A Thesis for the Degree of Master of Science

Synthesis of Estradiol in Intestinal Lymphoid Tissues of Pigs

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ABSTRACT

Estrogens are primarily synthesized in the gonads both in males and females and regulate the development and function of reproductive organs, undoubtedly as gonadal sex hormones. Recent studies however find that estrogens also play important roles in regulating normal and patho-biological processes in non-reproductive organs that are critical for health in humans and animals alike. Furthermore, recent findings show that estrogens are also synthesized in extra-gonadal sites and play an equally important role in non-reproductive systems. These exciting discoveries highlight a great potential for the use of estrogen as a therapeutic target in disease prevention and treatment. Yet developing a targeting strategy remains challenging because knowledge of extra-gonadal estrogen biosynthesis, and the mechanisms by which estrogens act are very limited. In a previous study, we discovered that 17β-estradiol (E2) is synthesized *de novo* in the intestinal lymphoid tissues in mice and plays a role in regulating leukocyte homeostasis. This finding led us to hypothesize that E2 is synthesized in the intestinal lymphoid tissues

across the entire mammalian species. In this study, we investigate procine E2 synthesis in the intestinal lymphoid tissues. Porcine species are similar to humans in genetic, physiological, anatomical and developmental aspects of gastrointestinal tract and immune systems, making them an excellent model system for medical research on human. My study found that cultured mesenteric lymph node (mLN) and Peyer's patch (Pp) synthesized estradiol whereas ileum did not. mLN showed and Pp had 60% and 30% estradiol synthetic capacity compared to that of ovary, respectively. Addition of androstenedione, precursor of estradiol, further increased E2 synthesis. Messenger RNAs for steriodogenic enzymes (*StAR, Hsd17b, Hsd3b, Cyp17, Cyp19*) were found in both mLN and Pp, comparable levels to the those in the ovary. In the mLN, aromatase (*Cyp19)* protein was expressed the endothelial cells of high endothelial venule (HEV). Taken together, HEV endothelial cells in intestinal lymphoid tissues of porcine GI tract synthesize E2 *de novo*.

Keywords: Estradiol; Ileum; Peyer's Patch, Mesenteric Lymph node; Aromatase; High Endothelial Venule; Pig

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CONTENTS

Ⅰ. INTRODUCTION

Estrogen is a well-known reproductive hormone that is synthesized primarily by gonads that regulates development and function of the reproductive system. Previous studies have noted estrogen's additional functions in cell proliferation[1], anti-inflammation[2], tissue survival[3], cardiovascular health[4], memory[5] and mood[6]. Therefore, it is not surprising that estrogen deficiency aggravate acne[7], ovulatory dysfunction[8], menopause[9], insulin resistance[10], Alzheimer's disease[11], bone loss[12]. As estrogen function in extra-gonadal sites gets attention, researchers also started to search for the possibilities of estrogen synthesis in extra-gonadal sites. Subsequently, bone, brain, liver pancreas, adipose tissue, skin, blood vessel and spleen are reported to be sites of estrogen synthesis [13]. The most active endogenous form of estrogens is 17β–estradiol (E2). Synthesis of 17β–estradiol involves 6 steroidogenic enzymes in order to convert cholesterol into 17β–estradiol. 17β–estradiol is a steroid hormone that

easily diffuse away from its synthesis site. Therefore, locally concentrated estradiol implies estradiol synthesis in local tissue. Among the 6 steroidogenic enzymes, aromatase (CYP19), a member of cytochrome P450 enzymes, is a critical rate-limiting enzyme for 17β–estradiol synthesis. Therefore, expression of CYP19 in a tissue is considered to be evidence of 17β–estradiol synthesis in that tissue [13]. Estrogens play a role in the digestive system, by influencing gut microbiome, immune cells and many others[14, 15]. Specifically, 17β–estradiol is known to regulate appetite[13], prevents cancer in the colon[13], alleviates IBS symptoms[16] and decrease metabolic syndrome in menopausal women[17]. However, a full map of estrogen function in the gut is not yet determined. Recently, we generated Cyp19-RFP transgenic mice that express red fluorescence protein (RFP) in aromatase expressing cells[18]. In mouse GI tract, we found that RFP is expressed in the Peyer's patch (Pp) and mesenteric lymph node (mLN)[18]. Pp and mLN are located inside of and near to small intestines where they regulate innate and adaptive immunity of GI tract.

To broaden our studies on mice to mammalian species, pigs GI tract and its secondary lymphoid tissues were investigated in this experiment. Pigs are genetically, physiologically, anatomically and developmentally similar to human[19]. They are the most commonly used animal models for studying GI tract and its immune function. Therefore, pigs are important for the study of nutrition of agricultural animals and medical research. Porcine model in research may be applied to both human health sciences and agricultural industry[19]. In this study, we report mesenteric lymph nodes (mLN) and Peyer's patches (Pp) as novel sites of 17β -estradiol in the pigs.

Ⅱ. MATERIALS AND METHODS

1. Chemicals and reagents

Medium alpha (α-MEM) was purchased from Invitrogen, fetal bovine serum FBS) from ATCC, insulin-transferin-selenium (ITS) from VWS and androstenedione from Sigma. Sensitive estradiol ELISA kit, testosterone ELISA kit, progesterone ELISA kit, androstenedione ELISA) kits were purchased from DRG international.

2. Animals.

This study was performed in accordance with recommendations in *Guide for the Care and Use of Laboratory Animals of the National Institutes of Health*. Animal handling was done in accordance with the protocols approved by the University of Illinois at Urbana Champagne's Institutional Animal Care and Use Committee (IACUC). Wild type female piglets of *Sus scrofa domesticus* species in age week 3 and week 12 were used. For analysis, porcine organs and circulation, tissues (Pps, mLNs, ileum, gonads and Liver) and serum were collected, weighed, and

immediately frozen. Following primers were used for RT-PCR.

HSd17b (58.5℃, 25cycle) 5’-AGCCAGAATATGTGGCACCC-3’/ 5’-CAACAAGTCCTGATGGGGCT-3’;

Cyp19a1 (57℃, 35 cycle) 5’- GGAAATCCAGACTGTTGTTG-3’/ 5’-GCTGGAAGTACCTGTAAGGA-3’;

StAR (58.5℃, 30cycle) 5’- GACTTTGTCGGCTGT-3’/ 5’-ATCCCTTGAGGTCAATGCTC-3’;

Hsd3b1 (58.5 ℃, 33cycle) 5’-CGTCCTGACACACAACTCCAA-3’/5’-CCACGTTGCCGACGTAGA-3’;

CYP17a1 (58 ℃, 35cycle) 5’- TCCGAGAGGTGCTTCGATTC-3’/ 5’- GGCGCTCCTTGATCTTCACT-3’;

Rpl19 (60℃, 25cycle) 5'-CCT GAA GGT CAA AGG GAA TGT G-3'/ 5'-GTC TGC CTT CAG CTT GTG GAT -3'.

3. Primary cell culture

Mesenteric lymph node was isolated from animal and washed with PBS. With fine forceps and scissors, tissue was teased into smaller pieces. CDS (Collagenase/DNase) solution was added into the tube. The

content of the tube was moved to 20ml scintillation vial and incubated at 37°C for 30 minutes. The digested tissues were filtered through a 40um/70um filter and the cell flow-through to centrifuge at 250xg for 5 min. Cell pellet was re-suspended with appropriate media and placed to cell culture flask for culture.

4. Hormone measurement

17β -estradiol concentrations were measured in the blood and local tissues (Pps, mLNs, ileum, gonads and Liver). Briefly, total lipids were extracted from the samples following a standard procedure [20] and stored at -80C until use for hormone assay. To measure 17β -estradiol synthetic capacity in the mLN and Pps, individual Pps and mLNs were dissected from pigs, weighed, and cultured overnight in steroid-free media [Medium alpha (α-MEM) supplemented with 1% ITS (10ng/mL Insulin, 5.5ng/mL Transferrin, 5.5ng/mL Selenium), 5% Fetal bovine serum (FBS) and penicillin-streptomycin], and then washed with fresh media to remove any steroid that might be released to culture media. Tissue samples were further cultured in the absence (Vehicle) or

presence of androstenedione (200nM). Media were removed at 24, 48 and 72 hours, lipids extracted from them, and the lipids kept at –80°C until use for hormone assay. The concentrations of 17β-estradiol in the lipids were measured by enzyme-linked immunosorbent assays ELISA (EIA-4399, DRG International, Germany). The final 17β-estradiol concentrations were expressed by weight per unit volume or weight of the tissues, sera, or culture media (pg/ml or pg/mg). The samples were run in triplicates and had intra- and inter-assay coefficients of variability were below 10% and the detection range was 0.1–30pg/ml.

5. Histology

Collected tissues were fixed in 4% paraformaldehyde, embedded in paraffin wax[21], sectioned at 5 μm thickness, and stained with H&E. Immunofluorescent labeling of Cyp19 and PNAd was performed as previously described [21] using goat polyclonal anti-Cyp19 (sc-14244; Santa Cruz Biotechnology) or Purified Rat anti-mouse PNAd carbohydrate epitope (MECA-79) (553863; BD Biosciences) followed by incubation with FITC Mouse anti-rat IgM (553887; BD Biosciences)

and Texas red conjugated Chicken anti-goat IgG (sc-3923; Santa Cruz Biotechnology)

6. Statistical analysis

Datasets were first tested for normality and homogeneity of variance. When appropriate, data were transformed before statistical analysis. Non-transformed data are depicted in all the figures. Statistical analyses were performed by two-tailed Student *t* test or one-way analysis of variance (ANOVA) followed by Tukey's HSD.

Ⅲ. RESULTS

1. Peyer's patch and mLN contain concentrated estradiol and the tissue estradiol synthetic capacity increase during pre-pubertal growth period

Tissue concentration of estradiol was measured in 3 weeks-old pigs (Fig 1A). Among various tissues collected from intestinal area, mesenteric lymph node estradiol concentration was 0.66pg/mg which is three times higher than serum estradiol concentration (0.27pg/ml). In order to compare E2 synthetic capacity with relationship to the development, two age period to represent early(3weeks) and late(12weeks) pre-pubertal period was chosen. 3weeks and 12weeks porcine tissues(Ova, iLPp, mLN) were cultured for 48hrs to compare their E2 production (Fig 1B). In porcine species, Ova, iLPp and mLN showed higher capacity in week 12, especially in mLN and iLPp 12 week tissues showed 10 fold higher E2 production. In porcine guilt sexual maturation is determined by the onset of first estrus. Varying greatly on genetic and nutritional factors, porcine

guilt age at puberty range from 80 to 220 days of birth[22]. Meanwhile, from birth to 12weeks of age, porcine ovarian weight proportionally increases[23].

Figure 1

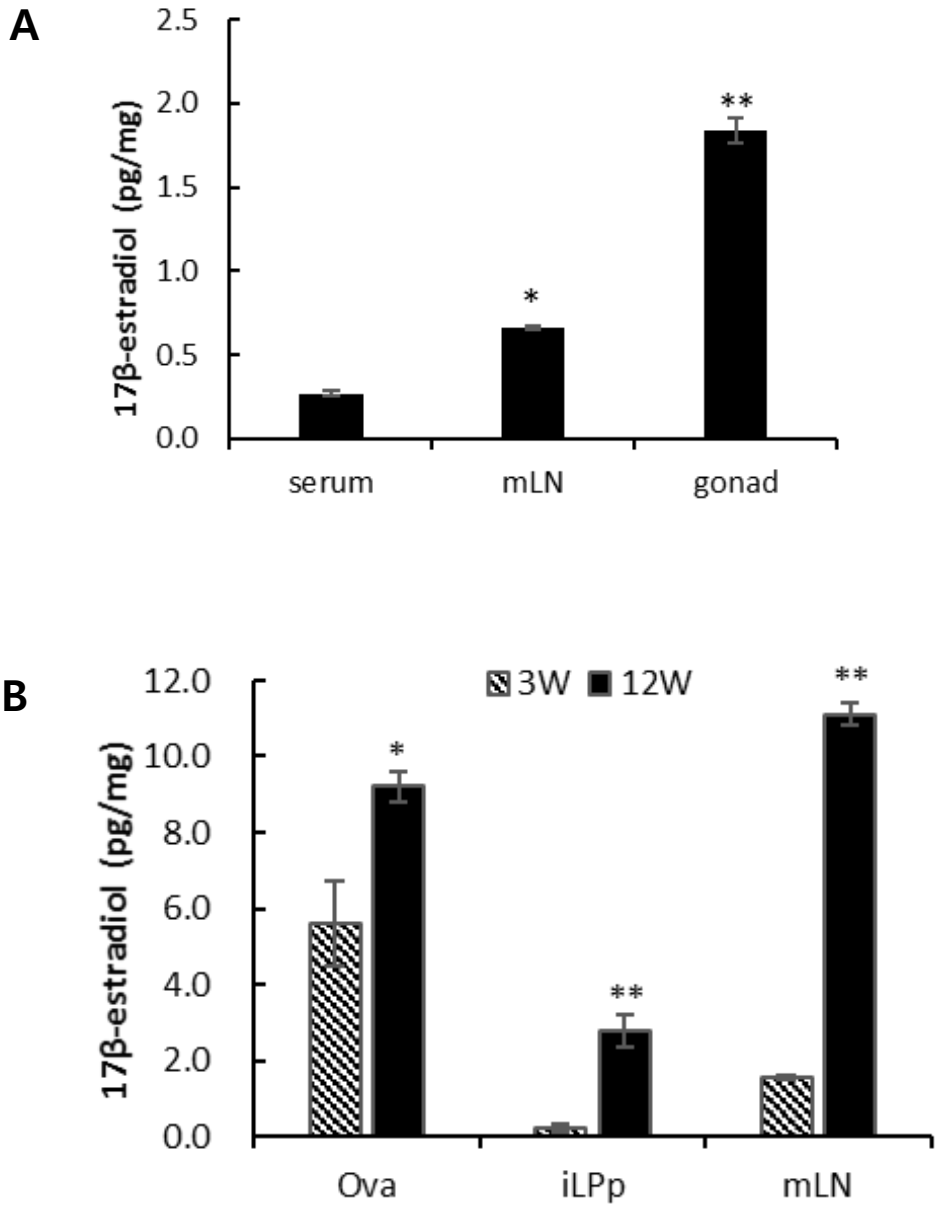

A
17β-estradiol (pg/mg)
2.5
2.0
1.5
1.0
0.5
0.0
*
**
serum
mLN
gonad
B
17β-estradiol (pg/mg)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
3W
12W
*
**
**
Ova
iLPp
mLN

Figure 1. Peyer's patch and mLN contain concentrated 17b-estradiol.

A. Tissue concentrations of 17b-estradiol in the sera, mLN and the ovary (gonad) of 3-week old female piglets (n=4). *, $p<0.01$; **, $p<0.005$ vs. serum. Note a significantly higher 17b-estradiol concentration in the mLN compared to serum. **B.** Tissue 17b-estradiol concentrations were compared in the ovaries (Ova), iLPp and mLN of 3-week (3W) and 12-week (12W) old mice (n=3). *, $p<0.01$; **, $p<0.005$ vs. 3 weeks old. Note that 12 weeks old iLPp (middle) and mLN (right) tissues contain > 10 folds higher 17b-estradiol compared to those of 3 weeks old.

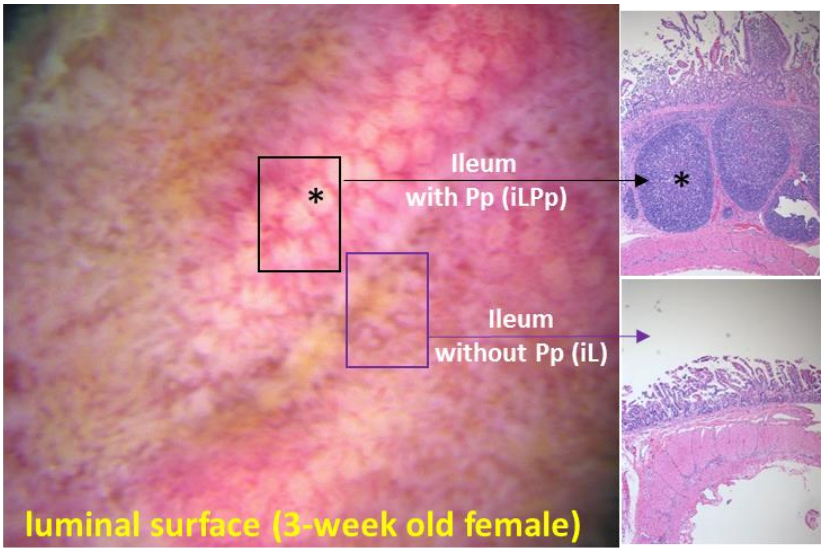
2. Peyer's patch and mesenteric lymph node synthesize estradiol in culture

In porcine species, Peyer's patches are located in the ileum. In 3 weeks old porcine ilia, Peyer's patch are microscopically identifiable as white dots underneath of luminal epithelium. Ileal tissues that contain Peyer's patch (iL) and ilial tissues without Peyer's patch (iLPp) were dissected out and were stained H&E (Fig. 2A). Peyer's patch is lymphocyte rich region and therefore were stained purple (marked by asterisk). Isolated iL and iLPP estradiol synthetic capacity were compared in culture for 72 hours (Fig. 2B). Ileum with Peyer's patch estradiol concentration increased for 72 hours while ileum without Peyer's patch did not, indicating that Peyer's patch is the organ responsible for E2 production in ileum. Time course culture of ovary (C), iLPp (D) and mLN (E) iLPp and mLN are E2 concentration proportionally increased with time (Fig 2. C-E). Addition of androstenedione, the precursor of estradiol, further increased estradiol concentrationin ovary and iLPp, yet holding statistical significance. Remarkably, in 72hour culture time, mLN estradiol

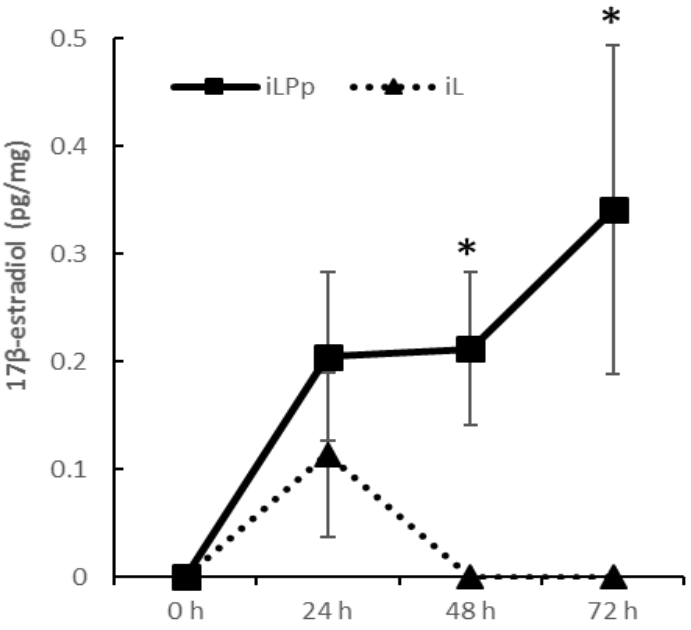
concentration (12.36pg/mg) was comparable to that of the ovary (12.45pg/mg).

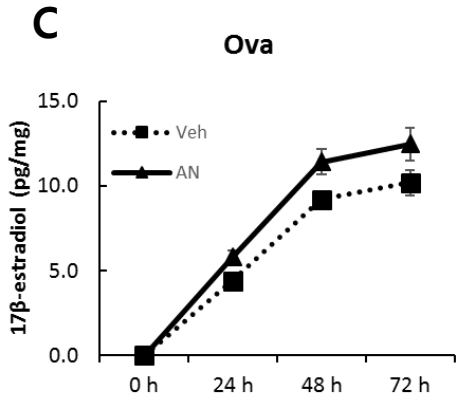
Figure 2

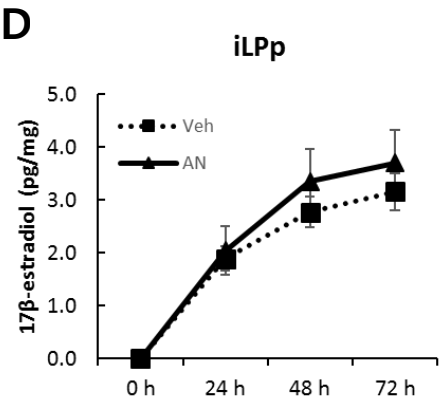
A


*
Ileum
with Pp (iLPp)
*
Ileum
without Pp (iL)
luminal surface (3-week old female)

B


17β-estradiol (pg/mg)
0.5
0.4
0.3
0.2
0.1
0
iLPp
iL
*
*
0 h
24 h
48 h
72 h


C
Ova
17β-estradiol (pg/mg)
15.0
10.0
5.0
0.0
Veh
AN
0 h
24 h
48 h
72 h


D
iLPp
17β-estradiol (pg/mg)
5.0
4.0
3.0
2.0
1.0
0.0
Veh
AN
0 h
24 h
48 h
72 h

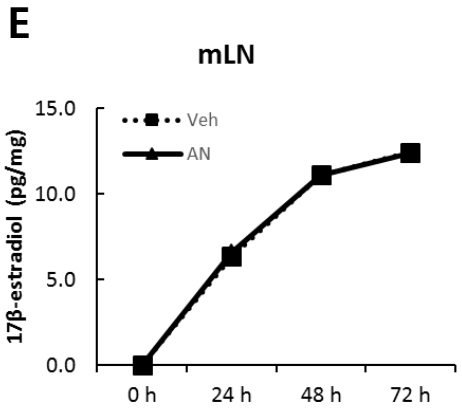

E
mLN
17β-estradiol (pg/mg)
15.0
10.0
5.0
0.0
Veh
AN
0 h
24 h
48 h
72 h

Figure 2. Peyer's patches and mesenteric lymph node synthesize estradiol in culture. A. Localization of Peyer's patch in ileal luminal surface (iLPp and ileal tissues without Peyer's patch (iL). Peyer's patches are indicated by asterisks. **B.** iLPp and iL tissues were dissected from 3 week old piglets (n=4), cultured *in vitro* and the amount of 17b-estradiol released from the tissues was measured over time. *, $p<0.005$ vs. iL. **C-D.** Ovaries (Ova), iLPp and mesenteric lymph node (mLN) were dissected from 12-week old female piglets, cut into into small pieces (1 mm, diameter), and cultured *in vitro* in the presence (AN, 200 nM) or absence (Veh) of androstenedione (n=4). Note that mLN (E) produce 17b-estradiol (pg/mg) that is comparable that of the ovary (C).

3. High endothelial venule (HEV) cells of mesenteric lymph node express aromatase (CYP19)

Primary cell culture and immunofluorescence staining of cultured mLN was performed to determine cell type responsible for CYP19 (aromatase) expression (Fig 3). sites. CYP19 antibody (red) was localized in peripheral surface of cells co-stained with MECA-79 antibody (green). MECA-79 antibody binds to peripheral lymph node addressin (PNAd), a marker protein for HEV endothelial cells. Co-staining of CYP19 and MECA79 indicates that HEV endothelial cells express CYP19.

Figure 3

A

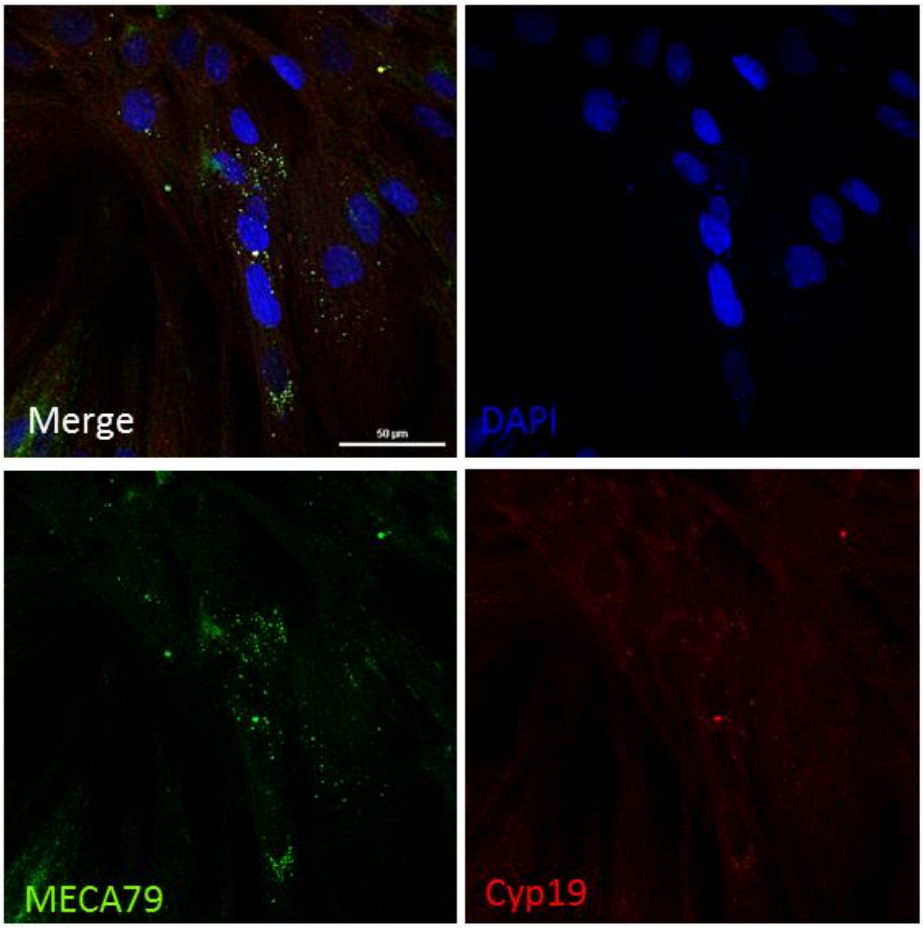


Figure 3. HEV endothelial cells of mLN synthesizes estradiol in culture. mLN of 12-week old female piglets by FACS were isolated and cultured for 72h. The cultured cells were stained antibodies against MECA-79 (green, HEV marker) and CYP19 (red aromatase) and Hoechst (nuclear marker).

4. Mesenteric lymph node and Peyer's patch express Hsd17b and aromatase (Cyp19).

Cyp19 and *Hsd17β* are steroidogenic enzyme gene necessary for conversion of precursor, androstenedione into final product, estradiol (Fig 4A). Expresssions of *Cyp19* and *Hsd17β* mRNAs in the iLPp and mLN were identified by RT-PCR. Relative quantification of *Cyp19* and *Hsd17β* mRNAs were analyzed using *Rpl19* as internal control (Fig 4B). Quantified expression level of*Cyp19* mRNA in iLPp and mLN was 0.58 ($p<0.05$) and 0.78 ($p<0.1$) respectively. Both iLPp and mLN showed lower mRNA level compared to 1.35 in ovary.. *Hsd17β* mRNA expression in ovary, iLPp and mLN were 0.68, 1.12 and 1.06 respectively. iLPp and mLN *Hsd17β* mRNA expression was two fold higher compared to the ovary with statistical significance ($p<0.1$).

Figure 4

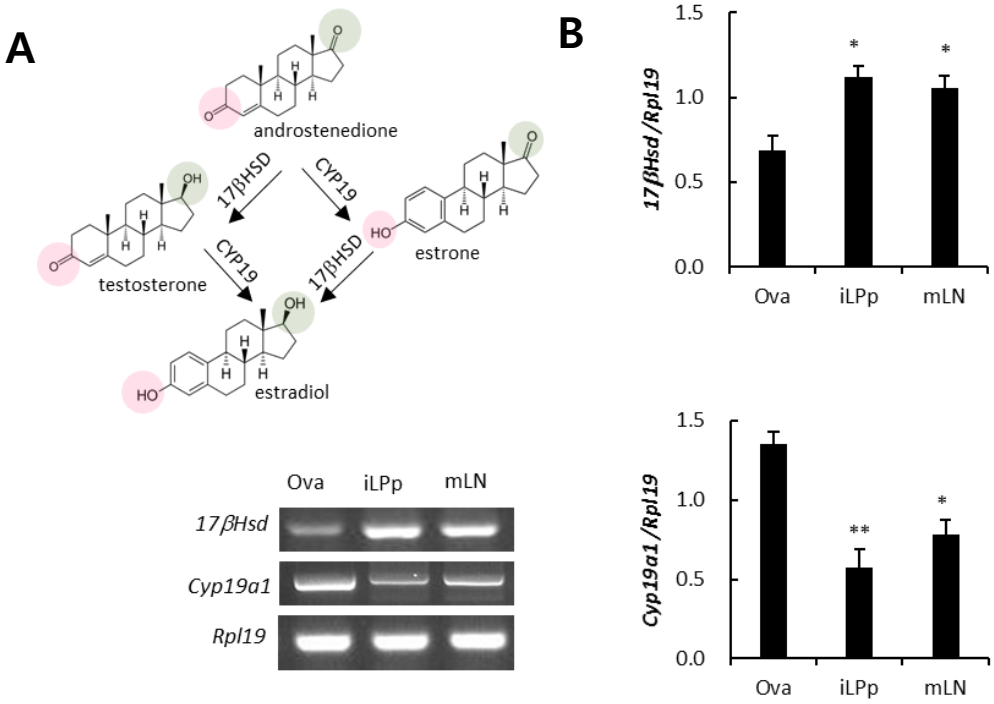

A
androstenedione
17βHSD
CYP19
testosterone
estrone
CYP19
17βHSD
estradiol
Ova
iLPp
mLN
17βHsd
Cyp19a1
Rpl19
B
17βHsd/Rpl19
1.5
1.0
0.5
0.0
*
*
Ova
iLPp
mLN
Cyp19a1/Rpl19
1.5
1.0
0.5
0.0
**
*
Ova
iLPp
mLN

Figure 4. Peyer's patch and mesenteric lymph node express rate limiting enzymes necessary for 17β-estradiol synthesis. A. The rate limiting estradiol synthetic steps are indicated with responsible enzymes; CYP19 and 17β-HSD. Representative agarose gel images of the RT-PCR products of the mRNAs for *Cyp19a1*, *17β-hsd* and *Rpl19* (internal control) in the ovary (Ova), ileal Pyer's patch (iLPp) and mesenteric lymph node (mLN) of 12-week old female piglets are shown. **B.** Quantitation of the relative mRNA expression of *Cyp19a1*, *17β-hsd.* *, $p<0.01$; **, $p<0.005$ vs. ovary (n=4). Note that *17β-hsd* expression is higher in the iLPp and mLN compared to ovary, but *Cyp19a1* expression is lower in these lymphoid tissues than in the ovary, respectively.

5. Peyer's patch and mesenteric lymph node express StAR, HSD3β and CYP17

StAR, P450scc, Hsd3β and *Cyp17* are genes of sterodogenic enzymesto synthesize androstenedione, the precursor of estradiol (Fig 5A). Relative quantification of *StAR, Hsd3β* and *Cyp17* mRNAs were analyzed using *Rpl19* as internal control, by dividing *Rpl19* mRNA result to the result of each gene (Fig 5B). Both iLPp and mLN express *StAR, HSD3β* and *CYP17* in similar pattern. *StAR* and *CYP 17* mRNA expression of iLPp and mLN was similar to that of the ovary, while *HSD3β* mRNA expression in both iLPp and mLN were significantly lower compared to the ovary.

Figure 5

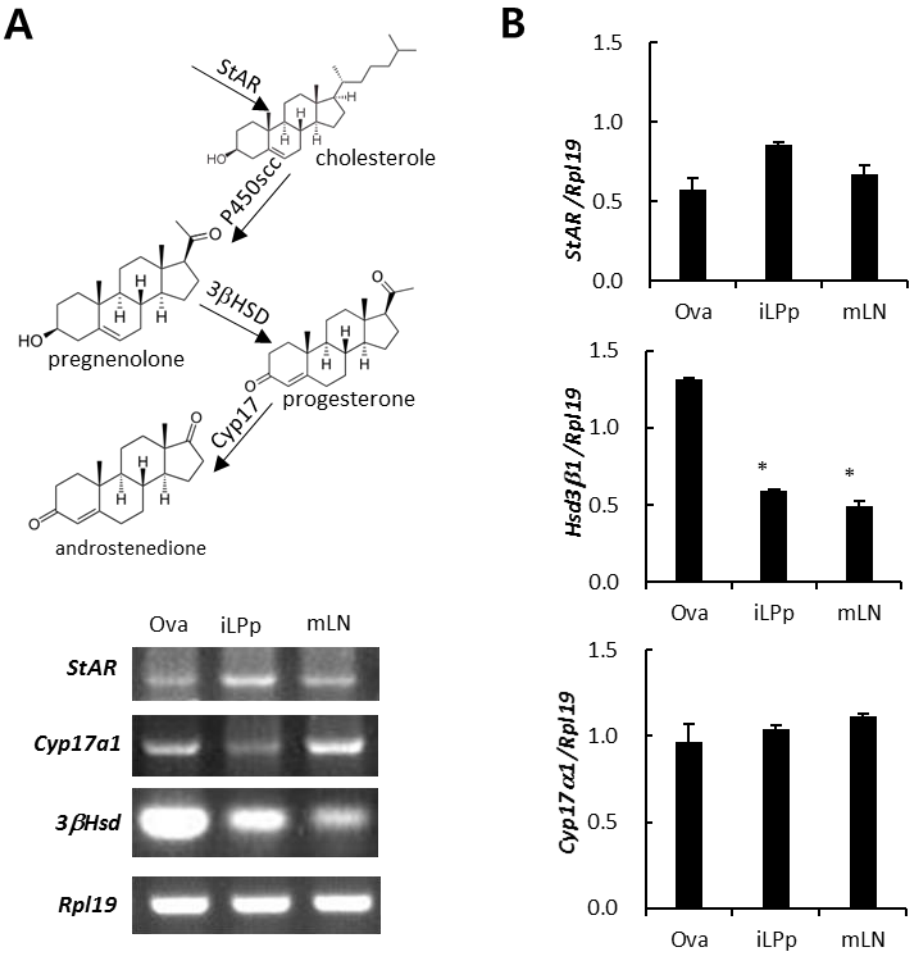

A
StAR
cholesterole
P450scc
3βHSD
pregnenolone
progesterone
Cyp17
androstenedione
Ova
iLPp
mLN
StAR
Cyp17a1
3βHsd
Rpl19
B
StAR /Rpl19
Hsd3β1/Rpl19
Cyp17α1/Rpl19
1.5
1.0
0.5
0.0
Ova
iLPp
mLN
*

Figure 5. Peyer's patch and mesenteric lymph node mLN express enzymes necessary for synthesizing precursors for 17β-estradiol synthesis. A. Key enzymes responsible for precursor synthesis (StAR, P450scc, 3β-HSD and CYP17) are indicated. Representative agarose gel images of the RT-PCR products of the mRNAs for *StAR*, *P450scc*, *3β-hsd, Cyp17a1* and *Rpl19* (internal control) in the ovary (Ova), ileal Pyer's patch (iLPp) and mesenteric lymph node (mLN) of 12-week old female piglets are shown. **B.** Quantitation of the relative mRNA expression of *StAR*, *P450scc*, *3β-hsd* and *Cyp17a1*. *, $p<0.005$ vs. ovary (n=4).

Ⅳ. DISCUSSION

Estradiol has long been recognized as female reproductive hormone in history. For such reason study of estrogen production and function was focused on ovarian studies. Estrogen deficient models such as aromatase knock-out mice (ArKO) and estrogen receptor knock-out mice (ERαKO, ERβKO) were developed for estrogen studies. Such models revealed estrogen function and synthesis in non-reproductive organs such as brain[24, 25], adrenal gland[26, 27], bone[28] and adipose tissue[29]. Estrogen synthesized in extra-gonadal sites, estrogen acts as paracrine or intracrine regulator overriding its well-known reproductive function[30].

Recent studies highlighted estrogen action in inflammatory response such as development, proliferation, migration, and apoptosis of immune cells[31]. Lymphocytes express estrogen receptors, ERα and ERβ, although expression level varies depending on the cell type[32]. Estrogen inhibit B- and T- lymphopoiesis [33, 34] and production of Th1

cytokines (IL-12, TNF-α and IFN-γ), while stimulating Th2 anti-inflammatory cytokine (IL-10, IL-4, and TGF-β)[35]. In addition, E2 al so modulates maturation, migration and differentiation of myeloid cells [36-39]. Studies support that E2 influence both the innate and the adaptive immune systems. Peyer's patch and mesenteric lymph node are first set of lymphoid tissues programmed to maintain homeostasis in gut[40, 41]. With growing importance on estrogen function on immune system, our current study is first to identify intestinal lymphoid tissues (Peyer's patch, mesenteric lymph node) as a novel 17β-estradiol synthesis site in porcine species. Supporting our previous findings in mouse studies[18], pigs Peyer's patch and mesenteric lymph node in gut synthesize estradiol. Peyer's patch and mesenteric lymph node in pigs hold steroidogenic enzymes for estradiol conversion (*Cyp19, Hsd17b*) and estradiol precursor production (*StAR, Hsd3b, Cyp17*) as shown in figure 4 and figure 5. From such result we can lead to conclusion that in pig's Peyer's patch and mesenteric lymph node may synthesize all types of steroidogenic hormones, starting from cholesterol to final

product of 17β-estradiol. Considering that total mass of mLN is more than 10 times larger than the ovary, we may also assume that mLN might hold stronger estradiol synthetic capacity than ovary in total.

We also discovered that High endothelial venule (HEV) cells in intestinal lymphoid tissues express Cyp19(aromatase), which is responsible for final conversion of 17β-estradiol (fig 3). Estradiol function in immune regulation and lymphoid organ function has also been studied in various aspects[42]. Meanwhile, estradiol's anti-inflammatory function remains debatable as estradiol is also called 'double-edged sword', since high concentration of estradiol may on the other hand promote inflammation[35].

Our current research designated intestinal lymphoid tissues and the specific cell that are capable of E2 synthesis. However, we still have little understanding of the exact mechanism of action of locally produced 17β-estradiol and its synthesis regulation. Aromatase expression level co-related with 17β-estradiol production[43]. In order to fully understand the regulative mechanism and function of 17β-

estradiol in gut immunity, functional studies should follow. Current finding in pig species indicates greater possibility of 17β-estradiol synthetic capacity of intestinal lymphoid tissues (Peyer's patch, mesenteric lymph node) across the entire mammalian species. In addition, clinical studies are required to apply current knowledge for prevention of related disease and therapeutic application in human.

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Ⅵ. 국문 초록

에스트로겐은 성호르몬으로 생식기관의 발달과 기능을 조절한다. 최근 많은 연구에서 기존에 알려진 주요 성호르몬 합성 기관인 생식기관 (난소, 정소) 이외의 기관에서도 에스트로겐이 합성되며 이는 생식계 이외의 체내 시스템에서도 면역 체계 조절, 종양 형성 억제 등 중요한 역할을 함이 밝혀지고 있다. 이러한 트렌드에 맞추어 우리는 본 연구의 선행 연구에서 17β-estradiol (E2)가 마우스의 장내 면역 기관에서 합성된다는 것을 신규 발견하였으며 백혈구 항상성 유지에 관여함을 발견하였다. 마우스에서의 연구를 토대로 모든 포유류의 장내 면역 기관에서 에스트로겐을 합성할 수 있을 것이라는 가설을 새웠다. 돼지는 인간과 유전학적, 생리학적, 해부학적 그리고 발달상의 긴밀한 유사성을 가지므로 인간의 위장관 그리고 면역에 대한 의학적 연구에 적합한 모델이다. 본 연구는 돼지의 장간막림프절(mLN)와 페이에르판(Pp)에서 에스트라디올 합성을 한다는 것을 신규 발견하였다. In vitro 조직 배양 조건에서 장간막림프절과 페이에르판은 난소 대비 각각 60%

와 30%의 17β-estradiol 합성을 보였다. 두 기관에서 17β-estradiol 합성의 메커니즘을 규명하기 위하여 스테로이드 합성 효소(StAR, Hsd17b, Hsd3b, Cyp17, Cyp19)의 mRNA 발현을 확인하였다. 장간막림프절과 페이에르판 모두 스테로이드 합성 효소(StAR, Hsd17b, Hsd3b, Cyp17, Cyp19)를 가지고 있음을 확인하였으며, 특이적으로 난소 대비 Hsd17b의 증가를 확인하였다. 또한 장간막림프절의 high endothelial venule (HEV)에서 에스트라디올 합성 효소인 Cyp19의 특이적 발현을 규명하였다.