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석사학위청구논문

Role of fibroblast growth factor during pre- and peri-implantation: a type I diabetes mellitus NOD mice

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성신여자대학교 대학원

생 물 학 과

황 희 경

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이 논문을 석사학위논문으로 제출함

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황희경의 석사학위 논문으로 인준함.

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논 문 개 요

배아의 착상과정은 스테로이드 호르몬과 다른 조절인자들의 상호공조에 의해 조절되는 것으로 알려져 있다. Fibroblast growth factor (FGF)는 착상 이후 배 발생과 임신유지에 관여하는 것으로 보고 된 인자로써 착상 전 및 착상과정에서의 기능에 대하여 알려져 있지 않다. DNA microarray 방법을 이용하여 착상 전 후 시기에 발현되는 것으로 동정된 유전자 중 FGFs 가 시기 특이적으로 발현되는 것을 알 수가 있었다. 이러한 결과는 FGFs 가 착상 이후 배 발달과 관련된 기능이 있을 뿐 아니라 착상 전에도 배 발생과 착상관련 자궁분화에 대한 조절기능이 있음을 의미한다. 본 실험에서는 FGF 가 착상 전 후 시기의 배 발생과 착상관련 자궁분화에서의 역할을 알아보고자 nonobeses diabetes(NOD) 생쥐를 사용하였다. NOD 생쥐는 제 1 형 당뇨 모델로 널리 사용되고 있으며 본 실험에서는 착상 및 생존 가능한 배아의 비율감소 현상과 기초실험 결과를 이용하여 착상 전 후 배 발생에서 FGF 의 역할을 알아보았다. 당뇨가 유발된 NOD 생쥐 에서 3 주 후에 생식주기가 나타나지 않고 비발정시기 상태가 지속적으로 유지되었다. 이러한 생식주기의 변화는 당뇨발병에 의해 성스테로이드 호르몬 분비가 원활히 진행되지 않음을 의미한다. 하지만 당뇨 유발 후 인슐린에 의해 생식주기가 정상군에서처럼 회복되었다. 당뇨유발 3 주된 NOD 생쥐가 과배란에 유도에 반응하여 배란하는 난자의 수가 유의하게 감소하였다. 그러나 인위적으로 성스테로이드 호르몬을 이용하여 임신 가능 조건을 유지한 상태에서 탈락막 신호를 주게 되면 탈락막 반응이 유도되었다. 그러나 대조군에 비해 반응정도가 미미하였다. FGFs 와 FGFRs 은 대조군과 당뇨유발 NOD 생쥐의 착상 전 후 배아와 자궁에서 모두 발현하였다.

그러나 임신시기 및 탈락막 반응 유도 후 시간특이적으로 특정 FGFs 와 FGFRs 의 발현증감을 보였다. 인위적 조건을 유지한 상태에서 탈락막 반응을 유도한 당뇨유발 NOD 생쥐 자궁에서는 탈락막 반응 특이적 FGFs 또는 FGFRs 발현을 탐지 하였다. 위의 결과를 바탕으로 당뇨 유발 NOD 생쥐 자궁의 탈락막 반응성의 감소가 특정 FGF 의 발현과 그 수용체의 발현 정도 변화에 의한 것이며 또한 이러한 변화가 착상 이후 유산율의 증가에도 관여할 것으로 추정 할 수 있다.

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INTRODUCTION

Fibroblast growth factor (FGF) family consists of more than 22 members both the human and mouse FGF families comprise twenty two and four members, respectively (Itoh, 2007). All the members of the family are structurally related signal molecules. They are involved in various biological processes during development and adult life, including integration of growth and patterning during early post-implantation, and formation of tissues and organs, such as brain, ear, limb, hair and skeletal system (Wilkie *et al*. 1995 Yamaguchi *et al*, 1995, Goldfarb, 1996).

FGF action is mediated by FGF receptors (FGFRs) which have different affinity to the ligands (Table 1). Signal mediation in cell surface is to be a ternary complex formed between FGF, FGFR subunits and two heparin sulfate proteoglycan (Klagsbrun and Baird, 1991, Fig. 1). There are four members of FGFRs (Table 2). In addition alternate mRNA splicing gives rise to multiple mRNA variants. Analysis of the alternative splicing pattern of these receptors demonstrates that the utilization of either the exon b or c is dependent upon cell lineage. The b isoform is preferentially expressed in epithelial tissues, whereas the c isoform is expressed in mesenchymal tissues (Ornitz *et al*, 1996).

During embryonic development, FGFRs expressed with developmental stage specific and tissue specific manners. Most expression patterns of FGFRs have been investigated in postimplantation embryos (Orr-Urtreger *et al,* 1991). FGF and FGFR mediated signaling is active in endoderm, mesoderm and ectoderm (Labonne *et al*, 1997). FGFR1 is expressed in the

primitive streak during gastrulation in murine and FGFR2 is expressed in the primitive ectoderm of day 5.5 post coitum (p.c) (Orr-Urtreger *et al,* 1991; Yamaguchi *et al,* 1994). FGFR3 could be detected in embryonic stem cells and fetus (Dvorak *et al*, 2005; Nakajima *et al*, 2003). FGFR4 also detect during organogenesis including muscle differentiation and neural differentiation (Marics *et al*, 2002; Hardcastle *et al*, 2000). In the case of their ligands, they also express in various cells. (Papadaki *et al*, 2007; Berisha and Schams, 2005; Ford-perriss *et al,* 2001). FGF1, FGF2 and FGF7 were expressed in trophoblast giant cells during trophoblast invasion (Pfarrer *et al*, 2006). FGF2 expressed in embryonic stem cell and it is suggested that FGF signaling pathway can be implicated in self-renewal or differentiation of human embryonic stem cells (hESCs) (Dvorak *et al*, 2005).

Using homologous recombination methodology, the physiological roles of FGFs have been examined. FGF3 has mild defects in inner ear, skeletal (tail) development but there is no defect in reproduction (Mansour, 1994). FGF4 null mice showed embryo lethal in embryonic day (E) 4-5 and defect proliferation ICM (Feldman *et al*, 1995). FGF5 has long hair and angora mutation (Mansour, 1994) FGF8 null mice showed embryo lethal in E7 and defects in central nerves system (CNS) and limb development (Sun *et al*, 1999; Shanmugalingam *et al*, 2000; Meyers *et al*, 1998; Reifers *et al*; 1998). FGF10 has lethal in postnatal day 0 and has defect in development of multiple organs, including limb (Ohuchi *et al*, 2000; Min *et al*; 1998, Sekine *et al*, 1999). In the other members, FGF1, 2, 4, 7, 9, 12, 14, 15, 16, 17, 18, 21 and 23 Knock-out (KO) mice have various phenotypes, ranged from early embryonic development and lethality to subtle change in adult mice. From

these FGF KO mice, it is revealed that FGFs play critical role in development.

According to the microarray database (Cheon *et al*, 2002), several FGFs related genes are dramatically changed at the time of implantation. In addition FGF2 is suspected to involve in uterine cell proliferation differentiation and embryo implantation (Carlone and Rider, 1993). These are suggested that FGFs could involve in development of pre- or peri-implantation stage embryos and uterine modulation for implantation.

The nonobese diabetic (NOD) mouse is one of the best-studied animal models for Type 1 diabetes (T1D) as it spontaneously develops the disease. Unlike human Type 1 diabetes, ketoaidosis is relatively mild and affected animals can survive for 30 weeks without the administration of insulin (Atkinson *et al*, 1999). Diabetes mellitus mice showed developmental defects in oocyte development, increase granulosa / cumulus cell apoptosis and meiotic disorganization (Moley *et al*, 1991; Kim *et al*, 2007). And it is revealed that NOD mice have reduced number of implantation embryos (Otani *et al*, 1991).

Based on them it is respected that FGFs are one of the regulators in early embryonic development or endometrial differentiation associated with embryonic implantation. However, it is not known the role of FGF in embryo or endometrial differentiation. In this study we analyzed possible roles of FGF and their receptors using NOD mice model in pre- or peri-implantation stage embryos and differentiation in implantation stage uterus.

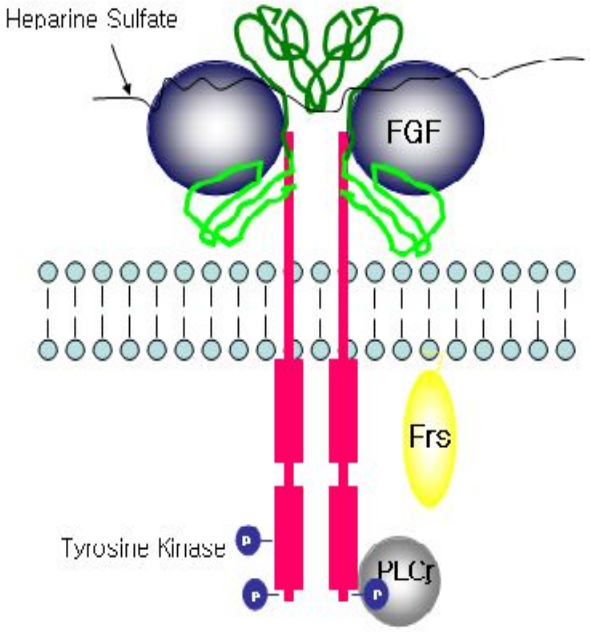

Figure 1. Diagram of FGF signal pathway

Heparine sulfate (HS) is indispensable for FGF signaling, HS enables FGF and FGFR to assemble into an active dimeric complex. Two FGFs, two FGFRs and two heparin oligosaccharides cooperate with each other to assemble into a symmetric functional dimeric unit. Each FGF is bivalent and has two remarkable degree of cooperation and synergism between the variance protein-protein and heparin-protein interfaces in the dimer. The ability of the HS chain to simultaneously assemble with a specific FGF and FGFR substrates may be highly regulated in FGF signaling (Rapraeger *et al*, 1995; Schlessinger *et al*, 2000).

Table 1 Some of FGF ligands and their KO-phenotypes which were screened molecules

FGF family	NCBI Gene bank	Function	High-affinity R	KO-phenotype	Reference
FGF1 (Acidic FGF)	M30641	mitogenic for vascular endothelial cells	FGFR1, 2, 3, 4	have no individual phenotype in pre/post implantation	Olivia *et al*.1991 Zhong *et al* 2006
FGF3 (Int-2)	Y00848	formed mesoderm leaving the primitive streak, commencing at E7	FGFR1, 2	have no individual phenotype in pre/post implantation	Olivia haub *et al*.1991 Mansour, 1994
FGF5	U42385	endothelial cell mitogen	FGFR1, 2	have no individual phenotype in pre/post implantation	Olivia *et al*.1991 Ornitz *et al*. 2001
FGF8 (AIGF)	D12483	outgrowth and pattern of different regions of the embryo	FGFR1, 3, 4	lethality in E7	Philip and Gail 1995 Ornitz *et al*. 2001
FGF10 (KGF-2)	D89080	important role in branching morphogenesis	FGFR2	lethality in P0	Ohuchi *et al* 2000 Ornitz *et al*. 2001

E: embryonic day, P: postnatal day

Table 2 FGF receptors and their substrates which were screeced moecules

FGFR	NCBI Gene bank	Function	KO phenotype	Reference
FGF receptor 1	NM010206 NM001079908 NM001079909	embryonic growth and mesodermal patterning during mouse gastrulation	display severe growth retardation both in vitro and in vivo lethality	Yamaguchi *et al,* 1994
FGF receptor 2	NM010207 NM201601	early postimplantation development between implantation the formation of the egg cylinder and contributes to the outgrowth, differentiation, maintenance of the inner cell mass	trophoblast defect, lethal periimplantation	Arman *et al*, 1998, Xu *et al,* 1998, Rappolee 1988
FGF receptor 3	M81342	mitogenic signal in the embryo related in trophoblast proliferation (blastocyts out growth)	has no lethal periimplantation	Chai *et al,* 1998 Rappolee 1988
FGF receptor 4	X59927	mitogenic signal in the embryo related in trophoblast proliferation (blastocyts out growth)	arrested later in development due to gastrulation defects caused by impairment in cell movement from the primitive streak	Chai *et al,* 1998
FGF receptor substrate 2	NM177798	control of cell movement through the primitive streak during gastrulation	mouse embryo die -E7.5 with defects in extra embryonic development	Hadari *et al,* 2001 Gotoh *et al,* 2005
FGF receptor substrate 3	NM144939		have not been reported yet	

MATERIALS AND METHODS

Animals

NOD mice were used to study the role of FGFs and FGFRs associated with pre-/peri-implantation embryo development and implantation. For the recovery study, diabetic NOD mice were administered with insulin (5IU/kg BW, SC). All experimental animals studied followed to the Guide for the Care and Use of Laborotory Animals published by National Institutes of Health. Animals were maintained under standard conditions at Sungshin Women`s University. Animals were feed a standard rodent diet and water ad libitum from weaning at 21 days of age. Animals were tested twice weekly for urine glucose determination. In suspect diabetic NOD mice, blood glucose was determined using a glucose meter. Diabetes was defined by 21hrs fasting hyperglycemia (>300mg/dl) with weight loss. Age-matched non diabetic NOD and CD-1 mice were used as control.

Embryos collection and uterus sampling

We got uteri on day 4, 5 and 6 of gestation and then embryos were removed from the uterine lumen by flushing. Various stage preimplantation embryos (embryonic day 0.5, 1.5, 2.0, 2.5, 3.0 and 3.5) were collected from oviducts or uteri by flushing with phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Uteri and embryos were quickly frozen using liquid nitrogen and stored at -80℃ in freezer until used.

Vaginal smear

To check the detrimental effects on physiological changes by diabetes mellitus in reproduction estrus cycle was measured with vaginal smear. Briefly vaginal luminal fluid was collected using pipette (100 μl sterilized saline), smeared on slide glass and dried. By the purpose, the slides were stained with hematoxylin and eosin.

Superovulation induction

Diabetic NOD mice were superovulated by injected to 5IU of pregnant mares serum gonadoprophin (PMSG), followed by injection of 5IU of human chronic gonadotrophin (hCG) 48hr. There were mated with wild stud after hCG injection. The next morning of finding a vaginal plug was defined day 1 of pregnancy.

Artificial induction of decidualization

Artificial decidualization was induced as described previously (Cheon *et al*, 2004). Briefly, female mice were subjected to bilateral ovariectomy and, 2 weeks later, were injected subcutaneously with 100ng 17β-estradiol / 0.1ml sesame oil for 3 days. Following two days the mice kept free from hormonal administration. After then 6.7ng 17β-estradiol and 1mg progesterone was administered everyday until sacrificed. At third day, artificial decidualization was induced with mechanical trauma: insertion of a blunt needle into the uterine horn just proximal to the cervix and longitudinally scratching the entire length of the uterine horn along the antimesometrial side. Induced female mice were sacrificed 12hr, 24hr and 48hr, after signaling. For

histological study, half of the stimulated or unstimulated horns were fixed with 10% neutral buffered formalin during 16hrs and treated for histology. To isolate RNA the other parts were quickly frozen with liquid nitrogen and kept at -80℃ freezer until used.

Total RNA extract and first cDNA synthesis

Total RNAs of uteri were extracted using TRIzol Reagent (Invitrogen cat # 15596-018, Carlsbad, CA, USA) following to the manual of manufacture with modification. Briefly, the uterine tissues in 1ml /100mg TRIzol Reagent were homogenized and stored for 10min at room temperature (RT). After then, homogenates were centrifuged 12000g for 20 min at 4℃, kept for 10min at RT and centrifuged 12000g for 20min at 4℃. The clear supernatant was transfer to new tube, added 0.5ml isoprophanol/1ml TRIzol Reagent, mixed well softly for 10min and centrifuged 12000g for 20min at 4℃. The supernatant was removed, added 1ml 80% (ice) ethanol /1ml TRIzol Reagent to wash, vortexed for 10sec and centrifuged 12000g at 4℃ for 10min. The supernatant discarded, dried completely to remove ethanol and added 100μl DEPC treated water. In case of embryos, the collected embryos lysised with Sidestep™ Lysis & Stabilization Buffer (Stratagene cat #. 400901-21, CA, USA) 15 μl. Total RNA measured OD value and stored at -80℃ freezer until used.

Screening the mRNA expression for FGFs, FGFRs and FRs

First strand cDNA was synthesized using First-strand synthesis system (Stratagene Cat #.200420, CA, USA). We used the following mixture for

first-strand cDNA synthesis; reaction reagent is 2.0μl standard buffer (10X), 1.5μl oligo (dT) primer (0.5μg/μl), 0.5μl random primers (0.1μg/μl), 0.8μl dNTP mix, 7μl total RNA (5μg/μl), 8.2μl RNase free water. Reaction mixture was incubated at 65℃ for 5min, place the tube at RT to allow the primers to anneal to the RNA for 10min, after then added 1μl Stratascript RT (50IU/μl) and 1μl RNase block. The tubes were placed at 42℃ for 1hr, incubated at 70℃ for 15min and place the completed first-strand cDNA synthesis reaction on the ice. Target genes transcripts were amplified using PCR method with the FGFs and FGFRs specific primers (Table 3). Primers were synthesized Sigama-Genosys (Santarouis, MO, USA). The primer parameters were 50% GC contents, avoiding repeat base pair and lengthening 20-24 mer. PCR product was analyzed using 1% agarose containing ethidium bromide by electrophoresis.

Histological study

Fixation of uterine sample was done for overnight and the samples were transferred to 70% ethanol. To make paraffin block, water was removed; 100% ethanol 1hr twice, 1:1 100% ethanol, xylene 40min twice and xylene 50min twice. After then, the tissues were put into paraffin for overnight. Using microtome the blocks cut with 4μm and adhered on slides. To stain, paraffin was removed by xylene and rehydrated with alcohole. The sections were rinsed by flowed tap water and stained with hematoxylin and eosin, 4min and 1min, respectively. Samples were dehydrated and mounted with Permount (Fisher Scientific SP 15-500 Toluen solution UN, USA).

Statistics

The t-test was used to evaluate the difference between controls and experiment groups. Results were presented as MEAN ± SD. A p-value less than 0.05 were considered to be a significant difference.

Table 3 Sequences of primers

FGF		Primer sequence (5`-3`)	Amplified length (bp)
FGF1	A	ATGGCTGAAGGGGAGATCACA	423bp
	AS	GCCATAGTGAGTCCGAGGACC	
FGF3	A	ACGCAGAGTGTGAGTTGGTGGAAC	315bp
	AS	TCTGCTTCTTCTGCCTCCGCT	
FGF5	A	CAAACATAAGCGGGAAGCGGAT	488bp
	AS	ACCTCTGACCACCACTAAGCTCA	
FGF8	A	GACAGTGTCCTGCCTAAAGTCACA	320bp
	AS	TCTGTGAATACGCAGTCCTTGC	
FGF10	A	ATCACCTCCAAGGAGATGTCCG	364bp
	AS	TGTTTTTGGTCCTCTCCTGGGAGC	
FGFR1 transcript variant 1	A	CAAGTGAGAGTCAGCTTGCGAA	600bp
	AS	GCTACAGGCCTACGGTTTGGTTT	
transcript variant 2	A	CAAACCAAACCCTGTAGCTCCCT	393bp
	AS	CACATGAACTGGACATTGCTGC	
transcript variant 3	A	CAAGTGAGAGTCAGCTTGCGAA	283bp
	AS	GCTACAGGCCTACGGTTTGGTTT	
FGFR2 transcript variant 1	A	CCAGAAGAGCCACCAACCAAATAC	349bp
	AS	GGTTGCTCCTCTTCTCACTGA	
transcript variant 2	A	CAACACTGTGAAGTTCCGCTGT	460bp
	AS	CAGCACTTCTGCATTGGAGCTA	
FGFR3	A	CCACTCAGTTGTGCGTGTAACAGA	366bp
	AS	ACATCCAGTGTGTATGTCTGCCG	
FGFR4	A	CCACTGGCTCAAGGATGGACA	539bp
	AS	TTGCTGTTGTACGTGAGGT	
FRs2	A	TAGGCTCTGGTGTGATGGAACTCA	349bp
	AS	CCATTAGGTAAGTTCTGAGCACCG	
FRs3	A	CGCTTATGGCTACGACTCCAATCT	412bp
	AS	CCCAGTTGGTGTGTTGACATAGGT	
FRl-1	A	GTTGACCATGTGGACCAAAGATG	383bp
	AS	CTTCATCCACATGATGTCTGG	
36B4	A	CGACCTGGAAGTCCAACTACTTCCT	303bp
	AS	GCACCTTATTGGCCAACAGCA	
ß-actin	A	CAGGGTGTGATGGTGGGAAT	287bp
	AS	TGTGGTACGACCAGAGGCATACA	

RESULT

Diabetic NOD mice lost their ovarian function

Generally, in physiological conditions, the estrus cycle happens rhythmically during supporting fertility. However the cycle can be disturbed by physiological stress. The estrus cycle was studied by vaginal smear for 29 days. Diabetic NOD mice could not maintain the estrus cycle. From 3 weeks after outbreak of DM, there was no more cycle and kept diestrus condition. (Fig. 2). However if insulin was treated, in diabetic NOD mice change of estrus cycle was maintained like in wild type mice (Fig. 2A, B).

Histological changes in pre-implantation stage NOD mouse uterus

To confirm the disturbance in ovarian function by DM, the diabetic NOD was attended in superovulation induction as mentioned in Materials and Methods. The number of ovulated oocytes were significantly decreased (Table 3). Pregnant diabetic NOD mice were sacrificed on day 6 to determine whether pregnancy was occurred or not. The implanted embryos could not detect in diabetic NOD mice (Fig. 3). In addition, the ovary had many corpus albicans. Those results showed that responsibility to the embryo was in very low in DM NOD mice.

To determine the uterine responsibility to the embryo artificial decidualization was induced as mentioned in materials and methods. Decidual response in stimulated horn was induced by trauma in the uteri of diabetic NOD mice and control (Fig 4). The uteri of diabetic NOD were developed glandular epithelial

cells and luminal epithelial cells after 48hr and were developed decidua cells after 72hr. However, the responsibility was decreased compared with control (Fig. 4E, F).

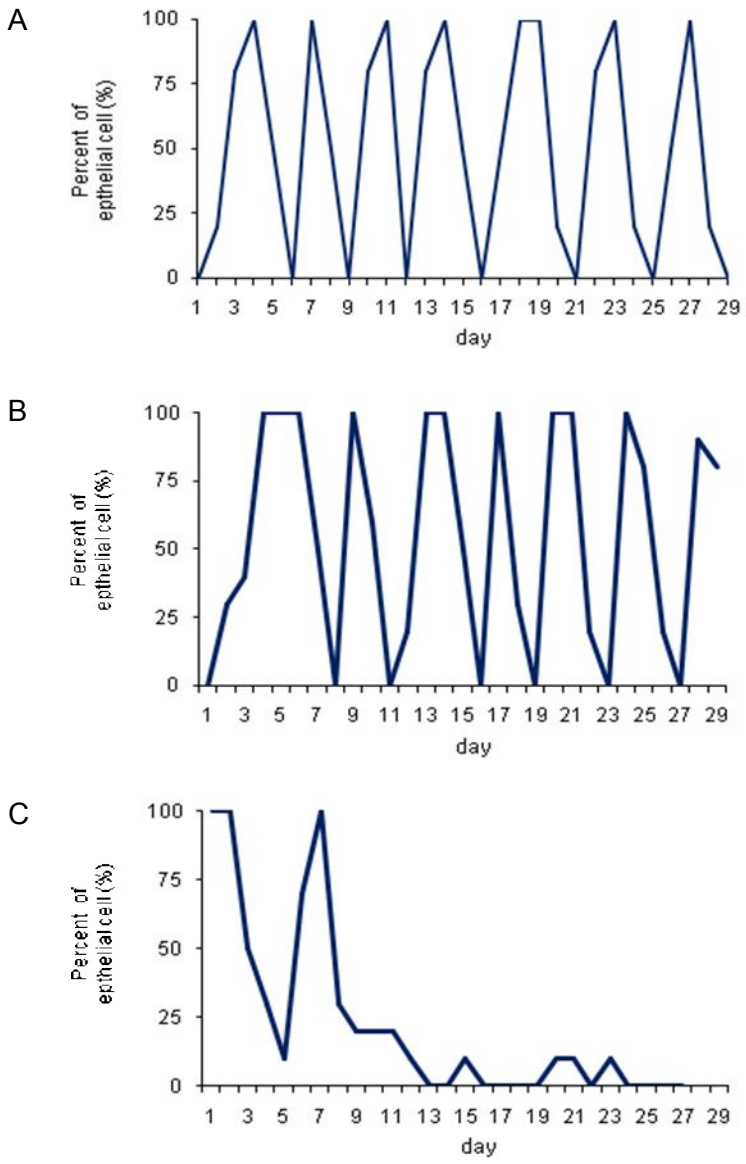

Figure 2. Chronic effects of diabetes mellitus on estrous cycle in NOD mice A. Wild type mice, B. insulin-treated diabetic NOD mice, C. diabetic NOD mice

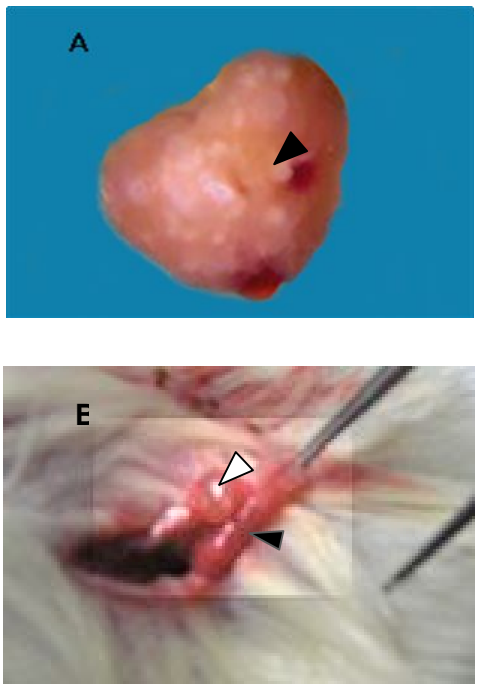

Figure 3. Photograph of ovary and uterus in superovulation-induced NOD mice at day 6 of pregnancy

A. A lot of corpus albicans (arrow head) existed in ovary of diabetic NOD mice.

B. Uteri at day 6 of pregnancy were pulled out to check the implantation sites after operation: empty arrowhead showed ovary and occupied arrowhead showed uterus.

Table 4. The number of ovulated oocytes after superovulation induction

	Control (No)	DM NOD (No)
Number of ovulated oocytes	28 ± 4.5 (24)	4.4 ± 8.8 (4)*

The numerical numbers in parentheses indicate a percentage of the total of the animals examined.

Values are mean ± SD (n=4)

* $p<0.05$ control vs DM NOD

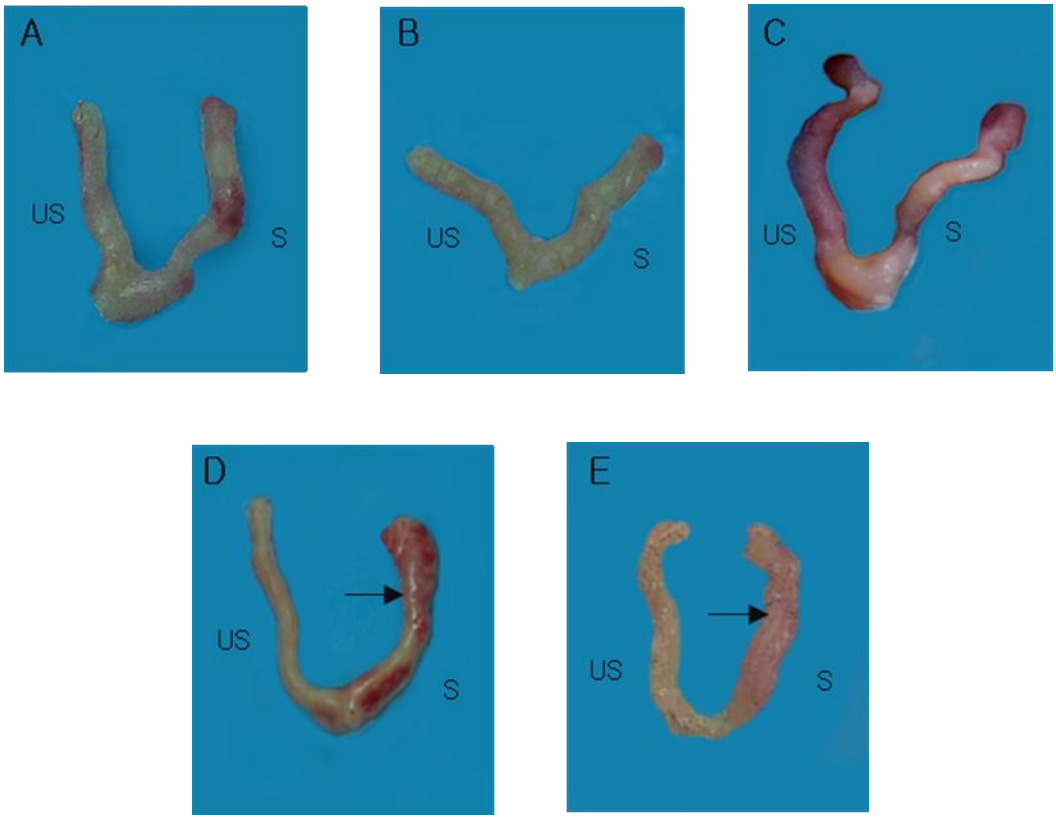

Figure 4. Artificial-decidual induction in diabetic NOD mice

Artificial decidualization induced by trauma and sacrificed at 12hr (A), 24hr (B), 48hr (C) and 72hr (D). E is decidualized uterus in control mice. US:unstimulated, S:stimulated

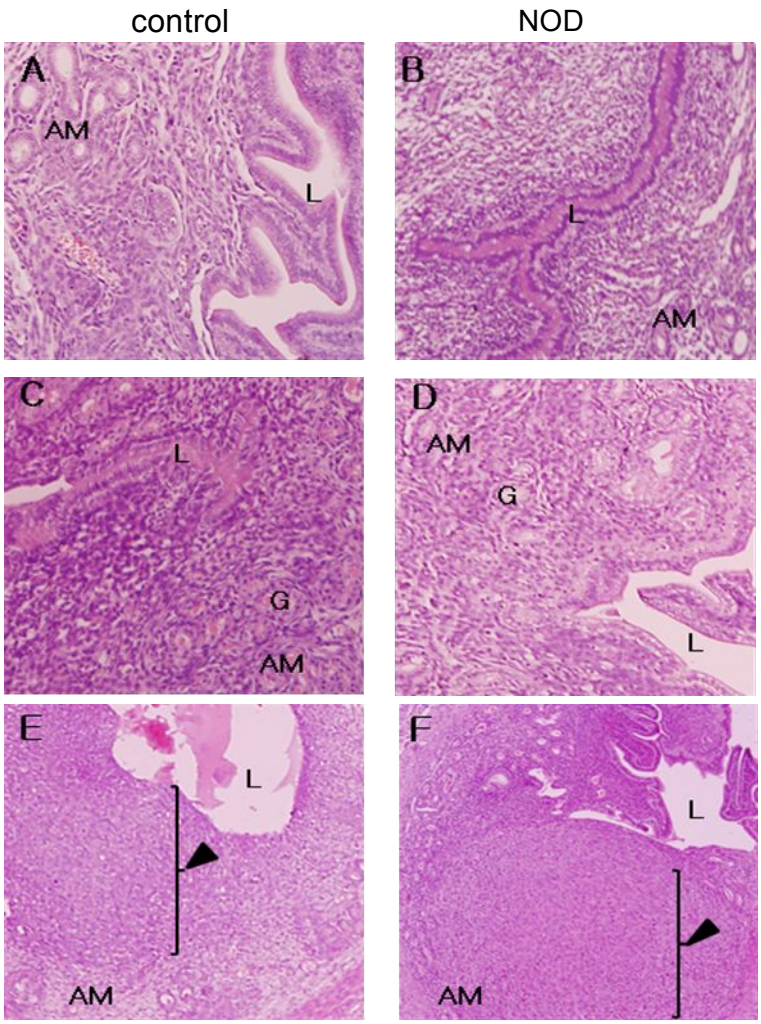

Figure 5. Photomicrograph of uteri in decidualized uteri in diabetic NOD mice

Daibetic NOD mice were ovariectomized and administed with steroid hormons as mentioned in Materials and Methods. After artificial Decidual induction control and deciduas induced uteri were collected at 24hr (A, B), 48hr (C, D) and 72hr (E, F). A, C and E are control and B, D and F are deciduas induced uteri. AM:antimesometium, L:lumen, G:gland

FGF ligands profile both control and diabetic NOD during pregnancy

FGF1, FGF3, FGF5, FGF8 and FGF10 were screened in various physiological status of uteri after pregnancy. FGF1 expressed all day except on day 2 of gestation and FGF5 expressed since on day 3. FGF3 expressed on day 5, 6 and 7 of gestation. FGF8 expressed on day 3, 5, 6 and 7 of gestation and FGF10 expressed on day 1, 4, 6 and 7 of gestation (Fig.6). Also, FGF expression pattern experimented in artificial decidualization induced diabetic NOD mice uteri to compare with control (Fig. 7). In the condition for artificial decidual induction, FGF5, FGF8, FGF10 expressed at 24hr, 48hr and 72hr after stimulation and FGF1 expressed at 24hr and 48hr, FGF3 expressed at 72hr stimulated uteri (Fig. 7A). In the case of diabetic NOD mice, FGF10 expressed at 24hr, 48hr and 72hr regardless of stimulation. FGF1, FGF5 expressed all that time in control uteri, but only expressed at 24hr after stimulation in diabetic NOD uteri. FGF3 expressed 24hr stimulated diabetic NOD uteri. But FGF8 did not observed expression both stimulation and control diabetic NOD uteri (Fig. 7B).

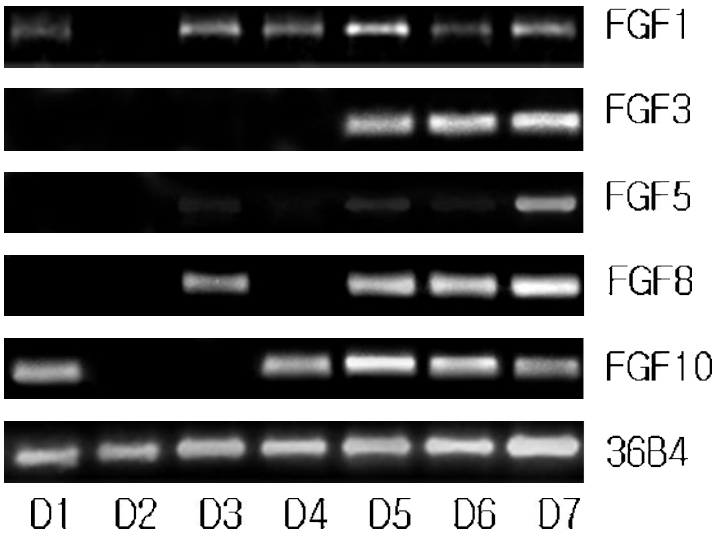

Figure 6. Profiles of expression of FGF ligands expressed in mouse uterus Uteri were collected in pregnant mice on day1 (D1), day2 (D2), day3 (D3), day4 (D4), day5 (D5), day (D6) and day7 (D7).

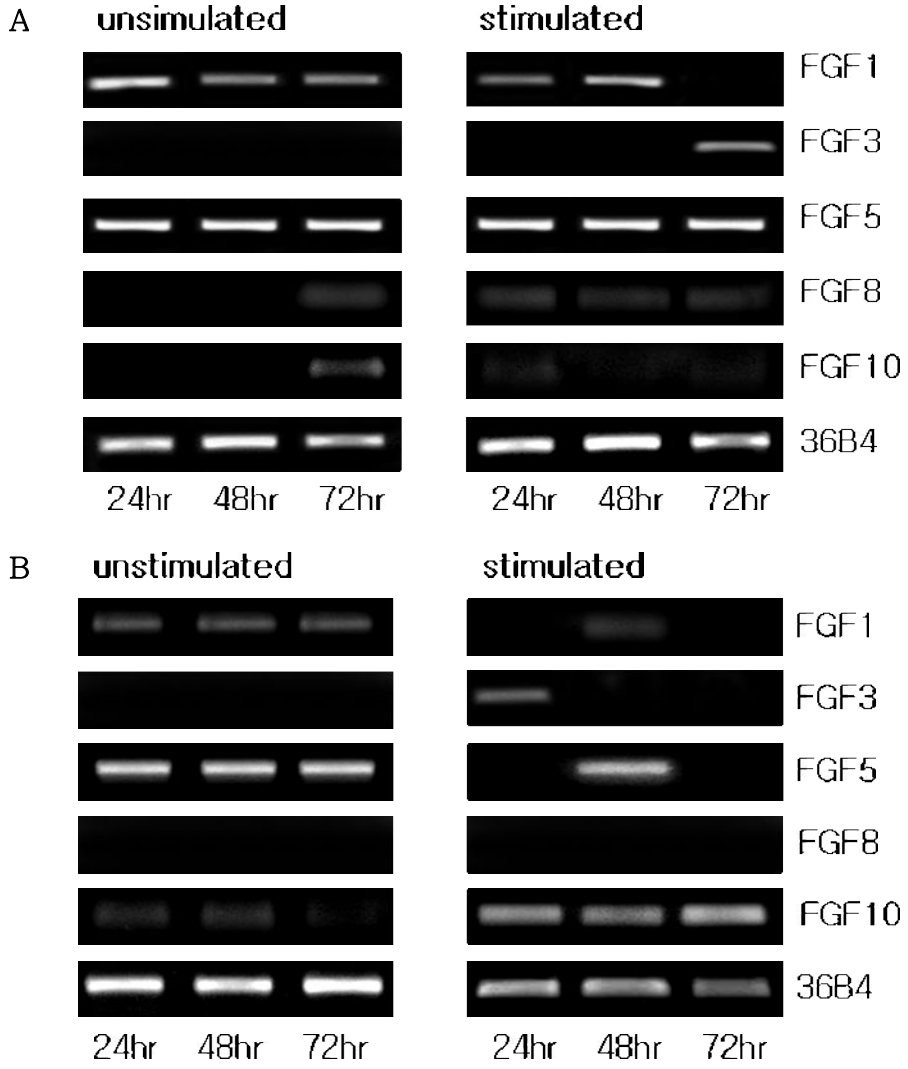

Figure 7. Profiles of expression FGF ligands in mouse uteri artificial-decidual induction

Expression profiles of FGF ligands in control (A) and diabetic NOD mice (B). Uteri were collected of 24hr, 48hr and 72hr after decidual stimulation. Trauma was given in left horn (stimulated) and right horn was used as control (unstimulated).

FGFRs profile both control and NOD mouse during pregnancy

FGFR1-1 and FGFR1-3 expressed in the uteri of day 7 of gestation. FGFR2-1 expressed just except day 2 and 3. FGFR3 expressed all the samples, FGFR4 expressed only at day 3 and 7 of gestation (Fig. 8). FGFR1-2 and FGFR2-2 did not express. FRs2 and FRs3. FRs2 expressed on day 6 of gestation but FRs3 did not express during early pregnancy (Fig. 8). In control mice, FGFRs expression pattern was similar between stimulated uteri and unstimulated uteri (Fig. 9A) except FGFR4. FRs 3 expression could not defect in 72hr stimulated samples. Among others, FGFR4 expressed from 24hr to 72hr and FRs3 did not express at 72hr after stimulation. But, in diabetic NOD mice, expression profiles of FGFRs were dramatically different from control mice. Only FGFR2-1 could be detected in stimulated uteri.

FGF profile in embryo development

FGF1, 8 and 10 expressed from pronucleus to blastocyst stage but FGF3 and 5 could not detect in pre-implantation stage embryos (Fig. 10A). FGFR1, FGFR transevariant 1, 2 and 3 did not expressed in embryo. FGFR2-1, FGFR3 and FGFR4 expressed in preimplantation stage embryo. FGF receptors like-1 (FRl-1) did not express in embryo (Fig. 10B).

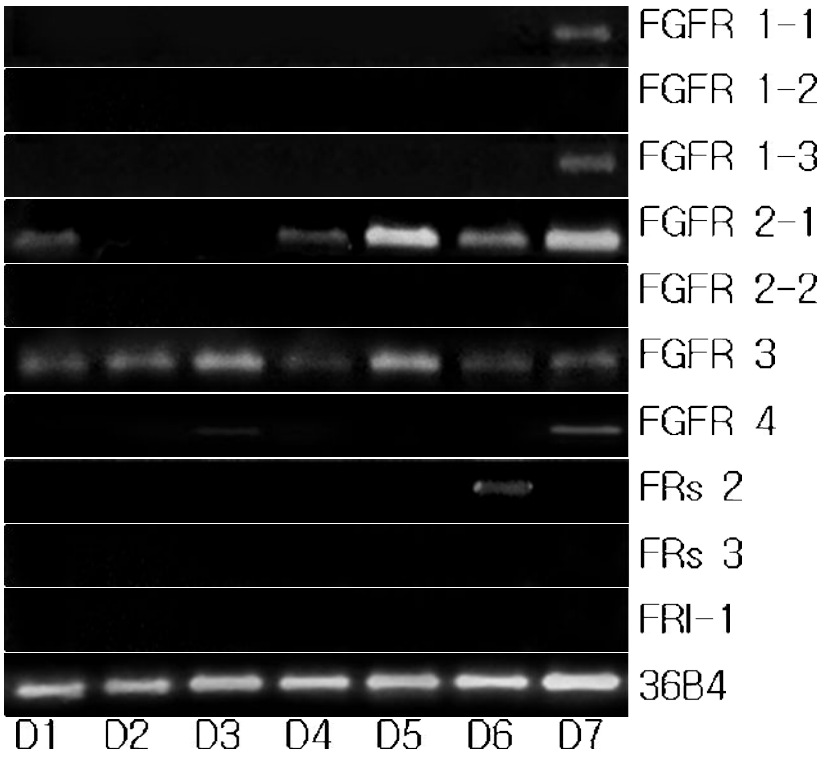

Figure 8. Profiles of expressed FGF receptors in pregnant uteri

Uteri were collected in pregnant mice on day1 (D1), day2 (D2), day3 (D3), day4 (D4), day5 (D5), day (D6) and day7 (D7). FGFR: Fibroblast growth factor receptor, FRs: FGFR substrate, FRI-1: FGFR like-1

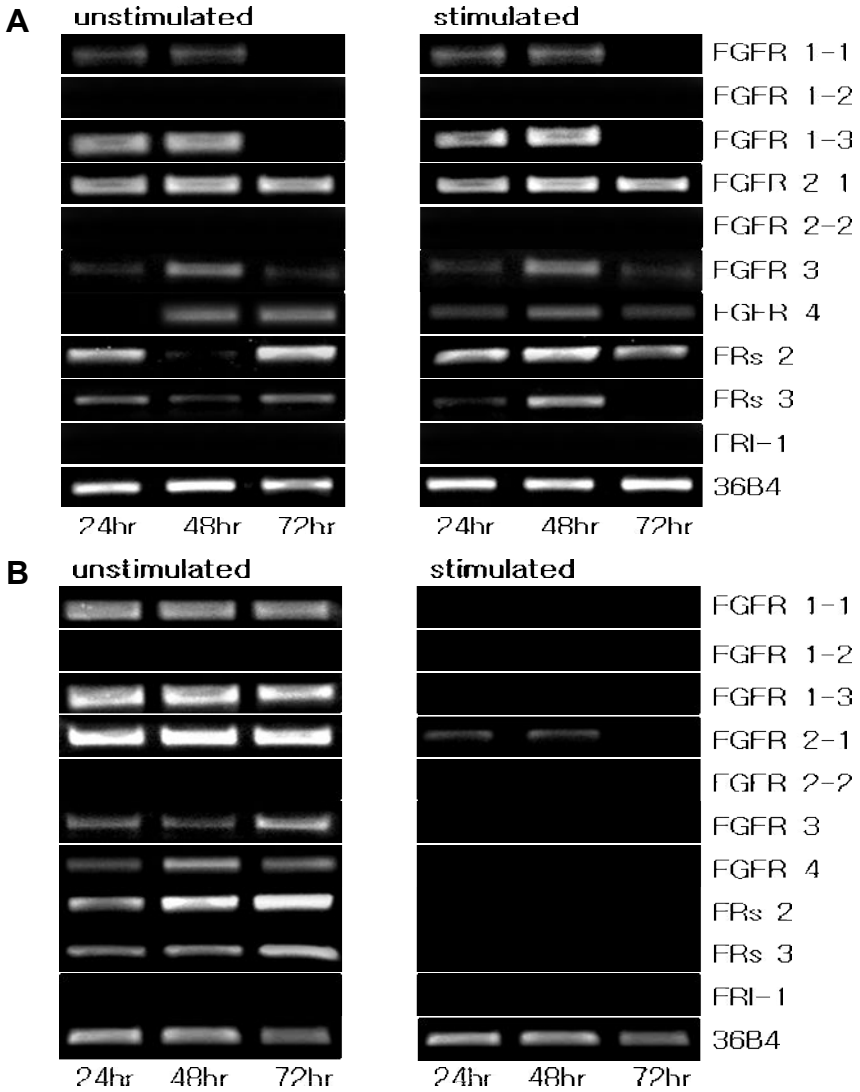

Figure 9. Profiles of expression FGF ligands in mouse uteri after artificial-decidual induction

Expression profiles of FGF ligands in control (A) and diabetic NOD mice (B). Uteri were collected of 24hr, 48hr and 72hr after decidual stimulation. Trauma was given in left horn (stimulated) and right horn was used as control (unstimulated). FGFR: Fibroblast growth factor receptor, FRs: FGFR substrate, FRI-1: FGFR like-1

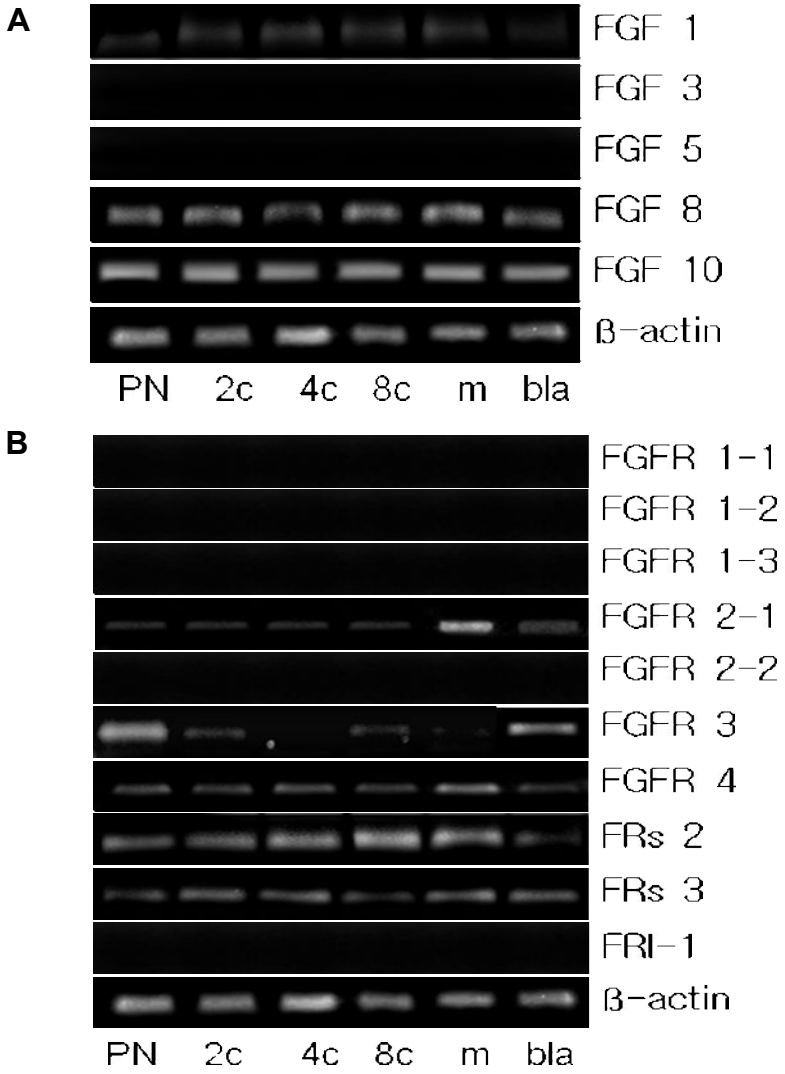

Figure 10. Profiles of expression FGF ligands and FGF receptors in mouse pre-implantation embryo

A. FGF ligands expression in embryo, B. FGFRs expression in embryo; PN: pronucleus cell, 2c: 2 cell, 4c: 4 cell, 8c: 8 cell, m: morula, bla: blastocyst FGFR: Fibroblast growth factor receptor, FRs: FGFR substrate, FRl-1: FGFR like-1

DISCUSSION

Diabetic NOD mice lost their fertility by defection in ovarian function. In the present study chronic diabetic NOD mice lost their rhythmical reproductive cycle and finally stopped the cycle. The number of ovulated oocytes significantly decreased in chronic diabetic NOD. It has been suggested that chronic DM is a cause of disruption in meiosis and follicular genesis (Kim *et al*, 2007). Our results support that suggestion. In the case of uterus, it could response to decidual signals in the artificial conditions. The uteri of diabetic NOD mice existed gland and epithelial cells at 24hr and 48hr. It is reported that on day 4 of pregnancy epithelial cells cease to proliferate and become differentiated. With the initiation of blastocyst attachment reaction, stromal cells at sites of blastocyst apposition undergo proliferation and differentiation into decidual cells (Huet-Hudson *et al,* 1989). In pregnant mice, the stimulus for decidualization is the implanting blastocyst. On day 5 at the beginning of decidual cell reaction, only stromal cells surrounding the implanting blastocyst proliferate. It means that uterus have ability to carry embryo. but there were different from control at 72hr in decidual cell and reaction. On day 6, the stromal cells in immediate proximity to blastocyst apposition cease to proliferate and form the decidual zone (Dey, 1996). Therefore, it is suggested that the reduced site of decidua results in the lower proliferation rate in DM NOD mice.

FGF ligands expressed peri-implantation stage uterus except FGF3, but FGF 8, FGF 10 expression decreased in trauma stimulated NOD mice uterus. In

middle of FGFR, only FGFR 2 expressed in stimulated uterus. In this result, FGF ligands and FGFR expression were mimic in pre-implantation uterus. Like wise, when from day 1 until day 3 of gestation before becoming pregnant seeing with pre-implantation stage, FGF1 and FGF10 expressed in uterus after fertilization time and implantation day. FGF1, FGF5, FGF8, FGF10 expressed in before implantation uterus and in after implantation period. FGF 8 and FGF 10 may associate with preimplantation and peri-implantation. It is reported that FGF10 expressed in preimplantation uterus. FGF2 and FGF10 showed unique patterns in the stroma from the time of implantation. FGF2 transcripts were localized in the antimesometrial stroma at the site of implantation. By contrast, the expression of FGF10 was localized mesometrially. The expression of FGF4 in the uterus was insignificant during the peri-implantation period (Bibhash C *et al*, 2001). In the case of FGFR, FGFR2 and FGFR3 to be expressed from before implantation to preimplantation and the remainder FGFR is visible the aspect which is been expressed in post implantation. In human, expression of FGF10, FGFR1 and FGFR4 in the maternal–fetal interphase suggests their role in decidual-trophoblast interaction. Thus FGF1, FGF5, FGF8, FGF10, FGFR2 and FGFR3 may involved in uterine differentiation during pre- and peri-implantation endometrium.

FGF1, 8 and 10 were expressed from PN stage to blastocyst stage. Also the FGFR 2-1, FGFR3, FGFR5, FRs2 and FRs3 expressed from PN stage. In diabetic NOD mice, FGF10 and FGFR2 expressed in stimulated uterus. FGF10 is considered to be the major ligand for FGFR2, and it is usually expressed in the mesenchyme surrounding the developing FGFR2 positive

epithelia (Ohuchi *et al*, 2000). FGF8 and FGFR3 which related pre-implantation in our result did not express, the knock mice phenotype of FGFR3 has no lethality in pre-implantation but defected cell proliferation, FGF8 has lethality in E7 and expressed pre-implantation uterus. It was demonstrated that FGF8 and FGFR3 has high-affinity and FGFR3 expression is in contact with the FGF8 expression domain in the rostroventral hindbrain (Blak *et al*, 2005). FGF1 decreased expression in NOD mice. FGF1 is the only ligand that binds to all FGF receptor as well as to FGFR4 (Ornitz *et al*, 1996; Orr-Urtreger *et al*, 1993). In the case of FGFR has no lethality except of FGFR2, but FGFR related in cell proliferation and mitogetic factor and important role in implantation as well as embryo development (Yamaguchi *et al,* 1994; Arman *et al*, 1998; Xu *et al,* 1998; Rappolee, 1998; Chai *et al,* 1998; Hadari *et al,* 2001; Gotoh *et al,* 2005). Immunoreactive FGFR1 has also been identified in glandular epithelial cells, stromal fibroblasts, and endothelial cells in the human endometrium, raising the possibility that this growth factor exerts numerous intracrine, autocrine, and/or paracrine interactions in the uterus (Sangha *et al*, 1997). These suggest that to defect FGFR in uterus may have negative influence on embryo development and uterine preparation implantation.

On summary, in our model mice have and response to decidual signals. However the size of decidua was smaller than that of control. In model mice, FGFs patterns of expression were different from the control. Based on, it is suggested that FGFs and FGFRs may associate with uterine differentiation for embryonic implantation and embryo development.

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Role of fibroblast growth factor during pre- and peri-implantation: A type I diabetes mellitus (DM) NOD mouse

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The process of embryo implantation is highly controlled by the coordinated action of the steroid hormones and other regulation factors. FGF has many roles for maintain the pregnancy and it has been suspected as a regulator for embryo implantation. To know the roles of FGFs in embryo implantation, we used nonobese diabetic (NOD) mouse. NOD mice are one of the best-studied animal models for type 1 diabetes mellitus as it spontaneously develops the disease and has developmental abnormalities, such as diminished rates of implantation and viable embryos, and structural changes in the reproductive organs. Specific pattern of estrus cycle observed in normal mice was destroyed time-dependently after outbreak of diabetes mellitus (DM) with hyperglycemia. From 3 wks after outbreak of DM, estrus cycle became irregular, and finally stopped the rhythmical change and kept the diesturs stage. However insulin treated-NOD mouse did not change estrus cycle. In these DM mice, decidual response did not induced by trauma under the artificially maintained hormonal levels. FGFs and FGF receptors were detected spatiotemporally in pregnant uteri of various physiological conditions in control pregnant mice. FGFs also identified in the periimplantation stage embryos.

The spatiotemporal expression of FGFs and FGF receptors were abolished in the DM uteri under the condition of artificially induced decidual signal. From these results, it is suggested that FGFs-mediated signals may involved in control of embryo implantation and uterine responsibility.