Decreased Indian hedgehog signaling activates autophagy in endometriosis and adenomyosis

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Abstract

Indian hedgehog (Ihh) signaling regulates endometrial receptivity and is an indispensable mediator of embryonic implantation. Hedgehog signaling is known to regulate autophagy, and aberrant regulation of autophagy is critically implicated in the pathogenesis of endometriosis and adenomyosis. However, potential dysregulation of Ihh signaling and its role in autophagy modulation in these diseases remain obscure. In this study, we found that components of Ihh signaling were significantly decreased, whereas the autophagy marker protein, LC3BII, was significantly increased in endometrial tissues of women with endometriosis or adenomyosis. Inhibition of Ihh signaling with the small-molecule inhibitor GANT61 or *Gli1* silencing in primary endometrial stromal cells increased autophagic activity, as measured by LC3 turnover assay and tandem mCherry-eGFP-LC3B fluorescence microscopy. Furthermore, we observed that GANT61 treatment significantly attenuated hydrogen peroxide-induced cell death, whereas disruption of autophagy with chloroquine diminished this effect. Collectively, these findings reveal that Ihh signaling is suppressed in endometrial tissues of patients with endometriosis or adenomyosis. This abnormal decrease may contribute to endometrial autophagy activation, which may promote aberrant survival of endometrial cells in ectopic sites in these two gynecological diseases.

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Introduction

Endometriosis and adenomyosis are common, refractory gynecological disorders affecting 10–19.5% of fertile women worldwide (Choi *et al.* 2015, Garcia-Solares *et al.* 2018, Zondervan *et al.* 2020). They present clinically with chronic progressive pelvic pain, dysmenorrhea, and infertility. Endometriosis is characterized by the presence of functional endometrial tissue outside the uterine cavity, whereas adenomyosis is defined by the presence of ectopic endometrial glands and stroma within the myometrium (Vannuccini *et al.* 2017, Kobayashi *et al.* 2020, Zondervan *et al.* 2020). These diseases exhibit differences in risk factors, some clinical features, pathological characteristics, and pathogenesis. However, they also share many similarities, including estrogen dependency, progesterone resistance, and multiple cellular and molecular aberrations (Vannuccini *et al.* 2017, Kobayashi *et al.* 2020, Zhu *et al.* 2016). For many years, adenomyosis was considered a specific form of endometriosis called endometriosis *interna* (Vannuccini *et al.* 2017, Che *et al.* 2020). Although endometriosis and adenomyosis cause severe symptoms in patients, they are generally believed to be chronic benign uterine diseases. However, ectopic endometrial

cells also have certain malignant biological behaviors, such as aggressive invasion, migration, unrestrained proliferation, and reduced susceptibility to apoptosis (Liu *et al.* 2017, 2019b, Che *et al.* 2020). These features are important for the pathogenesis of endometriosis and adenomyosis.

Autophagy is a dynamic, multistage process that includes the formation of the phagophore, the autophagosome, and the autolysosome. The phagophore is the initial sequestering double-membrane compartment, which expands and surrounds the targeted cytoplasmic material to form the autophagosome. The completion of autophagic flux occurs when autophagosomes fuse with lysosomes to generate autolysosomes, resulting in the degradation of the contents (Klionsky *et al.* 2016, Yim & Mizushima 2020). Autophagy is essential for cellular homeostasis and adaptation to stress (Leidal *et al.* 2018, Mizushima 2018). Autophagy in adenomyosis patients is poorly understood, and its role in endometriosis remains controversial (Allavena *et al.* 2015, Mei *et al.* 2015, Matsuzaki *et al.* 2018, Zhan *et al.* 2018, Liu *et al.* 2019b). However, recent evidence has demonstrated that autophagic dysregulation may be implicated in the pathogenesis of endometriosis and adenomyosis (Ren *et al.* 2010, Allavena *et al.* 2015, Mei *et al.* 2015,

Matsuzaki *et al.* 2018, Zhan *et al.* 2018, Liu *et al.* 2019b). Despite a significant increase in our knowledge regarding autophagy, the mechanism responsible for its regulation remains largely unknown in endometrial tissues of women with endometriosis or adenomyosis.

The hedgehog (Hh) signaling pathway regulates embryonic development, cell proliferation, differentiation, and cell–cell communication (Lee *et al.* 2006, Wang *et al.* 2013). Hh ligands, including Indian Hh (Ihh), Sonic Hh, and Desert Hh, bind to patched receptors (Ptch1 and Ptch2), subsequently abolish Ptch-induced smoothened (Smo) inhibition, and activate the glioma zinc finger transcriptional factors (Gli1, Gli2, and Gli3) (Wang *et al.* 2013). Ihh is essential for normal progesterone action, estrogen signaling, and uterine epithelium and stroma cellular communication (Lee *et al.* 2006, Franco *et al.* 2010, Yoo *et al.* 2017). Studies have demonstrated that the expression of Ihh and Gli1 may be decreased in endometriosis (Smith *et al.* 2011, Yoo *et al.* 2017). In contrast, other studies have indicated that Hh signaling may be enhanced in endometriosis (He *et al.* 2018, Liu *et al.* 2019a). Currently, there is no relevant study with respect to the role of Ihh signaling pathway in adenomyosis. Therefore, the regulation of Ihh signaling in endometriosis and adenomyosis requires further clarification.

Hh signaling is intimately associated with autophagy regulation; however, whether Hh pathway inhibits or activates autophagy may be context-dependent. Accumulating evidence has demonstrated that Hh signaling negatively regulates autophagy (Jimenez-Sanchez *et al.* 2012, Wang *et al.* 2013). In some cell types, such as hippocampal neurons and vascular endothelial cells, Hh signaling promotes autophagy (Li *et al.* 2012, Petralia *et al.* 2013). To our knowledge, it has not been determined whether Ihh signaling is involved in the autophagic regulation of endometrial cells in endometriosis or adenomyosis patients.

In this study, we determined whether autophagy and Ihh signaling are aberrantly altered in the endometria of patients with endometriosis or adenomyosis and elucidated the role of Ihh pathway in endometrial autophagy regulation in these two diseases.

Materials and methods

Ethical approval and tissue collection

This study was approved by the Institutional Ethics Committee of Taizhou First People's Hospital (approval number: 2019-KY006-01). All procedures were performed in accordance with the Declaration of Helsinki and the relevant guidelines and regulations. Written informed consent was obtained from all patients before endometrial tissue sample collection.

Patients of reproductive age (23–50 years old) with leiomyoma, endometriosis, or adenomyosis who attended the Department of Obstetrics and Gynecology in our hospital were recruited for this study. None of the study participants

had taken any contraceptives, used intrauterine devices, or received hormonal therapy for at least 3 months prior to surgery. Endometrial samples were collected during the proliferative stage of the menstrual cycle, which was confirmed by histological criteria. Control endometrial tissues (Con-E) were collected from patients with leiomyoma ($n=15$) who underwent laparoscopic myomectomy. We collected 11 cases of ovarian endometriotic tissues (EDM-ecE) from women who underwent ovarian cystectomy by laparoscopy. Specimens of EDM-ecE were carefully stripped from the inner wall of the ovarian endometriotic cysts. Ten cases of eutopic endometria (EDM-euE) were obtained from another group of patients with ovarian endometriosis. Samples from patients with adenomyosis, including the eutopic endometrium (ADM-euE) and the corresponding ectopic lesions of adenomyosis (ADM-ecE), were collected from patients ($n=18$) who underwent hysterectomy. These samples were used for quantitative real-time PCR (qPCR), immunohistochemical staining, and Western blot analysis. However, the ectopic endometrial tissue of adenomyosis usually presents as small focal or diffuse lesions. Therefore, we were unable to obtain sufficient ectopic endometrium for qPCR and immunoblot detection. An additional 14 cases of eutopic endometrial tissue from patients with adenomyosis (6 cases) and endometriosis (8 cases) were collected for isolation and cultivation of endometrial stromal cells.

Isolation and identification of endometrial stromal cells

Eutopic endometrial stromal cells (EuESCs) were isolated and purified as previously described (Liu *et al.* 2017). Briefly, fresh endometrial tissues were washed with PBS to remove blood, and then the tissues were minced with sterile surgical scissors and digested in DMEM/F12 medium (SH30023.01, Sigma-Aldrich) containing 2.5 mg/mL of type I collagenase (17100017, Gibco, Thermo Fisher Scientific) at 37°C for 90 min. EuESCs were obtained from two sequential filtrations with a 100- μ m and a 40- μ m cell strainer (15-1100 and 15-1040, Biologix) and were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) (RY-F22-05, Roy Biotechnology Company, Lanzhou, Gansu, China) and 1% (v/v) penicillin/streptomycin (10378016, Gibco) in a humidified atmosphere of 5% CO₂ at 37°C overnight. The medium was then discarded, and the cells were gently washed with PBS to remove blood cells and debris. The purity of EuESCs in our experiment was >95%, as determined by positive immunostaining for vimentin and negative staining for cytokeratins-19 (Choi *et al.* 2015, Che *et al.* 2020). Second and third passages of cells were used for subsequent experiments.

In vitro experiments

EuESCs were seeded into six-well cell culture plates with DMEM/F12 supplemented with 10% FBS (complete medium) and cultured for 24 h. The cells were then treated with a small-molecule inhibitor of Hh signaling, GANT61 (0–20 μ M; S8075, Selleck Chemicals), in serum-free DMEM/F12 for 24 h. For the LC3 turnover assay (Mizushima *et al.* 2010), which is one of

the principal methods to monitor autophagic flux, EuESCs were cultured in serum-free medium with 10 μ M GANT61 for 24 h in the absence or presence of 3-methyladenine (3-MA) (2.5 mM; S2767, Selleck) or chloroquine (CQ) (10 μ M; S4157, Selleck). To clarify the role of autophagy in oxidative stress-induced cell death, EuESCs were treated with 10 μ M GANT61 for 24 h in the absence or presence of 10 μ M CQ, the medium was then removed, and the cells were exposed to 200 μ M of hydrogen peroxide (H_2O_2) in serum-free medium for 2 h. The cells were washed twice with PBS and harvested for Western blotting analysis.

qPCR

EuESCs were directly scraped and harvested using RNAiso Plus reagent (9109, TakaRa), and tissues were homogenized with this reagent using a glass tissue grinder on ice. Total RNA was extracted and purified according to the manufacturer's instructions. cDNA was synthesized using a PrimeScriptTM RT Master Mix Kit (RR036A, TakaRa). Quantitative PCR was performed on an ABI Prism[®] 7500 real-time PCR detection system (Applied Biosystems, Thermo Fisher Scientific) using a TB Green[®] Premix Ex TaqTM (Tli RNaseH Plus) kit (RR420A, TakaRa). The following primers were used for the qPCR analysis: *Ihh*, 5'-CATTGAGACTTGACTGGGCAAC-3' and 5'-AGAGCAGGCTGAGTTGGGAGTCGC-3'; *Ptch1*, 5'-GGC-TCCCTCATCTCGTAAC-3' and 5'-ACTATGCTGTGGGTATTTCTGG-3'; *Smo*, 5'-GCTACTTCCTCATCCGAGGAGTCA-3' and 5'-GGCGCAGCATGGTCTCGTT-3'; *Gli1*, 5'-TCCTACCAGAGTCCCAAGTT-3' and 5'-CCCTATGTGAAGCCCTATT-3'; and *GAPDH*, 5'-TGACGCTGGGGCTGGCATTG-3' and 5'-GGCTGGTGGTCCAGGGGTCT-3'. Each sample was measured in triplicate, and the reactions without any cDNA template were used as negative controls. The $2^{-\Delta\Delta C_t}$ method was used to calculate the expression of each target gene, as previously described (Livak & Schmittgen 2001), and *GAPDH* served as a loading control.

Immunohistochemical analysis

Immunohistochemical staining was performed on thin paraffin sections with the following primary antibodies: mouse anti-Gli1 (1:100; 66905-1-Ig, Proteintech) and rabbit anti-Gli2 (1:400; A16864, ABclonal technology). Sections were dewaxed and rehydrated in graded ethanol (80–100%). Antigen retrieval was achieved using an EDTA antigen retrieval solution (pH 8.0) in a pressure cooker for 2.5 min at full pressure. Sections were then treated twice with 3% H_2O_2 for 5 min each to eliminate endogenous peroxidase activity and then blocked with normal goat serum for 30 min at room temperature. Sections were subsequently incubated with primary antibodies overnight at 4°C. After rinsing three times with PBS, sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (ready-to-use; DS-0004, ZSGB-BIO Company, Beijing, China) for 30 min. Finally, all slides were incubated with DAB Substrate and counterstained in hematoxylin before they were dehydrated and mounted. Dark-brown staining indicated a positive reaction. Results were imaged using an inverted microscope

(Olympus, IX53). Quantitative analysis was performed on three to five random fields at 400X magnification for each endometrial slice. The intensity of the dark-brown staining was analyzed using ImageJ software (NIH).

Western blot analysis

To prepare protein extracts, tissues were homogenized in radioimmunoprecipitation assay lysis buffer (R0020, Solarbio, Beijing, China) containing 1% protease inhibitor cocktail (FD1001, Fude Biological Technology, Hangzhou, Zhejiang, China) using a glass tissue grinder on ice. EuESCs were directly scraped in the lysis buffer to obtain cellular proteins. Samples were subjected to electrophoresis with SDS-PAGE denaturing gels, and the separated proteins were transferred to polyvinylidene difluoride membranes (146588, Millipore). The membranes were blocked in 5% nonfat milk at room temperature for 1 h and then incubated overnight with primary antibodies at 4°C. Subsequently, the membranes were washed with Tris-buffered saline containing 0.1% Tween-20 and incubated for 1 h with the corresponding HRP-conjugated secondary antibodies (1:1000; A0216 and A0208, Beyotime) at room temperature. Western ECL Substrate (1705060, ClarityTM, Bio-Rad) was used for protein band visualization, and the bands were detected and analyzed with the ChemiDoxTM XRS+ system (Bio-Rad) and Image LabTM software (Bio-Rad). The following primary antibodies were used for these experiments: rabbit anti-LC3B (1:1000; #3868, Cell Signaling Technology (CST)), rabbit anti-Beclin1 (1:1000; #3495, CST), mouse anti-Gli1 (1:1000; 66905-1-Ig, Proteintech), rabbit anti-Gli2 (1:1000; A16864, Proteintech), rabbit anti-GAPDH (1:1000; #2118, CST), and mouse anti- β -actin (1:1000; 60008-1-Ig, Proteintech).

Cytotoxicity analysis

EuESCs (5×10^3 cells/well) were cultured in 96-well cell culture plates with complete medium for 24 h. After the supernatant was aspirated, cells were treated with GANT61 (0–20 μ M) in serum-free DMEM/F12 for 24 h. The medium was then discarded, and 100 μ M of DMEM/F12 with 10% Cell Counting Kit-8 (CK04, Dojindo) was added to each well and incubated at 37°C for 1 h. The absorbance (optical density) at 450 nm was measured using a microplate spectrophotometer (Multiskan FC, Thermo Fisher Scientific). Cell viability measurements are presented as the proportion of control optical density.

Nuclear morphology analysis after cell staining with DAPI

EuESCs (3×10^4 cells/well) were cultured in 24-well cell culture plates for 24 h. The cells were then incubated with GANT61 (10 μ M) alone or in combination with CQ (10 μ M) in serum-free DMEM/F12 for 24 h. Subsequently, the medium was removed, and the cells were exposed to 200 μ M H_2O_2 in the serum-free medium for 2 h. EuESCs were then fixed with 4% paraformaldehyde for 15 min at room temperature, followed by incubation for 5 min with 1 μ g/mL DAPI (D9542, Sigma-Aldrich) in the darkness. Cell nuclei were observed using an inverted fluorescence microscope (Olympus,

IX53). Generally, normal nuclei are round, clear-edged, and uniformly stained, whereas apoptotic nuclei exhibit irregular edges around the nucleus and are more intensely stained and pyknotic. For apoptotic cell analysis, at least five views from each well were imaged, and the ratio of condensed nuclei to total nuclei was calculated and expressed as the apoptosis rate for each group.

Tandem mCherry-eGFP-LC3B fluorescence microscopy

Recombinant adenovirus vectors packing a tandem monomeric mCherry-eGFP-tagged LC3B construct for autophagic flux monitoring were generated by Vigene Biosciences (Jinan, Shandong, China). According to the manufacturer's instructions, 5×10^5 EuESCs were seeded in a 35-mm glass-bottomed dish (D35C4-20-1-N, Cellvis) and maintained for 24 h in complete medium. At approximately 60–70% confluence, the cells were cultured in 500 μ L of serum-free DMEM/F12 containing 2 μ L of recombinant adenovirus (3.2×10^8 pfu/mL). After 24 h, the medium was discarded, and the cells were treated with 10 μ M GANT61 in DMEM/F12 for 24 h. The cells were then observed and imaged using confocal microscopy (Olympus, FV3000). Autophagic flux was determined by evaluating the number of mCherry+eGFP– and mCherry+eGFP+ puncta (puncta/cell were counted).

siRNA transfection

Gli1 siRNA (5'-CCGAAGGACAGGUAUGUAATT-3') and a negative control (NC) siRNA (5'-GCGACGAUCUGCCUAA-GAUGdTdT-3') were designed and synthesized by GenePharma (Shanghai, China). EuESCs (5×10^5 cells/well) were cultured in 6-well cell culture plates in complete medium for 24 h. According to the manufacturer's instructions, si-*Gli1* or NC (100 nM) working solutions were prepared with Lipofectamine™ 3000 (L3000001, Thermo Fisher Scientific) and Opti-MEM™ (31985070, Gibco). At approximately 60–70% confluence, EuESCs were incubated with siRNA working solution for 6 h. Then the cells were maintained in complete medium for 24 h. Afterward, the cells were cultured in serum-free DMEM/F12 in the absence or presence of 10 μ M CQ for 24 h.

Statistical analysis

All data were expressed as means $\pm$ S.E.M. For statistical analysis, the Student's t-test was used for data with only two groups; the one-way ANOVA followed by Tukey's multiple comparisons test was used for data containing more than two groups. $P < 0.05$ was considered statistically significant.

Results

Ihh signaling is inhibited in endometrial tissues of endometriosis patients

We evaluated the expression levels of *Ihh* signaling pathway components in eutopic and ectopic endometrial tissues isolated from patients with endometriosis by

qPCR. As shown in Fig. 1A, B, C and D, the mRNA levels of *Ihh*, *Ptch1*, *Smo*, and *Gli1* tended to decrease in both eutopic and ectopic endometrial tissues. Though there was no statistically significant difference between control and eutopic endometrial tissues, inhibition of *Ihh*, *Ptch1*, and *Smo* expression in ectopic tissues was evident (Fig. 1A, B and C). The transcription factor *Gli1*, which functions as an amplifier of the pathway (Dai *et al.* 1999, Ruiz I Altaba 1999, Gorojankina 2016, Shi & Long 2017), was significantly suppressed in both eutopic and ectopic endometrium (Fig. 1D). *Gli2* predominantly acts as a transcriptional activator and is considered a marker of *Ihh* pathway activation (Jimenez-Sanchez *et al.* 2012, Shi & Long 2017). Therefore, we further analyzed the expression levels of *Gli1* and *Gli2* proteins in endometrial tissues from women with endometriosis. Western blot analysis revealed that *Gli1* expression tended to decrease in the eutopic endometrium and was significantly inhibited in the ectopic endometrium (Fig. 1E and F). In addition, *Gli2* expression was also markedly suppressed in ectopic endometriotic tissues, whereas there was no significant difference in *Gli2* expression between the eutopic and control endometrium (Fig. 1E

Figure 1 *Ihh* signaling is inhibited in endometrial tissues of endometriosis patients. (A, B, C and D) *Ihh*, *Ptch1*, *Smo* and *Gli1* mRNA expression levels were assessed by qPCR in Con-E, EDM-euE and EDM-ecE; $n = 10$. (E) Representative immunoblots of *Gli1* and *Gli2* in Con-E, EDM-euE and EDM-ecE. (F and G) Densitometric quantification of *Gli1* and *Gli2* in Con-E, EDM-euE and EDM-ecE; $n = 10$ or 11. Quantitative results are expressed as means $\pm$ S.E.M.; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Con-E: endometrium from patients with leiomyoma; EDM-euE and EDM-ecE: eutopic and ectopic endometrial tissues of endometriosis patients, respectively.

and G). These data indicate that Ihh signaling is inhibited in the endometrium of endometriosis patients.

Ihh signaling is inhibited in endometrial tissues of women with adenomyosis

We next examined the expression of Ihh pathway components in eutopic endometrial tissues of patients with adenomyosis. We found that the expression levels of *Ihh*, *Ptch1*, *Smo*, and *Gli1* in eutopic endometrium were decreased by approximately 50% compared with that of the control endometrium (Fig. 2A, B, C and D). Immunohistochemical staining of Gli1 revealed that it was predominantly localized in the cytoplasm of glandular and stromal cells, and the staining levels were decreased either in eutopic endometrium or in ectopic endometrial cells compared with the control group (Fig. 2E and F). Gli2 was observed in the cytoplasm and nuclei of glandular and stromal cells in the functional layer of the endometrium (Fig. 2G). It was also predominantly localized to the cytoplasm of glandular and stromal cells in the basal layer of the endometrium and ectopic tissues (Fig. 2G). Furthermore, Gli2 expression was significantly decreased in the basal layer of control endometrium (Con-E-B) and the basal layer of eutopic tissues from patients with adenomyosis (ADM-euE-B) compared with the respective endometrial functional layer (Con-E-F or ADM-euE-F) (Fig. 2G and H). Although no significant differences were observed between the control and the eutopic endometrial tissues, Gli2 expression was somewhat decreased in tissues of adenomyosis patients and was significantly inhibited in ectopic tissues (ADM-ecE) compared with Con-E-F or ADM-euE-F (Fig. 2G and H). To further examine Gli1 and Gli2 expression in endometria from patients with adenomyosis, we quantified these proteins in eutopic tissues by Western blot analysis. The data indicated that Gli1 and Gli2 were significantly downregulated in women with adenomyosis (Fig. 2I, J and K). These findings suggest that Ihh pathway is suppressed in endometrium of adenomyosis patients.

LC3BII expression is elevated in endometrium of adenomyosis and endometriosis patients

LC3 is a structural protein of the autophagosomal membrane and exists as four isoforms (LC3A, LC3B, LC3B2, and LC3C) in mammalian cells (Klionsky et al. 2016). LC3BII is converted from LC3BI by phosphatidylethanolamine conjugation and its expression correlates with autophagy activation (Mei et al. 2015, Felip et al. 2019). Therefore, we measured LC3BII levels by Western blot analysis as a surrogate marker for endometrial cell autophagy. In samples from endometriosis patients, we found that LC3BII expressions in eutopic and endometriotic tissues were nearly 1.4-fold and 2.6-fold of that in the control, respectively (Fig. 3A and C). Moreover, we observed

that the LC3BII expression level in the eutopic tissues of adenomyosis patients was approximately 1.4-fold of that in the controls (Fig. 3B and D). These results indicate that autophagy may be activated in the endometrium of women with endometriosis or adenomyosis.

Decreased Ihh signaling activates autophagy in endometrial stromal cells

To clarify the relationship between Ihh signaling and autophagy in endometrial cells, a small-molecule Hh signaling inhibitor GANT61, which directly blocks Gli-activated transcription, was employed in our *in vitro* experiments (Lauth et al. 2007). After GANT61 treatment, the expression level of LC3BII was found to be enhanced in EuESCs in a dose-dependent manner (Fig. 4A). Moreover, the expression of Beclin1, an essential protein in the autophagy interactome that signals the onset of autophagy (Klionsky et al. 2016), was significantly increased (Fig. 4A). The LC3 turnover assay was used to further evaluate the effects of GANT61 treatment on autophagy in EuESCs. 3-MA inhibits the initial steps of autophagosome formation, and CQ neutralizes lysosomal pH to block autophagolysosomal degradation. GANT61 treatment promoted LC3BII expression, whereas 3-MA significantly inhibited GANT61-induced LC3BII accumulation (Fig. 4B). In addition, LC3BII expression was elevated following treatment with CQ and GANT61 to a greater extent compared with treatment with either compound alone (Fig. 4B). Furthermore, we infected EuESCs with recombinant adenoviral vectors encoding a tandem sensor mCherry-eGFP-LC3B construct to monitor autophagic flux. eGFP is readily degraded under low pH and proteolytic conditions in the lysosome, whereas mCherry is more stable (Klionsky et al. 2016). Thus, yellow fluorescence generated by the colocalization of eGFP and mCherry indicates a phagophore or an autophagosome that has not fused with a lysosome (Klionsky et al. 2016). In contrast, autolysosomes only produce red signals (Klionsky et al. 2016). We observed that GANT61 (10 μ M) treatment significantly increased the number of red puncta in EuESCs (Fig. 4C and D). These results suggest that GANT61 treatment increases autophagy flux in EuESCs. To further confirm these results, we disrupted Ihh signaling by *Gli1* knockdown. The results showed that *Gli1* siRNA treatment suppressed Gli1 and Gli2 expression (Fig. 4E, F, G and H) but did not disrupt *Ptch1* expression (data not shown). Importantly, we found that *Gli1* knockdown significantly upregulated the expression of LC3BII and Beclin1 in EuESCs (Fig. 4G and H). The LC3 turnover assay showed that CQ treatment augmented *Gli1* knockdown-induced LC3BII accumulation in EuESCs (Fig. 4I). Taken together, our data suggest that suppressed Ihh signaling activates autophagy in endometrial stromal cells.

Figure 2 *Ihh* signaling is inhibited in endometrial tissues of women with adenomyosis. (A, B, C and D) *Ihh*, *Ptch1*, *Smo* and *Gli1* mRNA expression levels were assessed via qPCR in Con-E and ADM-euE; $n=7$. (E and F) Representative immunohistochemical images of Gli1, and densitometric quantification of Gli1 staining levels in Con-E ($n=15$), ADM-euE ($n=18$) and ADM-ecE ($n=18$). Scale bar = 50 μ m. (G and H) Representative immunohistochemical images of Gli2, and densitometric quantification of Gli2 staining levels in Con-E ($n=15$), ADM-euE ($n=18$) and ADM-ecE ($n=18$). Scale bar = 50 μ m. (I–K) Representative immunoblots and densitometric quantifications of Gli1 and Gli2 in Con-E ($n=8$) and ADM-euE ($n=5$). Quantitative results are expressed as means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Con-E: endometrium from patients with leiomyoma; ADM-euE and ADM-ecE: eutopic and ectopic endometrial tissues from adenomyosis patients, respectively.

Suppressed *Ihh* signaling promotes cell survival via autophagy

It is unknown whether autophagy is beneficial or detrimental to the survival of endometriotic cells. Previous reports indicate that ectopic endometrial cells in endometriotic ovarian cysts may experience strong and persistent oxidative stress (Yamaguchi *et al.* 2008, Sanchez *et al.* 2014, Allavena *et al.* 2015). Therefore,

we used H_2O_2 to induce intracellular oxidative stress to determine the role of autophagy in EuESCs. GANT61 (0–20 μ M) did not affect the viability of EuESCs over 24 h (Fig. 5A). The LC3 turnover assay showed that LC3BII production in response to 2 h of H_2O_2 (200 μ M) treatment after CQ (10 μ M) preincubation for 24 h was significantly increased compared with that in the control, CQ, or H_2O_2 treatment groups, indicating that

Figure 3 LC3BII expression is elevated in endometrium of adenomyosis and endometriosis patients. (A and C) Representative immunoblots and densitometric quantifications of LC3BII levels in Con-E ($n = 8$), EDM-euE ($n = 10$) and EDM-ecE ($n = 11$). (B and D) Representative immunoblots and densitometric quantifications of LC3BII levels in Con-E ($n = 12$) and ADM-euE ($n = 10$). Quantitative results are expressed as means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$. #Nonspecific band. Con-E: endometrium from patient with leiomyoma; EDM-euE and EDM-ecE: eutopic and ectopic endometrial tissues from endometriosis patients, respectively. ADM-euE: eutopic endometrial tissues from adenomyosis patients.

H_2O_2 may activate autophagy in EuESCs (Fig. 5B). In addition, we found that 2 h of H_2O_2 exposure following pretreatment with GANT61 (10 μ M) and CQ for 24 h significantly augmented LC3BII accumulation compared with H_2O_2 incubation after GANT61 pretreatment. This suggests that oxidative stress may increase Hh signaling inhibition-induced autophagic flux in EuESCs (Fig. 5B). The extent of nuclear condensation in EuESCs following exposure to H_2O_2 for 2 h was assessed by DAPI staining. The number of pyknotic nuclei was significantly increased following H_2O_2 treatment (Fig. 5C, white arrowheads; and Fig. 5D). GANT61 treatment decreased the number of H_2O_2 -induced pyknotic nuclei, whereas CQ treatment diminished GANT61-induced cell protection in H_2O_2 -stressed EuESCs (Fig. 5C and D). The results suggest that autophagy activated by Hh signaling inhibition may contribute to increased cell survival under oxidative stress in ectopic sites.

Discussion

A previously reported study has demonstrated that many upstream molecular aberrations such as KRAS activation and overexpression of SIRT1/BCL6 in the endometrium (Yoo *et al.* 2017), may disrupt endometrial Ihh signaling. In this study, we found that the Ihh signaling was significantly inhibited in endometrial tissues collected from women with endometriosis, which is consistent with several previous reports (Smith *et al.* 2011, Yoo *et al.* 2017). The same alteration of Ihh signaling was also observed in the proliferative

phase of tissues isolated from adenomyosis patients. Given that Ihh signaling plays an important role in the progesterone reaction, impaired Ihh signaling may result in progesterone resistance and contribute to infertility in women with endometriosis or adenomyosis (Lee *et al.* 2006, Smith *et al.* 2011, Yoo *et al.* 2017). Endometriosis and adenomyosis are viewed as estrogen-dependent diseases and are associated with increased local estrogen levels (Vannuccini *et al.* 2017, Zondervan *et al.* 2020). Moreover, fibrosis generally occurs in ectopic lesions during adenomyosis development (Shen *et al.* 2016, Vannuccini *et al.* 2017, Kobayashi *et al.* 2020). It has been reported that conditional ablation of *Ihh* stimulated estrogen signaling and enhanced collagen production in the murine uterus (Franco *et al.* 2010). This indicates that Ihh signaling inhibition may be implicated in local aberrant estrogen activity and fibrogenesis in eutopic or ectopic endometrium of endometriosis or adenomyosis patients.

Immunohistochemical analysis demonstrated that the distribution of endometrial Gli2 was spatially different as its expression was markedly increased in the functional layer compared with the basal endometrium layer. Gli2 functions as a transcriptional activator of Hh signaling pathway (Jimenez-Sanchez *et al.* 2012). Therefore, Ihh signaling in the functional endometrial layer may be more active, which implies that the progesterone signaling in the functional endometrium layer may be more active than that in the basal endometrium layer.

Many reports have indicated that autophagy may be decreased in endometrial tissues from endometriosis or adenomyosis patients (Ren *et al.* 2010, Mei *et al.* 2015, Zhan *et al.* 2018). However, evidence has also demonstrated that endometrial autophagy may be upregulated in the endometria of women with endometriosis (Allavena *et al.* 2015, Liu *et al.* 2017, 2019b, Zheng *et al.* 2018). In this study, we evaluated the autophagy activity by assessing the most widely used marker, LC3BII (Klionsky *et al.* 2016), and confirmed that autophagy may be activated in endometrial tissues of women with endometriosis or adenomyosis. Autophagy frequently occurs in response to various stimuli, including starvation, endoplasmic reticulum stress, hypoxia, and oxidative stress (Allavena *et al.* 2015, Liu *et al.* 2017, Zhang *et al.* 2020). In fact, previous reports have demonstrated that endometriotic tissues may experience hypoxia in early implanted ectopic endometrial lesions and suffer overwhelming oxidative stress in old ectopic lesions (Yamaguchi *et al.* 2008, Allavena *et al.* 2015, Xiong *et al.* 2016).

The Hh pathway may be primarily considered an inhibitory cell-signaling with respect to autophagy (Jimenez-Sanchez *et al.* 2012). However, whether decreased Ihh signaling in the endometrium promotes autophagy remains to be determined. Our results demonstrated that suppression of Ihh signaling either

Figure 4 Decreased Ihh signaling activates autophagy in endometrial stromal cells. (A) LC3BII and Beclin1 protein levels were measured by Western blot after EuESCs were treated with GANT61 (0–20 μM) for 24 h. (B) LC3BII levels were measured by Western blot after EuESCs were treated with 10 μM GANT61 for 24 h in the absence or presence of 2.5 mM 3-MA or 10 μM CQ. (C) Recombinant adenovirus vectors encoding mCherry-eGFP-LC3B were incubated with EuESCs for 24 h. The cells were then treated with 10 μM GANT61 for 24 h and analyzed by laser scanning confocal microscopy. Con., control; scale bar=20 μm. (D) Quantification of mCherry+eGFP– (red) and mCherry+eGFP+ (yellow) signals (puncta). Quantitative results are expressed as means ± S.E.M. of at least four independent experiments. ** $P < 0.01$, *** $P < 0.001$. (E, F, G, H and I) EuESCs were transfected with siRNA targeting *Gli1* and received CQ treatment as described in the ‘Materials and methods’ section. *Gli1*, *Gli2*, LC3BII, and Beclin1 levels were then evaluated with qPCR and/or Western blot. Quantitative results are expressed as means ± S.E.M. of at least five independent experiments. * $P < 0.05$, ** $P < 0.01$.

with the small-molecule inhibitor, GANT61, or by *Gli1* silencing markedly enhanced autophagic flux in endometrial stromal cells. These results, with the increased endometrial LC3BII expression observed in eutopic and ectopic tissues, indicate that inhibition of endometrial Ihh signaling contributes to the functional

activation of autophagy in endometrial tissues from endometriosis and adenomyosis patients.

Repeated monthly hemorrhage during endometriosis and adenomyosis causes high levels of local catalytic ferrous iron deposition in ectopic lesions, which can generate a large number of hydroxyl radicals via the

Figure 5 Suppressed Ihh signaling promotes cell survival via autophagy. (A) EuESCs were treated with GANT61 (0–20 μM) for 24 h. Cell viability was evaluated with the Cell Counting Kit-8 ($n=6$). (B) After EuESCs were treated with GANT61 (10 μM) alone or in combination with CQ (10 μM) for 24 h, cells were exposed to 200 μM H₂O₂ for 2 h. Then, LC3BII protein levels were measured by Western blot. (C) Morphological changes in the apoptosis-like nuclei in EuESCs were evaluated by DAPI staining; white arrowheads indicate pyknotic nuclei that underwent apoptosis. Scale bar = 50 μm; Con., control. (D) The ratio of apoptotic nuclei to total nuclei was calculated and expressed as the apoptosis rate; each value is presented as the mean $\pm$ S.E.M. of at least five fields of view under the microscope. * $P < 0.05$, *** $P < 0.001$; Con., control.

Fenton reaction (Yamaguchi *et al.* 2008, Mori *et al.* 2015). This may contribute to oxidative stress in the local microenvironment of ectopic tissues. For ectopic endometrial cells immersed in the local fluid environment, the most challenging objective is to survive. However, the underlying mechanism of survival has not been fully elucidated. Autophagy is generally regarded as a beneficial mechanism for cell survival (Leidal *et al.* 2018, Mizushima 2018). Our study demonstrated that GANT61-induced autophagy significantly decreased oxidative stress-induced cell apoptosis, indicating that Ihh signaling inhibition may restore intracellular homeostasis through autophagy in ectopic endometrial cells. Our study provides experimental evidence to support the hypothesis that endometrial autophagy activation in endometriosis or adenomyosis may be regarded as an adaptive response that contributes to the survival of endometrial cells in ectopic sites as well as lesion maintenance (Allavena *et al.* 2015). However, this hypothesis requires further validation in animal models.

Collectively, as depicted in Fig. 6, our study demonstrated that Ihh signaling is inhibited in endometriosis and adenomyosis patients, and this decrease may be implicated in the activation of pro-survival autophagy in ectopic tissues. The current study

Figure 6 Proposed model for the roles of Ihh signaling and autophagy in the pathogenesis of endometriosis and adenomyosis. Various kinds of inhibiting factors can disrupt Ihh signaling in the endometrium. Decreased Ihh signaling increases autophagic activity in ectopic endometria of endometriosis and adenomyosis patients. Autophagy may decrease oxidative stress-induced cell death in ectopic lesions, thus promoting the survival of endometriotic cells in ectopic sites and contributing to lesion maintenance.

expands our understanding of the roles of Ihh signaling and autophagy in the pathogenesis of endometriosis and adenomyosis and may lead to new therapeutic interventions.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Ethical approval and informed consent

This study was approved by the local ethical committee and the written informed consent was obtained from all patients.

Author contribution statement

Y Z, Y P and Q X performed Western blot analysis, immunohistochemical staining and cell experiments. L Z and Y C carried out qPCR analysis. D Y and H Z collected tissues and contributed critical discussions. X Z and J L designed the study, analyzed the data and wrote the manuscript. All authors approved the final manuscript.

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