $^{***}P < 0.001$, $^{\#}P = 0.077$

3.3 | GSI treatment induces a downregulation of LIFR protein expression and of stemness-related ALDH activity and colony formation capacity

To assess if the observed transcriptional downregulation of stemness-associated markers also affected protein expression, we used flow cytometry. GSI treatment of 12Z cells did not only affect *LIFR* mRNA expression, it also substantially reduced LIFR protein expression in 12Z cells by >50% (Figure 4A). Notch signaling regulates stem cell functions, which may promote the pathogenesis of endometriosis by supporting unlimited proliferation and developmental plasticity, so promoting persistence of the ectopic lesion.[6-8]

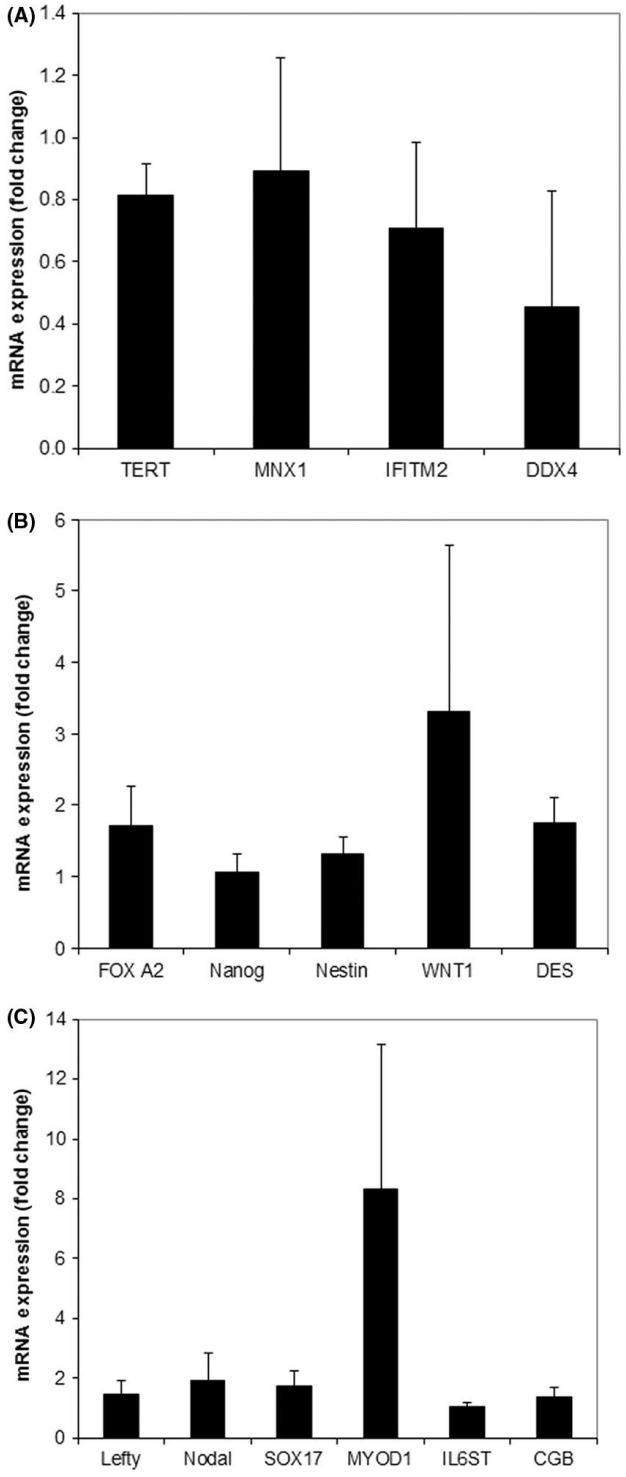

FIGURE 3 Quantitative PCR expression analysis of selected stemness-related and lineage-specific gene products in γ-secretase inhibitor (GSI) -treated vs DMSO-treated 12Z cells (1 μmol/L GSI, 24 hours). Target genes were selected based on results of a TaqMan Low-density array screening (human stem cell panel, ABI), which initially revealed GSI-dependent expression changes. Analysis of independent 12Z biological replicates subjected to GSI/DMSO-control treatment revealed no significant changes in the expression of (A) *TERT*, *MNX1*, *IFITM2*, *DDX4*, (B) *FOXA2*, *Nestin*, *nanog*, *WNT1*, *DES*, *(C) Lefty*, *nodal*, *SOX17*, *MYOD1*, *IL6ST*, *and CGB. n ≥ 5*, *error bars = SEM*

Therefore, we assessed the impact of GSI treatment on ALDH activity as a surrogate stem cell marker.[20] GSI treatment significantly reduced the ALDH-positive fraction (Figure 4B). Finally, we evaluated the impact of GSI treatment on colony formation as a readout of stemness properties.[15] Both continuous treatment and 24 hours of pretreatment with 1 μmol/L GSI resulted in a marked and significant reduction of colony formation, indicating an impact of notch inhibition on the stem cell phenotype (Figure 4C). Overall, these data point out novel GSI-sensitive stemness-related pathways, which are associated with altered viability, apoptosis, and ALDH activity in endometriotic cells.

4 | DISCUSSION

In this in vitro study, we have investigated the effect of a GSI on viability, apoptosis, and stem cell properties of the human endometriotic model cell line 12Z. The 12Z cells are a suitable model system for endometriotic stem cell studies, as we have previously shown that these cells possess several stem cell markers, including the surrogate markers side population phenotype and ALDH activity,[5,20] and expression of the stem cell markers *OCT4*, *SOX2*, *KLF4*, and *MSI2*.[5] In line with reduced ALDH activity and reduced colony formation ability, expression of the stemness-associated factors *LIFR*, *SOX2*, and *IFITM1* was reduced both in 12Z cells and in patient-derived stroma cells isolated from endometriotic lesions following treatment with GSI. The suppression of ALDH activity may be of therapeutic relevance, as this enzyme is involved in mediating drug resistance.[20] The notch signaling pathway has emerged as an important target in this context, as both notch and its modulators Msi1 and numb are dysregulated in endometriosis.[6-8] Our data extend the functional target range of the notch pathway from endocrine and antiangiogenic aspects to stem cell properties of endometriotic lesions.

Apart from a modulation of the stem cell phenotype, we observed a dose-dependent increase in apoptosis, and a decrease in viability/proliferation of 12Z cells and primary endometriotic stroma cells upon GSI treatment. Although short-term treatments resulted in moderate effects, our colony formation assays indicate that prolongation of the treatment substantially enhances treatment efficacy. A decrease in cell proliferation was recently also observed in an endometrial stroma cell line upon treatment with a different GSI, DAPT, indicating that γ-secretase inhibition affects both the epithelial and stromal compartments of the endometrium.[10] Decreased proliferation would be in line with a reduction in stem cell-like properties, as stem cells exhibit an unlimited proliferative potential.[6-8]

The reduction in cell proliferation may also be partially due to the observed shift of the cell cycle to the G2/M phase (Figure 1). Apparently, notch inhibition can have an—albeit cell-type specific—effect on the cell cycle: while studies on osteosarcoma and MCF-7 breast cancer cells reported a G1 cell cycle block upon GSI treatment, studies in gastric cancer cells and MDA-MB-231 breast cancer cells have reported a GSI-induced increase of the G2/M cell cycle phase, similar to our work.[21,22]

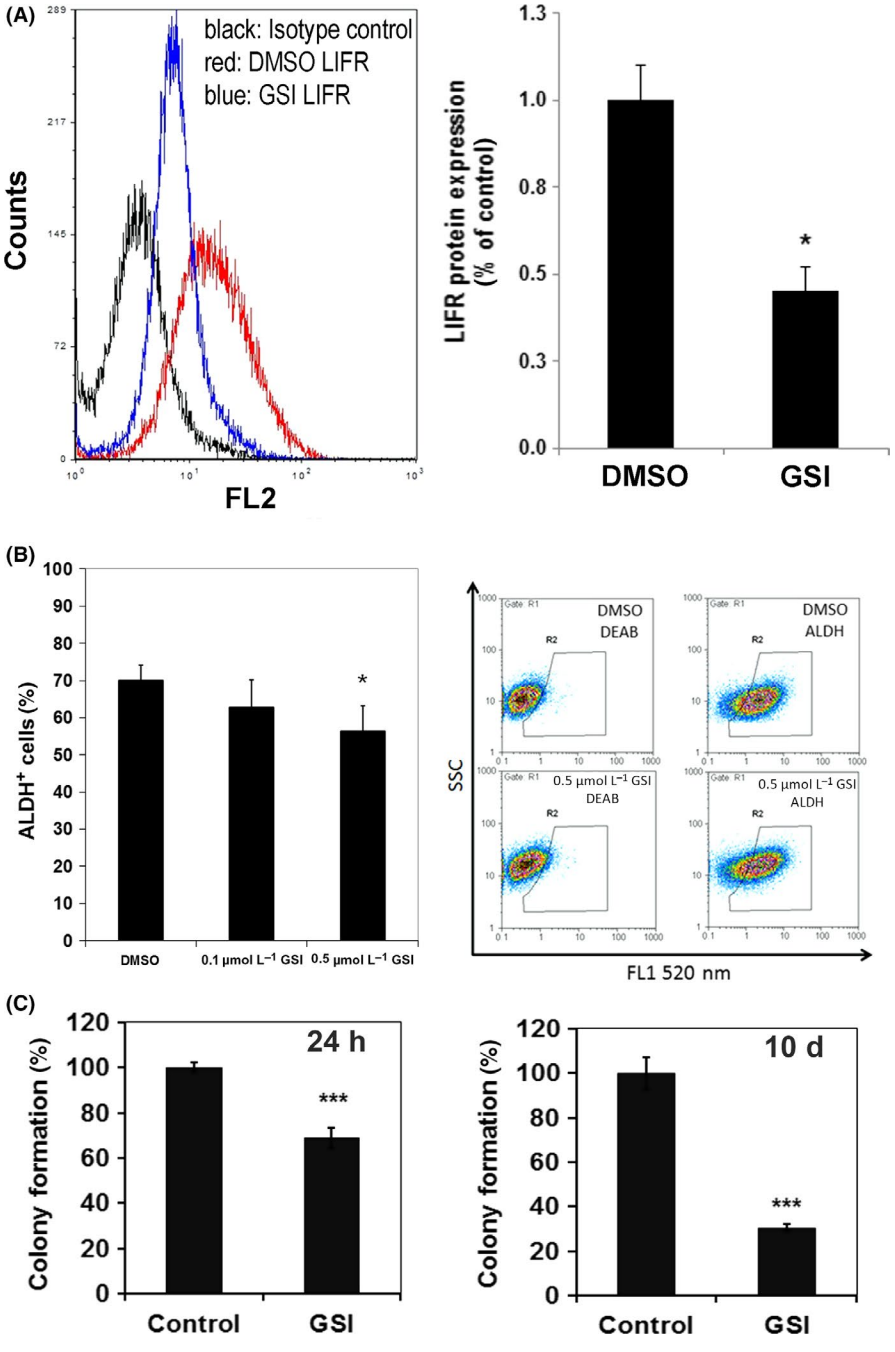

FIGURE 4 γ-Secretase inhibitor (GSI) treatment affects LIFR protein expression, aldehyde dehydrogenase (ALDH) activity, and colony formation potential of the endometriotic cell line 12Z. (A) GSI treatment of 12Z endometriotic cells results in LIFR downregulation at the protein level. 12Z cells were treated as indicated in the legend of Figure 2, followed by flow cytometric analysis of LIFR expression. Left panel shows representative experiment. Right panel shows quantitative analysis of 3 independent experiments. $P < 0.05$. (B) GSI treatment reduces the proportion of ALDH- cells as readout of stem cell activity in 12Z cells. The 12Z cells were treated with the indicated concentrations of GSI for 24 hours and subjected to a determination of the $ALDH^+$ cell population; 0.5 µmol/L GSI significantly reduced the ALDH subpopulation by 20%. Error bars = SEM. n ≥ 3. $^*P < 0.05$. (C) GSI treatment [1 µmol/L] for 24 hours (left panel) or for 10 days (right panel) inhibits colony formation as a readout of stem cell activity. 12Z cells were plated at low density in 3.5-cm Petri dishes, subjected to DMSO vehicle or 1 µmol/L GSI treatment for 24 hours (left panel) or during the complete assay period of 10 days (right panel), followed by counting of colonies containing >50 cells. Error bars = SEM. n = 3. $^{***}P < 0.001$ [Colour figure can be viewed at wileyonlinelibrary.com]

Previous studies on endometrial and endometriotic stroma cells employing the GSI DAPT had demonstrated reduced generation and nuclear localization of the intracellular domain of notch, respectively.[6,10] The application of an unbiased screening approach has produced a second important outcome of our study, as it revealed an impact of GSI on stemness-related genes other than notch. Among the identified stem cell markers, the pluripotency-associated transcription factor *SOX2* has a documented role in endometriosis.[5,23] Indeed, stromal *SOX2* expression is increased in the proliferative phase compared with the secretory phase of the menstrual cycle, and *SOX2*-positive cells are more frequent in endometriotic tissue compared with healthy control endometrium.[23] At the functional level, *SOX2* downregulation by microRNA miR-145 both in 12Z cells and primary endometrial stroma cells was associated with a decreased stem cell phenotype.[5] Our finding of GSI-dependent notch regulation conforms with the observation that notch signaling maintains *SOX2* expression levels in ovarian cancer stem cells.[24] Overall, our observation of *SOX2* downregulation by GSI expands the list of molecular targets relevant to endometriosis for this possible mode of pharmacological intervention.

Less is known about the second GSI-regulated stemness-related target identified in this study, *IFITM1*. *IFITM1* (Leu13) is a member of the family of interferon-inducible transmembrane proteins, and mediates anti-proliferative signals of the cytokine interferon-γ in addition to promoting cell adhesion.[25] *IFITM1* has a documented role in primordial germ cell migration in mice.[26] It has been proposed as a marker of endometriotic stromal cells in ovarian and extragenital endometriosis,[27]

and may be implicated in immunoregulation during implantation.[28] The novel observation of a γ-secretase-dependent expression of *IFITM1* in endometriosis expands the range of possible therapeutic effects of GSI treatment to *IFITM1*-dependent processes such as proliferation, migration, immune modulation, and angiogenesis.[25-28]

The third novel GSI-regulated target identified in this study is the *LIFR*. *LIF* is a member of the interleukin-6 family of cytokines, which induce signaling events linked to pluripotency, as evidenced by the essential role for LIF for culturing murine embryonic stem cells.[29] In the endometrium, LIF and LIFR expression are particularly prominent in the secretory phase of the menstrual cycle, indicating a potential role in embryo implantation.[30] Indeed, expression of LIFR, LIF, and its coreceptor gp130 is reduced in subfertile endometriosis patients compared with healthy controls,[31] and a reduction of LIFR expression is associated with reduced downstream signaling via signal transducer and activator of transcription 3 in women with adenomyosis during the window of implantation.[32] Therefore, although GSI-induced inhibition of LIFR expression can be expected to aid in targeting stem cell properties in endometriotic lesions, it may cause unwanted fertility-related side effects.

Some caveats are associated with our in vitro study. One limitation of our in vitro system is the use of cell lines and primary cell cultures, which may not fully reflect the complex situation in vivo. Moreover, immortalization of the 12Z cell line may have potentially caused alterations in the phenotype. On the other hand, 12Z cells have been extensively characterized at the molecular level,[14] and have been shown to reflect major phenotypic traits compared with primary cells. Indeed, a previous study on the stem cell phenotype of 12Z cells showed consistent microRNA-dependent regulation of several markers in the cell line and patient-derived cells.[5] Regarding the primary endometriotic cells, it has to be considered that they are derived from the stromal compartment, whereas 12Z cells are of epithelial origin.[14] In addition, the primary cells were derived from different ectopic locations and revised American Society for Reproductive Medicine stages, which may have masked some results. Nevertheless, we observed a consistent, yet quantitatively different extent in the regulation of GSI-dependent markers (Figure 2). Finally, no complete data on recurrence and extension of the disease were available for the donors of endometriotic biopsies, which could have acted as a confounder. Nevertheless, we obtained consistent and reproducible results with respect to GSI-induced transcriptional changes. Although the notch transcriptional target *HEY1* was downregulated by GSI treatment in our cell models, we cannot exclude that additional targets of GSI were regulated in our study. For example, cell surface receptors of the syndecan family, which act as regulators of invasive growth in endometriosis,[3,33] have been shown to be sensitive to GSIs.[34] Additional caveats are associated with a potential clinical application of GSIs in an endometriosis setting. These include possible fertility-related side effects linked to a downregulation of LIFR, a possible angiogenesis-promoting effect of some GSIs,[11] and gastrointestinal side effects previously reported in clinical trials on GSIs.[13] Moreover, notch overexpression is particularly prominent in the clinically challenging group of patients suffering from deep infiltrating endometriosis.[6,8] Alternative modes of notch inhibition, including vegetable- and fruit-derived natural compounds[35] may be associated with a more favorable side effects profile. For example, a recent study employing the flavonoid 3,6-dihydroxyflavone could demonstrate beneficial in vitro and in vivo effects on endometriotic cell migration and invasion, which were attributed to notch pathway inhibition.[35]

5 | CONCLUSION

Our preclinical in vitro study has demonstrated that application of GSI targets several pathogenesis-related properties in endometriotic cells, affecting viability, apoptosis, and several stem cell traits. Overall, these data suggest that the notch signaling pathway and some of the associated pathways identified in this study are worthwhile therapeutic targets in endometriosis; however, the mode of inhibition requires more study with respect to the side effects profile.

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CONFLICT OF INTEREST

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ORCID

Martin Götte https://orcid.org/0000-0003-2360-2496

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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