大韓不妊學會誌 : 第30卷 第4號 2003
Kor. J. Fertil. Steril., Vol. 30, No. 4, 2003, 12

생쥐자궁에서 에스트로겐에 의해 조절되는 Fra1과 Fra2 유전자의 발현양상

한양대학교 자연과학대학 생명과학과[1], 울산대학교 의과대학 서울아산병원 산부인과[2]

이지윤[1,2] · 홍석호[1,2] · 나희영[2] · 김성훈[2] · 채희동[2] · 김정훈[2] · 강병문[2] · 김문규[1]

Expression of Fra1 and Fra2 Genes are regulated by Estrogen in the Mouse Uterus

Ji Yoon Lee[1,2], Seok Ho Hong[1,2], Hee Young Nah[2], Sung Hoon Kim[2], Hee Dong Chae[2], Chung Hoon Kim[2], Byung Moon Kang[2], Moon Kyoo Kim[1]

[1]Department of Life Science, College of Natural Sciences, Hanyang University, Seoul,
[2]Department of Obstetrics and Gynecology, College of Medicine, Ulsan University, Asan Medical Center, Seoul, Korea.

연구목적: 스테로이드 계통의 에스트로겐 호르몬은 막 수용체와 결합하고 DNA에 부착되어, 자궁조직에서 발현되는 많은 유전자들의 발현 양상을 조절하는 것으로 알려져 있다. 본 연구에서는 난소를 제거한 생쥐 모델을 이용하여 에스트로겐에 의해 조절되는 전사 관련 유전자(transcription factor)들을 동정하고, early up-regulation gene으로 확인된 Fos related antigen (Fra1과 Fra2) 유전자의 발현 양상을 RT-PCR과 면역염색방법으로 살펴보았다.

연구재료 및 방법: 난소 절제술을 시행한 생쥐에 에스트로겐을 피하주사하고 2, 4 ,6, 12시간 간격으로 자궁조직을 적출하였다. 대조군으로는 sesame oil만을 주사한 후 2시간째에 수획한 자궁조직을 사용하였으며, 시간대별로 채취한 자궁조직(n=4)에서 RT-PCR을 수행하였다. RT-PCR을 통해 early response gene으로 확인된 Fra1과 Fra2에 대한 에스트로겐의 영향을 살펴보기 위해 estrogen receptor antagonist인 ICI 182,780을 주사하여 유전자 발현 양상의 변화를 살펴보았다. 또한, 자궁조직내에서의 단백질 발현 부위를 관찰하기 위해 면역조직화학염색을 실시하였다.

결 과: 생쥐 자궁조직에서 에스트로겐에 의해 발현 양상의 변화가 확인된 유전자는 early up-regulation genes (CREB2, Fra-1,2, GATA5), late up-regulation gene (E2F1), no response genes (CREB1, ATF1, GLI3, E2F3), down-regulation genes (GLI2, E2F5, GATA-2,3,6) 등으로 구분할 수 있었다. 그 중 early up-regulation genes에 해당하는 Fra1과 Fra2 유전자는 ICI 182,780에 의해 그 발현이 유의하게 감소되는 것을 확인하였다($p < 0.01$). 이들 단백질은 생쥐 자궁조직의 상피세포층, 기질층, 근육층에서 고루 발현되었으며, 특히 근육층에서 강한 염색정도를 관찰할 수 있었다.

결 론: 이상의 결과를 통해 Fra1과 Fra2 유전자의 발현은 에스트로겐에 의해 조절됨을 알 수 있었으며, 이들의 강한 발현이 자궁조직의 근육층에서 관찰되어 이들의 기능에 대한 연구가 필요할 것으로 생각된다.

Key Words: Estrogen, Uterus, RT-PCR, Fra1, Fra2, Immunohistochemistry

주관책임자: 김문규, 우) 133-791 서울특별시 성동구 행당동 17, 한양대학교 자연과학대학 생명과학과 발생학 연구실
Tel: (02) 2290-0954, e-mail: kimmk@email.hanyang.ac.kr
연락저자: 이지윤, 우) 138-736 서울특별시 송파구 풍납동 388-1, 서울아산병원 산부인과 체외수정실
Tel: (02) 3010-5059, Fax: (02) 3010-5053, e-mail: leejy@amc.seoul.kr

The uterus is composed of a heterogeneous cell types that undergo continuous synchronized waves of proliferation and differentiation in response to the rise and fall of estrogen and progesterone during the estrous cycle. One of these hormones, estrogen plays an essential roles in the many aspects of reproduction, development, as well as DNA synthesis and cell proliferation in the mouse uterus.[1,2] It is known that the effect of estrogen is mediated through its ability to bind the estrogen receptors (ERs), which are members of the nuclear receptor superfamily of ligand activated transcription factors that control these physiological process, in large part through the regulation of gene transcription. These mechnism include 1) direct interaction of the ligand-occupied receptor with DNA at estrogen response elements, followed by transcriptional coregulator; 2) interaction of the ligand-occupied ER with other transcription factors, such as activating protein-1. Like this, transcription factor is important in uterus function.[3~5] Also, the induction of mRNA transcription by estrogen divides into two classes of genes; early response genes include important regulatory genes like transcriptional factors and late response genes. The uterotrophic changes, such as water imbibition and glucose metabolism, are stimulated by early response transcription factors during the acute uterine response.[6] However, several genes regulated by estrogen have been identified in the uterus and the expression pattern of cell types as well as the mechanisms underlying the physiological changes caused by estrogen are largely unknown. In order to clarify the mechanisms of estrogen function, it is essential to identify the genes affected by estrogen, especially during the early stages of the uterus responses.[7~9] The aim of this study was to identify the early response genes that are up-regulated by estrogen in the mouse uterus, the candidated 15 kinds of transcription factor genes were examined by RT-PCR. Among them, especially Fra1 and Fra2 were selected and analyzed the effect of estrogen receptor antagonist on their expression. Also, proteins of the Fra1 and Fra2 were localized in the mouse uterus by immuno-histochemistry.

MATERIAL AND METHODS

1. Animals and estrogen treatments

ICR mice were housed within temperature and light controlled conditions under the supervision of a licensed veterinarian. Mice were maintained on a 12L: 12D cycles. Female mice (6-8 weeks old) were ovariectomized under ketamine (50 mg/ ml) combined to xylazine (23.3 mg/ ml) without regard to the stage of the estrus cylce and rested for 14 days. The ovx mice were injected with 17β stradiol that was dissolved with sesame oil and injected subcutaneously (Sigma, St, Louis, MO; 300 ng/ 0.1 ml/ mouse) and the control mice received oil only. An estrogen receptor antagonist, ICI 182,780 was (500 μ 0.1 ml/ mouse) injected at 30 min before estrogen injection. The uterus tissues were collected at 0, 2, 4, 6 and 12 hr after estrogen injection, respectively. For screening transcription factors, uteri were pooled from 4 mice and placed immediately in LN_2. To confirm whether the these genes were mediated by estrogen receptor, uteri were collected into 4 groups ; control, estrogen, estrogen plus ICI 182,780 and ICI 182,780. For immunohistochemistry, uteri horns were placed in OCT compound. All animal experiments were performed in accordance with the guide of Ulsan university for care and use of laboratory animals.

2. RNA isolation

Total RNA extraction was conducted with each uteri from 4 mice for RT-PCR. The uteri were washed three times with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS, Gibco/BRL) and directly plunged into the LN_2 for RNA storage. The RNA extraction step was performed by using TRIzol (Invitrogen, Carlsbad, CA) and purified using RNeasy total RNA isolation kit (Qiagen, Valencia, CA) according to the manufactures' instruc-

tion. Total RNA was quantified by spectrophotometer and its integrity was assessed by running on a denaturing 0.8% agarose gel. Each RNA sample was validated by assaying for early response (HB-EGF) or late response (lactoferrin) as markers of estrogen efficacy.

3. RT - PCR analysis

Prepared 1 µ of RNA was reverse transcripted at 42 ℃ for 60 min in 20 µ reaction mixture consisting of oligo dT adaptor primer (Takara, Shiga, Japan) and AMV (avian myoblastosis virus) reverse transcriptase XL. The following PCR was performed in a total volume of 40 µ with 2 µ 25 mM $MgCl_2$, 4 µ 10X PCR buffer (100mM Tris-HCl, 500 mM KCl, PH 8.3), 4 µ 2.5 mM dNTP mixture, 10 pmole forward and reverse primer and 1.25 U Taq polymerase (Takara). The sequences of oligonucleotide primers for CREB-1,2 ; ATF-1 ; GATA-2,3,5,6 ; E2F-1,3,5 ; GLI-1,2,3 ; Fra-1,2 are given in Table I. An increasing number of cycles was tested to assess the best

Table 1. Primers used for RT-PCR and the size of their amplification products

Gene		Primer	Position on cDNA	Product Size (bp)
CREB-1	F R	cggatggggtacagggcctg aggaagtgctggggaggacg	728-747 941-960	233
CREB-2	F R	gccccacaacatgaccgaga ccaacatccaatctgtcccg	263-282 508-527	265
GATA-2	F R	aacgtgaagacatggaggcg aggggaatgatctgggagac	2041-2060 2293-2312	272
GATA-3	F R	cctatccgccctatgtgccc gtccctgctctccttgctgcc	866-885 1130-1150	286
GATA-5	F R	ggggcaaaggcaagacatgc aaaccaacgtggaagggatc	2589-2608 3081-3110	512
GATA-6	F R	gcgggctctatatgaaactc ggatgtgacttcggcagggg	963-982 1287-1306	344
E2F-1	F R	ctggctgggtttgcagggca cagactggagggtggggagg	1984-2003 2381-2400	417
E2F-3	F R	gtctgaggatgggggcatgc ctcttggagcaggggaggcag	411-430 748-968	559
E2F-5	F R	ggaagggtgtaggtgctggc gttgaggactgggaggaagg	326-345 739-758	433
ATF-1	F R	cagacctaccagatccgtacc gtcatctccattctccctcc	543-563 962-981	439
GLI-1	F R	gactccaccggattggcacc gcagggctctgactaactt	2647-2666 2980-2999	353
GLI-2	F R	ccaagtacacgcccaaagag acagggctctgactaactt	5107-5126 5424-5443	337
GLI-3	F R	ggtactagggcaggttggtg gagacgggaggagctgtggg	4206-4225 4772-4791	586
FRA-1	F R	ggatgagaaatcggggctgc ggggtcggaggaggggtcgc	619-638 969-988	370
FRA-2	F R	gcagaaggagagatgagcag gctgaggaagaggggctgtc	335-354 685-704	370

conditions of achieve linear amplification. The thermal cycling parameters were consisted of 22-26 cycles of denaturation (94 ℃, 30 sec); annealing (60 ℃, 30 sec); and extension (72 ℃, 30 sec). Subsequently, 8 μ of the PCR products was run on 1.2 % TBE agarose gel (Seakem LF agarose) electrophoresis in Tri-Borate-EDTA buffer, stained with ethidium bromide (EtBr), and photographed under UV light (SL20 image visualization). The image were quantified by densitometric analysis with Bio ID image analysis software (Vilber-Lourmat, Mama La Vallee, Cedex, France) and gene expression was normalized against the density of the corresponding ribosomal protein L7 (rpL7) PCR products as an internal control.

4. Immunohistochemistry

Immunohistochemistry was performed with Fra-1,2 (sc-183, sc-171; Santa Cruz Biotechnology) and the Vectastain ABC kit (Vector laboratories). Frozen uterus tissues were cut into 6-7 μ sections by a Leitz Kryostat model 1720 (Leitz) and placed on poly L-lysine (Sigma) coated slides. Sections were fixed with 4 % paraformaldehyde in PBS for 15 min at room temperature and washed with avidin biotinylated peroxidase complex (ABC) buffer (Na_2HPO_4 10 mM, NaCl 0.9 %) for 5 min. To remove endogenous peroxidase activity, peroxidase quenching was performed with 0.3 % H_2O_2 in methanol for 30 min and washed with ABC buffer for 5 min. Sections were blocked with solution (1.5 % normal goat serum in PBS) for 1 hr, followed by incubated with primary antibody (rabbit anti mouse IgG) diluted 1: 500 for 24 hr at 4 ℃ or incubated without primary antibody were used as a negative control. The slides were rinsed with ABC buffer and incubated in secondary antibody solution (biotinylated goat anti rabbit IgG; Vector) for 1 hr, then washed with ABC buffer. The slides were then incubated with avidin and biotinylated horseradish peroxidase complexes for 1 hr, washed twice for 5 min each in buffer, and developed in 0.04% 3,3-diaminobenzidine (DAB) solution for 2 min. Sections were then counterstained in hematoxylin, dehydrated through ethanol solution, cleared in xylene, and mounted with permount (Fisher Scientific, PA) for bright field microscopy. Intense deposits indicated the positive sites of immunostaining.

5. Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed by Student`s *t*-test and $P < 0.05$ was considered statistically significant.

RESULTS

1. Analysis of gene expression patterns in the ovariectomized mouse uterus

To screen estrogen-responsive genes in the uterus, we extracted total RNA at 0, 2, 4, 6, 12 hr after estrogen injection and examined in the ovx mouse uterus using the semi-qantitative RT-PCR. Experiments were repeated three times with independently RNA samples. Estrogen regulated genes were divided into four classes by their characteristics: 1) early up-regulation genes were CREB2, Fra1, Fra2 and GATA5; 2) response maintained genes were CREB1, ATF1, GLI3 and E2F3; 3) late up-regulation gene was E2F1; and 4) down-regulation genes were GLI2, E2F5, GATA2, GATA3 and GATA6. Their response patterns were illustrated in Figure 1.

2. Confirmation of early up - regulation genes by estrogen injection

The expression pattern of several genes up-regulated by estrogen were examined to verify the results of screening by RT-PCR. Early up-regulation genes contain a Fra1, Fra2, CREB2 and GATA5. Fra1 and Fra2 genes were randomly selected in four genes. The results of RT-PCR analysis were shown in Figure 2. After single injection of estrogen, the levels of Fra1, Fra2 mRNA rapidly increased. The level of Fra1 mRNA after estrogen injection peaked 2.35-fold

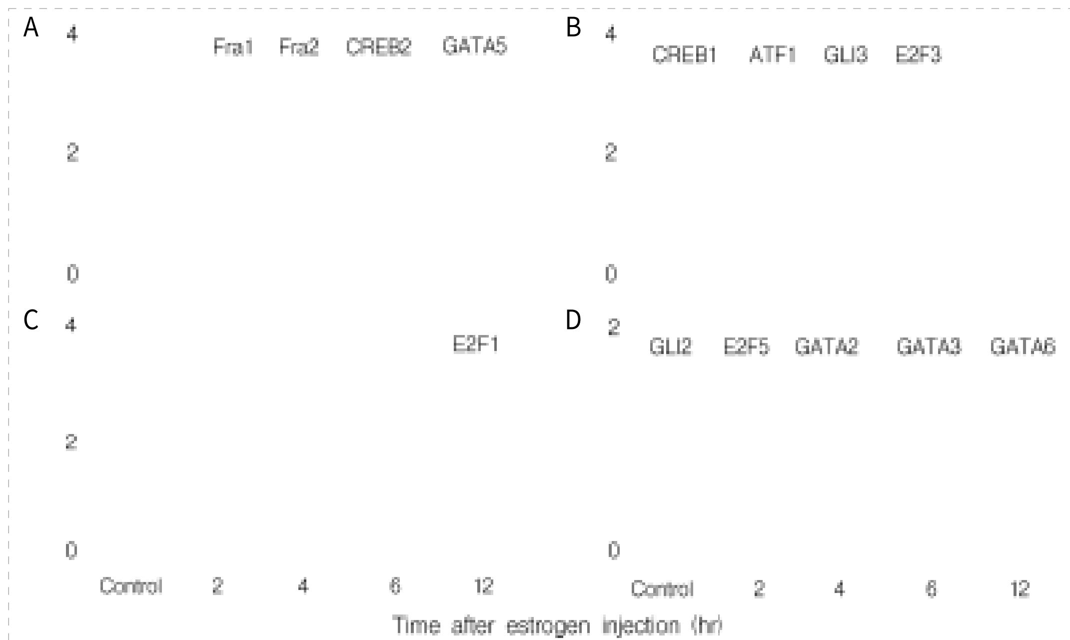

Figure 1. Semiquantitive RT-PCR analysis screening genes after estrogen injection. Values of each band were normalized to rPL7 for the same sample. Data were mean ± SEM from three replicate experiments.

A: Early response genes **B:** Response maintained genes
C: Late response genes **D:** Down regulation genes

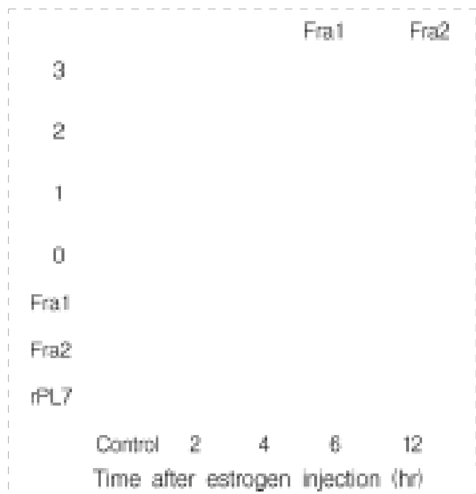

Figure 2. Expression patterns of selected early response genes, Fra1, Fra2 mRNA after estrogen injection. The expression level of LF and HB-EGF mRNA was examined as a positive marker gene. Values are represented by the mean ± SEM and significant differences by Student`s *t*-test are showed as **p<0.01 and *p<0.05 level.

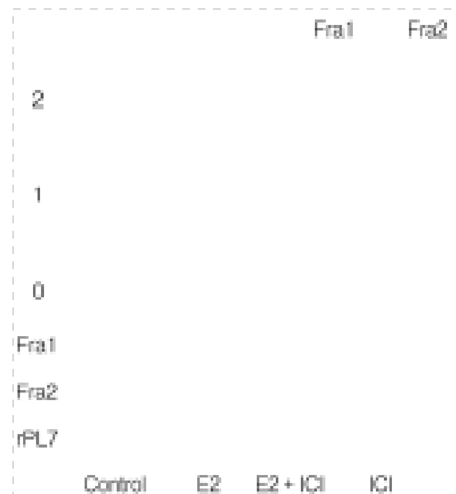

Figure 3. The expression pattern of Fra1 and Fra2 mRNA after injection of estrogen and ICI 182,780. Values are represented by the mean ± SEM. Significant differences by Student`s *t*-test compared to the control group are showed as **p<0.01 and *p<0.05 level. There is a significant difference of Fra2 mRNA between E2 + ICI and ICI as +p<0.05.

at 4 hr compared with the control level, the level of Fra2 mRNA expression peaked 3.24-fold at 2 hr, then gradually declined to the basal level after 12 hr. To determine whether the effects of estrogen on the induction of Fra1 and Fra2 mRNA in the ovx mouse uterus was mediated by ERs, ovx mice were injected with ICI 182,780 at 30 min before the injection of the estrogen. This experiment was confirmed that gene expression of Fra1 and Fra2 were under of control estrogen. The ICI 182,780 abrogated group showed a high level of Fra1 and Fra2 mRNA (Figure 3).

3. Immunohistochemistry of Fra1 and Fra2 protein

Microphotographs of representative longitudinal or cross uterine sections (7 μ) were taken at 0, 2, 4, 6 and 12 hr after estrogen injection. The Fra1 protein was expressed in the myometrium stronger than in luminal, glandular epithelium and stroma. The Fra2 protein was stained in the nuclei of stromal and myometrial, as well as epithelial cells, with maximal staining 2 hr, and maintained expression until 12 hr. Also, Fra2 protein was expressed in the myometrium stronger than luminal, glandular epithelium and stroma (Figure 4).

DISCUSSION

Although estrogen plays an crucial role in physiological events, few genes affected by estrogen have been identified. To search the early response genes up-regulated by estrogen, we examined candidated 15 kinds of transcription factor genes by RT-PCR and selected the early up-regulation genes, Fra1 and Fra2 genes, which expressed under the control of estrogen in mouse uterus. Their mRNA peaked 2.35-3.24 folds at 2-4 hr after estrogen injection and the estrogen effect was faded away by ICI 182,780, whoch is estrogen receptor antagonist. These results are compatible with the fact that steroids act on gene expression through their nuclear receptors.[10.11] The Fos related antigen 1,2 (Fra1 and Fra2) genes are

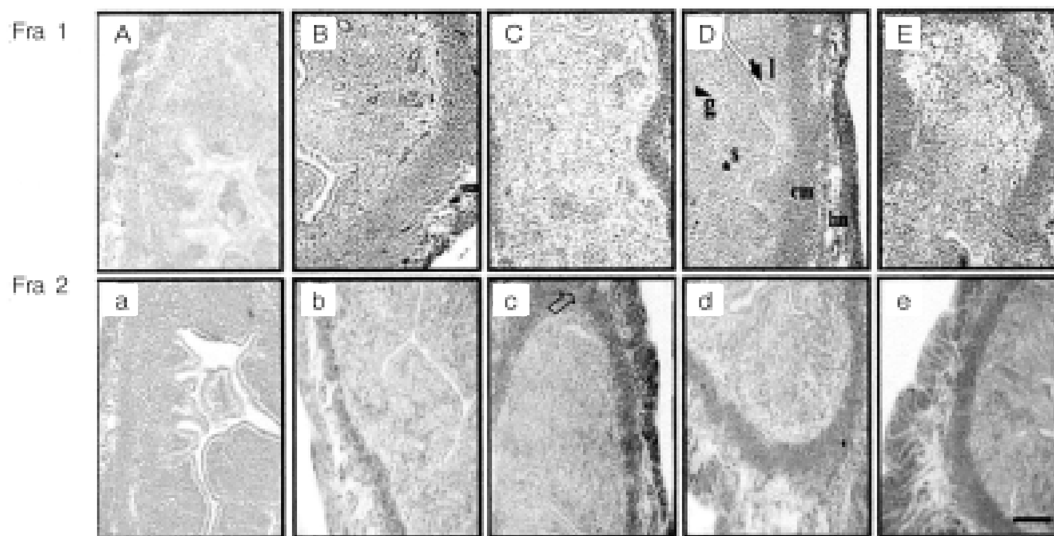

Figure 4. Immunohistochemical localization of Fra1 and Fra2 proteins after estrogen injection. Intense deposits(anopen arrow) under bright field indicate the site of each protein expression (100 x) Im: longitudinal myometrium Cm: circular myometrium S: stroma (asterisk) G: glandular epithelium (an arrow head) L: luminal epithelium (an arrow).

A: Fra1 (Negative control) **B:** Fra1 (2hr) **C:** Fra1 (4hr) **D:** Fra1 (6hr) **E:** Fra1 (12hr)
a : Fra2 (Negative control) **b:** Fra2 (2hr) **c :** Fra2 (4hr) **d :** Fra2 (6hr) **e :** Fra2 (12hr)
x 200. Bar indicates 100㎛.

related to the protooncogene c-fos encodes a member of the activated protein-1 (AP-1) family of transcription factor gene family.[12] Sigeo *et al* (1995) showed enhancing of c-fos mRNA and their oncoproteins in the mouse uterus treated with estrogen.[13.] It is suggest a similar effects for these genes in uterine estrogen response. Early responses genes of the uterus to estrogen injection include stimulation of amino acid uptake, increased nucleotide uptake, synthesis of metabolic enzymes, enhanced RNA polymerases I and II activities, increased total uterus RNA content, glucose oxidation, increased phospholipid synthesis, water imbibition and increased uterine weight related to reproductive total action.[14] The early response genes would include important regulatory genes such as transcriptional regulatory factor,s and it has been difficult to elucidate the early molecular events contributing to uterotrophic changes as stimulated by estrogen during the acute uterus response. So many studies recently performed cDNA microarray to identity the genes regulated by estrogen in the mouse and rat uterus.[15] Our results show that Fra1 and Fra2 as early response genes are regulated by estrogen. Although luminal epithelium is considered one of the major target cell types for estrogen stimulation in the rodent uterus,[16] estrogen stimulates the proliferation of luminal and glandular epithelial cells, in the adult ovx mouse uterus.[17~19] Our results show that Fra1 and Fra2 proteins are broadly expressed all cell types of uterus and staining intensity are stronger in the myometrium than those in luminal and glandular epithelium and stromal cells. One possibility suggest that Fra1 and Fra2 are not major players in the proliferate response of the luminal epithelial cells to estrogen or act on low levels in epithelial proliferation. On the other hand, these proteins may be suppressors of proliferation in the epithelium. In support of this suggestion, it can be speculated that Fra1 protein can either increase or decrease total AP-1 activity depending on the status of the other FOS and JUN protein in the cell, and has been proposed to function as a negative feed back regulator of AP-1. In addition, Fra2 protein also may inhibit AP-1 complexes and have antagonistic activity in steroid hormone-mediated events in other system.[20-21] It is possible that multiple signals culminating to Fra1 and Fra2 expression in the mouse uterus could not be excluded. In myometrium, the high levels of Fra1 and Fra2 gene may be related to smooth muscle contraction and relaxation of mouse uterus. In conclusion, the expression of Fra1 and Fra2 mRNA of the early response genes are up-regulated by estrogen in the mouse uterus and estrogen activates these genes expression by binding estrogen receptor. Myometrium showed strong expression of Fra1 and Fra2 in immunohistochemistry, thus, their function in the myometrium should be elucidated in further studies.

REFERENCES

1. Todd AT, Marisela MM, Bert WO, Orla MC. Mutual and intercompartmental regulation of estrogen receptor and progesterone receptor expression in the mouse uterus. Biol Reprod 998; 59: 1143-1152.
2. Holly L, Boettger T, Lata M and George MS. Cellular pattern of c-fos induction by estradiol in the immature rat uterus. Biol Reprod 1995; 53: 1398-1406.
3. April HC, Andrzej KB, Racheal LK, Hawkins AL Aldaz CM. Effects of estrogen on global gene expression : identification of novel targets of estrogen action. Cancer research 2000; 60: 5977-5983.
4. Brivanlou AH, James E and Darnell JH. Signal transduction and the control of gene exression. Science 2000; 295: 813-8.
5. Johha Frasor, Jeanne MD, Barry Komm, Ken CN, Benita SK. Profiling of estrogen up-and down-regulated gene expression in human breast cancer cells: Insight into gene networks and pathways underlying estrogenic control of proliferation

and cell phenotype. Endocrinology 2003; 144: 4562-72.

6. David KW, Bruce CM, Sohaib AK. estrogen induces expression of C-jun and jun-B proto oncogenes in specific a uterine cells. Endocrinology 1992; 133: 20-9.
7. Wang XN, Das SK, Klagsbrun MC, Dey SK. Differential regulation of heparin-binding Epidermal growth factor-like growth factor in the adult ovariectomized mouse uterus by progesterone and estrogen. Endocrinology 1994; 135: 1264-1271.
8. Jeff Reese, Sanjoy K Das, BC Paria, Sudhansu K Dey. Global gene expression analysis to identify molecular markers of uterine receptivity and embryo implantation. 2001. J of biol chem 2001; 47: 44137-44145.
10. Hou Q and Gorski J. estrogen receptor and progesterone receptor genes are expressed differentially in mouse embryos during preimplantation development. Proc Natl Acad Sci. 1993; 90: 9460-4.
11. Choe YS, Shim CS, Kim KJ. Expression of galectin-1 mRNA in the Mouse uterus is under the control of ovarian steroids during blastocyst implantation. Mol Reprod Develop 1997; 48: 261-266.
12. Carrasco D, Bravo R. Tissue-specific expression of the fos-related transcription factor Fra-2 during mouse development Oncogene 1995; 16: 10691079.
13. Sigeo M, Kenji N, Satoshi I, Teruhiko T. Overexpression of c-fos/ jun mRNA and their oncoproteins (FOS/JUN) in the mouse uterus treated with three natural estrogens. Cancer. letters 1995; 97: 225-231.
14. Clark JH, Mccormack SA, Padykula H, Hardin JW. Biochemical and morphologicla changes stimulated by the nuclear binding of the estrogen receptor. Bristolmyers Cancer Symposia, Academic press, Inc New York 1979; 382-418.
15. Watanabe H, Suzuki A, Mizutani T, Iguchi T. Genome-wide analysis of changes in early gene expression induced by oestrogen. Genes to cells 2002; 7: 497-507.
16. Kenneth PN, Moli T, Sohaib AK. estrogen differentially c-jun expression on uterine tissue compartments. Endocrinology 1993; 134: 1827- 1833.
17. Kenneth S. Korach J, James C. estrogen action in the mouse uterus ; differential nuclear localization of estradiol in uterine cell types. Endocrinology 1981; 108: 1989-1991.
18. Kenneth PN, George AP, Sohaib AK. Cellular localization of estradiol-induced c-fos messenger ribonucleic acid in the rat uterus ; c-fos expression and uterine cell proliferation do not correlate strictly. Endocrinology 1995; 136: 3008-3015.
19. Michele P, Vincenzo M, Alessandro W. estrogen induces c-fos expression specifically in the luminal and glandular epithelia of adult rat uterus. Biochem Biophy Res Comm. 1991; 175: 480-485.
20. Suzuki T, Okuno H, Yoshida T, Endo T, Nishina H, Iba H. Difference in transcriptional regulatory function between c-Fos and Fra-2. Nucleic Acids Res 1991; 25; 5537-42.
21. Schule R, Rangarajan P, Kliewer S, Ransone LJ, Bolado J, Yang N, Verma IM, Evans RM. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. Cell 1990; 21: 1217-26.