Molecular and preclinical basis to inhibit PGE_2 receptors EP2 and EP4 as a novel nonsteroidal therapy for endometriosis

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Edited by R. Michael Roberts, University of Missouri-Columbia, Columbia, MO, and approved June 26, 2015 (received for review April 23, 2015)

Endometriosis is a debilitating, estrogen-dependent, progesterone-resistant, inflammatory gynecological disease of reproductive age women. Two major clinical symptoms of endometriosis are chronic intolerable pelvic pain and subfertility or infertility, which profoundly affect the quality of life in women. Current hormonal therapies to induce a hypoestrogenic state are unsuccessful because of undesirable side effects, reproductive health concerns, and failure to prevent recurrence of disease. There is a fundamental need to identify nonestrogen or nonsteroidal targets for the treatment of endometriosis. Peritoneal fluid concentrations of prostaglandin E_2 (PGE_2) are higher in women with endometriosis, and this increased PGE_2 plays important role in survival and growth of endometriosis lesions. The objective of the present study was to determine the effects of pharmacological inhibition of PGE_2 receptors, EP2 and EP4, on molecular and cellular aspects of the pathogenesis of endometriosis and associated clinical symptoms. Using human fluorescent endometriotic cell lines and chimeric mouse model as preclinical testing platform, our results, to our knowledge for the first time, indicate that selective inhibition of EP2/EP4: (*i*) decreases growth and survival of endometriosis lesions; (*ii*) decreases angiogenesis and innervation of endometriosis lesions; (*iii*) suppresses proinflammatory state of dorsal root ganglia neurons to decrease pelvic pain; (*iv*) decreases proinflammatory, estrogen-dominant, and progesterone-resistant molecular environment of the endometrium and endometriosis lesions; and (*v*) restores endometrial functional receptivity through multiple mechanisms. Our novel findings provide a molecular and preclinical basis to formulate long-term nonestrogen or nonsteroidal therapy for endometriosis.

PGE2 signaling | endometriosis | pelvic pain | pain pathways | infertility

Endometriosis is a debilitating, chronic inflammatory gynecological disease of reproductive age women. Two major clinical symptoms of endometriosis are chronic intolerable pelvic pain and subfertility or infertility, which profoundly affect the quality life in women (1–3). The prevalence of the disease is 5–10% in reproductive age women, increases to 20–30% in women with subfertility, and to 40–60% in women with pain and infertility (1–3). Endometriosis remains as the single major cause for hysterectomy in reproductive age women in the United States, with an annual estimated societal cost of ~$69.4 billion (3). These significant individual and public health concerns underscore the importance of understanding the pathogenesis of endometriosis.

Although endometriosis has been traditionally viewed as an estrogen (E_2)-dependent and progesterone (P_4)-resistant disease (1–3), its pathogenesis remains an enigma in reproductive medicine. The most widely accepted hypothesis first advanced by Sampson in 1927 is that viable endometrial tissue fragments move in a retrograde fashion through the fallopian tubes into the pelvic cavity during menstruation (4). These ectopic endometrial cells invade the underlying peritoneum, survive for considerable time (~7–8 y), and establish peritoneal endometriosis characterized by heterogeneous lesions/phenotypes.

Current treatment strategies include surgical intervention, medical therapy, or a combination of both. After surgical removal of endometriosis lesions, the disease reestablishes within 3–5 y in ~30–50% of women. Surprisingly, the disease reoccurs in ~10% of women who have had the uterus and both ovaries removed (5). Hormonal therapy to induce a hypoestrogenic state through the use of oral contraceptives, progestagens, androgenic agents, and gonadotropin releasing hormone analogs can be prescribed only for a short time due to undesirable side-effects, pseudomenopause, and bone density loss in reproductive age women (1–3, 5). Nevertheless, the recurrence rate is ~50–60% after cessation of therapy within a year (5). Together, existing treatment modalities fail to prevent recurrence of disease, and affect pregnancy and reproductive health of women. There is a fundamental need to identify potential cell signaling pathways for nonestrogen or nonsteroidal targets for endometriosis.

Peritoneal fluid concentrations of prostaglandin E_2 (PGE_2) are higher in women with endometriosis, and this increased PGE_2 plays an important role in survival and growth of endometriosis lesions (6–9). Inhibition of PGE_2 biosynthesis impedes growth of endometriosis (9) and chronic pelvic pain in women (7), and decreases growth of endometriosis lesions in animal models (8). COX-2 is the

Significance

Endometriosis is an inflammatory gynecological disease of reproductive age women associated with chronic pelvic pain and infertility. Endometriosis remains as the single major cause for hysterectomy in reproductive age women in the United States. The pathogenesis of endometriosis remains an enigma in reproductive medicine. Current hormonal therapies cause undesirable side effects, reproductive health concerns, and are unable to prevent recurrence of disease. Results of the present study indicate that selective inhibition of prostaglandin E2 (PGE_2) receptors EP2 and EP4 suppresses the growth and survival of endometriosis lesions, decreases pelvic pain, and restores endometrial functional receptivity through multiple mechanisms. Our novel findings provide a molecular and preclinical basis to formulate long-term nonhormonal therapy for endometriosis.

Author contributions: J.A.A. and S.K.B. designed research; J.A.A., J.L., D.B., J.A.S., and S.K.B. performed research; J.A.A., C.R.L., M.W.M., K.G.O., K.L.B.-T., R.C.B., and A.S.-P. contributed new reagents/analytic tools; J.A.A. and S.K.B. analyzed data; and J.A.A., R.C.B., and S.K.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1507931112/-/DCSupplemental.

rate limiting enzyme that regulates biosynthesis of PGE_2. The biological actions of PGE_2 are mediated via G protein receptors EP1, EP2, EP3, and EP4 by integrating multiple cell signaling pathways (10). Recent studies from our laboratory indicate that selective inhibition of EP2 and EP4 inhibits adhesion, invasion, growth, and survival of human endometriotic epithelial and stromal cells by modulating integrins, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), cell cycle, survival, and apoptosis pathways in vitro (11–14).

The objectives of the present study were to determine the pharmacological effects of selective inhibition of EP2/EP4 on: (*i*) growth and survival of endometriosis lesions; (*ii*) molecular and cellular aspects of the pathogenesis of endometriosis; (*iii*) pelvic pain and molecular pain pathways; and (*iv*) endocrine and immune modulation in the endometrium, using chimeric mouse model of endometriosis as preclinical testing platform. Our novel results collectively indicate that inhibition of PGE_2-EP2/EP4 signaling could emerge as a potential nonestrogen or nonsteroidal therapy for endometriosis.

Results

Therapeutic Effects of Selective Inhibition of EP2/EP4 on Growth of Endometriosis Lesions. Xenograft of mixed populations of endometriotic epithelial cells 12Z-GFP and stromal cells 22B-RFP in ovariectomized and estrogen-treated nude mice induced peritoneal endometriosis characterized by heterogeneous white and bleeding red lesional phenotypes which is similar to the human peritoneal endometriosis (Fig. 1). Histomorphology of the endometriosis lesions showed that epithelial cells 12Z-GFP formed endometrial glands and stromal cells 22B-RFP formed stroma and thus established functional communicating endometrial glands and surrounding stroma similar to the human peritoneal endometriosis. Under gross examination, 8–10 endometriosis lesions/mouse were visible. Importantly, under fluorescence dissection microscopy, 20–25 clusters of endometriosis lesions/mouse were detectable. Among the visible peritoneal endometriosis lesions, ∼60% were white lesions and 40% were bleeding red lesions (Fig. 1 *A–D*). Selective inhibition of EP2/EP4 from days 15–28 of xenograft decreased ($P < 0.05$) the growth of endometriosis lesions up to 60% dose-dependently, and the maximal effect was observed at 25 mg/kg. Inhibition of EP2/EP4 decreased ($P < 0.05$) the growth of epithelial cells 12Z-GFP and stromal cells 22B-RFP up to 70% and 60% respectively (Fig. 1 *E–J*). Real-time in vivo bioimaging, fluorescence dissection microscopy imaging, and morphometry data together indicated that pharmacological inhibition of EP2/EP4 decreased ($P < 0.05$) growth (size and volume) of endometriosis lesions. In addition, EP2-I/EP4-I (25 mg/kg) did not affect functions of liver and kidney (Fig. 1*K*), and the treated mice were apparently healthy.

Inhibition of EP2/EP4-Induced Apoptosis of Endometriosis Lesions. Inhibition of EP2/EP4 increased ($P < 0.05$) TUNEL positive epithelial as well as stromal cells in endometriosis lesions (Fig. 2 *A1–A3*). Next, we determined epithelial-stromal cell specific apoptosis. Inhibition of EP2/EP4 induced ($P < 0.05$) expression of cl-caspase-3 (Fig. 2 *B1–B5*) and cl-PARP (Fig. 2 *C1–C5*) proteins in both epithelial and stromal cells of endometriosis lesions. We examined eight lesions per mouse, among them ∼60% lesions showed increased expression of cl-caspase-3 and cl-PARP proteins and increased TUNEL-positive cells. These lesions were classified as regressing lesions and selected for further study to investigate the underlying molecular pathways through which inhibition of EP2/EP4 induced apoptosis of endometriosis lesions.

Inhibition of EP2/EP4 Decreased Survival, Invasion, and Proinflammation Machinery Proteins in Endometriosis Lesions. Inhibition of EP2/EP4 decreased ($P < 0.05$) expression of PGE_2 biosynthesis and signaling proteins COX-2, and EP2 and EP4 respectively (Fig. 3 *A1–C3*); proinflammatory cytokine proteins IL1β, TNFα, and IL6 (Fig. 3 *D1–F3*); important intracellular survival pathway proteins p-AKT, p-ERK1/2, and active β-catenin (Fig. 3 *G1–I3*); and invasion pathway proteins MMP2 and MMP9 (Fig. 3 *J1–K3*) in an epithelial-stromal cell specific as well as protein-specific pattern in endometriosis lesions.

Inhibition of EP2/EP4 Decreased Estrogen Biosynthesis and Signaling and Increased Progesterone Signaling Machinery Proteins in Endometriosis Lesions. Cytochrome p450 aromatase, ERα, and ERβ proteins were abundantly expressed in epithelial and stromal cells of endometriosis lesions. Inhibition of EP2/EP4 decreased ($P < 0.05$) p450 aromatase, ERα, and ERβ proteins (Fig. 3 *L1–N3*) in both cell types of endometriosis lesions. Interestingly, PR protein was not expressed in epithelial cells and expressed at very low levels in the stromal cells, which supports the typical P4-resistance state of endometriosis. Inhibition of EP2/EP4 increased ($P < 0.05$) expression of PR both in epithelial and stromal cells of endometriosis lesions and restored P_4-responsive state (Fig. 3 *O1–O3*). Surprisingly, expression of one of the important transcription factors SF1 in epithelial and stromal cells of endometriosis lesions was not modulated by EP2/EP4 inhibition (Fig. 3 *P1–P3*), suggesting posttranslational mechanisms.

Inhibition of EP2/EP4 Decreased Angiogenesis of Endometriosis Lesions. The primary angiogenesis signal vascular endothelial growth factor (VEGF) was highly expressed in the stromal cells but not in

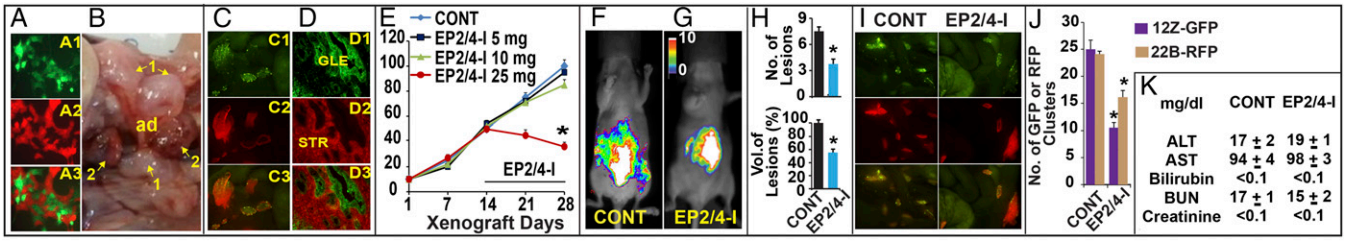

Fig. 1. Effects of inhibition of EP2 and EP4 (EP2/4-I) on growth and survival of endometriosis lesions. Fluorescence microscopy of (*A1*) human endometriotic epithelial cells 12Z-GFP and (*A2*) stromal cells 22B-RFP grown in coculture and imaged with red or green channels and (*A3*) overlay of both channels. (*B*) A mixture of epithelial cells 12Z-GFP and stromal cells 22B-RFP suspension was injected into the peritoneal cavity of nude mice, peritoneal endometriosis was induced (day 1), and necropsied on day 29 as detailed in *Materials and Methods*. Gross examination of white (1) and bleeding red (2) endometriosis lesions phenotypes with adhesions (ad). (*C1–C3*) Fluorescence zoomstereo microscopy examination of dissemination of 12Z-GFP and 22B-RFP cells of endometriosis lesions in the peritoneal cavity. Histomorphology of endometriosis lesions, (*D1*) 12Z-GFP cells formed the glands (GLE), (*D2*) 22B-RFP formed the stroma (STR), and (*D3*) established communicating glands and stroma. (*E*) Endometriosis nude mice were treated with EP2 (AH6809) and EP4 (AH23848) inhibitors (EP2/4-I) at 5, 10, or 25 mg/kg, i.p, at 24h intervals from days 15–28 of xenograft. Growth of endometriosis lesions was imaged in vivo real-time on days 1 (before), 7, 14, 21, and 28 of xenograft. Representative in vivo images for (*F*) control and (*G*) EP2/4-I at 25 mg/kg on day 28 are shown. (*H*) Gross number and volume of endometriosis lesions. (*I*) Fluorescence zoomstereo microscopy examination of peritoneal endometriosis lesions and (*J*) quantity of 12Z-GFP and 22B-RFP cells in these lesions. (*K*) Plasma biochemical parameters. *Control vs. EP2/4-I, $P < 0.05$, $n = 6$.

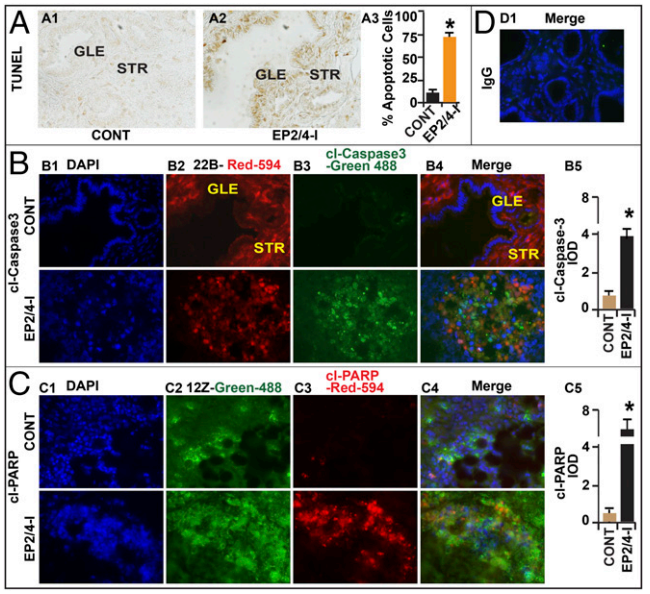

Fig. 2. Effects of inhibition of EP2 and EP4 (EP2/4-I) on induction of apoptosis and activation of caspase-3 and PARP proteins in endometriosis lesions. (*A*) TUNNEL assay. (*B*) The 22B-RFP stromal cells (STR) were labeled with Alexa 594 (red) and cl-caspase3 protein was labeled with Alexa 488 (green) antibodies. (*C*) The 12Z-GFP epithelial cells (GLE) were labeled with Alexa 488 (green), and cl-PARP protein was labeled with Alexa 594 (red) antibodies. Nuclei were stained with DAPI (blue). (*D*) IgG controls. Peritoneal endometriosis was induced in nude mice, treated with EP2 (AH6809) and EP4 (AH23848) inhibitors (EP2/4-I) at 25 mg/kg body weight, and necropsied as detailed in Fig. 1. *Control vs. EP2/4-I, $P < 0.05$, $n = 8$.

epithelial cells of endometriosis lesions. Inhibition of EP2/EP4 decreased ($P < 0.05$) expression of VEGF protein (Fig. 3 *Q1–Q3*) in stromal cells. Expression pattern of the endothelial cell marker protein Von Willebrand factor (vWF) indicated the density of endothelial cells around the endometriosis lesions. Inhibition of EP2/EP4 decreased ($P < 0.05$) expression of vWF, and in turn, decreased existing and newly developing angiogenesis network of endometriosis lesions (Fig. 3 *R1–R3*).

Inhibition of EP2/EP4 Decreased Pelvic Pain in Endometriosis. We used chimeric Rag2g(c) intact mouse model of endometriosis to investigate pain mechanisms. Xenograft 12Z-GFP and 22B-RFP cells induced peritoneal endometriosis characterized by heterogeneous white and red lesional phenotypes similar to that of nude mouse model as shown (Fig. 1 *B* and *I*). Vaginal cytology confirmed that peritoneal endometriosis progressively prolonged the E_2 phase of the estrous cycle after 3 wk of disease, suggesting a compromised estrous cycle. Inhibition of EP2/EP4 decreased ($P < 0.05$) the growth of endometriosis up to 60% (Fig. 4*A*) irrespective of E_2 or P_4 phase of the estrous cycle. Importantly, inhibition of EP2/EP4 decreased growth, survival, and dissemination of endometriosis lesions in such an E_2-dominant state. As shown in Fig. 3 above, the epithelial cells 12Z and stromal cells 22B of endometriosis lesions have inherent capacity to produce E_2. Therefore, the source of E_2-dominance appears to be the peritoneal endometriosis lesions rather than mouse ovaries in this model.

Mechanical hyperalgesia was assessed by stimulating the pelvic floor with von-Frey filaments and the threshold force required to elicit a behavioral withdrawal response was determined. Peritoneal endometriosis decreased ($P < 0.05$) pelvic floor withdrawal threshold (reflecting increased pain to von-Frey stimulus). Inhibition of EP2/EP4 increased ($P < 0.05$) pelvic floor pain threshold or decreased mechanical hyperalgesia irrespective of E_2 versus P_4 phase of the estrous cycle (Fig. 4*B*, data from E_2 phase is shown). Interestingly, the observed pelvic floor mechanical hyperalgesia is correlated with growth of endometriosis lesions.

We examined innervation (formation of new nerve fibers) of endometriosis lesions by determining the expression of PGP9.5 (pan neuronal marker), TRPV1 (afferent nerve marker), CGRP (C-sensory nerve fiber marker), SP (Aδ-sensory nerve fiber marker), and VMAT (sympathetic nerve fiber marker) proteins around the

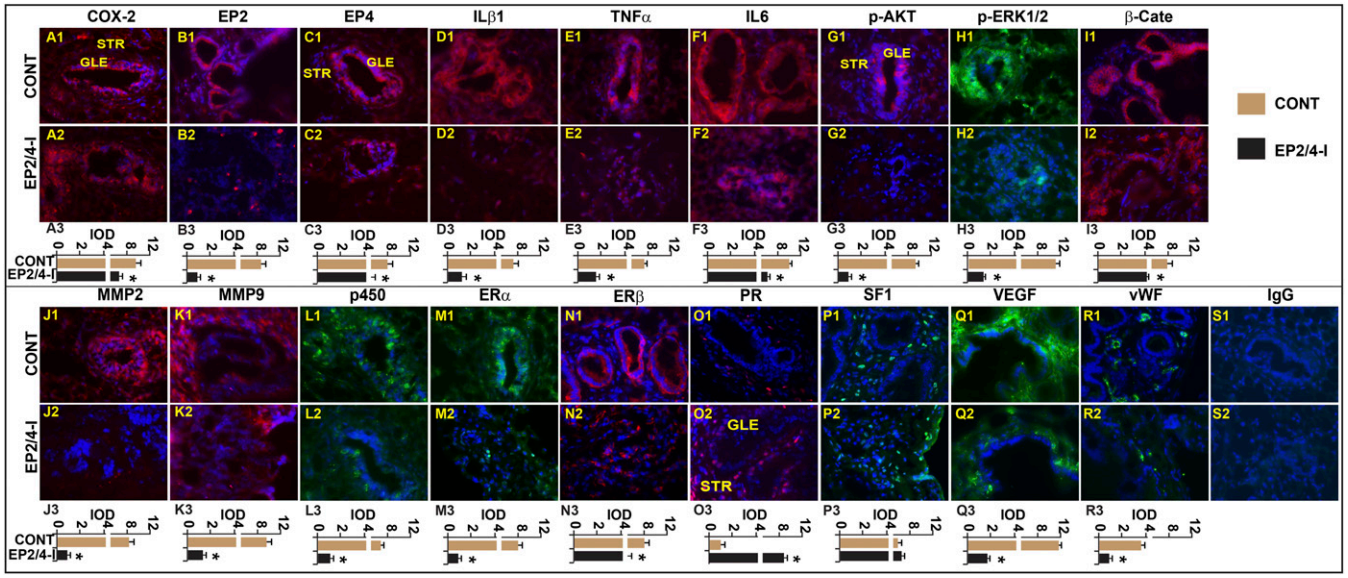

Fig. 3. Effects of inhibition of EP2 and EP4 (EP2/4-I) on regulation of key proteins involved in inflammation, survival, invasion, angiogenesis, and biosynthesis and signaling of PGE_2, E_2, and P_4 in endometriosis lesions. (*A1–A3*) COX-2. (*B1–B3*) EP2. (*C1–C3*) EP4. (*D1–D3*) IL1β. (*E1–E3*) TNFα. (*F1–F3*) IL6. (*G1–G3*) p-AKT. (*H1–H3*) p-ERK1/2. (*I1–I3*) β-catenin. (*J1–J3*) MMP2. (*K1–K3*) MMP9. (*L1–L3*) p450 aromatase. (*M1–M3*) ERα. (*N1–N3*) *ER*β. (*O1–O3*) PR. (*P1–P3*) SF-1. (*Q1–Q3*) VEGF. (*R1–R3*) vWF proteins. (*S1–S2*) IgG controls. Nuclei were stained with DAPI (blue), and each protein was stained using Alexa 488 (green) or Alexa 594 (red) secondary antibodies. Peritoneal endometriosis was induced in nude mice, treated with EP2 (AH6809) and EP4 (AH23848) inhibitors (EP2/4-I) at 25 mg/kg, and necropsied as detailed in Fig. 1. *Control vs. EP2/4-I, $P < 0.05$, $n = 8$.

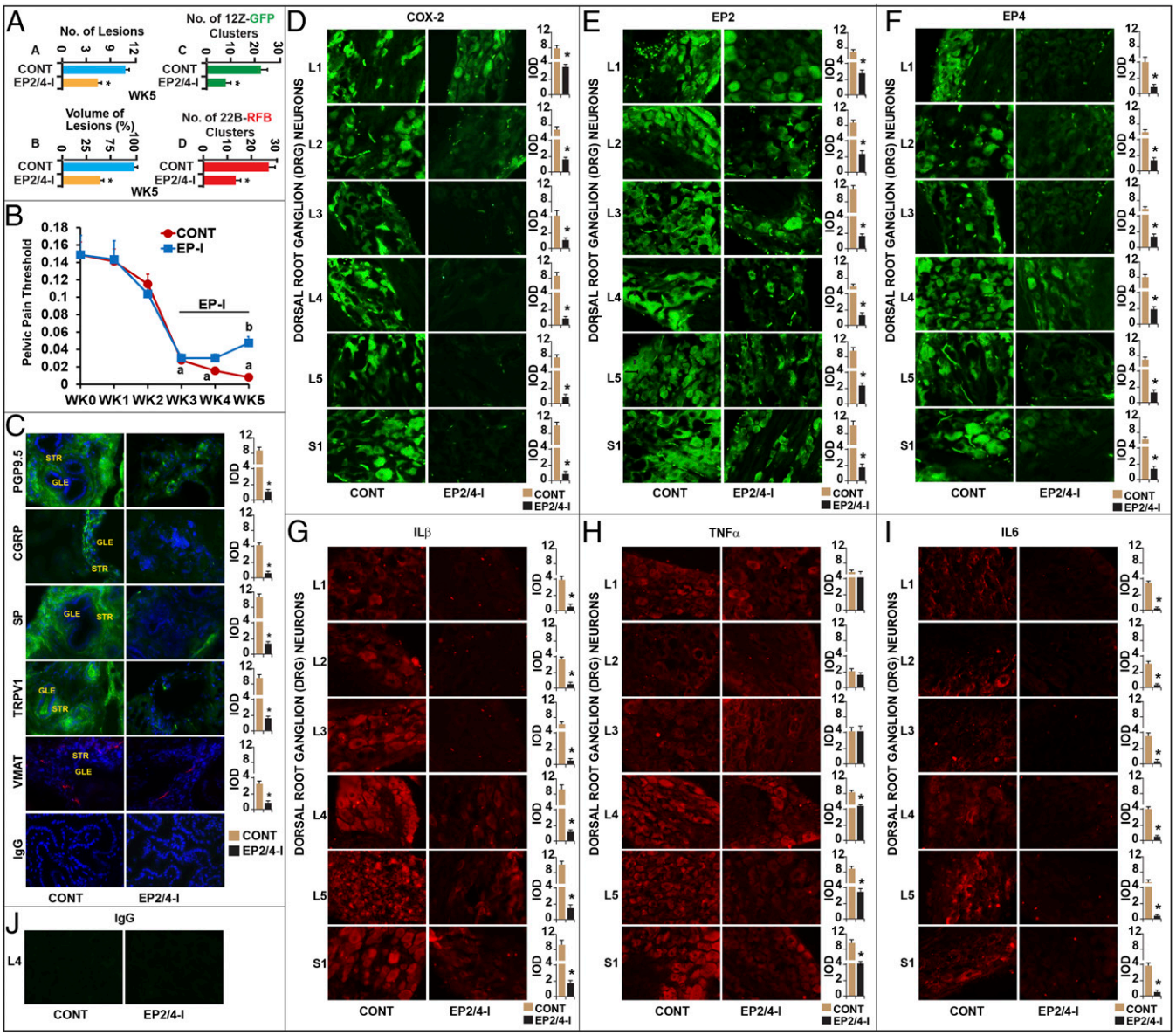

Fig. 4. Effects of inhibition of EP2 and EP4 (EP2/4-I) on innervation of endometriosis lesions, regulation of proinflammatory machinery proteins in DRG, and nociception of pelvic pain in endometriosis. (*A*) Growth of endometriosis lesions measured by gross examination and morphometry (A and B) and fluorescence zoomstereo microscopy (C and D). (*B*) Pelvic floor referred hyperalgesia using von-Frey test. (*C*) Expression of neuronal markers PGP9.5, CGRP, SP, TRPV1, and VMAT proteins in endometriosis lesions. Expression of COX-2 (*D*), EP2 (*E*), EP4 (*F*), ILβ (*G*), TNFα (*H*), and IL6 (*I*) proteins in DRG neurons L1, L2, L3, L4, L5, and S1. (*J*) IgG controls. GLE, glandular epithelium; STR, stromal cells. Nuclei stained with DAPI (blue), each protein stained with Alexa 488 (green) or Alexa 594 (red) secondary antibodies. *Control vs. EP2/4-I, $P < 0.05$, $n = 8$. After 3 wk of disease, endometriosis Rag2g(c) mice were treated with EP2 (AH6809) and EP4 (AH23848) inhibitors (EP2/4-I) at 25 mg/kg for 2 wk. At the end of 5 wk, the mice were necropsied at the E_2 versus P_4 phase of the estrous cycle as confirmed by vaginal cytology, and data from E_2 phase shown.

endometriosis lesions during E_2 phase of the estrous cycle. Results (Fig. 4*C*) indicated that endometriosis lesions developed their own innervations (PGP9.5-positive neurites), which include sensory/afferent nerve fibers (CGRP-, SP-, TRPV1-positive neurites) and sympathetic/efferent nerve fibers (VMAT-positive neurites). Inhibition of EP2/EP4 decreased ($P < 0.05$) the expression of PGP9.5, TRPV1, CGRP, SP, and VMAT proteins and in turn decreased C and Aδ sensory fiber innervation of endometriosis.

Visceral primary afferent neurons (nociceptors) detect and transmit information from the pelvic region into the spinal cord. The cell bodies of these primary afferent neurons are located in the lumbosacral dorsal root ganglia (DRG). Inflammation contributes to the sensitization of the primary afferent neurons leading to the enhancement of visceral sensitivity to mechanical stimulation. Pelvic organs are primarily innervated from L1, L2, L3, L4, L5, and S1 of the spinal cord. Therefore, we next examined regulation of proinflammatory molecular markers in the DRG neurons from L1 to S1. Results indicated that PGE_2 biosynthetic and signaling machinery proteins COX-2, EP2 and EP4 (Fig. 4 *D–F*) and proinflammatory cytokine proteins IL1β, TNFα, and IL6 (Fig. 4 *G–I*) were abundantly expressed in L1, L2, L3, L4, L5, and S1 DRG neurons. Importantly, inhibition of EP2/EP4 decreased ($P < 0.05$) expression of these proteins in DRG neurons L1-S1 selectively at different levels.

Inhibition of EP2/EP4 Decreased E_2-Dominance and P_4-Resistance in Eutopic Endometrium in Endometriosis. We used chimeric Rag2g(c) intact mouse model of endometriosis to investigate endocrine-

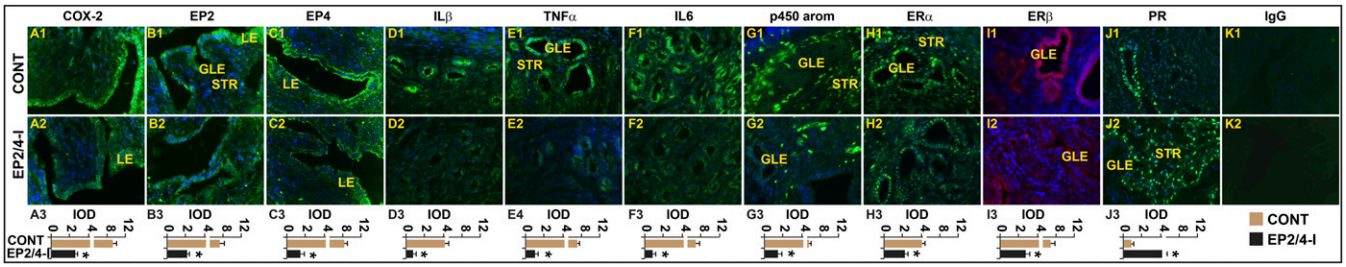

Fig. 5. Effects of inhibition of EP2 and EP4 (EP2/4-I) on regulation of key proteins involved in biosynthesis and signaling of PGE_2, E_2, and P_4, and proinflammation in eutopic endometrium in endometriosis. (*A1–A3*) COX-2. (*B1–B3*) EP2. (*C1–C3*) EP4. (*D1–D3*) ILβ. (*E1–E3*) TNFα. (*F1–F3*) IL6. (*G1–G3*) p450 aromatase (p450arom). (*H1–H3*) ERα. (*I1–I3*) ERβ. (*J1–J2*) PR protein. (*K1–K2*) IgG controls. Nuclei were stained with DAPI (blue), each protein was stained with Alexa 488 (green) secondary antibody. GLE, glandular epithelium; LE, luminal epithelium; STR, stromal cells. *Control vs. EP2/4-I, $P < 0.05$, $n = 8$. Peritoneal endometriosis was induced in Rag2g (c) mice, treated with EP2 (AH6809) and EP4 (AH23848) inhibitors (EP2/4-I) at 25 mg/kg, necropsied as detailed in Fig. 4, and data from E_2 phase shown.

immune modulations in the eutopic endometrium. Inhibition of EP2/EP4 decreased ($P < 0.05$) the expression of PGE_2 biosynthetic and signaling proteins COX-2, EP2, and EP4 (Fig. 5 *A1–C3*) and proinflammatory cytokine proteins IL1β, TNFα, and IL6 (Fig. 5 *D1–F3*) proteins in epithelial and stromal cells of endometrium. The key E_2 biosynthesis enzyme p450 aromatase protein was only expressed in stromal cells but not in the epithelial cells of endometrium. Interestingly, inhibition of EP2/EP4 decreased ($P < 0.05$) expression of p450 aromatase protein (Fig. 5 *G1–G3*) in stromal cells and concomitantly increased its expression in epithelial cells of endometrium. ERα protein (Fig. 5 *H1–H3*) was expressed in both epithelial and stromal cells, and inhibition of EP2 and EP4 decreased ($P < 0.05$) its expression in both cell types of endometrium. ERβ protein (Fig. 5 *I1–I3*) predominantly expressed in epithelial cells compared with stromal cells, and inhibition of EP2 and EP4 decreased ($P < 0.05$) its expression in both cell types of endometrium. PR protein (Fig. 5 *J1–J3*) was not expressed in stromal cells but was expressed in glandular epithelial cells. Importantly, inhibition of EP2/EP4 increased/restored ($P < 0.05$) its expression in stromal cells and concomitantly decreased its expression in glandular epithelial cells of endometrium.

Discussion

PGE_2 plays an important role in the pathogenesis of endometriosis (6–9). COX-2, EP2, EP4, p-ERK1/2, p-AKT, β-catenin, MMP2, and MMP9 proteins are highly expressed/activated in endometriosis lesions in women (11–15). PGE_2 increases SF1, StAR, and p450 aromatase genes and stimulates de novo biosynthesis of E_2 which in turn increases PGE_2 biosynthesis in endometriosis lesions (1). PGE_2 and E_2 (ERα and ERβ) interactive pathways appear to suppress PR expression leading to loss of P_4 action and P_4-resistance in endometriosis lesions (1, 16). In addition, endometriosis lesions secrete proinflammatory cytokines (17). COX-2/PGE_2 induces VEGF and promotes angiogenesis of endometriosis lesions (8).

Results of the present study indicate that inhibition of EP2/EP4 suppresses survival, invasion, biosynthesis, and signaling of PGE_2 and E_2, production of proinflammatory cytokines, and increases P_4 signaling machinery proteins in an epithelial and stromal cell-specific pattern in endometriosis lesions. These results together establish that inhibition of EP2/EP4 restores appropriate cross-talk among PGE_2, E_2, and P_4 pathways and converts the proinflammatory, E_2-dominant, and P_4-resistant state into a P_4-responsive state and thus decreases growth, survival, and dissemination of peritoneal endometriosis lesions. An inverse relationship among EP2, EP4, p450 aromatase, ERα, ERβ, and PR proteins in epithelial and stromal cells suggests that PGE_2 and E_2 interactive pathways are the important regulators of PR signaling in endometriosis lesions. However, the underlying molecular interactive mechanisms among EP2/EP4, ER, and PR in endometriosis lesions in an epithelial-stromal cell specific pattern are largely unknown.

The mechanisms underlying the detection and transmission of nociceptive signals from endometriosis lesions are also largely unknown. i.p. concentrations of PGE_2 (18), IL1β, and TNFα (19) are higher in women with endometriosis. PGE_2 acts on the peripheral nociceptors and increases responsiveness to peripheral stimuli through TRPV1 and induces chronic inflammatory pain through EP2 and EP4 (20, 21). Localized peripheral inflammation increases expression of COX-2 protein in the spinal cord (22) and DRG neurons (23) and EP4 protein in L5 DRG neurons (20, 21). Pharmacological and genomic inhibition of COX-2 (22) or EP4 (20, 21) decreases inflammatory pain hypersensitivity. Furthermore, activation of EP2 or EP4 increases TRPV1 activity in DRG neurons (20, 21). Inhibition of EP4 decreases PGE_2-induced sensitization of DRG neurons and release of neuropeptides SP and CGRP (20, 21). Advanced active endometriosis lesions are innervated by sympathetic and sensory C and Aδ fibers and they express CGRP and TRPV1 proteins in a rat allograft model (24).

Results of the present study indicate that endometriosis lesions induce innervation of C and Aδ afferent sensory and efferent nerve fibers around the endometriosis lesions, promote proinflammatory microenvironment of DRG neurons from L1-S1 which innervate pelvis and pelvic organs, and increase pelvic floor hyperalgesia. Inhibition of EP2/EP4 decreases innervation of endometriosis lesions, decreases the proinflammatory state of DRG neurons, and suppresses peripheral nociception. These results together establish that the inflammatory response of endometriosis lesions initiates a cascade of events resulting in enhancement of the responsiveness of nociceptors and DRG neurons, and inhibition of EP2/EP4 suppresses these pain pathways and decreases endometriosis-induced pelvic pain. In women with endometriosis, the pelvic pain can be assessed by bilateral hyperalgesia and allodynia from T9-S1 spinal segments (25) and mechanical hyperalgesia of the pelvis, where pain is reported in response to applied pressure. Although the von-Frey DRG mice models are widely used to measure hyperalgesia in pain models, we used this interactive approach, to our knowledge for the first time, to measure the pelvic floor pain threshold in endometriosis chimeric mouse model with translational relevance.

The underlying causes of endometriosis-induced infertility remain unclear and are likely multifactorial. In women with endometriosis, the microenvironment of the endometrium becomes proinflammatory, E_2-dominant, and P_4-resistant, and thereby impairs receptivity for establishment of pregnancy (1, 2, 26, 27). The endometrium first becomes hyperresponsive to E_2 and then resistant to P_4 (1, 16, 28). The loss of normal endometrial function becomes more evident with progression of disease (28). The disruption of these signaling processes in endometrial epithelial and stromal cells appears to be a consequence of growth of peritoneal

endometriosis lesions, which might be orchestrated by progressive deregulated interactions among PGE_2, E_2, and P_4.

Results of the present study indicate that inhibition of EP2/EP4 decreases PGE_2, E_2 biosynthesis and signaling, proinflammatory cytokine production, and increases P_4 signaling machinery proteins in an epithelial-stromal cell-specific pattern in the eutopic endometrium. These results together demonstrate that inhibition of EP2/EP4 suppresses E_2-dominant state and concomitantly increases P_4-responsive state of the endometrium. Thus, it restores the ability of the endometrium respond appropriately to PGE_2, E_2, and P_4 and its functional receptivity for establishment of pregnancy. Specific inverse regulatory relationship among COX-2, EP2/EP4, p450 aromatase, ERα, ERβ, and PR in epithelial and stromal cells of the endometrium suggest that PGE_2-E_2 interactive pathways are the important regulators of PR and p450 aromatase. However, the underlying molecular and cellular mechanisms that lead to restoration of PR and suppression of ER expression in an epithelial-stromal cell specific pattern are largely unknown.

In conclusion, results of the present study, to our knowledge for the first time, indicate that selective pharmacological inhibition of EP2/EP4: (*i*) suppresses the growth and survival of endometriosis lesions; (*ii*) decreases endometriosis-induced innervation and pelvic pain; (*iii*) decreases proinflammatory, E_2-dominant, and P_4-resistant molecular environment of the endometrium and endometriosis lesions; and (*iv*) restores endometrial functional receptivity through multiple mechanisms (Fig. S1). Our novel results provide molecular and preclinical basis to formulate personalized phenotype-based long-term nonestrogen or nonsteroidal therapy through inhibition of EP2/EP4 as a more effective alternative treatment to existing hormonal therapies for endometriosis. At present, no therapeutic agents are available to inhibit EP2/EP4 receptors for the treatment of endometriosis or other inflammatory diseases in human medicine. Discovery of specific targeted drugs, injectable antibodies or small molecules to inhibit EP2/EP4 is expected to provide new clinical trials and open new area of research in endometriosis. Findings of the present study provide new knowledge that fills an important gap in the current understanding of the pathogenesis, diagnosis, and treatment of endometriosis.

Materials and Methods

Human fluorescent endometriotic epithelial cell line 12Z and stromal cell line 22B were xenografted and peritoneal endometriosis was induced in immunocompromised mice. The experimental mice were treated with EP2 and EP4 inhibitors. Effects of treatment on growth, survival, pain, and infertility and the underlying molecular mechanisms were determined (29–37). All procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M University. Detail methods (Fig. S2 and Table S1) are provided in the *SI Materials and Methods*.

ACKNOWLEDGMENTS. This work is partially supported by National Institute of Child Health and Human Development Grants HD065138, HD066248, and HD079625, and National Institute of Environmental Health Sciences Grant ES014942.

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