

Fra1 Fra2

1, 2
 1,2 1,2 2 2 2 2 2 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 Nah², Sung Hoon Kim²,
 Hee Dong Chae², Chung Hoon Kim², Byung Moon Kang², Moon Kyoo Kim¹

¹Department of Life Science, College of Natural Sciences, Hanyang University, Seoul,

²Department of Obstetrics and Gynecology, College of Medicine, Ulsan University, Asan Medical Center, Seoul, Korea.

DNA (transcription factor) RT-PCR
 early up-regulation gene Fos related antigen (Fra1 Fra2) RT-PCR
 sesame oil (n=4) RT-PCR early response gene
 Fra1 Fra2 estrogen receptor antagonist ICI 182,780
 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) Fra1 Fra2 ICI 182,780 (p < 0.01).

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

Tel: (02) 2290-0954, e-mail: kimmk@email.hanyang.ac.kr 17,
 Tel: (02) 3010-5059, Fax: (02) 3010-5053, e-mail: leeje@amc.seoul.kr 388-1,

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 mechanism 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 immunohistochemistry.

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 cycle and rested for 14 days. The ovx mice were injected with 17 β estradiol 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 LN₂. 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 Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS, Gibco/BRL) and directly plunged into the LN₂ 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 transcribed 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 MgCl₂, 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	cggatggggtacaggcctg	728-747	233
	R	aggaagtgctggggaggacg	941-960	
CREB-2	F	gccccacaacatgaccgaga	263-282	265
	R	ccaacatccaatctgtcccg	508-527	
GATA-2	F	aacgtgaagacatggaggcg	2041-2060	272
	R	aggggaatgatctgggagac	2293-2312	
GATA-3	F	cctatccgccctatgtgcc	866-885	286
	R	gtccctgctctcctgtctgcc	1130-1150	
GATA-5	F	ggggcaaaggcaagacatgc	2589-2608	512
	R	aaaccaacgtggaagggatc	3081-3110	
GATA-6	F	gctggctctatatgaaactc	963-982	344
	R	ggatgtgactcggcagggg	1287-1306	
E2F-1	F	ctggctgggtttgcagggca	1984-2003	417
	R	cagactggagggtggggagg	2381-2400	
E2F-3	F	gtctgaggatggggcatgc	411-430	559
	R	ctcttgagcagggaggcag	748-968	
E2F-5	F	ggaagggttagtgctggc	326-345	433
	R	gttgaggactgggaggaagg	739-758	
ATF-1	F	cagacctaccagatccgtacc	543-563	439
	R	gtcatctccattctccctcc	962-981	
GLI-1	F	gactccaccgattggcacc	2647-2666	353
	R	gcagggtctgactaactt	2980-2999	
GLI-2	F	ccaagtacacgccaaagag	5107-5126	337
	R	acagggtctgactaactt	5424-5443	
GLI-3	F	ggtactagggcagggttggtg	4206-4225	586
	R	gagacgggaggagctgtggg	4772-4791	
FRA-1	F	ggatgagaaatcggggctgc	619-638	370
	R	ggggctcggaggagggtcgc	969-988	
FRA-2	F	gcagaaggagagatgagcag	335-354	370
	R	gctgaggaagagggtctgc	685-704	

conditions of achieve linear amplification. The thermal cycling parameters were consisted of 22-26 cycles of denaturation (94 °C, 30 sec); annealing (60 °C, 30 sec); and extension (72 °C, 30 sec). Subsequently, 8 µl 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 µm 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 (Na₂HPO₄ 10 mM, NaCl 0.9 %) for 5 min. To remove endogenous peroxidase activity, peroxidase quenching was performed with 0.3 % H₂O₂ 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 °C 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-quantitative 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

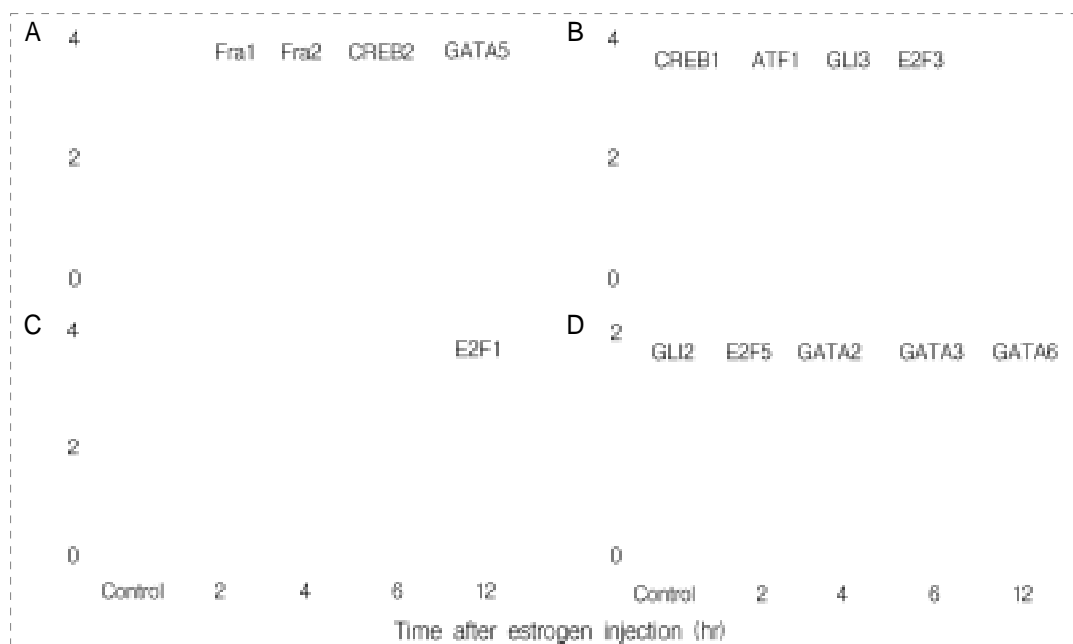


Figure 1. Semi-quantitative RT-PCR analysis screening genes after estrogen injection. Values of each band were normalized to rPL7 for the same sample. Data were mean \pm SEM from three replicate experiments.

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

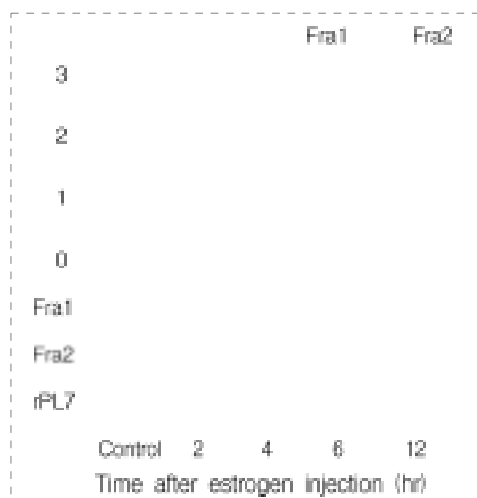


Figure 2. Expression patterns of selected early response genes, Fra1, Fra2 mRNA after estrogen injection. The expression level of LF and HB-EGF mRNA was examined as a positive marker gene. Values are represented by the mean \pm SEM and significant differences by Student's *t*-test are showed as ** $p < 0.01$ and * $p < 0.05$ level.

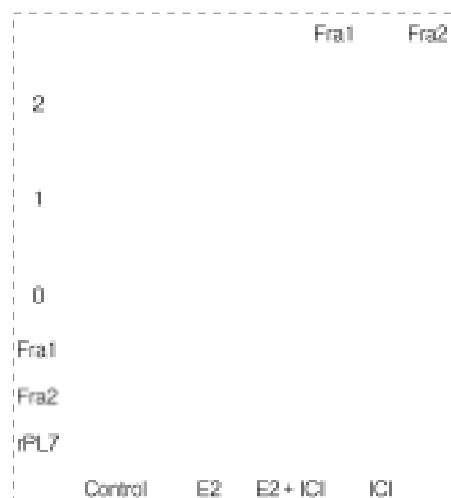


Figure 3. The expression pattern of Fra1 and Fra2 mRNA after injection of estrogen and ICI 182,780. Values are represented by the mean \pm SEM. Significant differences by Student's *t*-test compared to the control group are showed as ** $p < 0.01$ and * $p < 0.05$ level. There is a significant difference 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 candidate 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, which 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

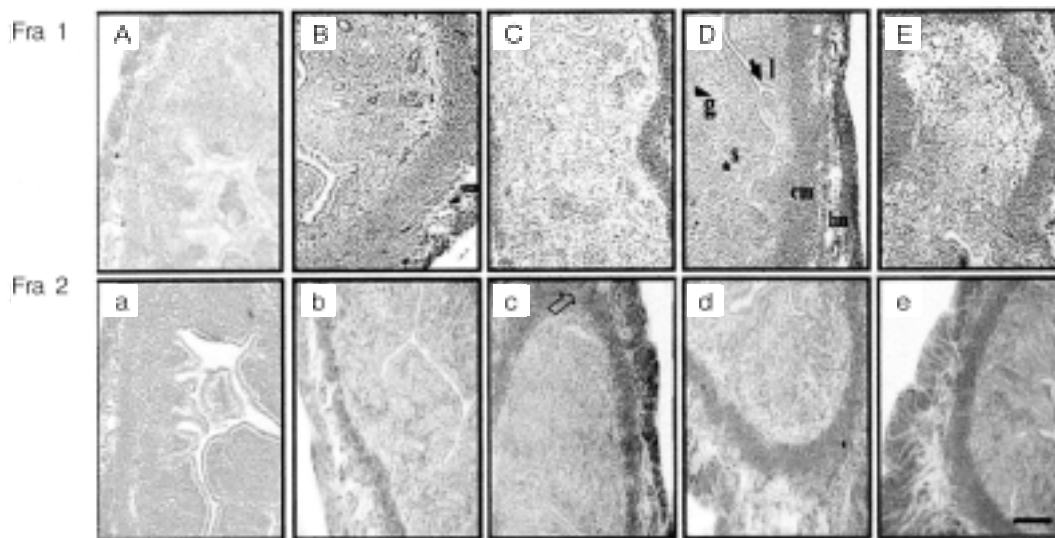


Figure 4. Immunohistochemical localization of Fra1 and Fra2 proteins after estrogen injection. Intense deposits (an open arrow) under bright field indicate the site of each protein expression (100 x). Im: longitudinal myometrium Cm: circular myometrium S: stroma (asterisk) G: glandular epithelium (an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

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* 1998; 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 expression. *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: 1069-1079.
 13. Sigeo M, Kenji N, Satoshi I, Teruhiko T. Over-expression 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.