Indian J Physiol Pharmacol 54 (5) 2010 : 27–32

Two Multipotential Transcription Factors, NF-kappaB and Stat-3, Play Critical and Hierarchal Roles for Implantation

Tadashi Kimura, Kazuhide Ogita, Keiichi Kumasawa, Shinsuke Koyama, Tateki Tsutsui and Hitomi Nakamura

Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, Osaka, Japan

Introduction

Implantation, the beginning of fetal life in utero, has complex and dynamic processes including apposition and attachment of embryo to the uterine endometrial surface, invasion, placentation and decidualization. In mice, the period when uterus is available to accept the conceptus implanted called "implantation window", is a very limited time, around day 3-5 post-coitum (p.c.).

Orchestral regulation of various molecules including cytokines, growth factors, and matrix-related molecules should play an important role in endometrium during implantation. Many reports focused on the strict physiological control of implantation within uterine endometrial epithelium and stroma. Some gene-targeting studies indicated several implantation failure models. However, these experimental settings often do not reflect clinical scenario (1). In women, most of the causes of implantation failure are supposed to be reversible process, because the definitive and hereditary implantation failure may be rare and the patient may conceive at some stage of cycle. It could be suggested that it is suitable to use a local and transient gene transfer system for investigation of the implantation mechanism, because the uterus can play different roles and its functions are dependent on short term events during life. Moreover, uterus is the applicable organ for local gene therapy without any operation, because it is tubular organ accessible from vagina. To modulate the uterine function during implantation period, we first established in vivo transient gene transfer system in murine uterus.

Transient in vivo gene transfer to the mouse uterus

For the study of reproductive physiology, gene transfer systems should fulfill the following conditions: (i) high efficiency of gene transfer into the target tissue; (ii) no adverse effect on pregnancy; (iii) no possibility of the gene being transferred to fetuses. Successful *in vivo* gene transfer to female reproductive tract had been reported with cationic liposomes (Lipofectamine, DOTAP, GenePorter) (2-4). Cationic liposomes show excellent entrapment of negatively charged macromolecules including DNA, and high transfection efficiency to in vitro cultured cells. However, *in vivo* conditions, the use of cationic lipids, instead of anionic lipids as a liposome component, severely reduced the transfection efficiency (5). Indeed in our experiments, cationic liposome did not provide enough efficiency (less than 1/120) compared with HVJ-E (Haemagglutinating virus of Japan-envelope) vector transfection system into murine uterine cavity. HVJ-E vector is derived from inactivated HVJ (Sendai virus), which has potent fusogenic proteins F and HN on its surface. The vector releases DNA, RNA and oligonucleotides incorporated inside of the

Address for correspondence : T. Kimura, Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 5650871, Japan. E-mail : tadashi@gyne.med.osaka-u.ac.jp

vector shell into the fused cells (6). Injection of HVJ-E vector into the uterine cavity on day 1.5 p.c. affected neither the entire course of pregnancy nor the parturition. This system also did not transfer the gene into the fetuses (7). Eighty micrograms of expression plasmid DNA entrapped by HVJ-E vector, suspended with 25 ml of HTF (human tubal fluid) medium, was injected into one uterine horn. We successfully extended this protocol to the pregnant uterus of on the day 14.5 (8) and 7.0 p.c. (manuscript in preparation). They did not disturb the entire course of pregnancy and fetal development. The duration of protein expression encoded in cDNA conjugated in the CMV-driven expression vector (pcDNA3) was approximately 3 days and localized in the luminal and glandular endometrial cells. Immunoreactivity of HVJ-F protein completely disappeared by 72 hours after transfection. These observations indicated that HVJ-E vector system is one of the ideal gene transfer system into murine pregnant uterus to investigate uterine reproductive physiology.

Multipotential nuclear transcription factor is a good target to modulate uterine function at implantation

Serial and orchestral regulation of various molecules is mandatory for implantation, although it is very short period during pregnancy. Who is the conductor? We hypothesized that a few multi-potential transcriptional factors, such as NF-kappaB, Stat-3 and AP-1, might regulate them to open the implantation window. Our previous study showed that NF-kappaB activity gradually increased during the course of implantation window opening in murine endometrial epithelium (9). Activation of Stat-3 at implantation window in murine and rat endometrium were also reported (10, 11). JunD and Fra-2, which are AP-1 components, were markedly increased in the decidua of early pregnancy. It regulates the decidua-specific enhancer element of human prolactin gene via AP-1 binding sites (12). However, the targeted disruption of these component molecules, i.e. p50, p65 (for NF-kappaB), Stat3, c-fos and c-jun (for AP-1) resulted in embryonic lethal or no phenotype on reproduction (13-16).

In order to examine the importance of specific transcription factor on implantation, we at first performed decoy-based screening. Decoy for NF-kappaB, Stat-3 and AP-1 were prepared with synthesized phosphorotioate-modified oligodeoxynucleotides (17-19). The sequences of these decoys are listed in Table I. Scramble decoys were prepared as negative controls. After annealing sense- and antisense-strands, the decoy was incorporated into HVJ-E vector and transferred into uterine cavity on day 1.5 p.c. as described (7). Among three decoys, AP-1 decoy did not affect implantation rate determined by injection of 0.5% Evans blue (20) to visualize implantation sites on day 5.0 p.c. (Fig. 1). Therefore we further investigated the function of NF-kappaB and Stat-3 activities on implantation.

If it is possible to design decoy sequences which can work *in vivo*, the decoy-based screening of the transcription factor function (21) combined with the high efficient gene transfer system using HVJ-E vector could provide straightforward answers.

Activation of NF-kappaB determines the timing of implantation window opening

In order to suppress the NF-kappaB activation in endometrium, we used I-kappaB a mutant cDNA, which is a dominant negative form of I-kappaB a and cannot dissociate from

Table I. Decoy sequences for multipotential nuclear transcription factor binding sites.

NF-kappaB	5'-CCTTGAAGGGATTTCCCTCC-3'
Stat-3	5'-CCTTCCGGGAATTCCTTCC GGGAATTC-3'
AP-1	5'-GGATCCATGACTCAGAAGACG-3'
Scramble#1	5'-TTGCCGTACCTGACTTAGCC-3'
Scramble#2	5'-AGTCCATTCGGCAGGCCTC TGCTCTAT-3'

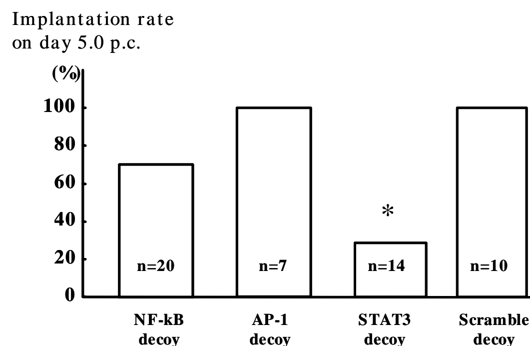

Fig. 1: Implantation rate after decoy treatment. Double strand decoy oligonucleotides were synthesized and entrapped by HVJ-E vector. Twenty-five ml of HVJ-E vector suspension was injected to one uterine horn on day 1.5 p.c. Implantation sites were visualized on day 5.0 p.c. by Evans blue injection method. On day 5.0 p.c. implantation partially began in the NF-kappaB decoy treated mice. Stat-3 treatment significantly disturbed implantation rate (*; $P<0.05$), however AP-1 decoy had no effect on implantation.

NF-kappaB. The gene transfection of cytomegalovirus promoter driven I-kappaB a mutant cDNA by HVJ-E vector on day 1.5 p.c. induced significant suppression of NF-kappaB activity until day 4.5 p.c. But it was recovered to be control level on day 5.5 p.c. On day 4.5 p.c., the numbers of implantation sites were significantly decreased in NF-kappaB suppression group. However, it was observed in histological study that the implantation had been just started on day 6.0 p.c. in uterus suppressed NF-kappaB activity. The pregnancy rate, litter size and birth weight of pups were similar, but the date of delivery was 1 day later than control mice. In the NF-kappaB disturbed uterus, expression level of LIF mRNA was suppressed on day 4.5 p.c. but it was recovered as control level on day 5.5 p.c. The simultaneous induction of LIF cDNA with I-kappaB a mutant cDNA partially offset the delay of implantation on day 4.5 p.c. These results suggested that the activation of NF-kappaB in uterine endometrium at the early period of pregnancy determined the timing of implantation window opening, partially via upregulation of LIF transcription (22).

Transient, partial and local inhibition of endometrial Stat-3 activation results in implantation failure independent of hormonal milieu

The gene transfection of Stat-3 decoy by HVJ-E vector on day 1.5 p.c. reduced uterine Stat-3 activity to be lesser half of control level on day 5.0 p.c.. Stat-3 decoy treated mice revealed implantation failure. On day 8.0 p.c., there were no conceptus observed and the morphology turned to be non-pregnant state in Stat-3 decoy transferred mice. The morphology of blastocysts flushed out from Stat-3 decoy treated uterus on day 4.0 p.c. was normal, and exogenous DNA was not transferred in embryos. Stat-3 decoy treatment inhibited decidualization in both pregnant and pseudopregnant mice. However, Stat-3 decoy treatment neither altered expression levels of progesterone receptor in the uterus nor reduced serum progesterone level. Approximately 50% of disturbance of Stat-3 activity in endometrium at the beginning of pregnancy induced implantation failure without altering progesterone system (23). In human unexplained infertility cases, the patients often reveal normal progesterone level with thin endometrium determined by endovaginal ultrasonography. Our preliminary study noticed that in some mid-luteal phase endometrium obtained from unexplained infertility patients, Stat-3 activation appeared to be disturbed (manuscript in preparation). LIF, IL-11 and other IL-6 family cytokines activate Stat-3 via heterodimerization of their specific receptors and gp130. Among them, both LIF and IL-11 system are necessary for successful implantation in gene targeting mouse model (24, 25). Mouse implantation failure model by interfering the activation of Stat-3 by Stat-3 decoy (23) or pharmacological Stat-3 antagonist (26) in endometrium might explain some part of pathophysiology of unexplained infertility.

Conclusion

Our series of experiments suggest the

hierarchy of multifunctional nuclear transcription factors. AP-1 would not play crucial role for implantation. NF-kappaB could determine the timing of implantation window opening, partially via LIF induction. It has been reported that uterine NF-kappaB is activated by estradiol administration (27). This observation could be reasonable. Because the injection of estradiol can release dormant state of embryo and induce implantation procedure in rodent model (28). LIF as well as another cytokines including IL-11 activate Stat-3, which is necessary for decidualization independent of progesterone. We suggest this cascade (Fig. 2) could be applied to human implantation physiology. We also hypothesize that Stat-3 could be good therapeutic target for the patients of unexplained infertility, especially those who are suffering multiple failure of IVF-ET (*in vitro* fertilization and embryo transfer) treatment.

Acknowledgement

We thank Prof. Yasufumi Kaneda (Department of Gene Therapy Science, Osaka University) for critical advice for our HVJ-E works, Prof. Yasuo Kiso (Yamaguchi University) for mouse histological studies, and prof. Virginia Rider (Pittburg University) for critical advice to assess decidualization. This work was supported in part by Grant-in-Aid for Scientific Research (Nos. 17591734, 20890120) from the Ministry of Education, Science, and Culture of Japan (Tokyo, Japan), Suzuken Medical Foundation (Naoya, Janap), Uehara Memorial Foundation (Tokyo, Japan) and Serono Foundation (Geneva, Switzerland). HVJ-E vector (GenomONE-NEOÒ) was provided by Ishihara Sangyo Co. Ltd. (Osaka, Japan).

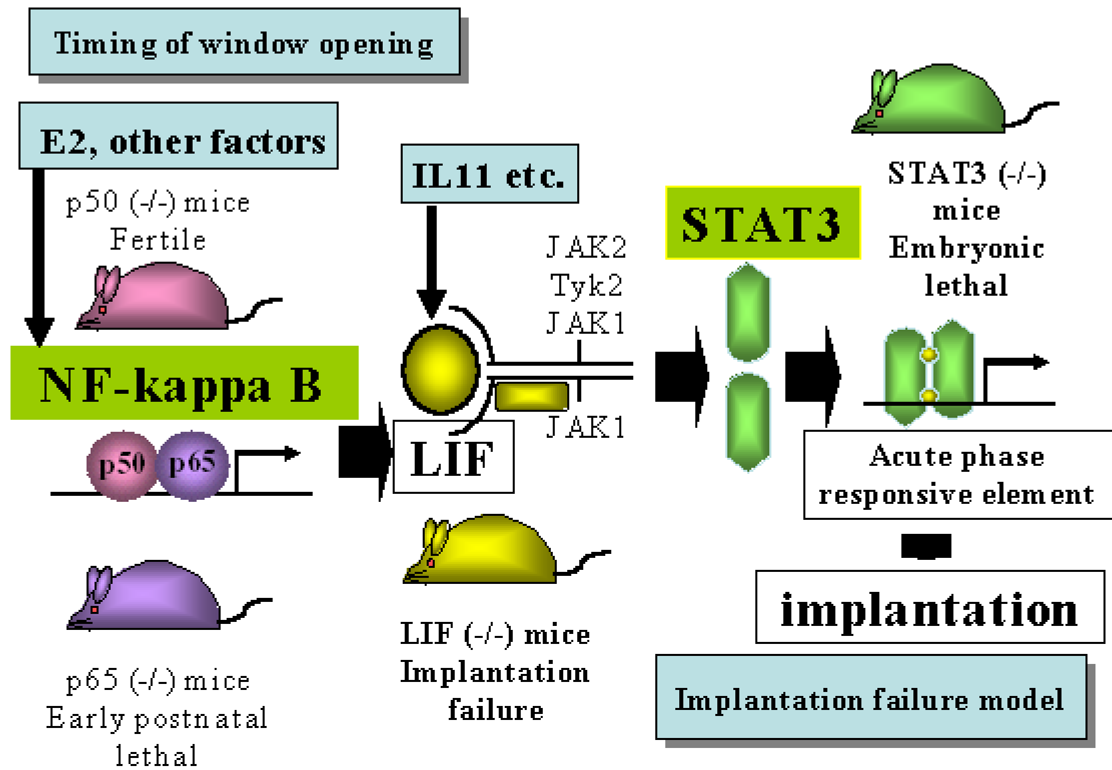

Fig. 2 : Hypothesis of hierarchy of transcription factor activation during implantation.

References

1. Lee KY, Jeong JW, Tsai SY, Lydon JP, DeMayo FJ. Mouse models of implantation. *Trends Endocrinol Metab* 2007; 18: 234–239.
2. Charnock-Jones DS, Sharkey AM, Jaggers DC, Yoo HJ, Heap RB, Smith SK. *In vivo* gene transfer to the uterine endometrium. *Hum Reprod* 1997; 12: 17–20.
3. Zhu L-J, Bagchi MK, Bagchi IC. Attenuation of calcitonin gene expression in pregnant rat uterus leads to block in embryonic implantation. *Endocrinology* 1998; 139: 330–339.
4. Hsieh Y-Y, Lin C-S, Sun Y-L, Chang C-C, Tsai H-D, Wu JC-H. In vivo gene transfer of leukemia inhibitory factor (LIF) into mouse endometrium. *J Assist Reprod Genet* 2002; 19: 79–83.
5. Saeki Y, Matsumoto N, Nakano Y, Mori M, Awai K, Kaneda Y. Development and characterization of cationic liposomes conjugated with HVJ (Sendai virus): reciprocal effect of cationic lipid for in vitro and *in vivo* gene transfer. *Hum Gene Ther* 1997; 8: 1965–1972.
6. Kaneda Y, Nakajima T, Nishikawa T, et al. Hemagglutinating virus of Japan (HVJ) envelope vector as a versatile gene delivery system. *Mol Ther* 2002; 6: 219–226.
7. Nakamura H, Kimura T, Ikegami H, et al. Highly efficient and minimally invasive *in-vivo* gene transfer to the mouse uterus using haemagglutinating virus of Japan (HVJ) envelope vector. *Hum Reprod* 2003; 9: 603–609.
8. Koyama S, Kimura T, Ogita K, et al. Simple and highly efficient in vivo gene transfer to mid-late pregnant mouse uterus. *J Reprod Immunol* 2006; 70: 59–69.
9. Nakamura H, Kimura T, Ogita K, et al. NF-kB activation at implantation window of the mouse uterus. *Am J Reprod Immunol* 2004; 51: 16–21.
10. Teng CB, Diao HL, Ma H, et al. Signal transducer and activator of transcription 3 (Stat3) expression and activation in rat uterus during early pregnancy. *Reproduction* 2004; 128: 197–205.
11. Cheng JG, Chen JR, Hernandez L, et al., Dual control of LIF expression and LIF receptor function regulate Stat3 activation at the onset of uterine receptivity and embryo implantation. *Proc Natl Acad Sci USA*. 2001; 98: 8680–8685.
12. Watanabe K, Kessler CA, Bachurski CJ, et al. Identification of a deciduas-specific enhancer on the human prolactin gene with two critical activator protein 1 (AP-1) binding sites. *Mol Endocrinol* 2001; 15: 638–653.
13. Sha WC, Liou HC, Tuomanen EI, Baltimore D. Targeted disruption of the p50 subunit of NF-kappaB leads to multifocal defects in immune responses. *Cell* 1995; 80: 321–330.
14. Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D. Embryonic lethality and liver degeneration in mice lacking the RelA component of NFkB. *Nature* 1995; 376: 167–170.
15. Takeda K, Noguchi K, Shi W, et al. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci USA* 1997; 94: 3801–3804.
16. Roffler-Tarlov V, Brown JJ, Tarlov E, et al. Programmed cell death in the absence of c-Fos and c-Jun. *Development* 1996; 122: 1–9.
17. Morishita R, Sugimoto T, Aoki M, et al. *In vivo* transfection of cis element "decoy" against nuclear factor-kappaB binding site prevents myocardial infarction. *Nat Med* 1997; 3: 894–899.
18. Akira S, Nishio Y, Tanaka T, et al. Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. *Cell* 1994; 77: 63–71.
19. Ahn JD, Morishita R, Kaneda Y, et al. Inhibitory effects of novel AP-1 decoy oligodeoxynucleotides on vascular smooth muscle cell proliferation in vitro and neointimal formation *in vivo*. *Circ Res* 2002; 90: 1325–1332.
20. Huet YM, Dey SK. Role of early and late oestrogenic effects on implantation in the mouse. *J Reprod Fertil* 1987; 81: 453–458.
21. Nakagami H, Tomita N, Kaneda Y, Ogihara T, Morishita R. Anti-oxidant gene therapy by NF kappaB decoy oligodeoxynucleotide. *Curr Pharm Biotechnol* 2006; 7: 95–100.
22. Nakamura H, Kimura T, Ogita K, et al.

Alteration of the timing of implantation by in vivo gene transfer: delay of implantation by suppression of nuclear factor kB activity and partial rescue by leukemia inhibitory factor. *Biochem Biophys Research Commun* 2004; 321: 886–892.

23. Nakamura H, Kimura T, Koyama S, et al. Mouse model of human infertility: transient and local inhibition of endometrial STAT-3 activation results in implantation failure. *FEBS Lett* 2006; 580: 2717–2722.

24. Stewart CL, Kaspar P, Brunet LJ, et al. Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. *Nature* 1992; 359: 76–79.

25. Robb L, Li R, Hartley L, Nandurkar HH, Koentgen F, Begley CG. Infertility in female mice lacking the receptor for interleukin 11 is due to a defective uterine response to implantation. *Nat Med* 1998; 4: 303–308.

26. Catalano RD, Johnson MH, Campbell EA, Charnock-Jones DS, Smith SK, Sharkey AM. Inhibition of Stat3 activation in the endometrium prevents implantation: a nonsteroidal approach to contraception. *Proc Natl Acad Sci USA* 2005; 102: 8585–8590.

27. Shyamala G, Guiot MC. Activation of kB-specific proteins by estradiol. *Proc Natl Acad Sci USA* 1992; 89: 10628–10632.

28. Lopes FL, Desmarais JA, Murphy BD. Embryonic diapause and its regulation. *Reproduction* 2004; 128: 669–678.