PI:	Title: Neuroangiogenesis in Deep Infiltrating Endometriosis	
Received: 03/03/2014	FOA:	Council: 10/2014
Competition ID: FORMS-C	FOA Title: NIH EXPLORATORY/DEVELOPMENTAL RESEARCH GRANT PROGRAM (PARENT R21)	
	Dual:	Accession Number:
IPF:	Organization:	
Former Number:	Department: Obstetrics and Gynecology	
IRG/SRG: ICER	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: 150,000 Year 2: 125,000	Animals: N Humans: Y Clinical Trial: N Current HS Code: 30 HESC: N	New Investigator: N Early Stage Investigator: N
Senior/Key Personnel:	*Organization:*	*Role Category:*

OMB Number: 4040-0001
Expiration Date: 06/30/2016

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	**State Application Identifier**

1. TYPE OF SUBMISSION*

❍ Pre-application ● Application ❍ Changed/Corrected Application

4.a. Federal Identifier

b. Agency Routing Number

c. Previous Grants.gov Tracking Number

2. DATE SUBMITTED 2014-03-03

Application Identifier

5. APPLICANT INFORMATION **Organizational DUNS*:**

Legal Name*:
Department:
Division:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
ZIP / Postal Code*:

Person to be contacted on matters involving this application

Prefix: First Name*: Middle Name: Last Name*: Suffix:

Position/Title:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
ZIP / Postal Code*:
Phone Number*: Fax Number: Email:

6. EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)**

7. TYPE OF APPLICANT* O: Private Institution of Higher Education

Other (Specify):

Small Business Organization Type ❍ Women Owned ❍ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION*

❍ New ● Resubmission
❍ Renewal ❍ Continuation ❍ Revision

If Revision, mark appropriate box(es).

❍ A. Increase Award ❍ B. Decrease Award ❍ C. Increase Duration
❍ D. Decrease Duration ❍ E. Other *(specify)* :

Is this application being submitted to other agencies?* ❍Yes ●No What other Agencies?

9. NAME OF FEDERAL AGENCY*
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
Neuroangiogenesis in Deep Infiltrating Endometriosis

12. PROPOSED PROJECT

Start Date* 09/01/2014
Ending Date* 08/31/2016

13. CONGRESSIONAL DISTRICTS OF APPLICANT

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name*: Middle Name: Last Name*: Suffix:
Position/Title:
Organization Name*:
Department:
Division:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
ZIP / Postal Code*:
Phone Number*: Fax Number: Email*:

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested*	$424,960.00
b. Total Non-Federal Funds*	$0.00
c. Total Federal & Non-Federal Funds*	$424,960.00
d. Estimated Program Income*	$0.00

16.IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ❍ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR

❍ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: First Name*: Middle Name: Last Name*: Suffix:
Position/Title*:
Organization Name*:
Department:
Division:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
ZIP / Postal Code*:
Phone Number*: Fax Number: Email*:

Signature of Authorized Representative*

Date Signed*
03/03/2014

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:Cover_Letter_Attachment.pdf

424 R&R and PHS-398 Specific Table Of Contents

OMB Number: 4040-0010
Expiration Date: 06/30/2016

Project/Performance Site Location(s)

Project/Performance Site Primary Location

❍ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name:
Duns Number:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
Zip / Postal Code*:
Project/Performance Site Congressional District*:

File Name

Additional Location(s)

Funding Opportunity Number: PA-13-303. Received Date: 2014-03-03T10:20:35.000-05:00

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ● **Yes** ❍ **No**

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ❍ Yes ● No

If YES, check appropriate exemption number: _ 1 _ 2 _ 3 _ 4 _ 5 _ 6

If NO, is the IRB review Pending? ❍ **Yes** ● **No**

IRB Approval Date: 01-06-2014

Human Subject Assurance Number 00001435

2. Are Vertebrate Animals Used?* ❍ **Yes** ● **No**

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ❍ Yes ❍ No

IACUC Approval Date:

Animal Welfare Assurance Number

3. Is proprietary/privileged information included in the application?* ❍ **Yes** ● **No**

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ❍ **Yes** ● **No**

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ❍ Yes ❍ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ❍ **Yes** ● **No**

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ❍ **Yes** ● **No**

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	abstract.pdf
8. Project Narrative*	projplan.pdf
9. Bibliography & References Cited	ref.pdf
10.Facilities & Other Resources	Facilities_Upload.pdf
11.Equipment	Major_Equipment_Upload.pdf

Funding Opportunity Number: PA-13-303. Received Date: 2014-03-03T10:20:35.000-05:00

PROJECT SUMMARY

The scientific goal of this proposal is to test the roles of estrogen and interleukin (IL)-1β, potent autacoids known to exacerbate inflammation, on a particularly severe cause of pelvic pain associated with deep infiltrating endometriosis (DIE). Endometriosis affects as many as 10% of reproductive age women and accounts for >$22 billion annually in direct and indirect medical costs here in the USA. DIE, wherein ectopic implants penetrate >5 mm into the subperitoneal space, represents the most painful manifestation of the disease. It is reported that ~95% of women with this type of endometriosis experience moderate to severe pain, commonly presenting with symptoms of dysmenorrhea (painful periods), dyschezia (painful bowel movements) and dyspareunia (painful intercourse). DIE lesions are imbued with dense nerve and vascular networks that we postulate arise via an integrated developmental program we have coined "neuroangiogenesis". We have identified one multifunctional protein, brain-derived neurotrophic factor (BDNF), that is a candidate neuroangiogenesis mediator in DIE, which we will investigate in detail under the ægis of this exploratory R21 grant. BDNF is mitogenic for both neurons and endothelial cells and it is overexpressed in ectopic and eutopic endometrium of women with endometriosis compared to controls without the disease. Moreover, both mRNA and protein of its cognate receptor, tyrosine kinase B (TrkB), are upregulated in endometriosis cases. Thus, BDNF exhibits the expression pattern and biochemical characteristics expected of a neuroangiogenesis mediator. We postulate that this protein contributes to DIE lesion growth, vascularization and pain. Preoperative pain and quality of life instruments will be collected for comparison with the biochemcial data. We will validate the cellular distribution of BDNF mRNA and protein in DIE biopsies using *in situ* molecular histochemical assays. Normal endometrium from women without endometriosis or pain will serve as a control. Isoforms of BDNF will be evaluated by blotting. The aforementioned descriptive studies will be accompanied by mechanistic experiments, using cell culture models that we developed and characterized. Control and endometriosis cell cultures will be used to evaluate the effects of estrogens and IL-1β on BDNF mRNA and protein regulation *in vitro,* including the intracellular processing of pro-BDNF to its mature, bioactive form. Our preliminary data indicate that estrogens and IL-1β stimulate BDNF production. Dose-response and time-course experiments, along with pharmacological inhibitors and siRNA knock down of ERα, ERβ, GPER or IL-1 type I receptor (using scrambled siRNA as a control) will identify mechanism-specific drug targets for neuroangiogenesis, so that effective therapeutics can be rationally designed for endometriosis-associated pain in the future. Two novel estrogen receptor antagonists, synthesized and provided by our collaborators, also will be evaluated. At the completion of the R21 period, an R01 application will be prepared to address the relevant biological activities of BDNF-induced nerve and capillary proliferation and migration and their correlation with pelvic pain symptoms.

PROJECT NARRATIVE

Deep infiltrating endometriosis (DIE) is one of its most distressing conditions in gynecology, with pain during menstruation and intercourse that interferes with a woman's activities. DIE lesions have dense nerves and blood vessels. The goal of this research is to understand the mediators of nerve and vessel growth and to design new drugs that disrupt their development and pain signals.

RESOURCES

Scientific Environment: Since the PI's recruitment to two years ago, we have established the superb clinical and laboratory-based collaborations necessary to carry out the high quality and innovative translational gynecologic research that our group has been recognized for. On the clinical side, and , outstanding surgeon-scientists who are referred and expertly treat women with DIE. Their operative skill with conventional and robot-assisted laparoscopic procedures, and their experience in carrying out and completing complex surgical clinical trials, promise that we can recruit, consent and collect tissue specimens from women suffering painful symptoms associated with DIE. The PI's clinical practice provides access to referred participants with chronic endometriosis symptoms as well as unaffected controls without pelvic pain. Our sample size analyses and clinical volumes have been carefully worked out as presented in the application. , will participate in subject recruitment, protocol compliance and administration of the B&B exams and EHP-30 questionnaires. In addition, clinical research support provided through the , in which serves as a senior faculty advisor, will be available as noted in the letter of support from . The facilitates research by assisting in participant recruitment, conducting study visits, administering questionnaires, collecting, preparing, and processing specimens, and developing study databases in REDcap to facilitate data analysis

On the laboratory side, we will be assisted by my colleague and collaborator of 8 years, , and aided by the outstanding expertise (and reagents) of and , renowned reproductive scientists from the . The latter will bring unique pharmaceutical compounds and deep expertise in estrogen receptor signaling science to our translational project. provides excellent core laboratory resources including flow cytometry and molecular microscopy that will be useful in the proposed application. The latter is housed in the Hypertension and Vascular Research Program, to which was elected as a full member last year.

The expertise of our team and the research facilities of our department will ensure the proper and timely execution of the study proposed here. As emphasized by Reviewer 1 of our original grant, we believe that "the translational application of the proposal is significant...if successful, the impact of this research would be exceptionally high...with possible major impact in the area of female reproductive health."

Facilities

Personnel: , has already begun work on this project and has technically mastered all of the proposed assays. and are active collaborators and named investigators on our IRB. The department of Ob/Gyn also has two full-time clinical research nurses; will be available to recruit patients for the present study. She has extensive expertise in patient recruitment and managing IRB proposals. As described in the Budget Justification, and , have graciously offered their own, unique novel estrogen receptor ligands OBHS and CLI, described in the application, as well as unsurpassed technical expertise in the design of cellular experiments to dissect the actions of ERα, -β and GPER on gene expression: in this case, BDNF. Their expertise and resources assure the optimal outcomes of these experiments.

Laboratories: The Research Laboratories of the Department of Obstetrics and Gynecology occupy approximately 6,300 sq. ft. of laboratory space in the . In addition, there are three equipment rooms, two cell culture facilities, one dark room, extensive -80 C freezer space, and a 4 C cold room. The laboratories are immediately available for use in the proposed study and contain all major items of equipment necessary for the experiments proposed (described below).

The laboratories house a BioRad real-time PCR machine that is available for use in the proposed study. Power supplies and gel electrophoresis apparati are accessible. Our department's cell culture laboratory is equipped with two laminar flow hoods, two water-jacketed CO_2 incubators, and a refrigerator/freezer. Two common tissue culture laboratory spaces are devoted to media and reagent preparation, storage of sterile glass- and plastic ware, as well as other cell culture supplies. A Zeiss microscope with fluorescence capabilities is available next to the PI's laboratory. A spectrophotometer and plate reader are available to analyze the

proposed ELISA experiments. The Department of Pathology at maintains a available to all researchers in the institution should it be useful.

Computer: In each individual laboratory there is at least one computer station connected to the Ob/Gyn department local area network and the medical school-wide network. Software available at each computer station is obtained either as a school broad license (Office 2007), department license (Adobe PhotoShop, Reference Manager, Adobe Acrobat) or individually purchased (Sigma Plot, Sigma Stat, Prism, Cruncher) and all are equipped to perform online literature searches.

Offices: Each faculty member has an academic office of ~120 sq. ft. within the Department of Ob/Gyn, three floors above. The offices are staffed by three secretaries, an assistant for manuscript and grant preparation and one administrative manager each for pre- and post-award management. The Department conference room is located in this space and allows the faculty, staff, and trainees from the Department and surrounding academic community to attend meetings related to discuss research and other matters important to the department. Virtual and teleconferencing capabilities exist and are used frequently.

Major equipment: The cell culture laboratory is equipped with two laminar flow hoods, two water-jacketed CO_2 incubators, a refrigerator/freezer and a Nikon inverted phase-contrast microscope with camera. We are in the process of building an additional cell culture facility to allow maintenance and provisioning of stock cell lines sequestered from explants and primary cell cultures. Two common tissue culture laboratory spaces are devoted to media and reagent preparation, storage of sterile glass- and plastic ware, as well as other cell culture supplies. Long-term cell storage in two liquid nitrogen tanks is available on site.

A centralized arrangement of resources offers the investigators maximum use of common space. The Department maintains a Sorvall RC-SB high speed centrifuge, a Sorvall OD50B ultracentrifuge, a Packard Liquid Scintillation counter, two Packard gamma counters, a SpeedVac concentrator, a Virtis lyophilizer, a Living System calcium imaging system, a Radnoti 8 chamber organ bath system, two myographs (Multi Myograph, Model 610M Danish Myo Technologies, a Spectronic 2000 spectrophotometer. In addition, the laboratories are equipped with pH meters, analytical balances, fume hoods, gel electrophoresis systems with power supply, refrigerators, -20°C freezers, ultracentrifuge, refrigerated low speed centrifuge, beta counter, Victor multi-label plate reader, microplate washer, end-point (Bio-Rad MyCycler) and real-time (ABI Systems 7500) PCR thermocyclers, water-jacketed CO2 incubators, an ice maker, 8 Ultralow (-70°C) freezers, gel scanner and digitizer with miscellaneous image analysis software, and the necessary equipment for pipetting and preparing solutions.

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: First Name*: Middle Name Last Name*: Suffix:

Position/Title*:
Organization Name*:
Department:
Division:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
Zip / Postal Code*:

Phone Number*: Fax Number: E-Mail*:

Credential, e.g., agency login:

Project Role*: PD/PI Other Project Role Category:

Degree Type: Degree Year: 1981

File Name

Attach Biographical Sketch*:

Attach Current & Pending Support:

PROFILE - Senior/Key Person

Prefix: First Name*: Middle Name Last Name*: Suffix:

Position/Title*:
Organization Name*:
Department:
Division:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
Zip / Postal Code*:

Phone Number*: Fax Number: E-Mail*:

Credential, e.g., agency login:

Project Role*: Co-Investigator Other Project Role Category:

Degree Type: Degree Year:

File Name

Attach Biographical Sketch*:

Attach Current & Pending Support:

Funding Opportunity Number: PA-13-303 . Received Date: 2014-03-03T10:20:35.000-05:00

PROFILE - Senior/Key Person

Prefix: First Name*: Middle Name Last Name*: Suffix:

Position/Title*:
Organization Name*:
Department:
Division:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
Zip / Postal Code*:

Phone Number*: Fax Number: E-Mail*:

Credential, e.g., agency login:

Project Role*: Co-Investigator Other Project Role Category:

Degree Type: Degree Year:

File Name

Attach Biographical Sketch*:

Attach Current & Pending Support:

PROFILE - Senior/Key Person

Prefix: First Name*: Middle Name Last Name*: Suffix:

Position/Title*:
Organization Name*:
Department:
Division:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
Zip / Postal Code*:

Phone Number*: Fax Number: E-Mail*:

Credential, e.g., agency login:

Project Role*: Co-Investigator Other Project Role Category:

Degree Type: Degree Year:

File Name

Attach Biographical Sketch*:

Attach Current & Pending Support:

PROFILE - Senior/Key Person

Prefix: First Name*: Middle Name S. Last Name*: Suffix: PhD

Position/Title*:
Organization Name*:
Department:
Division:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
Zip / Postal Code*:

Phone Number*: Fax Number: E-Mail*:

Credential, e.g., agency login:

Project Role*: Other (Specify) Other Project Role Category: Other Significant Contributor

Degree Type: Degree Year:

File Name

Attach Biographical Sketch*:

Attach Current & Pending Support:

PROFILE - Senior/Key Person

Prefix: First Name*: Middle Name Last Name*: Suffix: PhD

Position/Title*:
Organization Name*:
Department:
Division:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
Zip / Postal Code*:

Phone Number*: Fax Number: E-Mail*:

Credential, e.g., agency login:

Project Role*: Other (Specify) Other Project Role Category: Other Significant Contributor

Degree Type: Degree Year:

File Name

Attach Biographical Sketch*:

Attach Current & Pending Support:

Funding Opportunity Number: PA-13-303 . Received Date: 2014-03-03T10:20:35.000-05:00

OMB No. 0925-0001/0002 (Rev. 08/12 Approved Through 8/31/2015)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY

A. Personal Statement

I have devoted my career to research in the field of women's health and have committed my own training to the application of biochemical, molecular and cellular models to obstetrical and gynecological conditions. Before "translational research" was popularized in the scientific lexicon, it was my focus and passion. I should emphasize that this orientation was not a particularly attractive career objective in the 1980s when I was establishing myself as an independent investigator. Fundamental sciences, especially molecular genetics and immunology, were touted to be the most expeditious paths to academic success. It is satisfying to look back on a career in which I have combined a genuine interest in clinical medicine, a profound exposure to human pathophysiology, and a mechanistic appreciation of the cell and molecular biology that underlie diseases in women, particularly endometriosis. I was a founding member of the Vanderbilt U54 SCCPIR grant, which was the first of now several U54s focused on the pathogenesis of endometriosis, and I continue to collaborate with members of the and other U54 groups. I was a longstanding member of the SCCPIR Endometrial Focus group.

My recent focus has been on the regulation of "neuroangiogenesis" in the endometrium and endometriosis. We put forward the hypothesis that coordinated neural and capillary growth into ectopic and eutopic endometrial tissues are mediated by endocrine and inflammatory pathways and identified brain-derived neurotrophic factor (BDNF) and neurotrophin (NT) 4/5 as candidate mediators of endometrial neurogenesis. In this R21 we plan to focus on deep infiltrating endometriosis (DIE) and specifically the role of BDNF, a mitogen for both neurons and endothelial cells. This focus is supported by our prior proteomics discoveries and further by the finding that the cognate receptor for BDNF, tyrosine kinase receptor B (TrkB), also is upregulated in endometriosis tissues. Our preliminary data indicate that estrogen and IL-1β stimulate endometrial cell production of BDNF, particularly the mature, bioactive isoform. Through a collaboration with the (see their letter of support) novel estrogen receptor ligands, as well as inhibitors of IL-1β signaling, will be tested as innovative therapeutic candidates. We postulate that the neuroangiotrophic axis established by BDNF and TrkB will yield innovative therapeutic opportunities to mitigate endometriosis-associated pain and lesion growth in DIE, and likely in pain manifestations of other endometriosis lesion subtypes. Our proposed in vivo and in vitro experiments reflect an important translational extension of our fundamental studies of endometrial neuroangiogenesis in endometriosis. Validation of BDNF as a specific therapeutic target will be a breakthrough for the largely underserved population of women suffering from the symptoms of DIE and likely applicable to other aspects of endometriosis-associated pelvic pain.

B. Positions and Employment

Honors and Awards:

C. Selected peer-reviewed publications (in chronological order among 200 papers):

Most relevant to the current application

1.

Additional publications of importance to the field

D. Research Support:

Ongoing Research Support:

Completed Research Support (Last 3 years):

OMB No. 0925-0001/0002 (Rev. 08/12 Approved Through 8/31/2015)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY

A. Personal Statement

The proposed research requires obtaining tissue specimens from patients with endometriosis as well as patients with normal pelvic anatomy. I participate in a busy, full service clinical reproductive endocrine practice that utilizes minimally-invasive surgical treatment of pelvic pathologies such as infertility related to endometriosis and pelvic pain. In addition to advanced laparoscopy, in 2010 I became certified to apply robotic assistance in laparoscopy as well. I have recently completed the REI board certification process and am now able to better focus attention on the clinical and translational study of endometriosis. On average, I perform 6 laparoscopies per month and 2-4 robotic assisted laparoscopies per month. My case-load for deep infiltrative endometriosis is not as great as my partner, , but I perform approximately 5 such cases each year. I am a co-investigator on to collect endometrium and endometriosis specimens and also a co-investigator on a Phase III Clinical Trial of a GnRH antagonist to treat endometriosis-associated pain. This topic has become my primary clinical research focus and I look forward to participating on the R21 project.

B. Positions and Honors

Positions and Employment:

Other Experience and Professional Memberships:

Honors:

C. Selected Peer-reviewed Publications

.

D. Research Support

Completed Research Support

OMB No. 0925-0001/0002 (Rev. 08/12 Approved Through 8/31/2015)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY

A. Personal Statement

As an academically-oriented reproductive endocrinology-infertility specialist for the past 21 years, I have dedicated my time to create a quality REI program and clinical research. In 1992, I designed and led the recruitment of 100 patients for a randomized double-blind study of 25 versus 50 mg/m^2 BSA of methotrexate for the treatment of ectopic pregnancy. My interactions with other clinical investigators led me to participate in all aspects of other, randomized multicenter trials, including PEACH (PID evaluation and Health) trial and STOP-DUB (Surgical Treatments Outcomes Project for Dysfunctional Uterine Bleeding) trial, in which I was also a Steering Committee member as the director of top-recruiting clinic. Both were AHRQ sponsored projects. I have the expertise, leadership and motivation necessary to successfully carry out the proposed role. I have a broad background in reproductive endocrinology and physiology as well as specific training and expertise in key research areas for this application. As a postdoctoral fellow at , I carried out bench research in steroid biosynthesis and sexual differentiation besides acquiring fundamentals of reproductive biology and clinical and surgical practice of reproductive endocrinology and infertility. Since then I have been practicing and teaching clinical aspects of this field in academic settings. I have been active in the design and administration of multicenter and multinational (such as, ESEP) trials, industry-sponsored (such as GnRH-agonist or selective progesterone receptor modulator use in leiomyoma and endometriosis) or investigator-initiated trials in reproductive health, recruiting participants and engaging in data analysis. As a result of these experiences, I am aware of the importance of developing and precise implementation of research policies, procedures and processes and follow-up of research patients. The current application fits logically into my extensive practice of treating infertility with multifactorial etiologies, including endometriosis.

Moreover, through the preparation of an R01 application on Pesticide Exposure and Reproductive Health among Latino Men, jointly with in the , which was scored favorably but not funded, taught me that I could bring together a team of expert collaborators from various departments demonstrated that subject recruitment challenges could be overcome by actively involving other physicians in our department.

I have a busy surgical practice, with a particular focus on robotic procedures, and manage more than 15 cases of deep infiltrative endometriosis annually. I see no difficulty in meeting the recruitment needed (18 cases over two years) for adequate power as indicated in R21 application.

B. Positions and Honors

Honors and Awards:

C. Selected Peer-Reviewed Publications

C. RESEARCH SUPPORT

Ongoing Research Support:

OMB No. 0925-0001/0002 (Rev. 08/12 Approved Through 8/31/2015)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY

A. Personal Statement

This project will test our hypothesis that the severe pain symptoms of deep infiltrative endometriosis (DIE) are associated with “neuroangiogenesis.” Despite years of investigation, and many attempted surgical and medical interventions, endometriosis remains a common cause of pelvic pain. This is particularly true of DIE. From clinical specimens of endometriosis patients, we have previously determined that one protein with nerve and capillary stimulating properties, brain-derived neurotrophic factor (BDNF) is more heavily expressed than in the endometrium of controls. I am an accomplished research investigator and mentor with more than 26 years experience in translational clinical research in a university setting. I have inherent organization and communication strengths compatible with successful coordination and implementation of research programs and teams. I have provided supervision, experimental instruction, and research mentoring to clinical fellows, PhD students, residents, undergraduate and medical students, and research staff.

I will function as a key member of the research team based on my expertise in the area of endometriosis, and in chronic inflammatory diseases in general, knowledge of scientific theory, and methods of analysis of data sets. I will coordinate various aspects of experiments performed; provide direction to trainees and staff; develop and conduct presentations at national and international meetings; co-author publications; and serve as the liaison for laboratory regulatory compliance. I will be responsible for isolating, characterizing and propagating the endometrial and endometriosis cell cultures, in which I have become an expert working with for the past 7.5 years.

With supervision I will the immunohistochemistry, blotting and in situ hybridization experiments proposed in the application. Moreover, I will assess the mechanisms of BDNF regulation by estradiol and IL-1β signaling pathways in the primary endometriosis cell culture systems, using a combination of biochemical, molecular biology and pharmacology tools described in our grant. Our findings are expected to improve the understanding of pathways leading to endometriosis-associated pain and are likely to suggest new diagnostic tests and interventions for future clinical applications.

B. Positions and Honors

Professional Experience:

Honors And Awards:

C. Selected Peer Reviewed Publications:

Abstracts

D. Research Support.
None.

OMB No. 0925-0001/0002 (Rev. 08/12 Approved Through 8/31/2015)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY

A. Personal Statement

My professional career has been directed to elucidating cellular and molecular mechanisms of steroid hormone-mediated regulation of the phenotypic properties of reproductive target tissues and of tumors that develop in these tissues, especially breast cancer. My research in endocrinology and in women's health has been especially focused on estrogen receptors and their mechanistic, physiological, and integrative actions, and on improving the effectiveness of endocrine therapies in breast cancer. My laboratory has published extensively on estrogen receptor and coregulator involvement in endometriosis, and on global gene expression analyses of estrogen action and genome-wide interactions of estrogen receptors and collaborating transcription factors and protein kinases in target cells, and mechanisms of nuclear receptor and growth factor signaling resulting in therapy resistance. We do basic and translational studies in uterine biology and endometriosis, and in breast cancer cells and tumor samples and have identified factors associated with aggressiveness and early time to recurrence and their regulation in different subtypes of breast cancer. I have trained over 80 graduate students and postdoctoral scientists, many of whom are leading distinguished careers in universities and medical centers, governmental agencies, and the pharmaceutical/biotechnology industry. I am very pleased to collaborate with on these integrative analyses of endometriosis and studies with estrogen receptor ligands that might be of especial benefit in this disease.

B. Positions and Honors

Honors

C. Relevant Peer-Reviewed Publications (from a total of 320-excluding abstracts)

D. Research Support

Ongoing Research Support

Completed Research Support

OMB No. 0925-0001/0002 (Rev. 08/12 Approved Through 8/31/2015)

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
eRA COMMONS USER NAME (credential, e.g., agency login) Jkatzene	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY

A. Personal Statement

I have had a longstanding interest in how estrogens act through the estrogen receptors, ERα and ERβ, and through both nuclear-initiated and extranuclear-initiated pathways, to regulate very distinctive gene transcription patterns in diverse target tissues. In particular, I have tried to understand how changes in the structure of estrogen receptor ligands can manifest themselves in distinct tissue-specific effects, which often result in patterns of activity that provide greater health benefits than that of estradiol itself. To study these intriguing pharmacological phenomena, we have taken a diverse approach that combines ligand synthesis, X-ray crystallography of ligand-receptor complexes, fluorescence and other biophysical measurements of ligand-receptor and receptor-coregulator interactions, receptor conformation and conformational dynamics, computational modeling, and cell and animal model experiments. Many of these studies are done through collaborators.

We have a particular interest in understanding how altering ER ligand structure can give a pattern of activity that differs from that of estradiol in the following ways: selective action through only one of the ER subtypes (generally ERβ) with minimal or no stimulation of reproductive tissues but effective suppression of inflammation. We have found two such compounds, each of which is backed up with a compound series: (a) OBHS is an ER ligand having a novel bicyclic core that is overall an antagonist, but one that induces a conformation of ER that is different from that of any other class of ER ligands that engenders potent anti-inflammatory activity *[See Refs 1 and 2]*, and (b) chlorindazole (CLI) , a very ERβ selective ligand that we have shown engenders potent neuroprotective activity that is clearly ERβ dependent but much more effective than that of other ERβ-selective ligands *[See Ref 3]*. Both of these compounds have promising effects in animal models of endometriosis.

We will be delighted to continue to supply OBHS and CLI for the studies proposed in the Project, and as we develop other members of these two interesting series, we will offer them to the Project participants for further investigations.

B. Positions and Honors

Positions and Employment

Other Experience and Professional Membership

Honors and Awards

C. Selected Peer-reviewed Publications (Selected from the last 5 years; total publications are ~500, excluding abstracts)

Most relevant to the current application

Additional recent publications of importance to the field (in chronological order)

D. Research Support

Ongoing Research Support

Completed Research Support

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

1. Project Director / Principal Investigator (PD/PI)

Prefix:
First Name*:
Middle Name:
Last Name*:
Suffix:

2. Human Subjects

Clinical Trial? ● No ○ Yes
Agency-Defined Phase III Clinical Trial?* ● No ○ Yes

3. Permission Statement*

If this application does not result in an award, is the Government permitted to disclose the title of your proposed project, and the name, address, telephone number and e-mail address of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information (e.g., possible collaborations, investment)?

● Yes ○ No

4. Program Income*

Is program income anticipated during the periods for which the grant support is requested? ○ Yes ● No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

Budget Period*	Anticipated Amount ($)*	Source(s)*

Funding Opportunity Number: PA-13-303. Received Date: 2014-03-03T10:20:35.000-05:00

PHS 398 Cover Page Supplement

5. Human Embryonic Stem Cells

Does the proposed project involve human embryonic stem cells?* ● No ❍ Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://grants.nih.gov/stem_cells/registry/current.htm. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

Cell Line(s): Specific stem cell line cannot be referenced at this time. One from the registry will be used.

6. Inventions and Patents (For renewal applications only)

Inventions and Patents*: ❍ Yes ● No

If the answer is "Yes" then please answer the following:

Previously Reported*: ❍ Yes ❍ No

7. Change of Investigator / Change of Institution Questions

❑ Change of principal investigator / program director

Name of former principal investigator / program director:

Prefix:

First Name*:

Middle Name:

Last Name*:

Suffix:

❑ Change of Grantee Institution

Name of former institution*:

Funding Opportunity Number: PA-13-303. Received Date: 2014-03-03T10:20:35.000-05:00

PHS 398 Modular Budget

OMB Number: 0925-0001

Budget Period: 1

Start Date: 09/01/2014 **End Date:** 08/31/2015

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium F&A*	150,000.00
Consortium F&A	0.00
Total Direct Costs*	**150,000.00**

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
1.	Organized Research	54.14	150,000.00	81,210.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Steven Zuraf, 301-492-4855

Indirect Cost Rate Agreement Date: 05/21/2013

Total Indirect Costs **81,210.00**

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($) **231,210.00**

Funding Opportunity Number: PA-13-303. Received Date: 2014-03-03T10:20:35.000-05:00

PHS 398 Modular Budget

Budget Period: 2

Start Date: 09/01/2015 **End Date:** 08/31/2016

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium F&A*	125,000.00
Consortium F&A	0.00
Total Direct Costs*	**125,000.00**

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
1.	Organized Research	55.00	125,000.00	68,750.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Steven Zuraf, 301-492-4855

Indirect Cost Rate Agreement Date: 05/21/2013

Total Indirect Costs **68,750.00**

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($) **193,750.00**

Funding Opportunity Number: PA-13-303. Received Date: 2014-03-03T10:20:35.000-05:00

PHS 398 Modular Budget

Cumulative Budget Information

1. Total Costs, Entire Project Period

Section A, Total Direct Cost less Consortium F&A for Entire Project Period ($)	275,000.00
Section A, Total Consortium F&A for Entire Project Period ($)	
Section A, Total Direct Costs for Entire Project Period ($)	275,000.00
Section B, Total Indirect Costs for Entire Project Period ($)	149,960.00
Section C, Total Direct and Indirect Costs (A+B) for Entire Project Period ($)	424,960.00

2. Budget Justifications

Personnel Justification	PersonnelJustification.pdf
Consortium Justification	
Additional Narrative Justification	

Funding Opportunity Number: PA-13-303. Received Date: 2014-03-03T10:20:35.000-05:00

PERSONNEL:

is PI of this project and will devote 2.4 person months (20% effort). He is

and an experienced physician-scientist and molecular endocrinologist, with 20 years' experience as an independent NIH investigator. He will supervise the overall project, design the experiments, analyze the data and prepare all reports and manuscripts.

, Co-I, is
. She will devote 4.8 person months (40%). She is a highly capable physician-scientist and molecular biologist who will be responsible for clinical specimen management and primary endometrial and endometriosis cell culture. Over the past 8 years she has worked with and has developed unique expertise, improving the efficiency and consistency of our ESC preparation and characterization. She will establish and maintain the primary endometrial and endometriosis and cell cultures from the subjects with DIE-associated pelvic pain or from control volunteers without pain. She has excellent western blotting, RT-PCR and immunohistochemistry experience and will conduct these important experiments. She will be involved in the analysis and interpretation of data and in preparation of all reports and manuscripts and will travel to one national meeting per year. She will contribute 4.8 person months (40% effort) to this project.

, Co-I, is
and board certified in Reproductive Endocrinology and Infertility with an interest in clinical investigation. She is an active reproductive surgeon with excellent laparoscopic and robotic surgical skills. She will contribute 0.48 person months (4% effort) to support subject recruitment, consenting and endometrial and endometriosis specimen collection at the . She will be actively engaged in research discussions, project execution, participant recruitment and manuscript preparation.

, Co-I, is a and
. He is Board certified in Obstetrics and Gynecology and REI and an
. He will devote 0.48 person months (4% effort) to this project. has served as Principal Investigator on several large clinical trials including the ESEP study, an international multicenter randomized controlled trial comparing salpingostomy *vs.* salpingectomy in women with ectopic tubal pregnancy, evaluating the impact on future fertility (Mol et al., 2008; Mol et al., 2014). responsibilities for this project will include subject recruitment, consenting and endometrial and endometriosis specimen collection. He will be actively engaged in research discussions, project execution, and manuscript preparation..

, , will recruit subjects for the clinical studies. She has >20 years experience at
, recruiting and following human subjects for several ongoing GOG trials and coordinated past clinical trials within the from 1991-2006. Her contacts and experience throughout the clinical care sites at are invaluable. Ms. will devote 1.2 person months (10% effort) administering the pain and quality of life questionnaires, coordinating human subject recruitment and assuring compliance oversight for the project.

. and , , collaborators, are renowned
, respectively, at the , . Their independent and collaborative contributions to molecular reproductive endocrinology and medicinal chemistry are legion. Each has over 300 exceedingly high-impact, peer-review publications and >30 years of fundamental contributions to the field of estrogen action in the female reproductive tract. The PI has had the privilege of collaborating with the for the past 7 years, under the aegis of a SCCPRR U54 grant entitled "Hormone-Regulated Pathways Controlling Implantation and Fertility." The have no direct effort in the budget, but they have graciously offered their own, unique novel estrogen receptor ligands OBHS and CLI, described in the application, as well as unsurpassed technical expertise in the design of cellular experiments to dissect the actions of ERα, -β and GPER on gene expression: in this case, BDNF. Their expertise and resources assure the optimal outcomes of these experiments.

PHS 398 Research Plan

Please attach applicable sections of the research plan, below.

OMB Number: 0925-0001

1. Introduction to Application (for RESUBMISSION or REVISION only)	intro_ra.pdf
2. Specific Aims	rplan_nar.pdf
3. Research Strategy*	rplan_rs.pdf
4. Progress Report Publication List	
Human Subjects Sections	
5. Protection of Human Subjects	rplan_hs.pdf
6. Inclusion of Women and Minorities	Inclusion_Women_Upload.pdf
7. Inclusion of Children	Inclusion_Children_Upload.pdf
Other Research Plan Sections	
8. Vertebrate Animals	
9. Select Agent Research	
10. Multiple PD/PI Leadership Plan	
11. Consortium/Contractual Arrangements	
12. Letters of Support	rplan_con.pdf
13. Resource Sharing Plan(s)	rplan_res.pdf
Appendix (if applicable)	
14. Appendix	

Funding Opportunity Number: PA-13-303. Received Date: 2014-03-03T10:20:35.000-05:00

1. INTRODUCTION

Response to the original review

We have carefully considered the critiques and appreciate the referees' perspectives of the relative merits of our proposal. Revised or new text since the initial submission appears in Georgia 11 font. All three Reviewers were highly complimentary of the investigative team we assembled. None of our clinical pain or quality of life endpoints, nor sample size calculations, were criticized.

We thank Reviewer 1 for her/his comments that "the translational application of the proposal is significant" and "if successful, the impact of this research would be exceptionally high...with possible major impact in the area of female reproductive health." The referee did note that "the techniques used are not very novel," a criticism shared by Reviewer 2. We have taken this lack of enthusiasm seriously and propose a new Aim (3) to evaluate the processing of pro-BDNF to the functional, mature protein that we postulate is accelerated in diseased *vs.* control cells. This innovative hypothesis extends our observation that the predominant protein isoform in endometriosis cells is mature BDNF (14 kD), whereas control endometrium has abundant pro-BDNF (28 kD). The experiments outlined will establish a proof-of-principle concept, befitting our research model, with new evidence of differential neuroangiogenic activity in endometriosis *vs.* control endometrium.

Reviewer 2 stated that "while the study is clinically significant, it is not clear that the approach will provide useful information on the precise role played by BDNF and potential avenues to treat endometriosis" and that "the studies proposed will not provide conclusive evidence for the role of BDNF in DIE (Deep infiltrating endometriosis)." Our group would submit that this reviewer has set a very high bar for the exploratory, 2-year, R21 granting mechanism. In our view, conclusive evidence for the role of BDNF in DIE symptoms in women can only be achieved by effectively antagonizing this neurotrophin in one arm of a placebo-controlled, randomized clinical trial and demonstrating a significant reduction in endometriosis-associated pain in the active treatment group. Admittedly, reversal of DIE lesions in rodent or nonhuman primate models would be highly suggestive, but not conclusive. Reviewer 2 indicated, quite correctly, that BDNF expression is not unique to endometriosis and is expressed in other clinical conditions. The hypothesis we proposed to test in Aim 1 does not require specificity of expression. Rather, the intent was to quantify relative BDNF levels in lesions *vs.* control endometrium to assure that this biomarker correlates with the clinical phenotype. S/He indicated, under Approach, that "other cytokines and chemokines...have not been taken into consideration." In our revised application we have made an effort to more clearly rationalize the selection of these particular pathways, based on our own preliminary data and inference from the literature. The limited budget and timeline of the R21 mechanism, the inherent complexity of the ER and IL-1 pathways (with multiple receptors as well as ligands), and our extensive experience conducting the necessary time-course, dose-response, and agonist and antagonist experiments required for these studies, in multiple cell types from different subjects, demand a feasible scope, focused on the most compelling signaling pathways.

Reviewer 3 comments that our "proposal addresses a previously unexplored aspect of endometriosis, namely the role of potential neuroangiogenic molecules in the pathobiology of DIE." We appreciate this point of view, which justifies our selection of the R21 mechanism to fund this new endeavor. The reviewer notes we have focused on the relative expression of BDNF in control eutopic endometrium vs. DIE lesions and that many of our proposed analyses omit direct comparisons with peritoneal and endometrioma lesions. While we agree that all are different manifestations of endometriosis and warrant examination, we have chosen to follow Ockham's Razor principle: "Plurality should not be posited without necessity." We have focused the application on the most extreme manifestation of endometriosis, using our most promising candidate factor. Extant evidence indicating that pain symptom severity is most pronounced in DIE, that nerve density is particularly high in DIE, and our preliminary findings that BDNF is highly concentrated in DIE lesions relative to surrounding tissue, support the hypothesis posed below and justify focused analyses comparing normal endometrial cells to those derived from eutopic and ectopic tissues from women with DIE. This referee also commented that "there is no effort to link the molecular analyses to either neurogenesis or angiogenesis in a functional way." Indeed, this point was the topic of the first paragraph in the original *Limitations and alternative strategies* section. We stand by the conviction that beyond some limited rodent pain models, conclusive evidence for a functional role of BDNF in human endometriosis pain can only be achieved by future randomized clinical trials, for which supportive preclinical experimentation, such as that proposed here, is an absolute prerequisite.

2. SPECIFIC AIMS

Deep infiltrating endometriosis (DIE) represents the most severely painful manifestation of this common gynecological syndrome. DIE lesions are imbued with dense nerve and vascular networks, postulated to arise via an integrated phenomenon that we coined “neuroangiogenesis” (1). While multiple, redundant uterine signals potentially contribute neuroangiogenic activities, an unbiased, proteomic approach (described below) allowed us to identify one multifunctional candidate, brain-derived neurotrophic factor (BDNF), that we propose to investigate in detail under the ægis of this exploratory R21 grant. BDNF is mitogenic for both neurons and endothelial cells (2-4) and it is overexpressed significantly in eutopic endometrium of endometriosis cases (5). Moreover, prior research indicates that both mRNA and protein of its cognate receptor, tyrosine kinase B (TrkB), also are upregulated in endometriosis cases (6). In sum, BDNF has the expression pattern and biochemical characteristics expected to mediate DIE lesion maintenance and pain. R21 funds will be used to validate the cellular distribution of BDNF mRNA and protein in DIE biopsies *in situ* using molecular histochemical assays. In addition, a well-characterized cell culture model will be used to evaluate the effects of estrogens and interleukin (IL)-1β, and their antagonists, on BDNF regulation *in vitro*. The latter experiments will identify mechanism-specific drug targets for rationally designed endometriosis therapeutics in the future.

We propose that coordinated neural and vascular growth represents an embryonic program coopted by endometriotic cells and defines the molecular pathways involved in lesion nociception (1). The **neuroangiogenesis hypothesis** predicts that paired ligand-receptor guidance molecules direct *de novo* nerve and capillary growth into nascent DIE lesions, leading to their growth, persistence and associated pain. This R21 proposal focuses on a single, but well-evidenced candidate neuroangiogenic factor, BDNF.

Aim 1. DIE nodule and eutopic endometrial biopsies will be collected surgically by and examined to confirm the *in vivo* distribution of nerves, capillaries, and BDNF protein using established immunohistochemical (IHC) methods. The localization of BDNF mRNA by *in situ* hybridization (ISH), will verify the cellular origin(s) of BDNF gene expression within adjacent microscopic sections of the same biopsies. Normal endometrial tissue will serve as a control. Validated pain and quality of life questionnaires will be collected preoperatively and compared among cases and controls.

Aim 2. Based on the localization of BDNF mRNA transcripts *in situ* (Aim 1), primary epithelial and/or stromal cell cultures will be established from subjects with DIE-associated pelvic pain and from control volunteers without pain. Eutopic and ectopic sources will be used for the endometriosis cases. The cell cultures will be interrogated using a combination of estrogens and cytokines, which our preliminary data indicate can modulate BDNF expression. The contributory roles of ERα, ERβ and nonclassical signaling via the cell membrane receptor GPER will be tested. Parallel experiments will be performed to identify interleukin (IL)-1 receptor activated pathways. Real-time (RT)-RT-PCR and ELISA methods will be used to quantify BDNF mRNA and protein, respectively. Multiple isoforms of BDNF protein are synthesized and processed in endometrial cells from high molecular weight precursors and manifest differential regulation and bioactivity. Western blotting will be used to resolve these. The *in vitro* models will be used to examine the effects of novel, selective ERα and -β receptor agonists and antagonists, as well as those for GPER and IL-1 receptor, on BDNF isoform expression; these mechanistic experiments should inform potential lead compounds for future endometriosis therapeutics.

Aim 3. Proprotein convertases (PCs), proteolytic enzymes involved in the processing of BDNF and other neural proteins and hormones, have been localized in normal human endometrium and endometrial cancer (7) but to date, no publications describe PCs in endometriosis. We will identify by RT-RT-PCR and Western blotting which isoforms of this nine-member family are predominantly expressed in DIE lesions and will quantify PC enzyme activity in isolated DIE *vs*. control cells via cleavage of fluoresecent substrates. This Aim will explore the innovative hypothesis that furin, PC5/6 or other PCs also are upregulated in DIE lesions, such that when BDNF is overexpressed (initially in its inactive pro-form [28 kD]) it is subsequently cleaved to mature, bioactive BDNF (14 kD), potentially offering another level of biological regulation leading to pain. The long-term objective of this line of inquiry is to develop PC inhibitors as novel targeted blockers of BDNF processing, to reduce neuroangiogenic activity locally.

3. RESEARCH STRATEGY

Significance

Endometriosis: epidemiology, pathogenesis and symptoms. Endometriosis is a common, chronic inflammatory gynecological disease that affects ~10% of all reproductive-aged women (close to 6 million in the US) and its prevalence is >30% among women with infertility and pelvic pain. In 2002 it was estimated that the direct and indirect costs of medical care for endometriosis in the US was over $22 billion, exceeding the costs of Crohn's disease in this country (8). Endometriosis is defined by the presence of endometrial cells outside the uterine cavity (ectopic endometrium), which classically is postulated to arise via retrograde menstrual flow, cœlomic metaplasia or vascular dissemination. These different etiologies have been critically reviewed (9) and will not be reiterated in this application, however the implantation hypothesis of Sampson is the most widely accepted (10), supported by observations that retrograde menstruation and intraperitoneal spillage of viable endometrial cells occur frequently in cycling women and more commonly in those with genital outflow tract obstruction.

In addition to the three etiopathogenetic theories described above, three principle clinical manifestations of disease are appreciated. Although cases with combined implant types are encountered, endometriosis typically presents as 1) superficial (peritoneal), 2) ovarian (endometrioma) or 3) deep infiltrating (subperitoneal extension >5 mm, often in the rectovaginal space (11)) lesions. Each has characteristic histomorphology, and may derive via distinct etiologies (12). However, based on their anatomic location and peritoneal fluid dynamics, arguments have been put forward that all three manifestations could arise as a result of menstrual regurgitation (13). Whether the histogenesis of DIE is truly distinct from superficial peritoneal endometriosis is hotly debated (14). What is widely accepted, however, is that pain associated with DIE is particularly severe (15). Without a noninvasive diagnostic test it is difficult to know the extent of asymptomatic endometriosis, but it has been estimated that ~95% of women with DIE (considerably higher than for other forms of endometriosis) have pain (16). Debilitating symptoms of dysmenorrhea, dyspareunia, bowel and/ urinary dysfunction are correlated with the location of DIE lesions (17) and are ameliorated by surgical excision (18).

Innovation

During the course of our pioneering studies of angiogenesis in endometriosis (19) we became aware of evidence that the growth of nerves, *pari passu* with capillaries, is associated with symptomatic endometriosis lesions (20). We reasoned that neuro- and angiogenic mitogens collude to stimulate coordinated nerve and blood vessel invasion in endometriosis, a phenomenon we termed "neuroangiogenesis," and would predispose to ectopic implantation and innervation of refluxed menstrual fragments (1). Thus, the eutopic endometrium represents the "mother" of all ectopic lesions, including DIE (21). Using an unbiased proteomic antibody microarray approach, we established that selected neurotropins and their receptors were differentially expressed in eutopic endometrial biopsies. The studies revealed that BDNF and neurotrophin (NT)-4/5 are preferentially expressed in endometriosis cases (22). Quantitative ELISAs showed that in 18 endometriosis cases, BDNF and NT-4/5, but not NGF, concentrations were significantly higher than in 15 controls (Fig. 1). Whereas vascular endothelial cell proliferation is not a characteristic of NT-4/5, a rich literature supports angiogenic effects of BDNF (2-4). Moreover, TrkB, the transmembrane receptor with the highest selectivity for BDNF, is upregulated >2-fold in eutopic tissue of endometriosis cases (23) and its mRNA is increased >4-fold in DIE lesions captured using laser microdissection (6). Amitriptyline and fluoxetine, antidepressants that are ligands for TrkB, have been used successfully for the treatment of neuropathic pain such as that observed in endometriosis (24). Hence, the expression patterns, tissue distribution, biochemical and signaling characteristics of BDNF fulfilled our predicted criteria for a mediator of neuroangiogenesis in DIE. Moreover, BDNF has been shown to contribute to pain in the irritable bowel syndrome (25). In a preliminary report of 37 subjects with endometriosis, plasma BDNF concentrations were significantly higher than in unaffected controls (26). In the revised proposal, we describe innovative experiments to assess whether enhanced processing of neurotrophin proteins by PCs, proteolytic enzymes originally identified in the pituitary that process BDNF and other neural hormones, might contribute to stimulated neuroangiogenic activity. Interestingly, the pro-BDNF isoform has been shown to be a potent *inhibitor* of neurite outgrowth, opposing the effects of the mature BDNF species (27). If our hypothesis is upheld, it would support the development of an entirely novel approach to endometriosis therapy, targeting selective PCs by peptides or small molecule drugs, to prevent functional BDNF processing and neuroangiogenesis.

Nerve and vessel density is high in DIE: Classical IHC methods, using antibodies to detect PGP9.5- and neurofilament (NF)-positive nerve fibers, indicate that their density is significantly higher (P<0.01) in DIE lesions compared to superficial peritoneal lesions (28). More detailed analyses indicate that these represent a mixture of sensory Aδ, sensory C, cholinergic and adrenergic nerves, consistent with their nociceptive function (29).

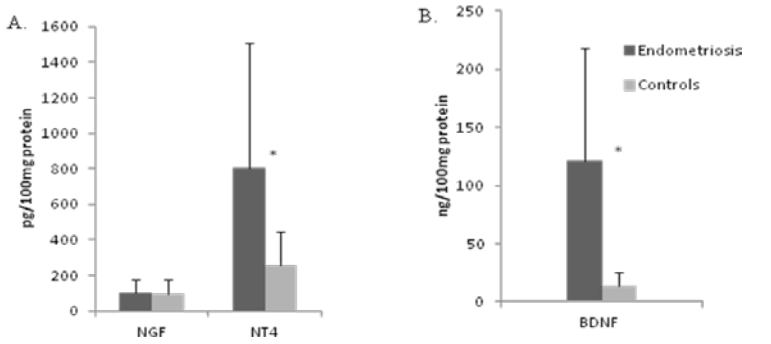


Fig. 1. NT-4 and BDNF levels (± SD) are elevated in the eutopic endometrium of women with endometriosis, relative to controls (5).

Most studies also support a higher concentration of blood vessels, angiogenic growth factors and their receptors in DIE (30). Vessel density, vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 (Flk-1) were all reported to be significantly higher in patients with DIE affecting the rectum (31), however Ramon et al. (32) described similar VEGF levels in peritoneal lesions and DIE. Clinical studies indicate a high correlation of pain with nerve fiber density in endometriosis lesions (33).

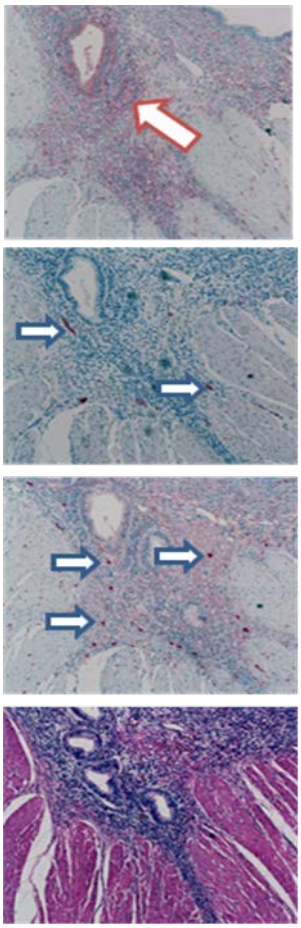


Fig. 2. IHC in bowel DIE. BDNF protein in glands and stroma (top panel, red arrow). Nerve fibers (NSE, 2nd panel and PGP9.5, 3rd panel, blue arrows) penetrate lesion. H&E stain (bottom) reveals DIE lesion invading into muscle of bowel wall.

Approach

Rationale: Molecular histology techniques can be used to accurately identify the cellular distribution and sources of specific mRNAs and proteins within properly prepared tissues. Once identified, we can derive and purify isolated cultures representative of those cell types, to systematically and directly test the mechanisms and signaling pathways involved in estradiol (E2) and IL-1β-mediated BDNF regulation *in vitro*. Finally, expression of PCs that regulate mature BDNF processing and their enzymatic activity can be determined in control and DIE tissues and cells to identify innovative drug targets for endometriosis pain.

Aim 1- **In situ *hybridization, immunohistochemistry and Western blotting for BDNF*:** We have extensive experience with mRNA and protein detection in endometrial and endometriosis tissues (34) including semiquantitative IHC of endometrial VEGF in fixed tissue sections (35). Based on our prior success we will use Histochoice®-fixed, paraffin-imbedded tissue with successive 5-8 µm sections cut through each block. Briefly, the slides are deparaffinized in xylene and rehydrated in graded concentrations of ethanol and exposed for 20 min to 0.1% saponin and 3% hydrogen peroxide in 0.05 M Tris-buffered saline (TBS) to quench endogenous hydrogen peroxide activity. Each sample is then washed in TBS and preincubated in 3% horse serum in TBS for 20 min. Positive control tissue sections (from archived brain and spinal cord specimens) will be included in each experiment to assure reliability and reproducibility of the IHC. Slides will be incubated in 10 mM citrate buffer (pH 6.0) for 45 minutes in a steam bath. Primary BDNF antibodies (Epitomics, dilution 1:100) in Tris buffer (pH 7.4) containing 0.5% casein as blocking reagent, will be applied and incubated overnight at 4 C. Biotinylated secondary F_c antibody will be used as a linking reagent and alkaline-phosphatase–conjugated streptavidin (Biogenex; San Ramon, CA) applied for labeling. The chromogen used is Vector Red (Vector Laboratories, Burlingame, CA) diluted in Tris (pH 8.5) and applied for 5-10 minutes at 30 C. Negative staining controls will be obtained by omitting the primary antibody or using identical concentrations of immunoabsorbed antibodies. Alternatively, an avidin-biotin peroxidase reaction (Elite

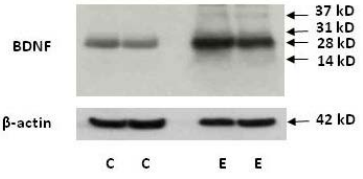


Fig. 3. Pro-BDNF precursor (28 kD) and mature BDNF (14 kD) are increased in eutopic endometrium of two endometriosis (E) subjects compared to two controls (C) (5).

Vectastain ABC, Vector Laboratories, Inc., Burlingame, CA), followed by a diaminobenzidine-black substrate reaction (Zymed) will be used. Similar IHC methods will be used to identify nerve fibers (using PGP9.5 and neuron specific enolase [NSE], see Fig. 2) and capillaries (anti-CD31 and -CD34) as we have published previously (35). Tissue regions rich in BDNF protein are associated with high density neurogenesis (Fig. 2).

We anticipate that mRNA *in situ* hybridization will confirm BDNF mRNA localization in the same regions, identifying those sites as sources of *de novo* BDNF gene expression. It is conceivable that despite the diffuse distribution of protein (Fig. 2, top panel), a specific cell type has a particularly high concentration of BDNF transcripts. Such a result would strongly inform our Aim 2 approach, below, by emphasizing the isolation of those specific cells. We will prepare antisense BDNF riboprobes from linearized T7T3D plasmids provided by colleagues at Emory University (36) but using digoxigenin-labeled nucleotides, as we successfully employed for the identification of VEGF transcripts in endometrial and endometriotic tissues. Alternatively, labeled oligonucleotides can be used. Sense probes will serve as negative controls, as described (19, 37).

Western blotting of endometrial homogenates, separated on 4%–12% SDS-polyacrylamide gradient gels and transferred to polyvinylidene fluoride filter paper, will be performed with mouse monoclonal anti-BDNF (Abcam, 1:500 dilution) with rotation at 4°C overnight. Mouse monoclonal anti-β-actin (Sigma, #A4700) antibodies at a dilution of 1:500 are used as an internal housekeeping control (Fig. 3). Our results demonstrate that the major intracellular BDNF isoform in eutopic tissue is the 28 kD pro-BDNF precursor of the mature (14 kD) neurotrophin and that its expression is higher in the eutopic endometrium of endometriosis than control subjects (5). In DIE lesions and endometriotic cells (Fig. 4) the 14 kD mature isoform appears to predominate.

Statistics and power analysis: Each experiment will be independently repeated at least three times. Descriptive data will be presented as mean ± SD. As our own preliminary data on endometrial BDNF IHC are too limited to direct a sample size analysis, we used Bruce Lessey's original endometrial integrin β3 IHC H-score analysis, demonstrating the under-expression of this integrin in eutopic endometrium of subjects with endometriosis and infertility, as an example (38). While the precision of H-score quantification is limited, this technique still offers to best approach to simultaneously localize and quantify transcript and protein expression. Based on the variance described in (40), and using α and β parameters of 0.05 and 0.20, 12 subjects in each group would be needed to detect a 50% difference in BDNF mRNA or protein H-scores. The calculation assumes normal data distribution and a two-tailed *t*-test model for comparison of the clinical categories (pain-free controls *vs*. DIE with pain). If the data prove to be non-Gaussian, Mann-Whitney nonparametric tests would be employed and a larger sample size would likely be necessary. This sample size calculation also is consistent with our published proteomics study, wherein 15 control and 18 endometriosis subjects were sufficient to demonstrate a significant increase in BDNF levels in eutopic endometrium of endometriosis cases (two-tailed $P<0.01$) (22). The target enrollment number for subjects was increased by 50% to account for subject drop-out and potential multiple comparisons if other analytes are assessed. All subjects will be fully informed of their responsibilities, the study aims and methods and the minimal risks of uterine perforation and infection (39). Validated Biberoglu and Behrman (B&B) scale evaluations and EHP-30 questionnaires will be administered preoperatively to all subjects (40). The results will be correlated with BDNF levels and nerve and vessel densities. We have the clinical volume to easily recruit 18 DIE with pain and 18 age- and race-matched, pain-free controls over the two-year duration of the exploratory R21.

Anticipated results and obstacles: We expect to find high BDNF mRNA and protein levels in DIE lesions, in epithelial, stromal and infiltrating nerve cells of the implants. Neuronal expression has been reported by others (41). It will not come as a surprise if tissue macrophages also express BDNF (42). We will identify the latter in serial sections or by double-antibody IHC using BDNF and CD68 antibodies, using methods published previously (43, 44). We are familiar with IHC and ISH and comfortable that we can troubleshoot any technical challenges by screening multiple antibodies or testing different riboprobe or if needed, oligonucleotide constructs. A good correlation of B&B and EHP-30 scores with nerve fiber density, as noted (33), is predicted.

In vivo *and* in vitro *models for endometriosis:* The application and utility of rodent models in endometriosis has been reviewed (45) and only recently has a DIE model been established in this species. The DIE mouse (46) uses xenografts into an immunodeficient host (nu/nu), which we feel is suboptimal given the important role of the inflammatory response in clinical endometriosis, particularly DIE. A nonhuman primate (baboon) DIE model was put forth (47), but this is feasible only in a limited number of facilities. Hence, we choose to use cell cultures for our mechanistic studies. *In vitro* models using SV40 T-antigen- (eg, 12Z (48)) or telomerase-immortalized cells have been described, but several of these "lines" are contaminated by other cell types (49). We created immortalized endometriosis cells with a temperature-sensitive T-antigen (50), but were

disappointed by their aneuploidy, loss of ERα and -β expression, and transformed phenotype, and abandoned that strategy. Instead, we pioneered the independent isolation and culture of primary endometriotic epithelial and stromal cells (51) and prefer the authenticity of this model: the cells express characteristic microfilament proteins, maintain functional steroid receptors under culture conditions (19) and respond to anti-inflammatory and anti-proliferative effects of several standard endometriosis treatments including danazol, progestins and GnRH analogs, as well as novel nonhormonal therapeutic interventions (52, 53).

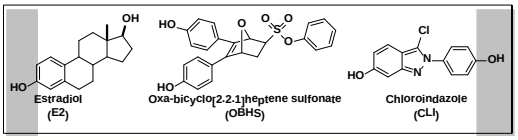
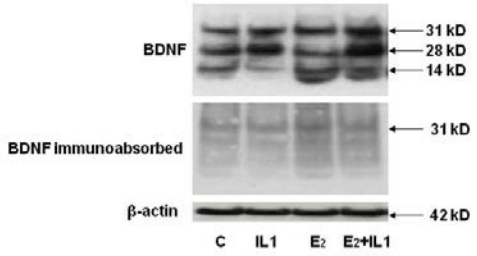


Fig. 5. E2 and novel ER ligands from the Katzenellenbogens will be used to assess ERα and -β effects on BDNF production.

Aim 2- Isolation of normal endometrial and endometriosis epithelial and stromal cells. Epithelial and stromal cells will be isolated from eutopic endometrial specimens using enzymatic digestion techniques developed and in our laboratory (51) and now used globally (54). Recently, the same methodology has been successfully applied for the isolation of cells from DIE lesions (55) and the authors report a 75% success rate of culturing epithelial and stromal cells from DIE lesions, similar to our own experience of a 56% yield for endometrioma-derived stromal cell cultures (51). We have found it optimal to isolate primary cells from early-mid proliferative phase tissue, so surgical intervention in the participants will be scheduled in these phases. This condition provides cells with very low *in vivo* exposure to progesterone, allowing us to better manipulate their proliferation and hormonal differentiation *in vitro* (56). Biopsied tissues will be dispersed after incubation with collagenase and stromal cells are separated from epithelial cells by sequential filtering through 100 µm and 40 µm sieves. Primary cultures are grown to 80% confluence using a 1:1 mixture of phenol-red free Dulbecco's modified Eagle medium and Ham's F-12 nutrient medium (DMEM/F-12) supplemented with 10% charcoal-stripped fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml). To eliminate endogenous leukocytes, the stromal cells are passaged twice. In previous studies, we verified by immunocytochemistry >93% purity of the respective cultures. To verify their phenotype, we showed that epithelial cells stain positively with cytokeratin and negatively with vimentin, whereas primary endometrial stromal cell cultures stain positively with vimentin and negatively with cytokeratin. Both preparations are negative for the CD45 (leukocyte) marker. Epithelial cells are studied in the first passage and stromal cells can be carried for up to 5 passages without noticeable loss of ER function (51).

Fig.4. BDNF isoforms expressed in endometriotic stromal cell culture treated with E2 and/or IL-1β for 48 h. The mature products are ~14 kD. All bands are reduced when antibody is immunoabsorbed.



Although endometriotic cells are targets for a panoply of steroid hormones, cytokines and chemokines, the narrow scope of the R21 grant compels us to focus on E2 and IL-1β signaling emblematic of endocrine and immune mediators of lesion activity (9). Moreover, E2 and IL-1β are both known to stimulate BDNF expression in neuronal and other non-neuronal tissues (57, 58). Primary epithelial and stromal cell cultures will be independently established from control (eutopic) endometrial Pipelle biopsies and from eutopic and lesion nodules of DIE subjects (55). As an alternative, if we fail to generate adequate DIE nodule cultures, we will resort to ovarian endometrioma epithelial and stromal cell cultures, with which we have extensive experience (51). Dose-response and time-course effects of E2 and/or IL-1β on BDNF mRNA and protein will be assessed in the established cultures by RT-RT-PCR, ELISA and Western blots, respectively. To establish the signaling pathways involved, ERα, -β and GPER selective agonists or inhibitors will be used. The IL-1 type I receptor (which we showed was 2.4-fold upregulated in endometriosis cells (59)) will be tested dose-responsively and in time-course studies using IL-1β and IL-1RA. BDNF isoforms will be resolved by running cell protein extracts (50 µg) on 4%–12% SDS-polyacrylamide gradient gels. Validated anti-BDNF antibodies will be used (Fig. 4). Multiple BDNF bands detected all appear specific when the antibody is immunoabsorbed with BDNF peptide. The bands reflect intracellular processing of high molecular weight precursors (31 and 28 kD) to mature (14 kD) BDNF, in contrast to the dominant 28 kD precusor observed in control endometrial homogenates (Fig. 3). E2 increases the 14 kD isoform, whereas IL-1β predominantly stimulates the 28 kD species (Fig. 4). E2 also upregulated BDNF in human umbilical stem cells (60).

The ERα and –β selective agonists, propyl pyrazole triol (PPT) and diarylpropionitrile (DPN), respectively, will be tested ± ICI 182,780 (an ERα and -β antagonist]) to verify the likely ER subtype(s) involved in BDNF regulation. We (61) and Bulun et al. (62) have reported previously that ERβ is overexpressed in endometriosis cells, making this an attractive target for lesion-selective pharmaceuticals. The Katzenellenbogens (see letter of collaboration and Biosketches), have developed two ER ligands of novel structure and activity (Fig. 5): oxabicycloheptene sulfonate (OBHS) has antagonist activity on ERα and ERβ (63), whereas chloroindazole (CLI) is selective for ERβ (64). In collaborative studies we showed that both have strong ER-dependent anti-inflammatory activities in human endometriosis cell cultures and in a murine model of endometriosis (65). In the latter, both OBHS and CLI significantly suppressed histological nerve density in the experimental lesions (Fig. 6). The Katzenellenbogens will guide us in determining the possible mechanisms of anti-neuroangiogenic activity by these compounds in human endometrial and endometriosis cell cultures.

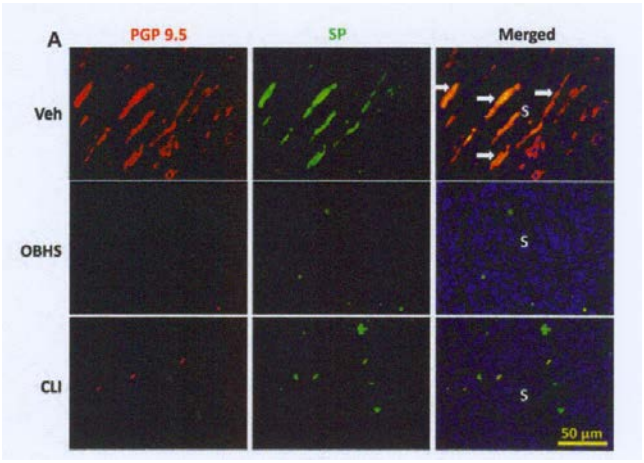


Fig. 6. Nociceptive nerves stained with PGP9.5 and substance P (SP) antibodies showed less invasion into experimental endometriosis lesions in mice treated with OBHS or CLI compared to vehicle (Veh) (65).

We reported that the putative G protein-coupled receptor for estrogen, GPER, is significantly upregulated in the eutopic endometrial glandular and luminal epithelium of women with endometriosis ((66), Fig. 7), where it is localized to epithelial and stromal cells. Its maximal expression is in the proliferative phase, suggesting that estrogen plays a role in its expression. GPER was independently identified in epithelial and stromal cells of DIE lesions by another group, with a predominant cytoplasmic distribution (67). Using Ishikawa adenocarcinoma cells as an endometrial epithelial model, E2, but not DPN (an ERβ agonist) or G1 (a GPER agonist ligand), induced GPER expression, suggesting that the estrogenic effect on GPER is mediated via ERα. We will probe the effects of the GPER pathway on BDNF expression by dose-response and time-course experiments with the GPER agonist (G1) and antagonist (G15) ligands (68) in primary endometrial and endometriotic cells. BDNF mRNA (RT-RT-PCR) and protein (by ELISA and Western blotting) will be quantified.

Verification of the pharmacology experiments above, will be confirmed by siRNA constructs to knock down ERα, ERβ, GPER or IL-1 type I receptor (or scrambled siRNA as a control, Ambion, Austin, TX), as we have done successfully for other transcription factors (56). Efficiency of knockdown will be confirmed by receptor protein Western blots and effects on BDNF expression will be quantified by RT-RT-PCR, ELISA and Westerns.

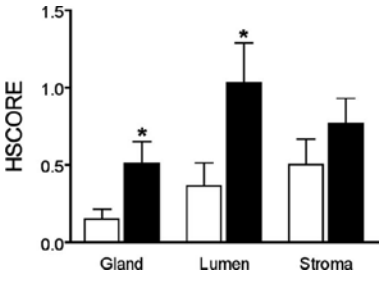


Fig. 7. GPER is increased in the eutopic epithelium of women with (black) *vs*. those without (white) endometriosis (*P<0.05) but not statistically different in stroma (66).

Anticipated results and obstacles: We expect to find that both epithelial and stromal cells express BDNF mRNA and protein *in vitro* and to reproduce our findings that eutopic cells derived from women with DIE have higher BDNF protein levels than eutopic cells of controls. Based on our previous findings of IL-6 and RANTES secretion (34, 69) we anticipate that cells derived from ectopic DIE lesions or endometriomas will express even higher levels of BDNF. The stimulation of BDNF by E2 and IL-1β also is likely to be more robust in ectopic cells than in control (eutopic) cells. (59, 70). Because we will validate BDNF effects by RT-RT-PCR, ELISA and Western we foresee few problems with data interpretation. As in our *in vivo* murine model, we expect that OBHS and CLI will inhibit BDNF expression *in vitro*. It will be of interest, for example, if we find that the effects of DIE are manifested predominantly at the level of steady-state mRNA levels *vs*. protein synthesis. Such findings can be resolved in future studies using BDNF gene promoter-reporter constructs or alternatively, assessment of ribosome accumulation on BDNF transcripts. We have experience conducting experiments of both types (59, 71). Based on our preliminary

observations, we expect that mature, pro-neurogenic BDNF (14 kD) will be preferentially produced in DIE cells, whereas the inhibitory pro-BDNF (28 kD) (27) will be the primary control cell product.

Aim 3- Characterization of proprotein convertases (PCs) responsible for BDNF processing. There are nine well characterized mammalian PCs that process neurotrophin precursors in the Golgi or at the cell surface. These enzymes, serine proteinases secreted themselves as zymogens, specialize in cleaving selected sites at paired basic amino acid residues (72). Based on their substrate specificity for pro-BDNF, furin and PC5/6 are candidate PCs for BDNF processing and both have been identified in human endometrium and endometrial carcinoma (although not yet in endometriosis) tissues (7, 73). We will validate their expression in control and DIE cell cultures by RT-RT-PCR and Western blotting using published primers and antibodies. To test our hypothesis that ectopic or eutopic epithelial or stromal DIE cells have more cell surface or intracellular PC activity than corresponding control eutopic cells, 50 μM fluorogenic substrate L-pyroGlu-Arg-Thr-Lys-AMC (pERTKR-AMC, R&D Systems, Minneapolis, MN) will be incubated in 25 mM Tris, 1 mM $CaCl_2$, pH 7.5 for 30 min at 37°C. Substrate conversion will be determined by excitation at 360 nm and emission fluorescence at 460 nm in a microplate reader. Activity in intact cells will indicate surface expression, whereas lysates will detect total cellular enzyme content.

Anticipated results and obstacles: We expect to identify candidate PCs in DIE cell lysates or expressed on the cell surface. Furin and PC5/6 are probable, but primer and antibody reagents for the other seven family members are available and will be screened. Enzyme activity measurements, using the sensitive fluorometric substrate method, should parallel differences in PC mass detected by RT-RT-PCR or Western blotting. Our hypothesis stands that BDNF processing is accelerated in DIE derived cells.

Limitations and alternative strategies: Throughout the application we have referred to BDNF as a potential neuroangiogenesis mediator. This is based on our findings that immunoactive BDNF (ie, that detected by ELISA or Western blot) is upregulated in endometriosis cells and tissues (5) and extensive literature showing and that the protein is mitogenic for both neurons and endothelial cells (2, 4). Moreover, we cite data that indicate that pro-BDNF can oppose the effects of the mature BDNF protein (27). Hence, understanding the ratio of precursor to product in each local setting is critical. We also have unpublished data indicating that endometriosis cell culture supernatants contain bioactivity(ies) that stimulates the proliferation of neurons and endothelial cells *in vitro*, which is partially inhibited by neutralizing anti-BDNF antibodies. We have chosen to use the exploratory R21 mechanism to focus on its cellular and molecular biology in our DIE models. Future independent R01 support will be sought to further characterize other neuroangiogenic bioactivities, using mitogenesis, proliferation and cell migration assays with which we have considerable experience (53, 74).

Timeline and benchmarks: The scope and detail of the proposed studies are commensurate with the two-year R21 mechanism. Both aims must be pursued simultaneously, as isolation of cells from fresh eutopic biopsies and DIE lesions is necessary. However, in funding year 01, we will emphasize the molecular histology experiments in Aim 1 to confirm the cellular distribution of BDNF in eutopic and ectopic epithelium and/or stroma and determine by *in situ* hybridization if BDNF mRNA predominates in one or both of those cell types. In year 02 we will continue emphasize the *in vitro* regulation of BDNF by estrogens and IL-1β in isolated cells. At the conclusion of the R21, an R01 application will be prepared to extend analysis of the relevant biological and biochemical activities of BDNF-induced and other mediators of nerve and capillary proliferation and migration. Establishing BDNF as a specific target should allow rational drug design for endometriosis-associated pain. The feasibility of therapeutically interfering with BDNF has already been established *in vitro* and *in vivo* using TrkB blocking peptides that suppress choriocarcinoma growth (75, 76) and PC inhibitors are under current development as possible therapeutics for hypercholesterolemia (72).

5. PROTECTION OF HUMAN SUBJECTS

This application meets the criteria for Human Research, but it is not a clinical trial. We have established highly efficient protocols for eutopic and ectopic endometrium collection that have worked well in multiple sites for 20 years. In all cases the samples are de-identified and coded without disclosure of private health information. Records linking specimens to the subjects are kept in encrypted computer files and paper records maintained in a locked file in office.

Inclusion Criteria: Generally healthy premenopausal women between 18-45 years of age inclusive with intact uterus and ovaries will be required of all subjects. The subjects will have regular ovulatory cycles with an interval of 25-35 days. A normal Pap smear within one year, blood hematocrit >36 and sufficient intelligence and motivation to comply with the consenting process are needed. They will be undergoing indicated laparoscopic and/or robotic surgery, and the clinical presence or absence of endometriosis, and characteristics of DIE or endometrioma, will be documented by the surgeon. Histopathological examination of excised implants will confirm the diagnostic groups.

Exclusion Criteria: Any history of thromboembolic disorders, chronic medical illness such as renal disorders, hepatic disease, diabetes, hypertension or thyroid disease, or past history of known or suspected neoplasia will exclude the study candidate. A history of irregular ovulation or use of any oral estrogen or progestin containing medication within a 3-month period or central nervous system active medications known to disrupt the menstrual cycle (eg, GnRH-analogs, clonidine, aldomet) will not be allowed. Given the postulated role of immune activation in women with endometriosis, subjects on potent immunosuppressants (eg, glucocorticoids, methotrexate, cyclosporin) will be excluded. A Pap smear indicating dysplasia or more significant abnormality and smoking more than 15 cigarettes/day (these individuals may metabolize estrogens more rapidly and have endocrine changes as a result). Subjects with a psychiatric history suggesting a functionally debilitating disturbance such as psychosis or major mood disorder will be excluded. To protect the laboratory personnel, patients with active or prior infection with hepatitis or HIV will be excluded. However, it should be noted that all tissues are handled and discarded as if they were potentially infected.

Research material will include demographic data and reproductive history, as well as cigarette, ethanol, and herbal product usage. Endometrial and endometriosis tissues will be collected under anesthesia in the proliferative phase of the cycle and this will be recorded based on menstrual dates and ultrasound appearance of the uterine cavity. Transvaginal ultrasound has no known short-term or long-term risks. The risk of uterine perforation using the Pipelle instrument is essentially nil. A single case is reported in the world's literature (39). Endometrial biopsies will be performed in the operating suite by trained personnel.

The recorded source of each tissue specimen (eg, rectovaginal DIE nodule) will be identified only by the patient's age, menstrual dating, date of collection and the presence or absence of endometriosis (confirmed at a later date by histopathology). The patient will have no potential loss of privacy or personal health information.

Risks to the Subjects: The subjects that will be recruited in protocol for specimen collection are relatively healthy women of reproductive age undergoing elective laparoscopic surgery. We have selected inclusive ages of 18-45 years (see 'Inclusion of Children' below) as the endpoints of our study are to understand the mediators of of endometriosis-associated pain. Thus, we want to study women within the age group that are commonly seeking care for pelvic pain. The patients admitted into our study will include the diverse ethnic mix represented in the Winston-Salem region. Inclusion of a broad range of ethnicities will enable us to identify markers with wide applicability to North American patient populations. Census records from our metropolitan area indicate an ethnic distribution consistent with the **Targeted Enrollment Form**, below.

Adequacy of Protection Against Risks: Subjects will be recruited through advertisements in the newspaper, posted fliers and radio announcements as well as through the referral practice of the investigators. A copy of the consent will be given to each subject. A second copy will be kept in a locked file. Each subject will be oriented to the objectives of the study, participant responsibilities, testing procedures and their risks and benefits.

Procedures will be followed for protection of confidentiality. All subject identities and records will remain strictly confidential. Individual subject data will not be associated with the subjects name or identifying record number. A key file linking the subject's name, identifying number and data will be kept locked in the PI's office. Only the Principal and Co-Investigators, or the PI's assigned designee, will have access to the linking key. None of the study information will be placed into the subject's medical record and that information only will be released after the express, written consent of the subject.

Potential Benefits of the Proposed Research to the Subjects and Others: No direct benefit for the subjects participating in this research is expected. Because the risks of this study are small and the information gathered will potentially have a positive impact on future clinical management of patients with DIE and endometriosis pain, the benefits outweigh any theoretical risks.

Importance of the Knowledge to be Gained: The knowledge gained from the studies will be new insights into the mechanisms of pelvic pain associated with endometriosis.

Data and Safety Monitoring Plan: This study is not a clinical trial. The study procedures, intraoperative endometrial and endometriosis biopsies, are very safe and will be monitored by the clinical Co-Is and PI. Should any complaint be lodged or complication arise, the PI will consult with senior members of the Department of Obstetrics and Gynecology at to assess the potential of future risks to study subjects.

Our studies involving endometrial specimen procurement and human subject research are approved under (initial approval date: 04/03/2012, recent renewal 01/06/2014,). The histological studies in this project will use de-identified tissues. Human endometrial and endometriosis cell cultures will be established at from de-identified fresh biopsy specimens.

6. INCLUSION OF WOMEN AND MINORITIES

Only women will participate in this study as endometriosis does not occur in men. There is no known racial predilection for endometriosis and it is relatively common among all women. The patients admitted into our study will include the diverse ethnic mix represented in the population. Inclusion of a broad range of ethnicities will enable us to identify endometrial markers with wide applicability to other North American populations.

All ethnic groups will be represented. No evidence exists to suggest that the results will be influenced by ethnic background. Thus, it is anticipated that the ethnic background of the patients studied will reflect the ethnic composition of . The ethnic/racial composition of the patients at the primary recruitment site is indicated in the Table. No attempt will be made to exclude any patient on the basis of ethnicity or race.

OMB Number: 0925-0002

Planned Enrollment Report

Study Title: Neuroangiogenesis in Deep Infiltrating Endometriosis

Domestic/Foreign: Domestic

Comments:

Racial Categories	Ethnic Categories				Total
	Not Hispanic or Latino		Hispanic or Latino		
	Female	Male	Female	Male	
American Indian/Alaska Native	0	0	0	0	**0**
Asian	4	0	0	0	**4**
Native Hawaiian or Other Pacific Islander	0	0	0	0	**0**
Black or African American	8	0	0	0	**8**
White	22	0	2	0	**24**
More than One Race	0	0	0	0	**0**
Total	**34**	**0**	**2**	**0**	**36**

Study 1 of 1

Funding Opportunity Number: PA-13-303. Received Date: 2014-03-03T10:20:35.000-05:00

7. INCLUSION OF CHILDREN

Children ages 18-21 years of age will be included as endometriosis does occur in young women, however it is unusual to find DIE in this young population.

REFERENCES CITED

1. Asante A, Taylor RN. Endometriosis: the role of neuroangiogenesis. Annu.Rev.Physiol 2011;73:163-82
2. Kermani P, Hempstead B. Brain-derived neurotrophic factor: a newly described mediator of angiogenesis. Trends Cardiovasc.Med 2007 May;17(4):140-3.
3. Newton SS, Duman RS. Regulation of neurogenesis and angiogenesis in depression. Curr.Neurovasc.Res. 2004 Jul;1(3):261-7
4. Long BL, Rekhi R, Abrego A, Jung J, Qutub AA. Cells as state machines: Cell behavior patterns arise during capillary formation as a function of BDNF and VEGF. J Theor.Biol. 2013 Jun;326:43-57
5. Browne AS, Yu J, Huang RP, Francisco AM, Sidell N, Taylor RN. Proteomic identification of neurotrophins in the eutopic endometrium of women with endometriosis. Fertil Steril. 2012 Sep;98(3):713-9.
6. Matsuzaki S, Canis M, Vaurs-Barriere C, Pouly JL, Boespflug-Tanguy O, Penault-Llorca F, Dechelotte P, Dastugue B, Okamura K, Mage G. DNA microarray analysis of gene expression profiles in deep endometriosis using laser capture microdissection. Mol.Hum.Reprod. 2004 Oct;10(10):719-28
7. Singh H, Heng S, Nicholls PK, Li Y, Tai LT, Jobling T, Salamonsen LA, Nie G. Proprotein convertases in post-menopausal endometrial cancer: distinctive regulation and non-invasive diagnosis. Biochem.Biophys.Res.Commun. 2012 Mar;419(4):809-14
8. Simoens S, Hummelshoj L, D'Hooghe T. Endometriosis: cost estimates and methodological perspective. Hum.Reprod.Update. 2007 Jul;13(4):395-404
9. Taylor RN, Lebovic DI. Endometriosis. In: Strauss JF, Barbieri RL, editors. *Yen and Jaffe's Reproductive Endocrinology*. 7th ed. Philadelphia: Saunders Elsevier; 2014. pp. 565-85.
10. Sampson JA. Peritoneal endometriosis due to menstrual dissemination of endometrial tissue into the peritoneal cavity. Am.J Obstet.Gynecol. 1927;14:442-69
11. Koninckx PR, Meuleman C, Demeyere S, Lesaffre E, Cornillie FJ. Suggestive evidence that pelvic endometriosis is a progressive disease, whereas deeply infiltrating endometriosis is associated with pelvic pain. Fertil.Steril. 1991 Apr;55(4):759-65
12. Nisolle M, Donnez J. Peritoneal endometriosis, ovarian endometriosis, and adenomyotic nodules of the rectovaginal septum are three different entities. Fertil.Steril. 1997 Oct;68(4):585-96
13. Bricou A, Batt RE, Chapron C. Peritoneal fluid flow influences anatomical distribution of endometriotic lesions: why Sampson seems to be right. Eur.J.Obstet.Gynecol.Reprod.Biol. 2008 Jun;138(2):127-34
14. Vercellini P, Aimi G, Panazza S, Vicentini S, Pisacreta A, Crosignani PG. Deep endometriosis conundrum: evidence in favor of a peritoneal origin. Fertil Steril. 2000 May;73(5):1043-6
15. Vercellini P. Endometriosis: what a pain it is. Semin.Reprod.Endocrinol. 1997;15(3):251-61
16. Koninckx PR, Ussia A, Adamyan L, Wattiez A, Donnez J. Deep endometriosis: definition, diagnosis, and treatment. Fertil.Steril. 2012 Sep;98(3):564-71
17. Seracchioli R, Mabrouk M, Guerrini M, Manuzzi L, Savelli L, Frasca C, Venturoli S. Dyschezia and posterior deep infiltrating endometriosis: analysis of 360 cases. J.Minim.Invasive.Gynecol. 2008 Nov;15(6):695-9
18. Bassi MA, Podgaec S, Dias JA, Jr., D'Amico FN, Petta CA, Abrao MS. Quality of life after segmental resection of the rectosigmoid by laparoscopy in patients with deep infiltrating endometriosis with bowel involvement. J Minim.Invasive.Gynecol 2011 Nov;18(6):730-3
19. Shifren JL, Tseng JF, Zaloudek CJ, Ryan IP, Meng YG, Ferrara N, Jaffe RB, Taylor RN. Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. J Clin.Endocrinol.Metab 1996 Aug;81(8):3112-8
20. Anaf V, Simon P, El Nakadi I, Fayt I, Buxant F, Simonart T, Peny MO, Noel JC. Relationship between endometriotic foci and nerves in rectovaginal endometriotic nodules. Hum.Reprod 2000 Aug;15(8):1744-50
21. Lessey BA, Lebovic DI, Taylor RN. Eutopic endometrium in women with endometriosis: 'Ground zero' for the study of implantation defects . Semin Reprod Med 2013; 31:109-124
22. Browne AS, Yu J, Huang RP, Francisco AM, Sidell N, Taylor RN. Proteomic identification of neurotrophins in the eutopic endometrium of women with endometriosis. Fertil Steril. 2012 Sep;98(3):713-9. PMCID:PMC3432681
23. Anger DL, Zhang B, Boutross-Tadross O, Foster WG. Tyrosine receptor kinase B (TrkB) protein expression in the human endometrium. Endocrine. 2007 Apr;31(2):167-73

24. Chavez NF, Zweizig SL, Stewart EA. Neuropathic uterine pain after hysterectomy. A case report. J.Reprod.Med. 2003 Jun;48(6):466-8
25. Yu YB, Zuo XL, Zhao QJ, Chen FX, Yang J, Dong YY, Wang P, Li YQ. Brain-derived neurotrophic factor contributes to abdominal pain in irritable bowel syndrome. Gut 2012 May;61(5):685-94
26. Wessels JM, Leyland NA, Agarwal S, Murji A, Foster WG. Can brain-derived neurotrophic factor be a clinical marker for endometriosis? American Society for Reproductive Medicine, Boston, Fertil Steril 100, S101-S102 (abst). 2013.
27. Sun Y, Lim Y, Li F, Liu S, Lu JJ, Haberberger R, Zhong JH, Zhou XF. ProBDNF collapses neurite outgrowth of primary neurons by activating RhoA. PLoS.One. 2012;7(4):e35883
28. Wang G, Tokushige N, Markham R, Fraser IS. Rich innervation of deep infiltrating endometriosis. Hum.Reprod. 2009 Apr;24(4):827-34
29. Berkley KJ, Rapkin AJ, Papka RE. The pains of endometriosis. Science 2005 Jun 10;308(5728):1587-9
30. Jondet M, Vacher-Lavenu MC, Chapron C. Image analysis measurements of the microvascularisation in endometrium, superficial and deep endometriotic tissues. Angiogenesis. 2006;9(4):177-82
31. Machado DE, Abrao MS, Berardo PT, Takiya CM, Nasciutti LE. Vascular density and distribution of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 (Flk-1) are significantly higher in patients with deeply infiltrating endometriosis affecting the rectum. Fertil.Steril. 2008 Jul;90(1):148-55
32. Ramon LA, Braza-Boils A, Gilabert-Estelles J, Gilabert J, Espana F, Chirivella M, Estelles A. microRNAs expression in endometriosis and their relation to angiogenic factors. Hum.Reprod. 2011 May;26(5):1082-90
33. Mechsner S, Kaiser A, Kopf A, Gericke C, Ebert A, Bartley J. A pilot study to evaluate the clinical relevance of endometriosis-associated nerve fibers in peritoneal endometriotic lesions. Fertil Steril. 2009 Dec;92(6):1856-61
34. Hornung D, Ryan IP, Chao VA, Vigne JL, Schriock ED, Taylor RN. Immunolocalization and regulation of the chemokine RANTES in human endometrial and endometriosis tissues and cells. J.Clin.Endocrinol.Metab 1997 May;82(5):1621-8
35. Pritts EA, Ryan IP, Mueller MD, Lebovic DI, Shifren JL, Zaloudek CJ, Korn AP, Darney PD, Taylor RN. Angiogenic effects of norplant contraception on endometrial histology and uterine bleeding. J.Clin.Endocrinol.Metab 2005 Apr;90(4):2142-7
36. Rattiner LM, Davis M, French CT, Ressler KJ. Brain-derived neurotrophic factor and tyrosine kinase receptor B involvement in amygdala-dependent fear conditioning. J.Neurosci. 2004 May 19;24(20):4796-806
37. Hornung D, Klingel K, Dohrn K, Kandolf R, Wallwiener D, Taylor RN. Regulated on activation, normal T-cell-expressed and -secreted mRNA expression in normal endometrium and endometriotic implants: assessment of autocrine/paracrine regulation by in situ hybridization. Am.J.Pathol. 2001 Jun;158(6):1949-54
38. Lessey BA, Castelbaum AJ, Sawin SW, Buck CA, Schinnar R, Bilker W, Strom BL. Aberrant integrin expression in the endometrium of women with endometriosis. J Clin.Endocrinol.Metab 1994 Aug;79(2):643-9
39. Koonings PP, Moyer DL, Grimes DA. A randomized clinical trial comparing Pipelle and Tis-u-trap for endometrial biopsy. Obstet.Gynecol. 1990 Feb;75(2):293-5
40. Jenkinson C, Kennedy S, Jones G. Evaluation of the American version of the 30-item Endometriosis Health Profile (EHP-30). Qual.Life Res. 2008 Nov;17(9):1147-52
41. Obata K, Yamanaka H, Kobayashi K, Dai Y, Mizushima T, Katsura H, Fukuoka T, Tokunaga A, Noguchi K. The effect of site and type of nerve injury on the expression of brain-derived neurotrophic factor in the dorsal root ganglion and on neuropathic pain behavior. Neuroscience 2006 Feb;137(3):961-70
42. Linker R, Gold R, Luhder F. Function of neurotrophic factors beyond the nervous system: inflammation and autoimmune demyelination. Crit Rev.Immunol. 2009;29(1):43-68
43. Mueller MD, Lebovic DI, Garrett E, Taylor RN. Neutrophils infiltrating the endometrium express vascular endothelial growth factor: potential role in endometrial angiogenesis. Fertil Steril. 2000 Jul;74(1):107-12
44. Dun EC, Hanley K, Wieser F, Bohman S, Yu J, Taylor RN. Infiltration of tumor-associated macrophages is increased in the epithelial and stromal compartments of endometrial carcinomas. Int J Gynecol Pathol 2013;32:576-584
45. Sharpe-Timms KL. Using rats as a research model for the study of endometriosis. Ann.N.Y.Acad.Sci. 2002 Mar;955:318-27

46. Leconte M, Nicco C, Ngo C, Chereau C, Chouzenoux S, Marut W, Guibourdenche J, Arkwright S, Weill B, Chapron C, et al. The mTOR/AKT inhibitor temsirolimus prevents deep infiltrating endometriosis in mice. Am.J Pathol 2011 Aug;179(2):880-9
47. Donnez O, Van Langendonckt A, Defrere S, Colette S, Van KO, Dehoux JP, Squifflet J, Donnez J. Induction of endometriotic nodules in an experimental baboon model mimicking human deep nodular lesions. Fertil.Steril. 2013 March;99(3):783-789
48. Grund EM, Kagan D, Tran CA, Zeitvogel A, Starzinski-Powitz A, Nataraja S, Palmer SS. Tumor necrosis factor-alpha regulates inflammatory and mesenchymal responses via mitogen-activated protein kinase kinase, p38, and nuclear factor kappaB in human endometriotic epithelial cells. Mol.Pharmacol. 2008 May;73(5):1394-404
49. Korch C, Spillman MA, Jackson TA, Jacobsen BM, Murphy SK, Lessey BA, Jordan VC, Bradford AP. DNA profiling analysis of endometrial and ovarian cell lines reveals misidentification, redundancy and contamination. Gynecol.Oncol. 2012 Oct;127(1):241-8
50. Lundeen SG, Lebovic DI, Carver JM, Taylor RN, Winneker RC. Establishment and characterization of endometriosis stromal cell lines with an extended life-span. VI World Congress on Endometriosis, Québec City , #25 (abst). 1998.
51. Ryan IP, Schriock ED, Taylor RN. Isolation, characterization, and comparison of human endometrial and endometriosis cells in vitro. J.Clin.Endocrinol.Metab 1994 Mar;78(3):642-9
52. Pritts EA, Zhao D, Ricke E, Waite L, Taylor RN. PPAR-gamma decreases endometrial stromal cell transcription and translation of RANTES in vitro. J Clin.Endocrinol.Metab 2002 Apr;87(4):1841-4
53. Wieser F, Yu J, Park J, Gaeddert A, Cohen M, Vigne JL, Taylor RN. A botanical extract from channel flow inhibits cell proliferation, induces apoptosis, and suppresses CCL5 in human endometriotic stromal cells. Biol.Reprod 2009 Aug;81(2):371-7
54. Noble LS, Takayama K, Zeitoun KM, Putman JM, Johns DA, Hinshelwood MM, Agarwal VR, Zhao Y, Carr BR, Bulun SE. Prostaglandin E2 stimulates aromatase expression in endometriosis-derived stromal cells. J.Clin.Endocrinol.Metab 1997 Feb;82(2):600-6
55. Ngo C, Nicco C, Leconte M, Chereau C, Weill B, Batteux F, Chapron C. Antiproliferative effects of anastrozole, methotrexate, and 5-fluorouracil on endometriosis in vitro and in vivo. Fertil Steril. 2010 Oct;94(5):1632-8
56. Wang W, Taylor RN, Bagchi IC, Bagchi MK. Regulation of human endometrial stromal proliferation and differentiation by C/EBPbeta involves cyclin E-cdk2 and STAT3. Mol.Endocrinol. 2012 Dec;26(12):2016-30
57. Pluchino N, Russo M, Santoro AN, Litta P, Cela V, Genazzani AR. Steroid hormones and BDNF. Neuroscience. 2013 Jun 3;239:271-9. doi: 10.1016/j.neuroscience.2013.01.025
58. Gruber HE, Hoelscher GL, Bethea S, Hanley EN, Jr. Interleukin 1-beta upregulates brain-derived neurotrophic factor, neurotrophin 3 and neuropilin 2 gene expression and NGF production in annulus cells. Biotech.Histochem. 2012 Nov;87(8):506-11
59. Lebovic DI, Bentzien F, Chao VA, Garrett EN, Meng YG, Taylor RN. Induction of an angiogenic phenotype in endometriotic stromal cell cultures by interleukin-1beta. Mol.Hum.Reprod. 2000 Mar;6(3):269-75
60. Kang JH, Lee CK, Kim JR, Yu SJ, Jo JH, Do BR, Kim HK, Kang SG. Estrogen stimulates the neuronal differentiation of human umbilical cord blood mesenchymal stem cells (CD34-). Neuroreport 2007 Jan 8;18(1):35-8
61. Brandenberger AW, Lebovic DI, Tee MK, Ryan IP, Tseng JF, Jaffe RB, Taylor RN. Oestrogen receptor (ER)-alpha and ER-beta isoforms in normal endometrial and endometriosis-derived stromal cells. Mol.Hum.Reprod. 1999 Jul;5(7):651-5
62. Bulun SE, Monsavais D, Pavone ME, Dyson M, Xue Q, Attar E, Tokunaga H, Su EJ. Role of estrogen receptor-beta in endometriosis. Semin Reprod Med 2012 Jan;30(1):39-45
63. Zhou HB, Comninos JS, Stossi F, Katzenellenbogen BS, Katzenellenbogen JA. Synthesis and evaluation of estrogen receptor ligands with bridged oxabicyclic cores containing a diarylethylene motif: estrogen antagonists of unusual structure. J.Med.Chem. 2005 Nov 17;48(23):7261-74
64. DeAngelis M, Abdel-Rahman WM, Stossi F, Carlson KA, Katzenellenbogen BS, Katzenellenbogen JA. Indazole estrogens: highly selective ligands for the estrogen receptor beta. J.Med.Chem. 2005 Feb 24;48(4):1132-44
65. Zhao Y, Gong P, Chen Y, Bagchi MK, Taylor RN, Nettles KW, Katzenellenbogen JA, Katzenellenbogen BS. Novel estrogen receptor ligands with anti-estrogenic and anti-inflammatory activity for prevention and

treatment of endometriosis: Studies in a mouse model. Endocrine Society, #OR44-4, San Francisco (abst). 2013
66. Plante BJ, Lessey BA, Taylor RN, Wang W, Bagchi MK, Yuan L, Scotchie J, Fritz MA, Young SL. G protein-coupled estrogen receptor (GPER) expression in normal and abnormal endometrium. Reprod.Sci. 2012 Jul;19(7):684-93
67. Samartzis N, Samartzis EP, Noske A, Fedier A, Dedes KJ, Caduff R, Fink D, Imesch P. Expression of the G protein-coupled estrogen receptor (GPER) in endometriosis: a tissue microarray study. Reprod.Biol.Endocrinol. 2012;10:30
68. Dennis MK, Burai R, Ramesh C, Petrie WK, Alcon SN, Nayak TK, Bologa CG, Leitao A, Brailoiu E, Deliu E, et al. In vivo effects of a GPR30 antagonist. Nat.Chem.Biol. 2009 Jun;5(6):421-7
69. Tseng JF, Ryan IP, Milam TD, Murai JT, Schriock ED, Landers DV, Taylor RN. Interleukin-6 secretion in vitro is up-regulated in ectopic and eutopic endometrial stromal cells from women with endometriosis. J.Clin.Endocrinol.Metab 1996 Mar;81(3):1118-22
70. Bulun SE, Cheng YH, Pavone ME, Xue Q, Attar E, Trukhacheva E, Tokunaga H, Utsunomiya H, Yin P, Luo X, et al. Estrogen receptor-beta, estrogen receptor-alpha, and progesterone resistance in endometriosis. Semin.Reprod.Med. 2010 Jan;28(1):36-43
71. Sidell N, Feng Y, Hao L, Wu J, Yu J, Kane MA, Napoli JL, Taylor RN. Retinoic acid is a cofactor for translational regulation of vascular endothelial growth factor in human endometrial stromal cells. Mol Endocrinol 2010 Jan;24(1):148-60
72. Seidah NG, Sadr MS, Chretien M, Mbikay M. The multifaceted proprotein convertases: their unique, redundant, complementary, and opposite functions. J.Biol.Chem. 2013 Jul 26;288(30):21473-81
73. Heng S, Cervero A, Simon C, Stephens AN, Li Y, Zhang J, Paule S, Rainczuk A, Singh H, Quinonero A, et al. Proprotein convertase 5/6 is critical for embryo implantation in women: regulating receptivity by cleaving EBP50, modulating ezrin binding, and membrane-cytoskeletal interactions. Endocrinology. 2011 Dec;152(12):5041-52
74. Hornung D, Waite LL, Ricke EA, Bentzien F, Wallwiener D, Taylor RN. Nuclear peroxisome proliferator-activated receptors alpha and gamma have opposing effects on monocyte chemotaxis in endometriosis. J.Clin.Endocrinol.Metab 2001 Jul;86(7):3108-14
75. Kawamura N, Kawamura K, Manabe M, Tanaka T. Inhibition of brain-derived neurotrophic factor/tyrosine kinase B signaling suppresses choriocarcinoma cell growth. Endocrinology 2010 Jul;151(7):3006-14
76. Kawamura K, Kawamura N, Kawagoe Y, Kumagai J, Fujimoto T, Terada Y. Suppression of hydatidiform molar growth by inhibiting endogenous brain-derived neurotrophic factor/tyrosine kinase B signaling. Endocrinology 2012 Aug;153(8):3972-81

Department of Molecular and Integrative Physiology

February 11, 2014

Dear ,

We are pleased to serve as collaborators on your NIH grant application entitled “Neuroangiogenesis in deep infiltrating endometriosis”. As you know, over the years we have synthesized and tested several novel estrogen receptor ligands. Some of these, particularly the cholorindazole (CLI) and oxabicycloheptene sulfonate (OBHS) compounds, have interesting antagonist effects on ER-α and –β that we believe could have important therapeutic activity in endometriosis and on the regulation of neurotrophins and nociceptive nerve fibers that you propose to investigate. We will be happy to provide you with these compounds and will assist in the design, interpretation and publication of your findings. We also can be helpful as you design experiments to test the effects of the ER-α and –β selective agonists, propyl pyrazole triol (PPT) and diarylpropionitrile (DPN), respectively, with which we have extensive experience. As we have successfully collaborated for several years, we are convinced that this too will be a fruitful interaction.

Best wishes for success in your project.

Sincerely,

February 28, 2014

Dear :

The is enthusiastic to support your research application titled "Neuroangiogenesis in Deep Infiltrating Endometriosis."

The TSI's mission is to improve the health of our region, state, and nation by facilitating the translation of knowledge to prevent, diagnose and treat disease; training leaders in clinical and translational science; and accelerating discovery by providing investigators with tools to complete clinical and translational research projects. Your project furthers these goals by studying the expression of BDNF in tissues and cells women with endometriosis versus healthy controls.

The is committed to supporting this research project, specifically with support of the Clinical Research Unit to assist in participant recruitment, conduct study visits, administer questionnaires, collect, prepare, and process specimens, and develop a clinical study database in REDcap to facilitate data analysis. We are confident that the new knowledge gained from this study will be invaluable in furthering the goals of the TSI and improving care of women in our region, state, and nation.

Sincerely,

13. RESOURCE SHARING PLAN(S)

The data generated in the NIH R21 Project will be made available to the public principally through publication in scientific research journals and presentations at scientific meetings. Once manuscripts are accepted for publication advance copy of the accepted manuscript can be obtained at the publisher's website or by contacting the Principal Investigator.

We will provide *in situ* hybridization plasmids and probes that are developed in connection with this project and described in publications and conference presentations to investigators who request them, once an appropriate materials transfer agreement has been executed. Details of specific techniques developed during this research will be provided upon request.

Raw data will be retained for at least three years in the laboratory notebooks of the key personnel participating in this project, and compilations of data generated to assist in the preparation of publications and presentations will be retained for at least three years in the files of the PI. These data will be made available to other investigators making appropriate inquiries. No transgenic animals or antibodies will be prepared as part of these studies.