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Primary Afferent Nociceptors and Visceral Pain

Victor Chaban
*Charles R. Drew University of Medicine and Science and
University of California, Los Angeles,
USA*

1. Introduction

Patients with chronic pelvic pain frequently have pain from several pelvic organs. The most common diagnoses include endometriosis, interstitial cystitis, irritable bowel disease, pelvic floor tension myalgia, vulvar vestibulitis, and vulvodynia. Frequently, pain does not correlate with pathologic findings at the time of laparoscopy in the case of endometriosis, while vulvodynia, irritable bowel syndrome and pelvic floor tension myalgia and neuropathy may have no clearly demonstrable pathologic tissue changes. Most diagnoses associated with chronic pelvic pain have a high rate of recurrence and all are considered to be chronic conditions with a relapsing course. Endometriosis is a complex, poorly understood chronic illness of women in their reproductive age and pain is the major concern of women with this disease. Despite a successful reduction of pain using during the novel treatments pain returns in up to 75% of treated women. Pain is strongly associated with this disease and the lack of awareness to its pathology is further illustrated by the fact that the average time duration between the onset of pain and the diagnosis of endometriosis is 3 to 11 years despite the fact that 25-30% of women with chronic pelvic pain suffer from this disease. In women with endometriosis (mainly of reproductive age) alterations in the limbic and sympathetic nervous system and hypothalamic-pituitary-adrenal axis mediate a cycle of hypervigilance for pain sensations from pelvic organs, which can lead to descending induction of pathologic changes in pelvic organs. Chronic pelvic pain patients frequently have multiple diagnoses. Vicero-somatic and viscero-viseral hyperalgesia and allodynia result in the spread of a perception of pain from an initial site to adjacent areas. Chronic pelvic pain patients may initially have only one pain source in the pelvis, such as the uterus in dysmenorrhea or endometriosis implants, but a multitude of mechanisms involving the peripheral and central nervous system can lead to the development of painful sensations from other adjacent organs. Often the etiology of visceral pain is not clear, as there are many symptoms of the reproductive system, gastrointestinal and urinary tracts, musculoskeletal, neurological and psychological systems that often co-occur in the same patient. The variation of pain symptoms and pain perception and behavioral responses to pain in these patients is poorly understood. The treating clinician is often tempted to take a unidimensional approach and focus on one organ system and ignore the psychological and behavioral manifestations of the chronic pain.

The incidence of persistent, episodic, or chronic visceral pain are more prevalent in females thus defining the site(s) and mechanisms through which female steroid hormones modulate

visceral nociception is an important step in understanding the gender differences in pain perception and in designing appropriate therapies for females. One such mechanism may be the convergence of nociceptive stimuli and estrogen input on the primary afferent neurons which innervate viscera. Based on our results, it is likely that estrogen receptors (ERs) expressed in primary afferent neurons modulate nociceptive signaling. Our recent data suggest that estrogen acting on primary afferent nociceptors modulates the response to pro- and anti-nociceptive signals associated with the clinical presentation of functional disorders such as endometriosis.

1.2 The nociception of endometriosis

Endometrial tissues outside the uterus can cause severe pain and this pain can be diminished with therapies that suppress estrogen production (Berkley *et al.* 2005). The mechanism of endometriosis-induced nociceptive signaling is poorly understood and in some cases pain can be exacerbated by co-morbidity with other chronic pelvic pain syndromes such as irritable bowel syndrome, painful bladder syndrome, vulvodynia and fibromyalgia. It has also been shown that ectopic implants develop sensory nerve supply both in women and in animal models of endometriosis. Sensory input arriving from the visceral organ to the spinal cord divergences at the level of primary sensory neurons which further transmit considerable information from periphery to the central nervous system. Visceral pain may be manifestation from a single organ such as uterus or may arise from algogenic conditions affecting more than one organ (Malykhina 2007). This type of pain is important not only because it is difficult to diagnose its clinical conditions but also for its therapeutic implications. It is quite possible to modulate pain from one viscus to another. Recent study by Giamberardino and others showed that the treatment of the endometriotic lesions results in the improvement of spontaneous and referred urinary symptoms (Giamberardino *et al.* 2010).

Cross-sensitization in the pelvis implies the transmission of noxious stimuli from one organ to another through an adjacent normal structure resulting in functional (rarely organic) changes. Pelvic organ cross-sensitization is considered as one of the factors contributing to chronic pelvic pain (Pezzone *et al.* 2005). Chronic pelvic pain (CPP) syndrome affects up to 25% of reproductive age women and results in dysmenorrhea, menstrual irregularities, back pain and reduced fecundity. One of the most common causes of CPP is endometriosis. Chronic pain adversely affects mood, social and professional life and general well being. Thus, assessing the impact of the pain on various domains of a patient's existence has become an important focus in the clinical management. Most women with complaints of pelvic pain will undergo laparoscopy to both diagnose and treat these diseases, but laparoscopy is often is unsuccessful due to lack of intraperitoneal pathology or altered pain processing. Pain out of proportion to identifiable pathology is the most immediate and dramatic consequence of disorders associated with CPP and is responsible for a highly negative impact on quality of life and substantial workforce loss. Results of a national survey determined that 15% of women in the United States have experienced CPP and only 10% of these consulted a gynecologist and 75% did not consult a health care provider of any type. Due to the alarming situation and unmet need, the USA and other countries have launched a call for more focused research on improving the diagnosis and treatment of CPP syndrome.

There is often no clear relationship between the severity of the chronic pelvic pain and pathology in the pelvic viscera, including reproductive tract (ovaries and uterus). It is still

poorly understood how endometriosis is associated with pain symptoms in different organs and how this nociceptive signaling is ameliorated by a hypoestrogenic state. One of the possible explanation can be that endometrial implants' sensory nerve supply and its potentially estradiol-modulated influence on the nociception.

Several researchers have investigated the presence of nerve fibers in endometriotic lesions in both human and animal study. Using different types of specific immunohistochemical neuronal markers such as substance P (SP) and calcitonin gene related peptide (CGRP) sensory nerve fibers markers) in human peritoneal endometriotic lesions from women with visually and biopsy proven endometriosis, investigators have demonstrated multiple, small unmyelinated nerve fibers are present in peritoneal endometriotic lesions, and these peritoneal endometriotic lesions contain both Aδ and C nerve fibers. Accumulating evidence has shown these nerve fibers may play a critical role in pain production in patients with endometriosis, and a close histological relationship has been identified between these nerve fibers and endometriosis associated pain. Tulandi *et al*. (2001) reported that the distance between endometriotic glands and nerve fibers in endometriotic lesions from women with pain was closer than in women with no pain. The density of nerve fibers in peritoneal endometriotic lesions was much greater than in normal peritoneum in women with no endometriosis. The nerve fiber density in endometriotic lesions can be markedly reduced by hormonal drugs such as gonadotropin releasing hormone (GnRH) analogues and combined oral contraceptives, which have been used efficaciously to treat endometriosis-associated pain, indicating that modulation of these nerve fibers might alter pelvic nociception. The fact that peritoneal endometriotic lesions are innervated by sensory Aδ, sensory C nerve fibers raises the intriguing questions, what kind of role do these nerve fibers play in the mechanisms by which endometriotic lesions produce pain and hyperalgesia, and how do they modulate pain perception in these condition?

The demonstration of Aδ and C sensory fiber innervations to peritoneal endometriotic lesions, suggesting these innervations contributes to both visceral hyperalgesia and pelvic pain that occur in patients with endometriosis brings up the interesting questions, how do these sensory fibers transmit and modulate visceral nociception in endometriosis? Immunohistochemical staining of these nerve fibers in endometriosis showed co-localization of SP, CGRP, implicating SP and CGRP might be involved in modulation of visceral nociception. Endometriosis is an inflammatory disease, which is known to contain pro-inflammatory cytokines, prostaglandins, and other neuroactive agents that could readily activate the CGRP- and SP-positive C-fiber nociceptive afferents found in the endometriotic lesions.

When these sensory nerve fibers are stimulated by inflammatory substances, neurotransmitters such as SP, CGRP can be secreted from sensory nerve endings. SP and CGRP can contribute to the inflammatory response by causing vasodilation, plasma extravasation and cellular infiltration by interacting with endothelial cells, arterioles, mast cells, neutrophils and immune cells. SP can also act on mast cells in the vicinity of sensory nerve endings to evoke de-granulation and the release of TNF-α, histamine, prostaglandin D2 (PGD2) and leukotriene, providing a positive feedback. CGRP has a wide range of biological activities, including sensory transmission, regulation of glandular secretion, and inhibiting SP degradation by a specific endopeptidase to enhance SP release, thereby amplifying the effects.

Dorsal root ganglion (DRG) neurons can be activated or modulated by the activation of chemosensitive receptors on peripheral terminals and ATP has been implicated in sensory transduction of noxious stimuli by activating purinergic P2X receptors (Dunn *et al.* 2001). Once released into the intercellular areas, the action of ATP is mediated by primarily P2X3 receptors which are expressed on primary afferent fibers and cell bodies within DRG (Burnstock 2001). The capsaicin-sensitive primary afferent neurons of small- and medium-diameter neurons mediate nociceptive-like behaviors suggesting that TRPV1 expressing neurons are nociceptors. Activation of purinergic (P2X3) and transient potential receptors family vcanilloid-1 (TRPV1) receptors results in the depolarization and opening of voltage-gated Ca^{2+} channels (VGCC) (Koshimizu *et al.* 2000). A sensation of pain is produced by depolarization of the peripheral nerve terminals.

1.3 Estrogen receptors and nociceptive signaling in primary afferent neurons

Defining the site(s) and mechanisms through which sex estrogen modulates visceral nociception is an important step in understanding the mechanisms in pain perception associated with endometriosis and in designing appropriate therapies. One such mechanism may be the convergence of nociceptive stimuli and estrogen input on the primary afferent neurons which innervate viscera (i.e. uterus). Estrogen may modulate female sensitivity to clinical and experimentally induced pain. Based on our preliminary results, it is likely that estrogen receptors (ERs) expressed in primary afferent neurons modulate chemical signaling associated with nociception. Nociception is a balance of pro- and anti-nociceptive inputs that is subject to regulation depending on the normal state of the organism. Sensitization of primary afferent neurons to stimulation may play a role in the enhanced perception of visceral sensation and pain. Chest pain from coronary heart disease, endometriosis, acute and recurrent/chronic pelvic pain in women or abdominal are all visceral pain sensations that may result in part from sensitization (Berkley *et al.* 2001; Mayer *et al.* 2001). Mechanisms of peripheral sensitization may involve increased transduction that is secondary to repeated stimulation or an increase in the excitability of the afferent nerves by molecules that decrease the excitation threshold (Zimmermann 2001)

The cell bodies of primary visceral spinal afferent neurons are located in DRG. Direct activation of chemosensitive receptors and ion channels on their peripheral terminals and modulation of neuronal excitability activates extrinsic primary afferent nerves. Nociceptors belong predominantly to small and medium size DRG neurons whose peripheral processes detect potentially damaging physical and chemical stimuli. The terminals of primary visceral afferent neurons are described as having no organs end or morphological specialization, but respond to different chemical stimuli. Visceral nociceptive C-fibers activated by ATP released by noxious stimuli from cells in target organs, have been implicated as mediators of noxious stimulus intensities (Burnstock 2000). Alteration in signal transduction of primary afferent neurons can result in enhanced perception of the visceral sensation that is common in patients with different disorders resulting in elevated pain perception. Acute and recurrent/chronic pelvic pain in women and abdominal pain from IBS are illustrative examples of visceral pain that undergo sensitization (Giamberardino *et al.* 2010).

Peripheral sensitization can develop in response to sustain stimulation, inflammation, and nerve injury. Visceral pain is different from cutaneous pain based on clinical,

neurophysiological and pharmacological characteristics (Chang and Heitkemper 2002). The pathophysiology of visceral hyperalgesia is less well-known than its cutaneous counterpart, and our understanding of visceral hyperalgesia is colored by comparison to cutaneous hyperalgesia, which is believed to arise as a consequence of the sensitization of peripheral nociceptors due to long-lasting changes in the excitability of spinal neurons. Endometriosis is currently defined as a chronic functional syndrome characterized by recurring symptoms of abdominal discomfort or pain. In the context of visceral pain, the TRPV1 receptor is a sensory neuron-specific cation channel which plays an important role in transporting thermal and inflammatory pain signals. Evidence for TRPV1's role is that mice lacking TRP1 receptor gene have deficits in thermal- or inflammatory-induced hyperalgesia (Davis *et al*. 2000). Activation of both TRPV1 and P2X receptors induce mobilization of $[Ca^{2+}]_i$ in cultured DRG neurons (Gschossmann *et al.* 2000).

Sex hormones and 17β-estradiol (E2) in particular may directly influence the functions of primary afferent neurons since both ERs are present on small-diameter DRG neurons (Papka and Storey-Workley 2002). Despite the broad spectrum of E2 effects in the nervous system, the mechanisms of hormonal pain modulation remain unclear. There are two subforms: estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) which were traditionally thought of as ligand-activated transcription factors. However, recent work has demonstrated multiplicity of E_2 actions (membrane, cytoplasmic and nuclear) (Nadal *et al.* 2001). ER distributed through CNS and PNS including regions that mediate nociception. For example, ERs are expressed in dorsal horn neurons of the spinal cord and DRG neurons. DRG neurons express both ERα and ERβ *in vivo* (Papka and Storey-Workley 2002) and *in vitro* (Chaban 2010). These findings suggest that E2 may modulate sensory input at the primary afferent level. E2 can alter gene transcription, resulting in pro-nociceptive (reducing β-endorphin expression) or anti-nociceptive (increasing enkephalin expression) changes of endogenous opioid peptides , opioid receptors (Micevych and Sinchak 2001) and, by increasing levels of CCK, an anti-nociceptive and anti-opioid molecule (Micevych *et al.* 2002).

E2 can modulate cellular activity by altering ion channel opening and second messenger signaling by stimulating G-proteins (Chaban *et al.* 2003) , the signal transduction pathways traditionally associated with membrane receptor activation. Many of these effects have been ascribed to membrane-associated receptors. The results from other laboratories (Lee *et al.* 2002) and our data (Chaban *et al.* 2003) indicate that E2 is acting to modulate L-type VGCC. The cloned TRPV1 receptor is a nonselective cation channel with a high permeability for Ca^{2+}. TRPV1's are distributed in peripheral sensory nerve endings and are involved in the transduction of different stimuli in sensory neurons. TRPV1 functions as molecular integrator of painful chemical and physical stimuli (noxious heat (>43° C) and low pH). Various inflammatory mediators such as prostaglandin E_2 (PGE_2) and bradykinin potentiate TRPV1. The potentiation of TRPV1 activity can be quantified by measuring the differences of capsaicin-induced Ca^{2+} concentration changes before and after receptor activation (Petruska *et al.* 2000). Significantly, a subset of DRG neurons respond to both capsaicin and ATP indicating that there may be cross-activation of these receptors that may underlie the sensitization of visceral nociceptors. Capsaicin-induced TPRV1 receptor-mediated changes in $[Ca^{2+}]_i$ may represent a level of DRG activation to noxious cutaneous stimulation while ATP-induced changes in $[Ca^{2+}]_i$ may reflect the level of DRG neuron sensitization to noxious visceral stimuli since ATP is released by noxious stimuli and tissue damage near the primary afferent nerve terminals (Burnstock 2001).

Most of the published reports about sex and hormone-related differences in pain have addressed the modulatory effect of E2 on central nervous system   mechanisms of nociception (Aloisi *et al.* 2000). Recent studies demonstrate that E2 has a significant role in modulating viscerosensitivity, indicating that E2-induced alterations in sensory processing may underlie sex-based differences in functional pain syndromes (Al-Chaer and Traub 2002). However, reports of E2 modulation of visceral and somatic nociceptive sensitivity are inconsistent. For example, elevated E2 levels have been reported to increase the threshold to cutaneous stimuli but decrease the percentage of escape responses to ureteral calculosis (Bradshaw and Berkley 2002). Additionally, nociceptive sensitivity increases when E2 levels are elevated (Holdcroft 2000; Bereiter 2001). Indeed in most clinical studies, women report more severe pain levels, more frequent pain and longer duration of pain than men. To help resolve these inconsistencies we propose to study E2 actions on the primary afferents.

Primary DRG neurons culture has been a useful model system for investigating sensory physiology and putative nociceptive signaling (Chaban *et al.* 2003). ATP-induced intracellular calcium concentration ($[Ca^{2+}]_i$) transients in cultured DRG neurons have been used to model the response of nociceptors to painful stimuli. In our laboratory we showed that E2, acting at the level of the plasma membrane, attenuates both ATP -induced $[Ca^{2+}]_i$ and capsaicin- induced $[Ca^{2+}]_i$ influx and that the expression of both P2X3 and TRPV1 depend on the expression of both ERs. Within the context of our hypothesis visceral nociception and nociceptor sensitization appear to be regulated by P2X3 and TRPV1. Estrogen attenuates DRG neurons response to ATP and capsaicin suggesting that visceral afferent nociceptors can be modulated by sex steroids at a new site at the level of primary afferent neurons. Our data suggest that E2 by itself appears to be anti-nociceptive but interferes with anti-nociceptive actions of other pain-modulating drugs (such as opioids). Thus, E2 acting on primary afferent nociceptors modulates the response to pro- and anti-nociceptive signals**.** Within the context of our cross-sensitization hypothesis, inflammation sensitizes non-inflamed viscera that are innervated by the same DRG and/or cross-sensitization occurs as a result of intra-DRG release of sensitizing mediators such as ATP or substance P in the DRG (Matsuka *et al.* 2001; Chaban 2008; Chaban 2010).

Lumbosacral DRG neurons (levels L6-S1) from wild type mice (WT) express estrogen receptors (ERα and ERβ), purinergic P2X3, vanilloid TRPV1, SP and methabotropic glutamate ($mGluR_{2/3}$) receptors. In our recent studies we also tested the difference in how somatic and visceral afferents are modulated by E2. Both short-term and long-term exposure to E2 significantly decreased the ATP and capsaicin-induced increase in $[Ca^{2+}]_i$.

2. Materials and methods

2.1 Animals

We have used 6~8 week female C57BL/6J, B6.129P2-Esr1tm1Ksk/J, and B6.129P2-Esr2tm1Unc/J mice were obtained the Jackson Laboratory (Bar Harbor, ME, USA). Upon arrival, mice were group housed in microisolator caging and maintained on a 12-h light/dark cycle in a temperature-controlled environment with access to food and water ad libitum. To test whether estrogen receptor α (ERα) or estrogen receptor β (ERβ) are involved in estradiol (E_2)-induced modification of $[Ca^{2+}]_i$ Wile type, estrogen receptor alpha knock-out (ERαKO) and estrogen receptor beta knock-out (ERβKO) mice will be used. The wild type, ERαKO and ERβKO mice will be obtained from the supplier and allowed to recover for two weeks. These studies were carried out in accordance with the guidelines of the Institutional Animal

Care and Use committee at the University of California and the NIH Guide for the Care and Use of Laboratory Animals.

2.2 Animal breeding

Experiments were performed on age-matched (8–10 wk old) heterozygous mutant mice lacking the gene male (ERα−/−) and female (ERα−/−) for ERα (ERα−/−), and the deficiency ERβ (ERβ−/−) mice were bred into heterozygous mutant female mice (ERβ−/−) and homozygous male mutant mice (ERβ−/−) (Jackson Laboratory, Bar Harbor, ME, USA). Mice were housed in climate-controlled rooms, and standard rodent chow and water were available *ad libitum* and were housed in accordance with the NIH Guide for the Care and Use of Laboratory Animals

2.3 Primary culture of DRG neurons

The isolation procedure and primary culture of mouse lumbosacral DRG has been published in detail (Chaban, Mayer et al. 2003). DRG tissues were obtained from c57/black 6J (The Jackson Laboratory; 30 g), ERαKO and ERβKO (Taconic; 20 g) transgenic types. Briefly, lumbosacral adult DRGs (level L1-S1) from Wt, ERαKO and ERβKO mice will be collected under sterile technique and placed in ice-cold medium Dulbecco's Modified Eagle's Medium (DMEM; Sigma Chemical Co., St. Louis, MO). Adhering fat and connective tissue will be removed and each DRG will be minced with scissors and place immediately in a medium consisting of 5 ml of DMEM containing 0.5 mg/ml of trypsin (Sigma, type III), 1 mg/ml of collagenase (Sigma, type IA) and 0.1 mg/ml of DNAase (Sigma, type III) and kept at 37°C for 30 minutes with agitation. After dissociation of the cell ganglia, soybean trypsin inhibitor (Sigma, type III) will be used to terminate cell dissociation. Cell suspension will be centrifuged for one minute at 1000 rpm and the cell pellet will be resuspended in DMEM supplemented with 5% fetal bovine serum, 2 mM glutamine-penicillin-streptomycin mixture, 1 µg/ml DNAase and 5 ng/ml NGF (Sigma). Cells will be plated on Matrigel® (Invitrogen)-coated 15-mm coverslips (Collaborative Research Co., Bedford, PA) and kept at 37° C in 5% CO_2 incubator for 24 hrs, given fresh media and maintained in primary culture until used for experimental procedures.

2.4 Western blot analysis

The expressions of TRPV1 and of P2X3 receptors in L1~S1 DRGs were studied by using Western blot analyses. Tissues from wild type (C57BL/6J), ERαKO, and ERβKO mice were quick frozen in tubes on dry ice during collection. L1~S1 DRG were combined, homogenized by mechanical disruption in ice-cold RIPA buffer plus protease inhibitors and incubated on ice for 30 minutes. Homogenates were then spun at 5000 g for 15 minutes and supernatants collected. Total protein was determined on the supernatants using the BCA microtiter method (Pierce, Rockford, Ill., USA). Samples containing equal amounts of protein (40µg) were electrophoresed under denaturing conditions using Novex Mini-cell system (San Diego, Calif., USA) and reagents (NuPage 4–12% Bis-Tris gel and MOPS running buffer). After electrophoretic transfer onto nitrocellulose membrane using the same system, the membrane was blocked with 5% non-fat dry milk (NFDM) in 25 mM TRIS buffered saline, pH 7.2, plus Tween 20 (TBST) for 1 hour at room temperature, followed by incubation with polyclonal rabbit antibody against TRPV1-N terminus (1:1000, Neuromics) and P2X3 receptor (1:1000, Neuromics) for overnight at 4oC. The membrane was then

washed in TBST plus NFDM, and incubated with secondary antibody, HRP conjugated and rabbit IgG (Santa Cruz Biotechnology) at 1:5,000 in the same buffer for 2 hours at room temperature. Following a final wash in TBST without NFDM, the membrane was incubated with ECL+ (Amersham, Arlington Heights, Ill., USA) substrate for HRP. Membranes were probed with primary antibody and corresponding secondary antibodies, signals were scanned and quantified by Image J version 1.28U and NIH Image 1.60 scan software. Following enhanced chemiluminescence (ECL) detection of proteins, the membranes were stripped and rehybridized with β-actin antibody as a loading control. At least three independent cell preparations were used.

2.5 Immunohistochemistry (IHC)

DRG tissues were obtained from C57/black 6J (The Jackson Laboratory; 30 g), ERαKO and ERβKO (Taconic; 20 g) transgenic types. Following decapitation, DRG from bilateral spinal levels L1-S2 were removed and fixed in 4% paraformaldehyde for overnight at 4oC, according to procedures approved by National Institutes of Health policy on Humane Care and Use of Laboratory Animals. DRGs were rinsed in Delbecco's Phosphate Buffered Saline (DPBS) before cryoprotection in sucrose (20%, 4oC) for two days, after which excess liquid was removed. DRG were quick snap frozen in 2-methylbutane, and store them at -70oC. Each DRG was mounted in Tissue-Tek® OCT embedding medium (Sakura Finetek), and sectioned at -20oC in a MICROM H505E cryostat. Sections were cut at 20μm and store 4oC until required. Sections of DRGs were collected in PBS. Endogenous tissue peroxidase activity was quenched by soaking the sections for 10 min in 3% hydrogen peroxide solution in 0.01 M PBS. The specimens were washed and then treated for 60 min in blocking solution, 0.01 M PBS containing 0.5% Triton X-100 and 1% normal donkey serum (NDS) at room temperature. They were processed for wild type (n=4), ERαKO (n=4), or ERβKO (n=4) immunohistochemistry by the free floating method using polyclonal rabbit TRPV1 antibody (1:50000, Neuromics) or P2X3 receptor antibody (1:15000, Neuromics) for overnight at 4oC, washed in 0.01 M phosphate-buffered saline (PBS) and 0.01M Tris Buffered Saline (TBS), followed by incubation in solutions of donkey anti-rabbit fluorophore-conjugated secondary antibodies (1:200, Invitrogen) in 0.01M Tris Buffered Saline (TBS) for 3 hours at room temperature. Cells showing no apparent or only faint membrane/intracellular labeling were considered to be negative for TRPV1 or P2X3. TRPV1-positive cells included those with strong plasma membrane labeling that formed a discernible clustered pattern, and those with strong intracellular labeling that formed a punctuate pattern. Some neurons showed both strong plasma membrane and intracellular labeling. P2X3-positive neurons showed diffuse membrane/intracellular labeling. Mounted sections were air dried and coverslipped with Aqua Poly Mount (Polisciences, Warrington, PA). Images from at least three sections in each level were taken using Leica DMLB M130X microscope. The total numbers of DRG neurons expressing TRPV1 and P2X3 were counted. TRPV1- or P2X3-positive neurons were categorized according to their labeling patterns and were expressed as a percentage of the total number of TRPV1- or P2X3-positive cells. Immunohistochemical signal percent was measured by computerized image analysis (Image Pro-Plus, Media Cybernetics, Silver Spring, MD, USA).

2.6 [Ca^{2+}]$_i$ fluorescence imaging

Ca^{2+} fluorescence imaging was carried out as previously described (Gschossmann *et al.* 2000, Chaban *et al.* 2001). DRG neurons were loaded with fluorescent dye Fura-2 AM (Invitrogen) for 45 min at 37°C in HBSS supplemented with 20 mM HEPES, pH 7.4. The coverslips will be

mounted on a fast-perfusion chamber P-4 (World Precision Instrument) and placed on a stage of Olympus IX51 inverted microscope. Observations were made at room temperature (20-23°C) with 20X UApo/340 objective. A fast superfusion system will be used to perfuse the cells with HBSS and rapidly apply E2 and other chemicals. Fluorescence intensity at 505 nm with excitation at 334 nm and 380 nm was captured as digital images (sampling rates of 0.1-2 s). Regions of interest were identified within the soma or neuritis from which quantitative measurements will be made by re-analysis of stored image sequences using Slidebook® Digital Microscopy software. $[Ca^{2+}]_i$ was determined by ratiometric method of Fura-2 fluorescence from calibration of series of buffered Ca^{2+} standards. We applied E2 acutely for five minutes onto the experimental chamber or the culture medium for 48 hours to study the prolonged effect of E2. Repeated applications of drugs were achieved by superfusion in a rapid mixing chamber into individual neurons for specific intervals (100-500 ms). Cells were perfused with experimental media (2 ml/min) using a Rainin® peristaltic pump.

2.7 Retrograde labeling

DRG neurons innervating viscera were identified by retrograde labeling. Briefly, mice were anesthetized with isoflurane. For colonic afferents, the descending colon was exposed and Fluorogold (5% solution in PBS; Molecular Probes, Eugene, OR) was injected into the intestinal muscle wall (10 µl injections of into five to six different sites) using a Hamilton syringe (Hamilton Co., Reno, NV) with a 26-gauge needle. In another experiments we used uterus-specific DRG neurons in which tetramethylrhodamine (TMR) dye was injected in the uterus. Injection sites were carefully swabbed, the colon and uterus were extensively rinsed with 0.9% sodium chloride solution and sealed with New Skin to prevent dye leakage. The abdomen was sutured and the animals monitored for signs of pain or discomfort during the survival period. All animals were allowed to survive one week to allow for maximal transport of retrograde markers and housed in groups of two under 12/12 hours light cycle with food and water available *ad libitum.*

2.8 Statistical analysis

The amplitude of $[Ca^{2+}]_i$ response represents the difference between baseline concentration and the transient peak response to drug stimulation. Significant differences in response to chemical stimulation will be obtained by comparing $[Ca^{2+}]_i$ increases during the first stimulation with the second. A cell will be judged responsive if E2 inhibits the second $[Ca^{2+}]_i$ transient by >30% of the first. This criterion was empirically derived in preliminary experiments. All of the data are expressed as the mean ± SEM. Statistical analysis was performed using Statistical Package for the Social Sciences 12.0 (SPSS, Chicago, IL, USA). To assess the significance among different groups, data were analyzed with one-way ANOVA followed by Schéffe post hoc test. A $P < 0.05$ was considered statistically significant.

3. Results

3.1 Role of P2X3 receptors in estrogen-induced nociceptive signaling in sensory neurons

P2X3 and TRPV1 receptors expression were examined by western blot analysis of lysates from wild type, ERαKO, and ERβKO DRG tissues using a P2X3 specific primary antiserum (Fig.1 (a)). An intense band representing a ~64 kDa protein (P2X$_3$) and a ~130 kDa (TRPV1) was seen

in DRG lysates from wild type animals. There was a dramatic decrease in intensity of this band using lysates made from the both knock out DRG tissues when compared with wild type control animals (>4 fold decrease of control Fig.1). When the density in the control group was standardized to 1.0, the average densities were 0.172 ± 0.08 of ERαKO and 0.262 ± 0.10 of ERβKO in P2X$_3$ receptors, and 0.59 ± 0.06 of ERαKO and 0.391 ± 0.04 of ERβKO in TRPV1 receptors, suggesting that both P2X$_3$ and TRPV1 protein decreased in DRG, $P<0.05$, n=10.
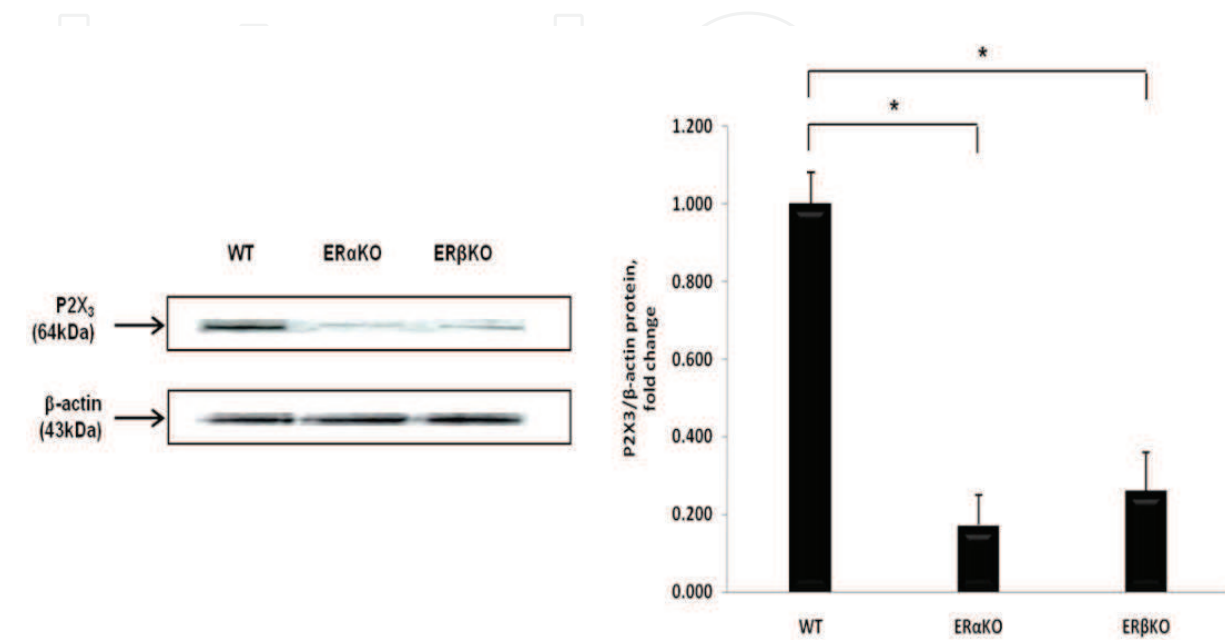
Fig. 1. Western blot analysis of DRG lysates shows reduced expression of P2X3 and TRPV1 in both knock-out mice.

Our study show that nociceptive capsaicin-sensitive TRPV1 receptors and ATP-sensitive P2X$_3$ receptors express in DRG neurons. DRGs section were immunostained with primary antibodies against P2X$_3$ and TRPV1. Neuronal profiles from each four mouse with ERαKO, ERβKO as well as wild type mice were quantified for each fluorescent probe. Both P2X$_3$ and TRPV1 receptors present in DRGs (Fig. 2).
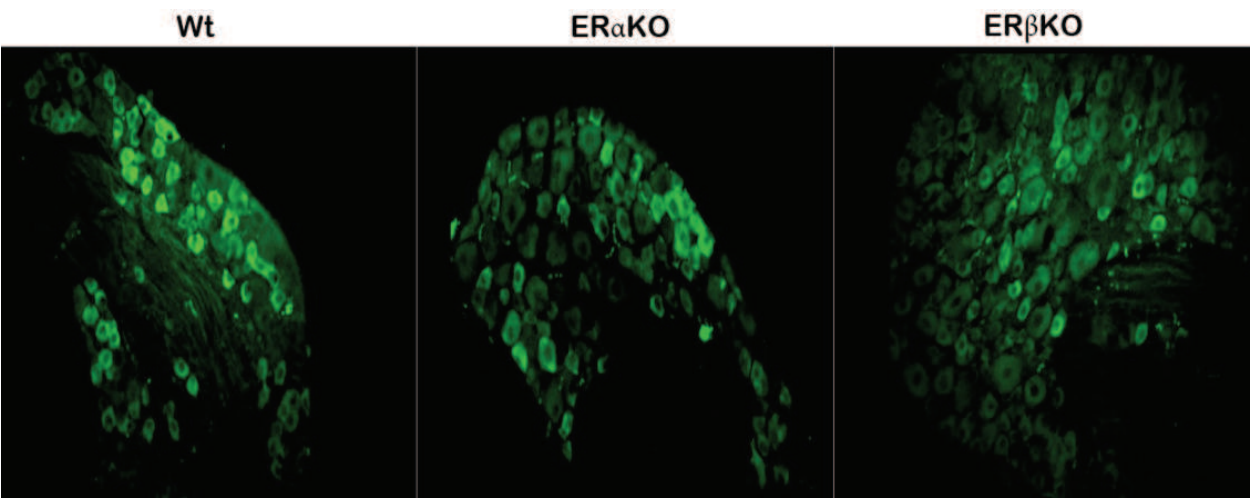
Fig. 2. Expression of P2X3 receptors in DRG neurons from wild type, ERαKO, and ERβKO *in vivo*

In our next experiments we evaluated P2X3 receptors modulation by ATP and E2 in sensory neurons. DRG neurons were loaded with fluorescent dye Fura-2 AM for one hour at 37°C in HBSS supplemented with 20 mM HEPES, pH 7.4. The coverslips were placed on a stage of Olympus IX51 inverted microscope. A fast superfusion system was used to perfuse the cells with HBSS and rapidly apply E_2 and other chemicals. Fluorescence intensity was captured as digital images (sampling rates of 0.1- 2s). Regions of interest were identified within the soma from which quantitative measurements were made by re-analysis of stored image sequences using Slidebook® Digital Microscopy software. $[Ca^{2+}]_i$ was determined by ratiometric method.
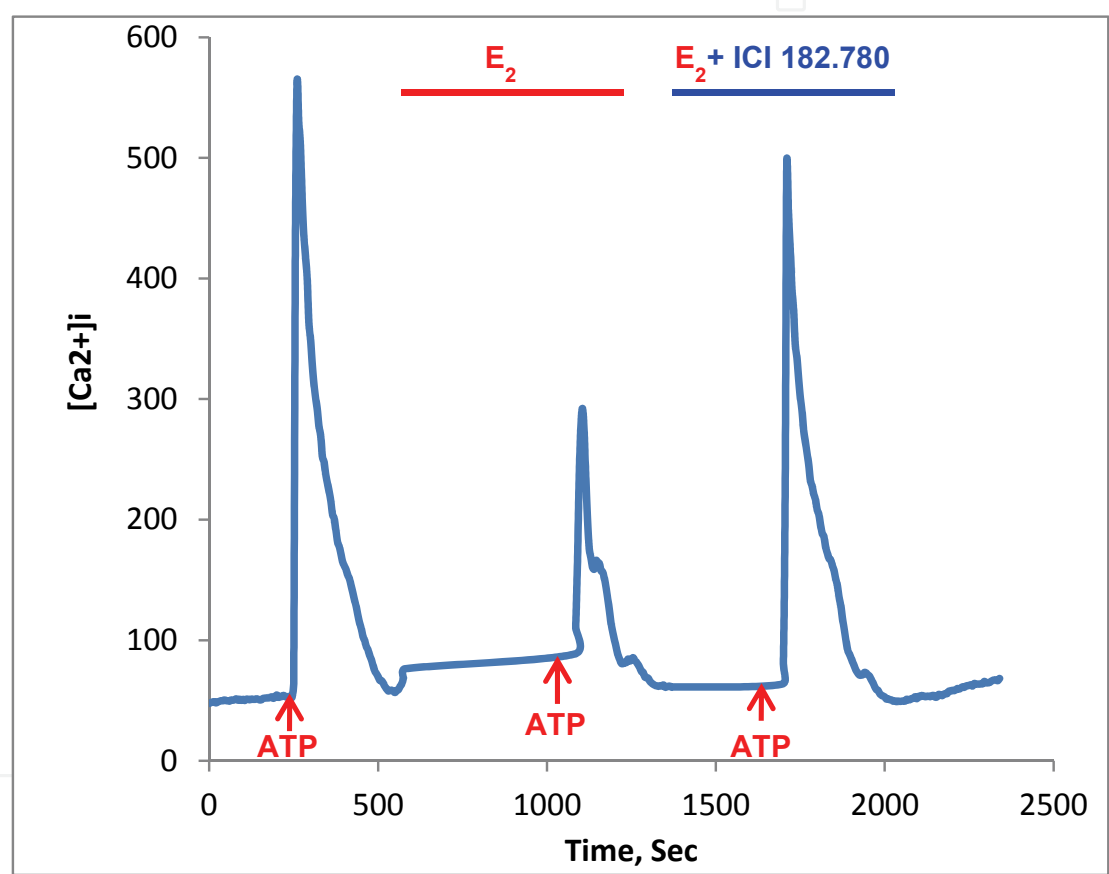
Fig. 3. 17 β-Estradiol (E2) significantly reduced ATP-induced $[Ca^{2+}]_i$ signaling *in vitro*. This effect was blocked by ER antagonist ICI 182 780

3.2 Role of TRPV1 receptors in estrogen-induced nociceptive signaling in sensory neurons

We found that nociceptive (small diameter) DRG neurons also express capsaicin-sensitive vanilloid (TRPV1) receptors. TRPV1positive neurons were categorized according to their labeling patterns and were expressed as a percentage of the total number of TRPV1 -positive cells. Immunohistochemical signal from ERαKO, ERβKO and WT mice was measured by computerized image analysis (Fig. 4)
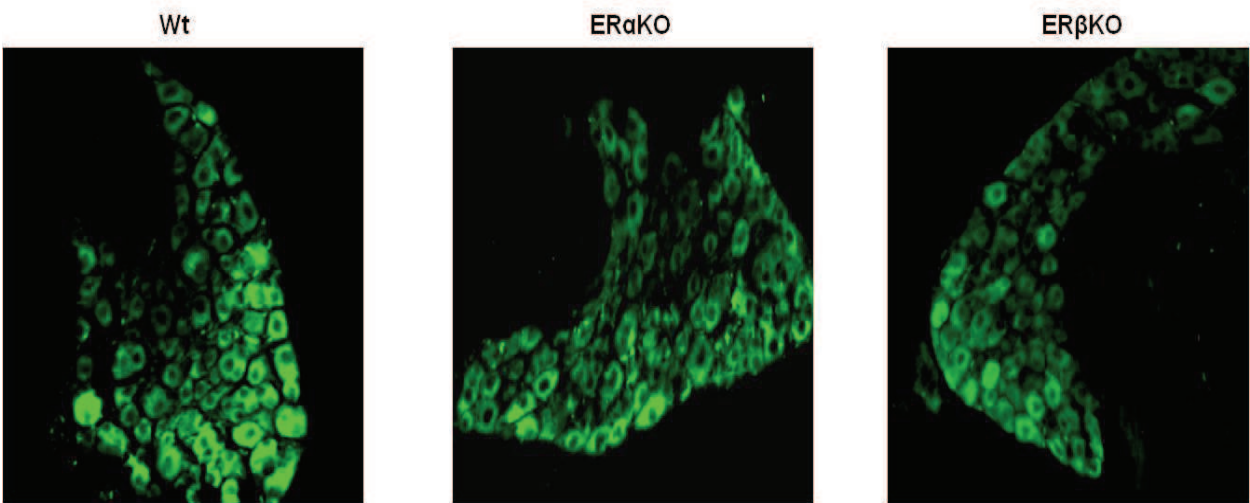
Fig. 4. Expression of TRPV1 receptors in dorsal root ganglion neurons from Wt, ERαKO, and ERβKO *in vivo*.

Capsaicin-induced TPRV1 receptor-mediated changes in $[Ca^{2+}]_i$ may represent a level of DRG activation to noxious cutaneous stimulation while ATP-induced changes in $[Ca^{2+}]_i$ may reflect the level of DRG neuron sensitization to noxious visceral stimuli since ATP is released by noxious stimuli and tissue damage near the primary afferent nerve terminals . In the view of this fact, TRPV1 receptor expression and activity might be considered as markers for a specific subtype of sensory neurons, and their activation by exogenous stimuli (e.g. capsaicin) could be a useful tool to exam the possible modulatory effects of pain-related substances.

3.3 Primary afferent sensory neurons receive input from different visceral organs

An important test of our hypothesis will be to establish that E2 modulates visceral afferents. A corollary of that hypothesis was that cutaneous pain may be differently modulated compared with visceral pain. We have proposed that E2 preferentially acts on visceral afferents to modulate the nociception. In a series of experiments using retrograde tract
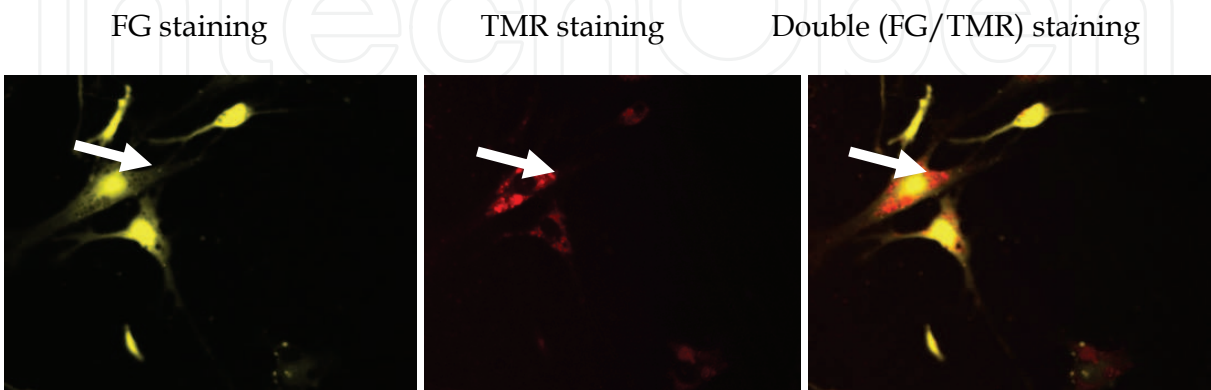

Fig. 5. Identification of colon-specific (with FG), uterus-specific (with TMR) and DRG receiving input from both organs *in vitro*

tracing we identified cutaneous and visceral afferent neurons *in vivo and in vitro*. We also found a subset of DRG neurons which innervate both visceral organs: uterus and colon.

4. Conclusion

Endometriosis is one of the most common benign gynecological diseases, characterized by the presence of endometrial tissue outside the uterine cavity, most commonly implanted over visceral and peritoneal surfaces within the female pelvis. Clinical studies have shown that it may occur in up to 10-15% of women of reproductive age. Symptoms of endometriosis are usually associated with pelvic pain, including recurrent painful periods, painful intercourse, and painful defecation during menstruation, chronic lower abdominal pain and hypersensitivity. Unfortunately, understanding of the mechanisms of endometriosis-associated pain and its management in women is currently insufficient. Studies have shown possible mechanisms of chronic pelvic pain associated with endometriosis could be due to persistent nociceptive input from endometrial tissues that lead to peripheral and central sensitisation resulting in increased responsiveness of dorsal root ganglion and dorsal horn neurons. Indeed, recent studies in human and animals have shown that peritoneal endometrial lesions are richly innervated by Aδ and C sensory nerve fibers, which positively stained by substance P or calcitonin gene-related peptide.

Several lines of evidence indicated that there is a close relationship between nerve fiber density and endometriosis-associated pain. There is a significant increase in nerve fiber density in women with endometriosis who reported pelvic pain, suggesting these nerve fibers may play an important role in the mechanisms of pain generation. Accumulating literatures described that SP presents in the myometrium and is involved in the inflammatory and pain responses, suggesting a possible role of SP nerve fibers in the generation of pain related to endometriosis. SP, which is synthesized and contained in 20–30% of DRG neurons, is involved in the transmission of nociceptive information to the central nerve system. SP is contained primarily in, and co-released from, small-diameter primary afferent fibers on noxious stimulation. Activation of nociceptive C and Aδ primary afferent fibers by electrical, chemical, or mechanical stimulation has been reported to release SP. Visceral nociceptive C-fibers can be activated by SP, representing an endogenous system regulating inflammatory, immune responses, and visceral hypersensitivity. SP afferent fibers play an important role in the pathogenesis of visceral hyperalgesia, suggesting critical role of SP in regulation of pelvic nociception associated with endometriosis. ATP is a peripheral mediator of pain which contributes to the activation of sensory afferents by activating ATP receptors following inflammation or nerve injury. It may correlate with SP release and play an important role in modulating nociception in primary sensory neurons. Local injections of ATP and ATP analogs to the rat hindpaw elicit spontaneous pain behaviors, hyperalgesia and allodynia which can be augmented by inflammation, indicating ATP might be involved in visceral hyperalgesia associated with endometriosis. Although these findings reveal the greater abundance of primary sensory nerve fibers clearly present within the peritoneal endometrial lesions in patients diagnosed with endometriosis, and these nerve fibers may play an important role

in pain generation associated with endometriosis, pain mechanisms associated with endometriosis are still not well known, and the role of these primary sensory nerve fibers has not been specifically determined.

Our data support the idea that E2 modulates nociceptive responses in pelvic pain syndromes such as endometriosis, however, whether E_2 is pro- or anti-nociceptive remains unresolved. Within the context of our hypothesis visceral nociception and nociceptor sensitization appear to be regulated by P2X3 and TRPV1. E2 modulates DRG neurons response to ATP and capsaicin suggesting that visceral afferent nociceptors are modulated by E2 in the DRG. The DRG is an important site of visceral afferent convergence and cross-sensitization. We have demonstrated that 17-β estradiol (E2), the most common form of estrogen act on functional properties of P2X3 and TRPV1 receptors in DRG neurons *in vitro*. DRG neurons from Wt and knock-out mice responded to P2X3 and TRPV1 activation. We also studied the long-term (chronic) exposure to E2 on sensory neurons that mimics the temporal pattern of circulating E2 levels in cycling female rodents which is equivalent to an E2 primal action on animal reproductive behavior. The localization of ER in DRG neurons and the attenuation of ATP/capsaicin- induce $[Ca^{2+}]_i$ strongly suggest that E2 modulates visceral pain processing peripherally. Moreover, E2 appears to have different actions on nociceptive signaling depending on the input. Adult DRG neurons in short-term culture retain the expression of receptors (P2X and TRPV1) which mediate the response to putative nociceptive signals. They continue to respond to ER agonists mimicking *in vivo* activation. An important advantage is that these neurons can be studied apart from endogenous signals. Our data clearly showed the new role of nociceptors in pathophysiological aspects of chronic pelvic pain and potential way of designing future therapies.

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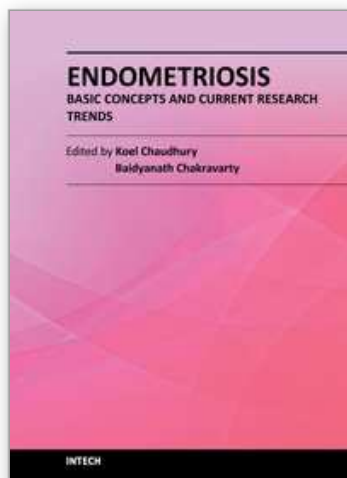
Endometriosis - Basic Concepts and Current Research Trends

Edited by Prof. Koel Chaudhury

This book provides an insight into the emerging trends in pathogenesis, diagnosis and management of endometriosis. Key features of the book include overviews of endometriosis; endometrial angiogenesis, stem cells involvement, immunological and hormonal aspects related to the disease pathogenesis; recent research reports on infertility, endometrial receptivity, ovarian cancer and altered gene expression associated with endometriosis; various predictive markers, and imaging modalities including MRI and ultrasound for efficient diagnosis; as well as current non-hormonal and hormonal treatment strategies This book is expected to be a valuable resource for clinicians, scientists and students who would like to have an improved understanding of endometriosis and also appreciate recent research trends associated with this disease.

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