Neonatal feeding of an estrogen receptor β agonist induces external adenomyosis-like lesions in ICR mouse

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Abstract
Objective: Despite the fact that adenomyosis is a fairly common gynecological disorder, its pathogenesis remains elusive. Several theories on the pathogenesis of adenomyosis have been proposed, but none of them has been proven experimentally. So far, the most used one is the neonatal feeding of tamoxifen (TAM) in ICR/CD-1 mouse. However, its underlying mechanism of action is unknown. To further delineate the mechanism of TAM-induced adenomyosis in ICR/CD-1 mouse with regard to specific estrogen receptor (ER), we conducted an experiment that neonatal mice were fed with either TAM, or 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT; an ERα agonist), or 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN; an ERβ agonist), or G-1 (a G-protein coupled receptor 30 agonist), or just vehicle, in an attempt to tease out which specific receptor plays a dominant role in the genesis of adenomyosis induced by neonatal feeding of TAM.

Methods: Forty female neonatal mice were randomly divided into 5 equal-sized groups: CTL (control), TAM, PPT, DPN, and G-1. Three months later, all mice were sacrificed and their uterine horns were harvested, weighed, and processed for histological evaluation.

Results: All mice in the TAM group developed adenomyosis, so did 4 mice (50%) in the DPN group, a result that should be considered significant given that mice in the CTL group would not develop adenomyosis. No mouse in the PPT or G-1 group developed adenomyosis. Remarkably, all lesions in the DPN group were seen exclusively near the uterine serosa, which are dramatically different from that of TAM mice and reminiscent of extrinsic or external adenomyosis in humans.

Conclusions: Neonatal feeding of DPN induces adenomyosis, but the adenomyotic lesions appear to be different from those induced by TAM. Thus, the cause of TAM-induced adenomyosis in ICR/CD-1 mouse cannot be attributable to one specific ER alone. This suggests that the extrinsic/external adenomyosis may have a pathogenesis that is different from other sub-types of adenomyosis.

Keywords: Adenomyosis, Estrogen receptor α agonist, Estrogen receptor β agonist, Mouse model, Neonatal, Tamoxifen

Introduction
Featuring the invasion of the endometrium into the myometrium[1], adenomyosis is a common gynecological disease affecting mostly women of reproductive age[2]. Traditionally viewed as an “elusive” disease[1,3] and still considered to be “a riddle, wrapped in mystery, inside an enigma” today[4], its pathogenesis remains elusive, despite that the first description of the disease dates back well over a century ago[5].

Currently, there are 2 widely accepted theories on the pathogenesis of adenomyosis, namely, metaplasia and invagination[6–8]. The metaplasia theory postulates that the endometrial cells in the muscular layer originate from the metaplasia of Müllerian remnants or stem cells[9–11]. In contrast, the invagination theory stipulates that the direct invasion of endometrium to the muscle layer results from the process of tissue injury and repair (TIAR) that leads to the formation of lesions[12–14]. One important building block within the invagination theory hinges on the TIAR hypothesis, proposed by Leyendecker et al[15] and Leyendecker and Wildt[16]. Unfortunately, so far there has been no experimental data to support or refute either of the theories, or the TIAR hypothesis[17].

Epidemiological data strongly suggest that iatrogenic uterine procedures are a risk factor for adenomyosis[16–20]. Taking this cue, we hypothesized that endometrial-myometrial interface disruption (EMID) resulting from iatrogenic uterine procedures may increase the risk of developing adenomyosis[15] and proved, by experimentation, that EMID can induce adenomyosis in mice[21]. However, it is apparent that not all patients with adenomyosis had the history of uterine procedures. Hence, there must be pathogenic factors other than EMID.
With no other apparent clue from epidemiologic studies, it may be profitable to turn to animal models of adenomyosis to gain insight into the pathogenesis of adenomyosis, since adenomyosis, unlike endometriosis, can and do occur spontaneously in many animal species, including, but not limited to, rodents[22–24]. This fact raises the prospect that the mechanisms underlying adenomyosis pathogenesis in mouse may also be at work in its human counterpart. At the very least, even if this is not true, we can still learn something about the pathogenesis if we can understand the mechanism of action for pathogenesis in animals.

There are numerous animal models of adenomyosis[22–24] and adenomyosis and arguably the most used one is the neonatal feeding of tamoxifen (TAM) in ICR/CD-1 mouse, established by Parrott et al[25] in 2001. Unfortunately, despite extensive investigation, the best we know about this model is the disruption of myometrium[26–28].

In many ways, the adenomyosis model by neonatal feeding of TAM is a very interesting model. It is strain- and also administration route-dependent, since the injection, instead of feeding of TAM, does not induce adenomyosis[29]. Neonatal feeding of TAM or toremifene (another selective estrogen receptor modulator [SERM]) can induce adenomyosis 42–90 days after dosing in ICR/CD-1 mice[25,26], but adenomyotic lesions can be seen as early as 10 days after dosing[27].

TAM is a member of the SERM family[30], known to display tissue-specific estrogen actions in different tissues[31]. In particular, it exhibits estrogenic effect in endometrium[32].

The action of estrogen is known to be mediated through estrogen receptors (ERs), which have 2 sub-types, ERα and ERβ. Both are expressed in multiple tissues such as uterus, ovary, breast, prostate, the cardiovascular system, brain, and other tissues/organs[33–37]. In particular, ERs are expressed in endometrial epithelial and stromal cells of both ICR and BALB/c mice at post-natal day 4[38]. In ICR mice, the entire uterine wall expresses ERα from post-natal day 2[39]. However, the tissue distribution and expression levels of ERα and ERβ within the same tissue vary and can be different. ERβ usually has a wider tissue distribution than ERα[39]. ERs and ERβ often have incongruent functional characteristics[40–42]. ERs promotes cellular proliferation, while ERβ inhibits proliferation[43] and the activity of ERα can be regulated by ERβ[44–46].

TAM can bind with both ERα and ERβ[47] and is used mainly for the treatment of ER-positive breast cancer[48,49]. However, TAM therapy has different efficacy in breast cancer with different ERα and ERβ distribution and has a higher efficacy for breast cancer with higher ERβ expression[46,48]. This seems to suggest that the 2 receptors have different binding affinity and responsiveness to TAM.

Estrogen and TAM can also activate ER by non-genomic mechanisms, such as through the G-protein coupled receptor 30 (GPR30) signaling pathway. TAM exerts its function as a GPR30 agonist that activates the epidermal growth factor receptor intracellular signaling, MAPK and PI3K/AKT signaling pathways, leading to TAM resistance in breast cancer[45–49]. Endometrial abnormalities, such as bleeding or endometrial thickness induced by TAM therapy, are also associated with TAM binding to GPR30[50]. TAM and estrogen can promote endometrial cancer cell migration and proliferation by triggering GPR30 activation[51].

In light of the above, it is conceivable that TAM induces adenomyosis through different ERs. To further delineate the mechanism of TAM-induced adenomyosis in ICR/CD-1 mouse with regard to specific ER, we conducted an experiment that neonatal mice were fed with either TAM, or an ERα agonist, or an ERβ agonist, or a GPR30 agonist, or just vehicle in an attempt to tease out which specific receptor plays a dominant role in the genesis of adenomyosis induced by neonatal feeding of TAM.

Methods and materials

Chemicals

TAM citrate was purchased from Fudan Forward Pharmaceutical Company (Shanghai, China). The estrogen ERα receptor agonist, 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT), and the ERβ receptor agonist, 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), were purchased from Tocris Bioscience (Bristol, UK). The membrane ER GPR30 agonist G-1 was purchased from Cayman Chemical Company (Ann Arbor, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, USA) unless stated otherwise.

Animals and experiment protocol

Eight female ICR mice at a gestational age of 17–18 days were purchased from Shanghai Slack Experimental Animal Co., Ltd (SCXK-2007-0005; Shanghai, China), and were housed individually in a single cage during the rest of the gestation period and the ensuring birth and nursing period. Sexing was performed to all the pups on the first day after birth (designating the day of birth as day 1) and the female pups were selected to be used in this study. Each dam and their litter of pups were housed in the same cage until weaned under controlled conditions with a 12 hours:12 hours light/dark cycle and had free access to chow and fresh water ad libitum. All animal care and procedures were conducted in accordance with the guidelines of the US National Research Council’s Guide for the Care and Use of Laboratory Animals[59], and were approved by the institutional experimental animal review board of the Shanghai OB/GYN Hospital, Fudan University.

Forty female neonatal mice were randomly divided into 5 equal-sized groups: CTL (control), TAM, PPT (an ERα agonist), DPN (an ERβ agonist), and G-1 (a GPR30 agonist). Following published reports[23,24,60–62], and as reported previously[60,62], mice in TAM group were dosed orally with 1 mg/kg TAM from day 2 to day 5 after birth. Following published reports[60,62], mice in the PPT, DPN and G-1 groups were dosed orally with 5 mg/kg PPT, 5 mg/kg DPN, and 6 mg/kg G-1, respectively, via capillary tubing on days 2–5 after birth, exactly identical to those in the TAM group. TAM, PPT, DPN, and G-1 were suspended in peanut oil/lecithin/condensed milk mixture in a 2:0:2:3 ratio (by volume) at a dose volume of 5 μL/g bodyweight each day, and mice in the CTL group were dosed in a similar fashion with the same volume of vehicle buffer only. The experiment design is shown schematically in Figure 1A.

Three weeks after birth, mice were weaned and maintained in routine condition. Three months after birth, all mice were sacrificed by cervical vertebra dislocation, and all their uterine horns were harvested, weighed and fixed in 4% neutral-buffered formalin for further histological analysis.

Hematoxylin–eosin and immunohistochemistry staining

The formalin-fixed uterine tissues were dehydrated and embedded in paraffin, then serial 4-μm sections were obtained from each tissue block. Routine deparaffinization and rehydration procedures were performed. Briefly, tissue slides were deparaffinized

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in xylene and rehydrated in a graded alcohol series, and then were subjected to hematoxylin–eosin (H&E) staining using H&E staining kit (Sun Biotec, Shanghai, China).

To show uterine smooth muscle, immunohistochemical staining of α-smooth muscle actin (α-SMA) was performed. After routine deparaffinization and dehydration, some sections were randomly selected to be heat-retrieved with citric acid (0.01 mol/L, pH 6.0) over 98°C for a total of 30 minutes. Then all sections were cooled naturally at room temperature, and then incubated with the primary rabbit polyclonal antibody against α-SMA (ab2765; Abcam, Hong Kong, China) at 4°C overnight. After the sections were rinsed with PBS, they were incubated with the secondary antibody (Sunpoly-HII; BioSun Technology Co., Ltd, Shanghai, China) for 30 minutes. The bound antibody complexes were stained for 3–5 minutes or until appropriate for microscopic examination with diaminobenzidine (BioSun Technology Co., Ltd) and then counterstained with hematoxylin.

Finally, slides were dehydrated, mounted and observed under 40×, 100× and, 200× magnification using Olympus microscope (Olympus, Tokyo, Japan) to confirm pathologic diagnosis.

**Uterine horn diameter measurement**

Uterine horn diameters were obtained by measuring the diameters of the uterine horn section images at 40 times of H&E staining sections. The average of the smallest and largest diameters of uterine horn cross-sections for each mouse was used as the diameter.

**Statistical analysis**

The uterine weight and the diameters of uterine horn cross-section data were presented in boxplots. In a boxplot, the line in the box represents the median, and the upper and lower sides represent the 3rd and the 1st quantile, respectively, while the upper and lower whiskers represent respectively the maximum and the minimum values in the data. The comparison of distributions of continuous variables between or among 2 or more groups was made using the Wilcoxon and Kruskal tests, respectively. Pearson’s correlation coefficient was used when evaluating correlations between the uterine weight and the diameters of uterine horn cross-sections.

*P* values of less than 0.05 were considered statistically significant. All computations were made with R 4.1.1 (www.R-project.org).

**Results**

**Uterine weight and the diameters of the uterine cross-sections**

All mice survived the experiment. We first evaluate the uterine weight and the diameters of the cross-sections of the harvested...
uterine horns for mice from all groups. We found that, compared with the CTL mice, the uterine weights of mice in the TAM and PPT groups were both significantly lower (both $P=0.00016$; Fig. 1B), while that from mice in the DPN and G-1 groups had no change (both $P \geq 0.92$; Fig. 1B). Consistently, the average diameter of the uterine horn cross-sections was reduced significantly in mice from TAM and PPT groups ($P=0.0003$ and $P=0.0009$, respectively), but not from the DPN and G-1 groups (both $P \geq 0.074$; Fig. 1C and Fig. 2). Indeed, the uterine weight and the uterine horn diameters correlated closely ($r=0.93$, $P<2.2 \times 10^{-16}$; Fig. 1D). Hence, as far as the uterine size is concerned, the ER$_a$ agonist acted in similar fashion as that of TAM, while the ER$_b$ and the GRP30 agonists did not.

Uterine morphology in CTL mice

In CTL mice, all uteri displayed distinct 4 layers: the endometrium, inner myometrium, outer longitudinal muscle layer, and serosa (Fig. 2). As expected, no adenomyotic lesion was found. The endometrial glands had branches and were confined within the endometrial layer, which was surrounded by the continuous inner myometrium with circular smooth muscles. Between the endometrium and inner myometrium, we could see abundant blood vessels. The outer longitudinal muscle layer consisted of uniformly distributed smooth muscle cell bundles with well-formed connective tissue sheaths (Fig. 3). The outmost serosa layer was well formed.

Uterine morphology in the TAM mice

As expected, adenomyosis was found in uterine specimens from all mice neonatally fed with TAM, consistent with results previously reported$^{[25,66]}$. The branches of endometrial glands were more complex and prominent than that of CTL mice. Some endometrial glands appeared in the inner myometrium, while others reached the serosa, forming sub-serosal cysts (Fig. 2). The continuity of the inner circular myometrium was disrupted by invasive glands. In particular, the inner myometrium showed marked loosening and increased intercellular space. In contrast, the outer myometrium appeared to be well developed with the apparent bundling, but was significantly thinner than CTL (Fig. 3).

Uterine morphology in the PPT mice

No mouse developed adenomyosis in this group. The branch of endometrial glands appeared to be similar to that of CTLs, and was confined within the endometrial layer (Fig. 2). Similar to the TAM mice, the inner circular myometrium showed marked loosening and increased intercellular space, but the outer myometrium seemed to be substantially thinner than that of CTLs (Fig. 3). This is consistent with the report that mice neonatally treated with PPT exhibit disrupted organization of uterine muscle layers and endometrial stromal cells, manifesting as hypoplasia of circular muscle and reduced density of longitudinal muscles$^{[63]}$. Taken together, neonatal administration of the ER$_a$ agonist PPT disrupted the uterine myometrium, but did not induce adenomyosis.

Uterine morphology in the DPN mice

We found adenomyotic lesions in 4 mice in this group, resulting in the incidence of adenomyosis of 50%, which is marginally

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**Fig. 2.** Representative photographs of H&E staining in different treatment mice. Representative photographs of H&E staining to show the morphology of uterine horns from mice in the CTL (control), TAM (tamoxifen), PPT (an ER$_a$ agonist), DPN (an ER$_b$ agonist), and G-1 (a GPR30 agonist) groups. Arrows show ectopic glands. Bar = 50 μm. DPN: 2,3-bis(4-hydroxyphenyl)-propionitrile; GPR30: G-protein coupled receptor 30; H&E: Hematoxylin–eosin; IM: Inner myometrium; OM: Outer myometrium; PPT: 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol.
higher than the CTL group ($P=0.077$). Remarkably, all lesions were seen exclusively near the uterine serosa (Fig. 2), which were dramatically different from that of TAM mice.

The branching of the gland was as complex as in TAM treatment mice, but in all cases all the branching glands were confined to the endometrial layer without any sign of breaching the continuity of inner myometrium (Fig. 2). In addition, the integrity of the inner myometrium appeared to be maintained, undisrupted by ectopic glands (Fig. 3). These findings are very similar to the extrinsic or external adenomyosis sub-type in human adenomyosis as described by Kishi et al. [67] and Bazot et al. [68]. The results suggest that ERβ agonist DPN may promote glandular branching and translocation, leading to the induction of adenomyosis.

**Uterine morphology in the G-1 mice**

No adenomyotic lesion was found in this group. Overall, the uterine morphology in this group of mice was similar to that of CTL mice. All branching glands were confined within the endometrium surrounded by well-formed inner myometrium (Fig. 2). The outer myometrium consisted of smooth muscle cell bundles with well-formed connective tissue sheaths (Fig. 3). These results indicated that neonatal feeding of GPR30 agonist G-1 did not exert much effect on uterine development.

**Discussion**

This study explored the roles of neonatal feeding of ERα, ERβ, and GPR30 agonists in the incidence of adenomyosis, in comparison, head to head, with the feeding of TAM and vehicle. While neonatal feeding of TAM induced adenomyosis in all mice, similar feeding of PPT and G-1 did not induce any visible adenomyosis. In contrast, neonatal feeding of DPN resulted in adenomyosis in 50% of the mice, but the adenomyotic lesions are located exclusively near the serosa, noticeably different from those induced by TAM but reminiscent of extrinsic/external adenomyosis in humans [67,68]. In view of these findings, it is concluded that the induction of adenomyosis by neonatal feeding of TAM in ICR/CD-1 mouse is not likely to be due to neonatal activation of either ERα, or ERβ, or GPR30 alone. In addition, the seemingly different presentation of adenomyosis in the DPN group in comparison with that of the TAM group appears to suggest that the extrinsic/external adenomyosis [67,68] may have a different pathogenesis than that of intrinsic/internal [67,68] adenomyosis.

While the incidence of adenomyosis in DPN-treated mice was only marginally significantly higher than that of CTL mice, the lack of statistical significance is most likely due to the lack of statistical power due to small sample sizes, especially in the CTL group. Since the CTL mice would never succumb to adenomyosis, we would still not see any mouse in that group to develop adenomyosis even if we had 100 or more mice. Therefore, the
difference should be viewed, arguably, as statistically significant since if we had 9, instead of 8, mice in the CTL group, the difference in incidence of adenomyosis between the DPN and CTL groups would become statistically significant (4 out of 8 vs. 0 out of 9; \( P = 0.029 \)). If the CTL group had 20 mice, the difference would be even more statistically significant (4 out of 8 vs. 0 out of 20; \( P = 0.003 \)).

Growing evidence indicates that extrinsic/external adenomyosis is quite different from the intrinsic/internal adenomyosis. The former is more likely to be associated with deep endometriosis[69,73] and likely to be responsible for dysmenorrhea and pelvic pain[67,69,72]. In contrast, intrinsic/internal adenomyosis is not associated with deep endometriosis[69,71] and is more likely to be associated with heavy menstrual bleeding[67,69,72]. Hence, extrinsic/external adenomyosis appears to have a different pathophysiology than that of intrinsic/internal adenomyosis. Our study suggests that the 2 sub-types may also have different pathogenesis. The upshot of our finding is that the classification of adenomyosis may not only help us to understand pathophysiology better but also help us to design better epidemiological studies in the future by better phenotyping of adenomyosis.

Mice neonatally fed with the ERβ agonist had similar changes in endometrial glands as those fed in similar manner with TAM. Mice neonatally fed with the ERαs agonist exhibited myometrial disruption similar to those fed with TAM. Those fed with the GPR30 agonist displayed unremarkable appearance in their uteri. These findings confirm distinct functional roles of different ERs in this short but critical developmental window. They are also consistent with the report that mice neonatally treated with ERs in this short but critical developmental window. They are only help us to understand pathophysiology better but also help us to design better epidemiological studies in the future by better phenotyping of adenomyosis.

The GPR30 is expressed primarily in epithelial cells and is closely involved in the progression of uterine, ovarian and breast cancers[76–78]. Activation of GPR30 can mediate the inhibition of the ERK1/2 pathway[78], which has been reported to be involved in the development of adenomyosis[79,80]. In addition, myometrial disruption could be seen in the PPT, DPN, and TAM mice while adenomyosis only developed, within 3 months after birth, in the TAM and DPN mice. Neonatal feeding of TAM also is reported to cause myometrial disruption but not adenomyosis in C57 mice[28]. Taken together, this seems to suggest that myometrial disruption is likely to be a necessary, but not a sufficient, condition for the development of adenomyosis.

ERβ is known to play an important role in endometrial physiology, benign endometrial diseases and endometrial cancers[80]. ERβ is involved in inflammation and angiogenesis[81,82]. Its expression levels in adenomyosis and endometriosis are reported to be elevated[83,84]. We found that activated platelets, which play an important role in the development of endometriosis, can induce ERβ upregulation in endometriotic stromal cells[85]. Our recent data showed that the expression of ERβ increased in mechanically induced EMID mice in 72 hours after surgery (Wang et al., unpublished data), probably resulting from platelet aggregation and activation following tissue injury. Adenomyosis developed in 100% mechanically induced EMID BALB/c mice[21]. Accordingly, ERβ may have an effect on the development or progression of adenomyosis.

In contrast to the TAM group, only 50% of mice in the DPN group developed adenomyosis. This reduced incidence is likely attributable to the dosage that was used in the experiment, which was not optimized. It has been reported that the DPN concentration in the plasma via oral administration is significantly lower than that of intra-muscular or subcutaneous injection[86]. Hence the incidence of adenomyosis in the DPN group could have been increased by either changing the administration route or increasing the dose of oral administration. Future studies using increased dosage or administration route other than oral would be needed to clarify this issue. In addition, whether this can induce similar adenomyosis in other mouse strains also remains to be investigated.

This study has several limitations. First, we only investigated the effect of different ER agonists in 1 mouse strain, that is, ICR mouse. Whether neonatal feeding of DPN can cause external adenomyosis in other mouse strains is unknown, and, as such, needs to be investigated. Second, while we investigated the effect of each individual ER agonist, we did not evaluate the outcome of using 2 or more agonists, such as PPT and DPN. Third, the absence of adenomyosis in mice neonatally fed PPT or G-1 does not mean that neither of them would never induce adenomyosis, since we only made our observation 3 months after the induction. It is possible that adenomyosis, at least microscopically visible, may arise later in these mice. But even if this were the case, our conclusion that neither PPT nor G-1 does produce adenomyosis is still valid. Lastly, this study only used histologic analysis and lacks molecular data. The molecular mechanisms underlying DPN-induced adenomyosis will need further investigation.

In conclusion, we found that, unlike TAM, neonatal feeding of the ERα ergonist or the GPR30 agonist did not induce any visible adenomyosis. Neonatal feeding of the ERβ agonist induced adenomyosis in 50% of the mice, but the adenomyotic lesions are exclusively under the serosa, reminiscent of extrinsic/external adenomyosis in humans and appear to be different from those induced by TAM. Thus, the cause of TAM-induced adenomyosis in ICR/CD-1 mouse cannot be attributable to one specific ER alone. Extrinsic/external adenomyosis thus appears to have a pathogenesis that is different from other sub-types of adenomyosis. These findings further highlight the complexity and heterogeneity of adenomyosis pathogenesis, and call for better phenotyping in future clinical and epidemiological studies of adenomyosis.

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None.

Author contributions

S.W.G. conceived and designed the study, performed data analysis and drafted the manuscript. Y.C. performed all the experiments and prepared all figures. X.W. and X.L. helped the experimentation. T.H. provided the G-1. All participated writing and approved the final manuscript.

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Conflicts of interest
Yunlei Cao, Xi Wang, Tasuku Harada, Xishi Liu, and Sun-Wei Guo declare that they have no conflict of interest.

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