Inhibiting NTRK2 signaling causes endometriotic lesion regression

Hsiu-Chi Lee¹, Shih-Chieh Lin², Meng-Hsing Wu¹ and Shaw-Jenq Tsai^{2,3}

¹Department of Obstetrics and Gynecology, College of Medicine, National Cheng Kung University, Tainan, Taiwan,

²Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan and

³Department of Physiology, College of Medicine, National Cheng Kung University, Tainan, Taiwan

Correspondence should be addressed to S-J Tsai; Email: seantsai@mail.ncku.edu.tw

Abstract

Endometriosis is a common gynecological disease in reproductive-age women. Although the hormone-dependent therapy is the first line treatment for endometriosis, it is not a curative regimen and associated with severe side-effects, which significantly decrease the life quality of affected individuals. To seek a target for treatment of endometriosis, we focused on plasma membrane proteins that are elevated in ectopic cells and exert beneficial effects in cell growth and survival. We performed bioinformatics analysis and identified the neurotrophic receptor tyrosine kinase 2 (NTRK2) as a potential candidate for treatment. The expression levels of NTRK2 were markedly upregulated in the lesions of clinical specimen as well as in the mouse endometriotic-like lesion. Mechanistic investigation demonstrated that upregulation of NTRK2 is induced by hypoxia in a hypoxia-inducible factor 1 alpha-dependent manner. Knockdown of NTRK2 or administration of ANA-12, a selective antagonist of NTRK2, significantly induced endometriotic stromal cells death, suggesting it may be a potential therapeutic agent. *In vivo* study using surgery-induced endometriosis mice model showed ANA-12 (1.5 mg/kg body weight) treatment induced apoptosis of endometriotic cells and caused the regression of ectopic lesions. Taken together, our findings suggest a possible mechanism responsible for the aberrant expression of NTRK2 in endometriotic lesions and this may be involved in the pathogenesis of endometriosis.

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Introduction

Endometriosis is a common gynecological disease defined as the growth of endometrial tissue outside of the uterus. About ten percent of women are persecuted by endometriosis. The clinical symptoms of this disease are chronic pelvic pain, dyspareunia, dysmenorrhea, and infertility; however, the exact cause of endometriosis is complex and largely unclear. Presently, the hormone-based treatments and surgical intervention are two main options for relieving the symptoms (Ferrero *et al.* 2018). Nevertheless, there are significant unmet needs due to the unfavorable side effects accompanied with the hormonal medication and the high recurrence after the surgery.

The etiology of endometriosis is incompletely understood, nevertheless, the retrograde menstruation theory has been proposed since 1927. In the menstrual period, endometrial cells flow back through the fallopian tubes and into the peritoneal cavity (Sampson 1927). To date, numerous evidences indicate that hypoxic peritoneal cavity and various growth factors, including cytokines and estrogen in the peritoneal fluid, can facilitate ectopic cell growth in the peritoneal cavity (Oosterlynck *et al.* 1994, Pizzo *et al.* 2002, Bulun 2009,

Wu *et al.* 2010, Young *et al.* 2013, Hsiao *et al.* 2015). Thus, characterizing mediators in the peritoneal fluid and peritoneal tissues may provide valuable information to identify potential candidate genes contributing to the pathophysiological processes of endometriosis. However, the components of peritoneal fluid are too complex to dissect mechanisms leading to the development of endometriosis. Therefore, an alternative way is to identify gene expression change between the eutopic and ectopic cells to find critical genes involved in promoting endometriosis development.

Neurotrophic receptor tyrosine kinase 2 (NTRK2), also known as tropomyosin receptor kinase B (TrkB), is a membrane-bound receptor for brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NTF-4). Upon the ligand binding, NTRK2 homodimerizes, autophosphorylates itself, and activates the downstream cascades. NTRK2 not only involves in the neuron differentiation through the GRB2-Ras-MAPK cascade but also regulates growth and survival through multiple signaling pathway (Yang *et al.* 2019). NTRK2 is expressed in many tissues, especially in brain tissues. It has been reported that heterozygous mutations of *NTRK2* can cause neurodevelopmental disorder such as early infantile epileptic encephalopathy-58 (EIEE58)

(Hamdan *et al.* 2017), and a disease named obesity, hyperphagia, and developmental delay (Yeo *et al.* 2004, Miller *et al.* 2017). The fusion of *NTRK2* with *QKI* due to chromosome translocation, t(6;9), results in the formation of pilocytic astrocytoma (Jones *et al.* 2013). Genetically engineered mouse study showed that mutations on different sites of *Ntrk2* possess unique and conserved biological functions in taste system (Koudelka *et al.* 2014). Moreover, the variants (Luberg *et al.* 2010) and SNP (Velez Edwards *et al.* 2013, Metrustry *et al.* 2014) also contribute to the complexity of the molecular functions and pathological impacts *NTRK2* exerts.

The expression and function of BDNF/*NTRK2* in reproductive system and related diseases were less investigated. BDNF mRNA was found to be increased in ovarian endometrioma (Borghese *et al.* 2010) but the protein level was not different in peritoneal fluid of women with endometriosis (Barcena de Arellano *et al.* 2013). In addition, it was reported that the serum mature BDNF is not a useful biomarker for endometriosis (Perricos *et al.* 2018). Previously, increased *NTRK2* expression was observed in the deep infiltrating endometriosis (Dewanto *et al.* 2016). Recent study indicates that *NTRK2* is increased in the secretory endometrium of women with adenomyosis and positively correlates with the serum CA125 concentration and dysmenorrhea (Huang *et al.* 2011). However, the function of *NTRK2* in endometriosis remains unclear.

In this study, we demonstrated that *NTRK2* expression was elevated in the ectopic endometriotic tissues and endometriotic stromal cells. In addition, we revealed the functional characteristics of *NTRK2* in the endometriosis. Furthermore, we showed that inhibition of *NTRK2* could be the alternative for treatment of endometriosis.

Materials and methods

Clinical specimens

Endometrial and endometriosis tissues were obtained from the Department of Obstetrics and Gynecology of National Cheng Kung University Hospital under a procedure approved by the Clinical Research Ethics Committee at the National Cheng Kung University Medical Center. Endometriosis was confirmed via the clinical diagnosis and pathology examination by a gynecologist and a pathologist. The revised American Society for Reproductive Medicine scoring system has been followed to classify the stages of endometriosis. Fifty-nine endometriosis specimens (paired tissues=26, non-paired endometriotic tissues=33) were obtained from women with mild endometriosis (stage II), moderate (stage III) or severe (stage IV) endometriosis (Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article). Written informed consent was obtained from each patient.

Cell culture and treatments

Experiments were performed in the isolated stromal cells from the endometrial and endometriotic tissues. The procedure for isolation of stromal cells was as previously described (Lin *et al.* 2019). In brief, tissues were digested with type IV collagenase (2 mg/mL) and DNase I (100 µg/mL) in PBS and shaken with 250 g for 60 min at 37°C. The stromal cells were selected through 70 µm pore size and then 40 µm pore size nylon cell strainer. Filtered cells were allowed to attach for 30 min in a T-75 flask. After 30 min, the tissue debris, blood cells and epithelial cells were removed by washing with PBS. For the purity, we routinely stained the isolated endometrial stromal cells with vimentin (positive marker) and keratin (epithelial cell marker for negative control) antibodies. Cells were cultured in DMEM/nutrient mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 1× GlutaMAX (Life Technologies, Inc) and 1× Antibiotic-Antimycotic Solution (Caisson Laboratories, Inc, North Logan, Utah) in a humidified atmosphere with 5% CO₂ at 37°C.

In the cytokines and estradiol (E₂) stimulation experiment, eutopic stromal cells were cultured in the phenol red-free DMEM/F12 medium supplemented with charcoal-stripped 1% FBS and various stimuli for 24 h. In the hypoxia treatment, eutopic stromal cells were cultured with DMEM/F12 supplemented with 10% FBS in a hypoxia chamber (1% O₂, 94% N₂) for 24 h.

Bioinformatics analysis

The expression profiles of endometriosis were downloaded from gene expression omnibus (GEO). We used the GSE23339 (Hawkins *et al.* 2011) and the GSE25628 (Crispi *et al.* 2013) datasets. The data were first analyzed using GEO2R for comparing the normal and endometriosis groups. After analysis, results were saved in a txt file format. The txt file was opened by excel and then selected the upregulating genes ($P < 0.05$, fold change ≥ 1.5). On the other hand, human genes with receptor activity were downloaded from AmiGO2 (Munoz-Torres & Carbon 2017) by using gene ontology term 'signaling receptor activity' (ID: GO:0038023, Alt ID: GO:0004872) with the following filters: organism: Homo sapiens, contributor: Uniprot. A total of 1289 genes were retrieved. Upregulated genes from two different endometriosis datasets and gene list of receptor were uploaded to a website tool (the Venn diagrams tool; <http://bioinformatics.psb.ugent.be/webtools/Venn/>) and the intersection of three datasets (GSE23339, GSE25628 and receptor gene list) were generated. The P values of the interest genes were calculated by an R package, *SuperExactTest* (<https://cran.r-project.org/web/packages/SuperExactTest/index.html>) (Wang *et al.* 2015). To evaluate the expression of *NTRK2*, another dataset GSE7305 (Hever *et al.* 2007) that contains mRNA expression levels of normal and diseased endometrial tissues were also downloaded for analysis.

Small interfering RNA (siRNA) knockdown

Eutopic stromal cells were transfected with either control siRNA or siRNA targeted to HIF-1α using Lipofetamine 2000

according to the manufacturer's instructions. After 24 h incubation, the cells were incubated under either normoxia or hypoxia (1% O₂) for another 24 h. Cells were then harvested for evaluation of mRNA expression level.

Ectopic stromal cells were transfected with either control siRNA or NTRK2 siRNA using Lipofetamine 2000, and then plated at a density of 2×10^5 cells per well in the six-well plate for further cell apoptosis assay.

RNA isolation and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

Total RNA was prepared from the cells according to the manufacturer's instructions (TRIpure; Bioline, London, United Kingdom). Five hundred nanogram of total RNA was reverse transcribed into cDNA and then quantified using real-time PCR to determine the gene expression levels. FastStart Universal SYBR Green Master (Roche) and the specific primers were used to amplify genes of interest. The 18S rRNA was used for normalization. The sequences of the primers used were: NTRK2: forward 5'-GGGACACCACGAACAGAAGT-3'; reverse 5'-CACCACAGCATAGACCGAGA-3'; BDNF: forward 5'-ATTCAGCCCATTTACACCT-3'; reverse 5'-TTTGCTTATCCCTCACCCTAC-3'; 18S rRNA: forward 5'-GTGTGCTACCCTACG-3'; reverse 5'-TGACCCGCACTTACTG-3'.

Immunocytochemistry

Sections of paraffin-embedded endometria were processed according to standard protocol. The sections were deparaffinized and rehydrated with xylene and serial ethanol (100, 95, 80, and 70%) followed by washing in deionized water. Sections underwent antigen retrieval in citric buffer (10 mM, pH 6.0) and immersed in Protein Block from Novolink Polymer Detection Systems (Leica Biosystems) for 1 h at room temperature to block nonspecific binding. Primary antibody against NTRK2 (1:250, Santa Cruz) or phosphorylated NTRK2 (pTyr515) were incubated with sections in humidified chamber at 4°C overnight. If the host of primary antibody is mouse, then the post primary block was added for 1 h at room temperature, followed by incubation with Novocasta polymer for 1 h at room temperature. If the host of primary antibody is rabbit, the sections were directly incubated with Novocasta polymer for 1 h at room temperature. Sections were washed and then developed by PolyDetector Liquid AEC HRP (Bio SB, Santa Barbara, California). The slide was counterstained with hematoxylin. Tissue sections were mounted on coverslips using glycerol gelatin mounting medium (Sigma).

For evaluating the staining, Histoscore (H-score) was calculated by a semi-quantitative assessment of staining intensities in positively-stained stromal cells ($3 \times$ percentage of strongly staining + $2 \times$ percentage of moderately staining + percentage of weakly staining nuclei, giving a range of 0 to 300) performed by two individuals. Or, the values were calculated by ImageJ, which assessed the staining area and intensity. It was showed as (positive staining area $\times$ intensity)/total area.

Apoptosis assay

Ectopic cells were treated with siRNA or different doses of ANA-12 (Sigma). After the treatment, both the floating and adhesive cells were collected and stained with annexin-V (BioLegend, San Diego, California) for 20 min at room temperature in dark. The annexin-V positive cells were determined using FACSCanto II Flow Cytometer (BD, Franklin Lakes, New Jersey) and analyzed using FlowJo® software (FlowJo, Ashland, Oregon).

Mouse model of endometriosis

Female C57BL/6NCrj mice (8–10 weeks old) were purchased from the Animal Center at the College of Medicine, National Cheng Kung University. To establish the mouse model of endometriosis, an estrogen capsule was imbedded in the female mouse back for 2 days. After E₂ stimulation, one side of the uterus was removed and divided into four equal-size pieces. Then the fragmented uterus was inoculated on the peritoneal wall in the same mouse for 1 month. Mice were then grouped randomly to receive vehicle or 1.5 mg/kg body weight ANA-12 compound intraperitoneally three times a week for additional 3 weeks. The weights of ectopic lesions in both groups were measured.

TUNEL assay

In vivo apoptosis detection was performed in the endometriotic-like lesions according to the manufacturer's instructions (Abcam plc). Sections of paraffin-embedded lesions were processed according to standard protocol. The sections were deparaffinized and rehydrated with xylene and serial ethanol (100, 95, 80, and 70%) followed by washing in 1× PBS. Permeabilization of specimen was performed by using proteinase K. Following by inactivation of endogenous peroxidases and equilibration, and then TdT labeling reaction was performed at room temperature for 1.5 h. After incubation, the entire specimen was covered by stop buffer and incubated at room temperature for 5 min followed by incubating with blocking buffer for 10 min. After blocking, 1× conjugate was added to the specimen and incubated for 30 min. DAB solution was used for color development and hematoxylin was used for counterstain. Tissue sections were mounted on coverslips using glycerol gelatin mounting medium (Sigma).

Statistical analysis

Results are expressed as mean $\pm$ S.E.M. and were analyzed using GraphPad Prism version 6 (GraphPad Software). Student's *t*-test was used to compare two groups or one-way ANOVA was used to compare three or more groups. The post-test analysis was performed using Dunnett's multiple comparisons test. A *P* value less than 0.05 was considered statistically significant.

Results

NTRK2 is increased in the endometriotic lesions

To screen a target candidate for treatment of endometriosis, we analyzed the gene expression omnibus (GEO)

datasets and cell membrane receptors list in view of the important role of cellular stimulus responses. We cross-referenced the upregulated genes in two GEO (GSE23339 and GSE25628) datasets and cell membrane receptors list (Fig. 1A). Nineteen candidates overexpressed in the endometriotic specimens were identified. NTRK2 is one of the candidates involved in the process of cell survival and proliferation. In addition, we further verified the expression of NTRK2 in another endometriosis GEO dataset (GSE7305). The results of the three GEO datasets (GSE23339, GSE25628, and GSE7305) indicated that NTRK2 was increased in the endometriotic lesions (Fig. 1B). Therefore, we hypothesized that NTRK2 may play a role in the development of endometriosis.

To determine whether NTRK2 signaling is involved in endometriosis pathogenesis, we quantified the expression levels of NTRK2 and its ligand, BDNF in eutopic and ectopic endometriotic cells. The results demonstrated that expression of NTRK2 and BDNF was elevated in the ectopic lesions (Fig. 1C) and primary culture endometriotic stromal cells (Fig. 1D) as compared to the eutopic counterparts. We further conducted the immunohistochemical staining for NTRK2 in clinical specimen. The results demonstrate that expression of NTRK2 was significantly increased in the ectopic lesions (Fig. 1E) even when paired samples were compared (Fig. 1F). Furthermore, the elevated level of NTRK2 was independent of menstrual cycle (Fig. 1G). These results indicated that BDNF-NTRK2 signaling cascade may play a role in the development of endometriosis.

Elevated expression of NTRK2 in the ectopic stromal cell was mediated by hypoxia

We then investigated the underlying mechanisms responsible for NTRK2 overexpression in endometriotic cells. As endometriosis is an inflammatory and estrogen-dependent disease, we treated endometrial stromal cells with proinflammatory cytokines (including IL-1 β , TNF α , TGF β , and IL-6) and estradiol (E $_2$). However, these stimuli did not increase the expression level of NTRK2 (Fig. 2A). We then examined whether aberrant expression of NTRK2 is mediated by hypoxia since it has been implicated to play critical roles in endometriosis (Hsiao et al. 2015, Wu et al. 2019). The result showed that the expression level of NTRK2 was elevated under hypoxic condition (Fig. 2B). In addition, knockdown of hypoxia inducible factor 1 α (HIF-1 α) attenuated hypoxia-induced NTRK2 expression (Fig. 2C), indicating hypoxia induces NTRK2 is mediated at the transcriptional level.

Inhibition of NTRK2 signaling induces apoptosis of the ectopic stromal cells

To understand the effect of the increased NTRK2 expression in the ectopic stromal cells, NTRK2 was knocked down by siRNA and cells were cultured for

48 h. The data showed that knockdown of NTRK2 increased number of apoptotic cells (Fig. 3A). Next, we treated ectopic endometriotic stromal cells with serial doses of ANA-12, a selective inhibitor for NTRK2 but not NTRK1 or NTRK3 (Cazorla et al. 2011), and found ANA-12 significantly induced apoptosis at the dose of 25 μ M (Fig. 3B).

Administration of NTRK2 causes endometriotic lesion regression in mouse model of endometriosis

To examine the potential of ANA-12 for treatment of endometriosis, mouse model of endometriosis was employed (Fig. 4A). Level of NTRK2 is dramatically upregulated in ectopic lesions of mice (Fig. 4A). Treatment with ANA-12 (1.5 mg/kg body weight) reduced the volume of endometriosis-like lesions as compared to vehicle-treated group (Fig. 4B). We determined the phosphorylated NTRK2 (Tyr515), the activated NTRK2, in the ectopic lesions and found the ANA-12 treatment tended to decrease the phosphorylation of NTRK2 (Fig. 4C). The *in situ* apoptotic analysis showed that treatment with ANA-12 induced apoptosis in endometriotic-like lesion but not in the eutopic endometrium (Fig. 4D).

Discussion

In the present study, we found NTRK2 was upregulated in ectopic endometriotic tissues and stromal cells compared to its eutopic counterpart likely due to hypoxic stress. Treatment with NTRK2 antagonist, ANA-12, induced apoptosis in endometriotic stromal cells and caused the regression of the endometriosis-like lesion in model animals. These data suggest aberrant expression of NTRK2 exerts protective effect of endometriotic stromal cells and warrant further investigation to explore the possibility of using NTRK2 as a molecular target for treating endometriosis.

NTRK2 is a conserved gene in human, chimpanzee, Rhesus monkey, dog, cow, mouse and is known as the BDNF receptor. It has been reported that the level of NTRK2 is higher in eutopic endometrium in women with endometriosis (Anger et al. 2007), adenomyosis (Huang et al. 2011), and deep infiltrating endometriosis (Matsuzaki et al. 2004), but not in ovarian endometriosis (Matsuzaki et al. 2006). Our results showed that NTRK2 was increased in ovarian endometriosis (Fig. 1 and Supplementary Table 1). Furthermore, our finding demonstrated that hypoxia induces NTRK2 through transcriptional regulation by HIF-1 α . The reason that our result is different from that of Matsuzaki's (Matsuzaki et al. 2006) may be due to different methods were used for evaluation. Matsuzaki et al. used RT-qPCR to detect the mRNA transcripts of NTRK2 while we performed IHC to identified NTRK2 protein. As there are various alternative splicing variants of NTRK2 (Luberg et al. 2010, Fenner 2012), the concordance of transcripts and

Figure 1 NTRK2 expression level is elevated in ectopic tissues. (A) Genes upregulated in endometriosis ($P < 0.05$ and fold change > 1.5) derived from public datasets (GSE23339 and GSE25628) were cross-referenced with a membrane receptor gene list from GO annotation. Venn diagram (left) and landscape chart with P values (right) were analyzed by Bioinformatic tools as described in materials and methods. (B) NTRK2 levels were analyzed in three public datasets (GSE23339, GSE 25628, and GSE7395). (C) The mRNA levels of NTRK2 were detected in paired endometrial tissues ($n=10$) and (D) paired endometrial stromal cells from patients with endometriosis ($n=7$). The levels of NTRK2 and BDNF mRNA were normalized to 18S rRNA. (E) NTRK2 expression levels were detected in clinical specimen ($n=26$ for eutopic tissues, $n=59$ for ectopic tissues) by immunohistochemistry. The same concentration of IgM antibody was hybridized as negative control. Quantification of the IHC result was shown in right panel. (F) Quantification of IHC result for paired tissues ($n=26$). (G) Analysis of the NTRK2 expression level in the different phases of menstrual cycle. Asterisks indicated significant difference at $P < 0.05$; Norm, normal endometrium; Endo, endometriosis; P phase, proliferative phase; S phase, secretory phase.

Figure 2 The NTRK2 induction is mediated by hypoxia. (A) The mRNA level of NTRK2 was detected in various cytokines and E₂-treated eutopic endometrial stromal cells ($n=8$). (B) NTRK2 level was measured in normoxia or hypoxia-treated eutopic stromal cells ($n=4$). (C) Eutopic stromal cells were transfected with control siRNA or siRNA against HIF-1 α and incubated under either normoxia or hypoxia for 24 h ($n=5$ biological repeats). The levels of NTRK2 mRNA were normalized to 18S rRNA. * $P < 0.05$; # $P < 0.05$ compared to hypoxia control siRNA group. IL1 β (1 ng/mL), interleukin-1 β ; TNF α (10 ng/mL), tumor necrosis factor- α ; TGF β (10 ng/mL), transforming growth factor beta 1; IL6 (10 ng/mL), interleukin-6; E₂ (1 nM), estradiol; PGE₂ (1 nM), Prostaglandin E₂; Nor, normoxia; Hyp, hypoxia.

proteins is ambiguous. Therefore, it is better and more representative to directly measure the level of protein instead of mRNA.

Although previous studies showed distinct expression of NTRK2 between normal endometrial and endometriotic cells (Matsuzaki *et al.* 2004, 2006, Anger *et al.* 2007, Huang *et al.* 2011); however, what causes NTRK2 overexpression in endometriotic cells has never been reported. Herein, we tested several potential factors that had been shown to play critical roles in the pathogenesis of endometriosis such as estradiol, PGE₂, and proinflammatory cytokines (Wu *et al.* 2010, Hsiao *et al.* 2015, Li *et al.* 2018) and found they did not contribute to aberrant expression of NTRK2 in endometriotic stromal cells. Our finding is consistent with a previous report that estradiol failed to affect NTRK2 expression (Wessels *et al.* 2015). On the other hand, we demonstrated that hypoxia is the critical factor inducing the expression of NTRK2. Since hypoxia is suggested to be the driving force of endometriosis (Wu *et al.* 2019), our current finding reveals another pathological process regulated by hypoxia.

BDNF-NTRK2 signaling cascades control neuron differentiation, growth, survival, and synaptic plasticity (Kaplan & Miller 2000, Huang & Reichardt 2001, 2003, Minichiello 2009). In endometriosis, effect of BDNF-

NTRK2 was reported to be associated with endometriosis-related pain (Barcena de Arellano *et al.* 2013, Ding *et al.* 2018). Our data showed that BDNF-NTRK2 signaling is important for anti-apoptosis as knockdown of NTRK2 increased apoptosis of endometriotic stromal cells. This finding agrees with that observed in cancer cells as accumulated evidence indicates that NTRK2 promotes tumor cell survival, drug resistance, and metastasis (Yu *et al.* 2008, Yuan *et al.* 2018). Based on this finding, we reasoned that blocking BDNF-NTRK2 signaling may be an alternative way to treat endometriosis. Indeed, treatment with ANA-12 enhanced the apoptosis of ectopic stromal cells. Furthermore, ANA-12 administration leads to endometriotic lesion regression in autotransplanted mouse model. Result from TUNEL assay indicates blocking NTRK2 signaling by ANA-12 increased apoptotic cell number in ectopic endometriotic lesion. Interestingly, intact eutopic endometrial cells were not affected by ANA-12 treatment in the same mouse. The reason for differential responses to ANA-12 is unknown and warrants further investigation.

ANA-12 was identified as a selective NTRK2 antagonist, which has been reported to reduce chronic pain in different experimental models (Tillu *et al.* 2015, Liu *et al.* 2018, Fan *et al.* 2020). Furthermore, ANA-12 has been used to target NTRK2 in studies

Figure 3 Elevated NTRK2 expression possesses the ability to promote anti-apoptosis of ectopic stromal cell. (A) The flow cytometry was performed in the ectopic stromal cells treated with control siRNA or NTRK2 siRNA for 48 h ($n=3$). (B) Ectopic endometriotic stromal cells were treated with ANA-12 for 48 h. The cells were collected and stained with annexin-V as an apoptotic marker and analyzed using flow cytometry ($n=3$). * $P < 0.05$ indicates significant different compared to control group. ANXA5 indicates Annexin A5 (or annexin V).

Figure 4 Specific NTRK2 inhibitor decreases the lesion growth in the endometriosis mouse model. (A) The diagram showing the procedures used to set up the animal model of endometriosis (left). The NTRK2 level was determined in surgical-induced mouse model using IHC staining (right). (B) Left panel: representative picture of ectopic lesions after DMSO or ANA-12 treatment (1.5 mg/kg body weight) for 1 month. The yellow dot-line indicated the location of lesions. Right panel: the volume of ectopic lesion was measured when mice were sacrificed (DMSO, $n=24$; ANA-12, $n=23$). (C) The level of phosphorylated NTRK2 (Tyr515) was detected in the ectopic lesions after DMSO or ANA-12 treatment (DMSO, $n=24$; ANA-12, $n=23$). (D) Apoptotic levels were determined in the ectopic lesions and uteri after DMSO or ANA-12 treatment (DMSO: lesion, $n=24$, uterus, $n=6$; ANA-12: $n=23$, uterus, $n=6$). Asterisks indicate $P < 0.05$.

of medulloblastoma (Thomaz *et al.* 2019), gliomas (Pinheiro *et al.* 2017), leukemia (Polakowski *et al.* 2014), lung adenocarcinoma metastasis (Sinkevicius *et al.* 2014) and lymphoid tissue neovascularization (Dalton *et al.* 2015). In our study, we demonstrated that ANA-12 treatment decreased the lesion size and increased the cell death in lesion but not in eutopic endometrium, suggesting that targeting BDNF-NTRK2 pathway may be a possible alternative for treating endometriosis. However, there are some limitations of current study. First, the ANA-12 was administered to an artificial mouse model of endometriosis, in which uterine tissue was sutured in the peritoneal cavity. This model may not recapitulate the real tissue microenvironment at the

time of retrograde menstruation and true inflammatory responses. In addition, despite that ANA-12 treatment induced about two -folds increase in cell death, the reduction of lesion size is not as great, suggesting other factors may be involved in lesion regression. Thus, the impact on the peritoneal microenvironment of ANA-12 administration needs further exploration.

In conclusion, NTRK2 is aberrantly expressed in endometriotic stromal cells due to hypoxic stress and contributes to anti-apoptosis. Treatment with selective NTRK2 antagonist causes regression of endometriotic-like lesion in mouse model suggests targeting NTRK2 signaling might be a potential choice for non-hormonal treatment of endometriosis.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0163>.

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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Author contribution statement

H C Lee and S C Lin conducted the experiment, collected and analyzed data. M H Wu and S J Tsai conceived the project and provided guidance of clinical and basic medical sciences, respectively. H C Lee and S J Tsai wrote the manuscript.

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