Title: Inhibition of KIF20A by BKS0349 reduces endometriotic lesions in a xenograft mouse model

Running title: BKS0349 reduces endometriotic lesions

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Abstract

Several studies have suggested a possible etiological association between ovarian endometriosis and ovarian cancer. Evidence has shown that KIF20A overexpression might confer a malignant phenotype to ovarian tumors by promoting proliferation and inhibiting apoptosis. However, no data about the role of KIF20A in endometriosis have been described. In this study human endometrium (n=4) was transfected by mCherry adenovirus and intraperitoneally implanted in mice. Subsequently, mice were divided in three groups (n=8/group) that were treated with Vehicle, BKS0349 (KIF20A-antagonist) or Cabergoline (dopamine receptor agonist) for 21 days. mCherry-labeled endometriotic lesions were monitored over time using the IVIS-Imaging System. Mice were sacrificed 72h after the last administration, proliferation was evaluated by immunohistochemistry and apoptosis by TUNEL. CCND1 gene expression (G1 phase-related gene) was measured by qRT-PCR. A significant reduction in mCherry-fluorescent signal was observed in the BKS0349 group after treatment ended (D24) compared to D0 (p-value=0.0313). Moreover, mCherry-signal on D24 showed a significant decrease in the BKS0349 group compared to controls (p-value=0.0303), along with significant size reduction of endometriotic lesions observed in BKS0349 group compared to control on D24 (p-value=0.0006). Functional studies showed a significant reduction in proliferating cells in BKS0349-treated group compared to controls (p-value=0.0082). In addition, CCND1 expression was decreased in BKS0349 group compared to control (p-value=0.049) at D24 and a significant increase in apoptotic cells among endometriotic lesions in BKS0349-treated mice was observed compared to control (p-value=0.0317). Based on these findings, we concluded that BKS0349 induces apoptosis and inhibits cell proliferation, reducing endometriotic lesion size and suggesting KIF20A inhibition by BKS0349 as a novel therapeutic treatment for endometriosis.

Keywords: KIF20A, endometriosis, cell proliferation, cell cycle, apoptosis
Introduction

Endometriosis is an estrogen-dependent gynecologic disorder characterized by the presence of endometrial tissue outside the uterus, most commonly in the ovaries and peritoneal cavity (Kennedy et al., 2005). The nature of this disease is heterogeneous and includes different anatomical entities such as ovarian, peritoneal, and deep infiltrating endometriosis, causing a range of severity of pelvic pain, dysmenorrhea, dyspareunia, painful defecation, and/or infertility (Practice Committee of the American Society for Reproductive Medicine, 2004). While endometriosis is diagnosed in 6–10% of all women, an estimated 35–50% of infertile women are affected by this disease (Giudice and Kao, 2004).

Although endometriosis is a chronic and recurrent condition, surgical removal of ectopic endometrial lesions has been the primary therapeutic approach for more than a century. However, this treatment approach provides only temporary relief as recurrence of endometriotic lesions can occur in up to 75% of cases within two years of corrective surgery (Giudice, 2010). Hormone treatments have also been used to reduce ectopic endometrial lesions; however, these treatments often fail to provide long-term relief and result in a hypoestrogenic state linked to secondary effects like osteoporosis and pseudo-menopause (Küpker et al., 2002), limiting the therapy duration. Therefore, there is a need for an efficient, non-surgical treatment that reduces ectopic endometrial lesions with lasting impact and minimal side effects.

Although endometriosis is a benign condition, it shares some characteristics of malignancy, such as local and distant dissemination, cell invasion, and damage of adjacent tissue (Sayasneh et al., 2011; Zafrakas et al., 2014). Ovarian endometriotic cysts have recently been suggested as the origin of ovarian clear cell carcinoma (CCC) and ovarian endometrioid cancer (EC) types, implicating a possible etiological association between endometriosis and ovarian cancer (Anglesio and Yong, 2017; Barreta et al., 2019). In this regard, an study about the frequency of endometriosis-associated ovarian carcinoma described that a 35% of CCC and 27% of EC have origin from endometriosis (Somigliana et al., 2006; Kajiyama et al., 2019). Indeed, endometriotic lesions can undergo immunohistological and molecular alterations similar to
those observed in ovarian tumors, suggesting that endometriosis may be an intermediate step in neoplastic progression (Komiyama et al., 2018).

*KIF20A* is an overexpressed gene in most ovarian carcinoma tissues and its expression is correlated with tumor progression, suggesting that *KIF20A* may be useful as an independent prognostic biomarker in ovarian carcinoma (Li et al., 2018). KIF20A, also known as RAM6KIFL/MKLP2, is a microtubule-associated motor protein in the kinesin-6 superfamily localized in the Golgi apparatus where it is involved in the dynamics of this organelle and consequently, implicated in the formation of the mitotic spindle and chromosome partitioning (Verhey and Hammond, 2009), cell adhesion, spreading, migration, proliferation, and intracellular transport (Echard et al., 1998; Li et al., 2018). KIF20A overexpression is correlated with progression of several human malignant tumors including bladder, breast, gastric, pancreatic, small cell lung, and hepatocellular carcinoma (Kikuchi et al., 2003; Claerhout et al., 2011; Imai et al., 2011; Ho et al., 2012; Shi et al., 2016; Zhao et al., 2018). These findings suggest that KIF20A is involved in tumor progression by promoting cell proliferation, angiogenesis, invasion, metastasis, and autophagy and inhibiting apoptosis. Thus, KIF20A is used as an indicator of prognosis for tumors in clinical practice (Zhao et al., 2018) due to its important role in cell cycle regulation.

Cell cycle is one of the molecular functions involved in cancer development, a dysregulation of this function can lead to an increased cell proliferation and consequently, tumor progression. In this regard, KIF20A affects cell proliferation by modulating the G1/S phase transition, which is a major checkpoint in cell cycle progression, and promotes apoptosis (Wang et al., 2017; Zhao et al., 2018). Recent evidence indicates that KIF20A inhibition can suppress the cell cycle transition from the G0/G1 phase to the S phase, causing cell cycle arrest and consequently, inhibition of cell proliferation. This implicates KIF20A as an anti-cancer drug target. Paprotrain (PAssenger PROteins TRAnsport Inhibitor; BKS0101) is reported to suppress cytokinesis by specifically inhibiting KIF20A (also known as MKLP2 inhibitor) (Tcherniuk et al., 2010; Labrière et al., 2016). In this regard, Paprotrain has been described as a potent inhibitor of
KIF20A in HL60 (Human myeloid leukemia cell line), leading to multinuclearity of these cells and suppressing cell growth (Morita et al., 2018), suggesting KIF20A inhibition could be one of the therapeutic targets of acute leukemia. In addition, Paprotrain may be a useful tool not only for the study of KIF20A function in cytokinesis, but also to validate cytokinesis-specific kinesins as potential targets for drug development in cancer chemotherapy. Accordingly, the in vitro inhibition of KIF20A in cancer cells by Paprotrain or BKS0349, a new KIF20A-specific inhibitor derived from Paprotrain with higher affinity (unpublished data), showed an inhibition from the Golgi complex and a delay in vesicle transport to the plasma membrane. These findings suggest that KIF20A is involved in the fission of RAB6-positive vesicles from Golgi membranes and movement of these vesicles along microtubules (Miserey-Lenkei et al., 2017).

Considering that endometriosis is etiologically associated with ovarian cancer, we hypothesize that KIF20A inhibition by BKS0349 could prevent cell proliferation and induce apoptosis in endometriotic lesions, similar to events that KIF20A inhibition causes in cancer cells. If so, BKS0349 could offer a viable therapeutic option for the treatment of endometriosis with lasting impact and minimal side effects. For this purpose, we studied the effect of BKS0349 on the development of endometriotic lesions generated in a xenograft mouse model of endometriosis and determined the effect of BKS0349 on lesion size, proliferation, apoptosis, and angiogenesis in vivo.

Material and Methods

Endometrial Tissue Collection

Human biopsies of eutopic endometrial tissue (n=4) at the late proliferative/early secretory phase were obtained from egg donors at the time of an oocyte retrieval procedure. Subsequently, endometrial biopsies were harvested in a maintenance medium (M199; Gibco, USA) containing 10% fetal bovine serum [(v/v) FBS; PAA Laboratories], 1% antibiotic-antimycotic solution [(v/v) Gibco, USA], and 10 mmol/L HEPES buffer solution (PAA Laboratories). This study
was approved by the IVI Valencia Clinical Ethics Committee (1606-FIVI-050-FD) and all participants provided written informed consent.

**Adenoviral Transfection of Endometrial Fragments**

Endometrial biopsies were cut into approximately 3-mm³ pieces with a scalpel, placed in 96-well plates (2-3 fragments per well) and transfected with Adenovirus mCherry (Ad-mCherry) as previously described (García-Pascual *et al.*, 2015). Briefly, fragments were incubated with Ad-mCherry (1·10⁸ plaque-forming units (PFU)/mL) diluted in antibiotic-free DMEM F-12 (Life Technologies) and 10% FBS (FBS Gold; PAA) medium overnight (12–18 h) at 37 ºC, with 5% CO₂ inside an incubator with gentle agitation. Tissue fragments were then rinsed with DMEM F12 twice and then replaced with fresh DMEM F12 medium containing 1% antibiotics (streptomycin and penicillin), fungizone (1 mg/mL) (Gibco), and 10% FBS. Fluorescence was observed in the red channel (568 nm) with the use of an inverted microscope (Eclipse; Nikon) and used to select 40–50 endometrial fragments per biopsy with optimal signal for subsequent engraftment.

**Generation of an Endometriosis Mouse Model**

A total of 24 six-week-old athymic nude female mice (Charles River Laboratories International) were used in this study. Mice were housed in pathogen-free conditions at 26 ºC with a 12-h light–12-h dark cycle and fed *ad libitum*. To avoid cycle-dependent variations, animals were ovariectomized and hormone levels were homogenized by 60-day-release pellets containing 18 mg of 17b-E2 (Innovative Research of America, USA) placed under the neck skin of ovariectomized mice. One week after surgery, four mCherry-labeled human endometrium fragments (each approx. 3 mm³) were implanted together in the peritoneum of each animal with a n-butyl-ester cyanoacrylate adhesive (3M Animal Care) to form a single endometriotic lesion (Figure 1). To allow for homogeneous distribution of human tissue, each biopsy (n=4) contributed one fragment per mouse to the engraftment. This study was approved by the Institutional Animal Care Committee at the University of Valencia (2017-16), and all
procedures were performed following the guidelines for the care and use of mammals from the
National Institutes of Health.

**Pharmacologic Interventions**

One week after tissue implantation, animals were randomly divided into three groups (n=8 per
group): 1) 50 mg/kg/week of Tween20 (vehicle group); 2) 200 mg/kg/week of BKS0349
(experimental group); and 3) 50 µg/kg/day of Cabergoline (Cb2) (positive control group).
Vehicle and different drugs were diluted in phosphate-buffered saline (PBS; Sigma-Aldrich,
USA). Vehicle and BKS0349 were administered via 100 µL tail vein injections once a week for
4 weeks and Cb2 was orally administrated every day for 21 days (Figure 1). Cb2 was chosen as
a positive control based on its effects on endometriosis observed in our previous study (Novella-
Maestre *et al.*, 2009). Mouse behavior and weight were monitored daily with no noticeable
variation (Figure 1). BKS0349 is a specific KIF20A inhibitor developed and synthesized by
Biokinesis (Biokinesis SAS, Paris, France, unpublished data). It is formulated as a
nanosuspension at 50 mg/mL in water with 12.5 mg/mL of Tween20.

**In Vivo Fluorescence Imaging**

Endometriotic lesions generated from mCherry-labeled endometrial tissue implanted into mice
were monitored over time using an IVIS Spectrum Preclinical *In Vivo* Imaging System (Perkin-
Elmer) and related software coupled to an isoflurane gas anesthesia machine (XG-8 Gas
Anesthesia System; Xenogen). Immunofluorescence images were acquired by epiluminescence
with a peak absorption/emission pair filter set at 587 nm and 610 nm, respectively. The field of
view was set at 10 cm until the maximum intensity was obtained as previously described
(García-Pascual *et al.*, 2015). Fluorescence was monitored twice weekly from the first day of
treatment (D0) until 72 hours after the end of treatment (D24) (Figure 1).

**Quantification of In Vivo Fluorescence Images**

Images were displayed as false-color photon counts superimposed on a grayscale anatomic
image with the optical intensity (photon flux) expressed as the average radiant efficiency in
photons/s/cm² as previously described (García-Pascual et al., 2015). Regions of interest (ROIs) corresponding to endometriotic lesions were automatically established by the software after manually setting a threshold over the lesion showing minimal intensity. Background fluorescence was automatically calculated and subtracted from the ROI data. Signals from each day were normalized relative to D0 as 100%.

**Recovery and Preprocessing of Lesions**

After 21 days of treatment, animals were euthanized via CO₂ inhalation, the peritoneal cavity was accessed, and a visual examination was performed. Lesions were recovered and examined macroscopically by scale precision caliper before being fixed in 4% neutral-buffered formalin overnight at 4 °C, embedded in paraffin, and cut into 3 µm sections for histologic, immunohistochemical, and immunofluorescent characterization or RNA extraction for gene expression analysis. The presence of gland-like structures mimicking human eutopic and ectopic endometrial tissue was confirmed with hematoxylin-eosin staining.

**Evaluation of Cellular Proliferation and KIF20A expression**

KIF20A expression and cell proliferation were assessed by immunohistochemistry for KIF20A (sc-374508, Santacruz Biotechnologies, USA) and Ki67 (AB9260, Millipore, USA), respectively. DAB staining was performed with an EnVision®+ Dual Link System-HRP (DAB+) Kit (Agilent, CA, USA). Briefly, after deparaffinization, antigen retrieval was performed with citrate buffer at 1 mM, pH 6, at 95 °C for 10 min and permeabilization was carried out with 1% PBS Normal Goat Serum (NGS) and 0.4% Triton X for 10 min. Slides were then blocked with Dual Endogenous Enzyme Block for 15 min and the primary antibody anti-Ki67 (5 µg/mL) or anti-KIF20A (4 µg/mL) were applied and incubated overnight at 4 °C. Subsequently, slides were covered with labeled-polymer horse radish peroxidase and incubated for 30 min. Afterward, slides were incubated with 3,3’-diaminobenzidine for 10 min and counterstained with hematoxylin. Samples were visualized and analyzed using a Nikon Eclipse
80 (Japan). Cellular proliferation, visualized by Ki67 expression, was quantitatively assessed with Image ProPlus (Media Cybernetics, USA).

**TUNEL Assay**

Apoptosis in endometriotic lesions from different experimental groups was evaluated with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining using fluorescein-labeled cell marker TMR red *in situ* cell death detection kit (Roche, Switzerland) according to the manufacturer’s instructions. Cell nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI, Life Technologies, USA). Samples were visualized and analyzed using fluorescence microscopy ZEISS-Axio Vert. A1 (Germany). Four fluorescence images per endometriotic lesion were quantitatively assessed with Image ProPlus (Media Cybernetics, USA) by analyzing each TUNEL image with its corresponding DAPI image and thereby, TUNEL signal was normalized with its corresponding background.

**qRT-PCR Analysis**

Total RNA from endometriotic lesions embedded in paraffin was extracted using a RNeasy Formalin-Fixed Paraffin-Embedded (FFPE) mini Kit (Qiagen, Germany) and cDNA was synthesized using a PrimeScript RT Reagent kit (Takara, Japan). *CCND1* gene expression, which is a G1 phase-related gene that provide information about cell cycle status, was measured by quantitative real-time polymerase chain reaction (qRT-PCR) using the following primers: 5’-TGGTGAACAAGCTCAAGTG-3’ (forward) and 5’-TTCATTTCCAATCCGCCC-3’ (reverse). The reaction was carried out using the StepOnePlus System (Applied Biosystems, CA) and PowerUp SYBR Green (ThermoFisher, MA). Qiagen Data Analysis Software was used to calculate fold regulation using *GAPDH* expression for normalization.

**Statistical Analysis**

GraphPad Prism 6.0 was used for statistical analyses and graphics generation (San Diego, CA, US). Data are presented as mean ± standard deviation (SD). A Wilcoxon test was performed for ROI analysis from different days between groups, immunohistochemistry of KIF20A and Ki67,
and TUNEL analysis in endometrial lesions. A paired t-test was performed for ROIS analysis from different days within the same group. Gene expression analyses of *CCND1* was carried out with Qiagen Data Analysis Software applying Student’s t-test. P value < 0.05 was considered statistically significant.

**RESULTS**

**Assessment of Endometriotic Lesions by Fluorescence Lifetime Imaging**

Endometriotic lesion size was determined by *in vivo* monitoring using fluorescence lifetime imaging (Figure 2A). We observed that the intensity of the fluorescent signals from endometriotic fragments in all groups were similar at the beginning of the experiment (D0) and normalized the signals from each day to the signal on D0. After the first week of treatment, a uniform reduction in the fluorescent signal was observed in all groups over time, as expected in accordance with the episomal and transient expression of adenoviral particles. However, this reduction was not pronounced in the control group, in which an increase was observed from day 17 to day 24 (Figure 2B), demonstrating an increase in endometriotic lesion growth. While the fluorescent signal continued to decline in treated groups, showing a significant decrease on D24 in the BKS0349 group (*p* value = 0.0313) and from D14 on the Cb2 group (*p* value = 0.0313 on days 14-21; *p* value = 0.0156 on day 24) compared to D0 (Figure 2C-D). In addition, when the signal intensity was compared between groups at different time points, a significant decrease was observed at 72 hours after the end of treatment (D24) in the BKS0349 group compared to the control group (40 ± 19.3% versus 81 ± 30.7%; mean ± SD n=8, *p* value = 0.0303) (Figure 2E). Differences in fluorescence intensity between the control and Cb2 groups, although noticeable (52 ± 31.2% versus 81 ± 30.7%; mean ± SD n=8), were not statistically significant at the end of the treatment period (Figure 2E).
Macroscopic and Histological Evaluation of Endometriotic Lesions

Seventy-two hours after the end of treatment, animals were euthanized, and lesions were recovered. Subsequently, lesion size on D24 was measured using a scale precision caliper. We found a statistically significant size reduction in endometriotic lesions in the BKS0349 group (0.073 mm² ± 0.022; mean ± SD n=8, p value = 0.0006) and in the positive control group (Cb2) (0.074 mm² ± 0.042; mean ± SD n=8, p value = 0.0260) compared to the control group (0.128 mm² ± 0.019; mean ± SD n=8) (Figure 3A-D). Moreover, histological examination of lesions revealed the presence of gland-like structures resembling human endometriotic lesions, confirming that human endometrial fragments were successfully implanted in mice (Figure 3E-G).

Cell Proliferation in Endometriotic Lesions

First, we corroborated that BKS0349 treatment effectively inhibits KIF20A protein in endometriotic lesions generated in our animal model. As expected, immunohistochemical analysis showed a significant reduction of KIF20A protein expression in the BKS0349 group compared to the control group (10 ± 1.4% versus 11 ± 3.5%; mean ± SD n=8, p value = 0.0258) and showed a reduction of KIF20A protein in the Cb2 group compared to controls 9 ± 1.6% versus 11 ± 3.5%; mean ± SD n=8, p value = 0.004, respectively) (Figure 4A-D). These results indicate that BKS0349 effectively inhibits KIF20A protein expression in endometriotic lesions.

Considering that KIF20A regulates cell proliferation due to its important role in cytokinesis, suppression of KIF20A could inhibit cell proliferation and thereby endometriotic lesion growth. For this reason, we assessed cell proliferation in the generated lesions from different animal groups by Ki67 immunohistochemistry and observed a significant reduction in proliferating cells in the BKS0349 group compared to the control group (4 ± 3.4% versus 9 ± 3.8%; mean ± SD n=8, p value = 0.0082) (Figure 4E-H) that was even more pronounced than in the positive control group (Cb2) (6 ± 5.8%; mean ± SD n=8). Finally, to assess whether KIF20A inhibition can cause cell cycle arrest in the G0/G1 phase, we analyzed expression of CCND1, a G1 phase-related gene. We observed that KIF20A inhibition significantly decreased CCND1 expression in
the BKS0349 group compared to the control group (Fold change = 0.2594; p value = 0.049) (Figure 4I).

Apoptosis in Endometriotic Lesions

A TUNEL assay was used to determine whether KIF20A inhibition can increase apoptosis in the endometriotic lesions in our animal model. Endometriotic lesions in the control group were practically devoid of apoptotic cells (25 ± 13.3%; mean ± SD n=8) (Figure 5A, D, G), while a statistical significant increase in apoptotic cells was observed in endometriotic lesions in the BKS0349 group (40 ± 23.0%) (Figure 5B, E, H, J). We also found an increase in apoptotic cells in the Cb2 group (38 ± 24.9%) (Figure 5C, F, I, J), although this increment was not significant.

Discussion

Accumulating evidence suggests that overexpression of KIF20A may confer a malignant phenotype to certain tumors (Kikuchi et al., 2003; Claerhout et al., 2011; Imai et al., 2011; Ho et al., 2012; Shi et al., 2016; Zhao et al., 2018), including ovarian tumors (Li et al., 2018), by promoting cell proliferation and inhibiting apoptosis. KIF20A downregulation inhibits cell proliferation and invasion, induces cell cycle arrest, and promotes apoptosis (Wang et al., 2017). Several studies have reported that endometriosis exhibits immunohistological and molecular alterations similar to those observed in ovarian tumors (Komiyama et al., 2018). However, no data are currently available regarding the role of KIF20A in endometriosis. This study describes for the first time the effect of KIF20A inhibition by a KIF20A-specific higher-affinity inhibitor derived from Paprotrain (Tcherniuk et al., 2010; Labrière et al., 2016), BKS0349, in endometriotic lesion development and highlights the possible role of KIF20A in the pathophysiology responsible for the establishment, development, and course of endometriosis.
In vivo monitoring of endometriotic lesions generated in our xenograft animal model showed a significant decrease in these lesions after BKS0349 treatment as well as controls. These findings suggest that KIF20A inhibition by BKS0349 leads to a significant reduction in endometriotic lesion size. In addition, macroscopic examination of endometriotic lesions after different treatments demonstrated that KIF20A inhibition by BKS0349 reduced endometriotic lesion size compared to the control group, even more than our positive control (Cb2), whose efficiency in decreasing endometriotic lesion size was previously described by our group (Novella-Maestre et al., 2009; Delgado-Rosas et al., 2011). These findings suggest that KIF20A inhibition produces the same significant size reduction in endometriotic lesions as occurs in cancers in which KIF20A downregulation significantly inhibits tumor development (Taniuchi et al., 2005; Wang et al., 2017; Zhao et al., 2018).

After in vivo evaluation, we corroborated in the endometriotic lesions generated that BKS0349 inhibited KIF20A expression. Surprisingly, we observed an inhibitory effect of Cabergoline on KIF20A that had never been described in endometriosis. This unexpected finding could bring a greater understanding about the mechanisms involved in the reduction of endometriotic lesions induced by dopamine agonists previously reported (Novella-Maestre et al., 2009). However, it is premature to conclude that dopamine agonists inhibit cytokine expression based on our results and further investigations should be performed to confirm the possible inhibitory effect of Cabergoline on cytokines expression.

Several cancer studies have suggested that the decrease in tumor growth induced by KIF20A downregulation could be due to the alteration of cell cycle regulation (Yan et al., 2012; Pishas et al., 2015; Wang et al., 2017; Youns and Abdel Halim Hegazy, 2017; Zhao et al., 2018), which affects cell proliferation, cell division and invasion (Wang et al., 2017). Therefore, we focused on cell proliferation in the endometriotic lesions generated in mice subjected to BKS0349 treatment compared to no treatment or to Cb2 treatment, which has proven efficacy in endometriotic lesion size reduction (Novella-Maestre et al., 2009). We demonstrated that KIF20A inhibition by BKS0349 significantly decreased cell proliferation in endometriotic
lesions generated, even more than Cb2 treatment, whose inhibitory effect on cell proliferation was previously demonstrated (Novella-Maestre et al., 2009), although this time was not statistically significant. In addition, KIF20A inhibition is reported to suppress expression of cell cycle genes, including cyclins (CCND1, CCNE1, TP21, and TP27), and thereby, induces cell cycle arrest in the G0/G1 phase and suppresses the cell cycle transition from the G0/G1 phase to the S phase, inhibiting cell proliferation (Zhao et al., 2018).

Cyclins function as regulators of the cell cycle. CCND1 forms a complex that functions as a regulatory subunit of cyclin-dependent kinase 4 (CDK4), or CDK6, whose activity is required for the G1/S transition (Lamb et al., 2003). To determine whether KIF20A inhibition can cause a cell cycle arrest in the G0/G1 phase, we assessed the expression of CCND1 in the endometriotic lesions generated in our mouse model. We observed that CCND1 expression was significantly decreased following BKS0349-mediated inhibition of KIF20A, suggesting that KIF20A inhibition appears to cause a cell cycle arrest at the G0/G1 phase in the endometriotic lesion, which consequently inhibits cell proliferation to reduce endometriotic lesion growth.

Several in vitro and in vivo studies have demonstrated that the inhibition of KIF20A contributes to cell apoptosis induction in cancer cells (Saito et al., 2017; Geng et al., 2018; Kawai et al., 2018). We found the same trend in our study, increasing the apoptosis rate in endometriotic lesions from animals treated with BKS0349 compared to controls. These findings also support previous studies in which apoptosis induction was demonstrated in cancer cells with KIF20A expression inhibited (Yan et al., 2012; Pishas et al., 2015; Saito et al., 2017; Youns and Abdel Halim Hegazy, 2017; Geng et al., 2018; Kawai et al., 2018).

In conclusion, data from the current study suggests that KIF20A inhibition by BKS0349 induces apoptosis and inhibits cell proliferation by cell cycle arrest at the G0/G1 phase and consequently reduces endometriotic lesion size in a xenograft mouse model of endometriosis. These findings imply that KIF20A expression could be an independent prognostic biomarker for endometriosis and may be useful as a novel therapeutic target for treating endometriosis due to its important role in cell cycle regulation and apoptosis. In this context, due to the suggested relevance of
KIF20A expression in endometriosis development, more extensive research of KIF20A expression levels in different types of endometrial tissues and functional studies such as KIF20A inhibition on proliferation, migration, invasion, and related mechanisms in endometrial cells would be required. In addition, further studies to determine the mechanism through which BKS0349 reduces cell proliferation and induces apoptosis in endometriotic lesions, as well as analyze the effects observed in these lesions in other tissues would be necessary before clinical translation to humans.

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Authors’ roles

H.F. was involved in study design, executed experiments, and wrote and edited the manuscript. A.C. was involved in experimental execution and wrote the manuscript. A.Q. were involved in animal model development as well as functional analysis. C.B. and P.P. gently provided BKS0349 and edited the manuscript. A.P. devised and supervised the study, contributed to data interpretation, and drafted the manuscript. F.D. coordinated the study design, contributed to data interpretation, and edited the manuscript. All authors reviewed the manuscript and provided critical feedback and discussion.
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Conflict of interest

The authors have no conflicts of interest to declare.
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Figure Legends

**FIGURE 1. Experimental design of endometriosis mouse model.** Six-week-old athymic nude female mice were ovariectomized and implanted with 60-day E2-release pellets. Subsequently, mCherry-labeled human endometrium fragments were implanted in the peritoneum of each animal. One week after tissue implantation, mice were divided into three treatment groups (n=8/group). Vehicle and BKS0349 were given by tail vein injection once a week, and Cabergoline (Cb2) was orally administered every day for 21 days. mCherry-labeled endometriotic lesions were monitored twice weekly from the start of treatment (D0) until 72 hours after the treatment stopped (D24) using an IVIS Spectrum Preclinical *In Vivo* Imaging System. After 21 days of treatment, mice were euthanized, and lesions were recovered.
FIGURE 2. *In vivo* monitoring of endometriotic lesions. Examples of the fluorescent signaling provided by mCherry-labeled endometriotic implants in control, BKS0349, and Cabergoline (Cb2) treated animals (n=8/group) (A). Quantitative analysis of mean ± SD fluorescence intensity provided by lesions generated in the control group (B), BKS0349 group (C), and the Cb2 group (D) from day 0 (D0) to D24 and in the three groups (E). Fluorescence intensity was expressed as the photon flux (photons/s) and normalized to the value on D0. * p value < 0.05; ** p value 0.01 compared to D0 (B,C,D) or control (E).
FIGURE 3. Effect of BKS0349 on macroscopic and histologic features of endometriotic lesions. Macroscopic observations and histologic evaluation of endometriotic lesions generated in vehicle-treated control (A, E), BKS0349-treated (B, F), and Cb2-treated (C, G) mice at the end of the 21-day treatment period (n=8/group). Lesion size is represented in mm². Black scale bars = 5 mm and white scale bars = 50 µm. * p < 0.05; ** p < 0.01.
FIGURE 4. Effect of BKS0349 on cell cycle and cell proliferation in endometriotic lesions.

Immunohistochemical staining of KIF20A protein in control (A), BKS0349 (B), and Cb2 (C) groups. Quantitative analysis of the percentage of KIF20A protein expression in the different groups (D), expressed as mean ± SD. Immunohistochemical staining of Ki67 (proliferation marker) in control (E), BKS0349 (F), and Cb2 (G) groups. Quantitative analysis of the percentage of Ki67-positive cells in the different groups (H) expressed as mean ± SD. CCND1 gene expression in each group (I) expressed as fold change. Red arrow shows example of positive staining. Scale bars = 50 µm. *p < 0.05.
FIGURE 5. Effect of BKS0349 on apoptosis in endometriotic lesions. Analysis of apoptosis by TUNEL staining in endometriotic lesions from different treatment groups. DAPI nuclear staining (blue) was used to stain nuclei (A, B, C). Apoptotic cells were identified by labeling DNA breaks induced by DNase I (red color) (D, E, F). Merge of TUNEL images in control (G), BKS0349 (H), and Cb2 (I) treatment groups. Quantitative analysis of the percentage of apoptotic cells in the different groups (J). Data are expressed as mean ± SD. Scale bars = 50 µm. * p < 0.05.