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# The roles of KROX-24 in ovulation in mouse

: Krox-24 in ovulation and expression  
regulation of putative downstream genes

2008

성신여자대학교 대학원  
생물학과  
이 자 명

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Krox-24 in ovulation and expression  
regulation of putative downstream genes

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성신여자대학교 대학원

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# The roles of KROX-24 in ovulation in mouse

Krox-24 in ovulation and expression  
regulation of putative downstream genes

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Submit a master's thesis

November, 2007

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이 자 명 의 석 사 학 위 논 문 으 로 인 준 함 .

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## 논문개요

난포형성 (folliculogenesis)은 성선자극호르몬, 스테로이드 호르몬, 자가-타가 조절요인의 조절을 통하여 진행되며, 궁극에 가서는 선택된 성숙난포에서 난자의 배란이 진행된다. 배란은 황체화 호르몬 (LH)에 의해난포세포에서 발현되는 단백질 분해효소, 면역세포조절물질 그리고 배란이 진행될 특정부위로 모이는 면역관련 세포들에 의해 진행되는 것으로 알려져 왔으나, 아직 많은 부분이 밝혀져 있지 않다. 따라서 황체화 호르몬 급증 이후 난자가 배란 되기까지 시기 특이적이고, 조직 특이적으로 난소에서 발현되는 유전자를 동정하기 위하여 DNA microarray 방법을 이용하였다. 황체화 호르몬급증 이후 시기 특이적으로 발현되는 유전자 중 배란과 관련된 유전자로 기대되는 유전자를 microarray 결과와 알려진 생물학적 기능을 근거로 하여 동정하였다. 그 중 Krox-24는 전사조절 인자로 뇌하수체에서 황체화 호르몬을 생성하는 것으로 잘 알려져 있기 때문에 본 연구에서는 난포형성과 배란에 있어서 Krox-24가 어떠한 역할을 할 것인가를 다양한 실험방법을 이용하여 알아보고자 하였다. 생쥐 난소의 Microarray data 결과와 동일하게 RT-PCR을 이용한 분석에서도 Krox-24는 RT-PCR에서 황체화 호르몬 주사 후 hCG 주사 후 시기 특이적으로 2~4시간대에 난소에서 발현 양이 급증하였다. 조직학적 분석에서 10개월 이상 된 Krox-24 형질전환 생쥐의 난소에서는 난포가 거의 없었으며 관찰된 난포는 혈액이 고이거나 또는 비정상적 형태를 보였다. 생식주기가 4~5일 단위로 규칙적으로 진행을 보인 대조군 생쥐와 달리 Krox-24 형질전환 생쥐에서는 주기적 변화가 관찰되지 않고 비발정(diestrus) 상태가 지속되었다. 황체화 호르몬을 인위적으로 주어 Krox-24 형질전환 생쥐의 뇌하수체에서의 문제점을 극복하고 배란에서의

역할을 알아보았다. 난포자극 호르몬 (FSH)과 황체화 호르몬을 이용한과배란 유도 실험에서 배란 전 난자의 수가 대조군에 비하여 유의하게 감소하였고, 난구세포 (cumulus)가 균테균테 엉킨 건강하지 않은 팽창 양상을 보였다. 또한 hCG 주사 후 배란이 진행 된 시간 이후 난소 조직 절편에서 난자가 배란되지 못하고 난포 내에 갇혀 있는 것을 확인할 수 있었다. Krox-24와 이 유전자에 의해서 조절되는 것으로 알려진 a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS-1)와 tissue growth factor- $\beta$  (TGF $\beta$ )의 배란 전후 발현 양을 알아보고자 RT-PCR을 수행하였다. 대조군에서 Krox-24는 hCG 주사 후 2~4시간째, ADAMTS-1은 12시간째, TGF $\beta$ 는 모든 실험 시점에서 발현하였다. 그러나 Krox-24 형질전환 생쥐에서는 ADAMTS-1은 hCG 주사 후 9, 12, 15시간에 강하게 발현하였다. 또한 ADAMTS-1 은 프로게스테론 수용체 (PR) 하부 및 다른 조직에서 Krox-24의 하부 유전자로 알려져 있어 Krox-24에 의한 프로게스테론 수용체의 발현 조절 가능성을 in silico 방법을 이용한 프로모터 (promoter)부위 분석을 통하여 추정하였다. 프로게스테론 수용체는 hCG 주사 후 4시간째에 강하게 발현하였는데, 형질전환 생쥐에서는 모든 시점에서 발현하였다. 프로게스테론 수용체의 프로모터 부위에 Krox-24 결합 부위 (binding domain)을 찾을 수 있었다. 이러한 결과를 바탕으로 Krox-24는 난포형성과정에서 난자-난구복합체의 정상적 분화에 관여할 것으로 추정된다. 또한 Krox-24는 프로게스테론 수용체를 매개로 ADAMTS-1 등의 하부 유전자 발현조절을 통하여 배란 조절에서 중요한 역할을 수행할 것을 알 수 있었다.

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## Introduction

Ovulation is a critical step in reproduction and is a cause of embryo-uterine communication. Ovulation looks like inflammation reaction but ovulation mechanisms could not explained with one factor because it is very complex. In ovulation process, luteinizing hormone (LH) surge works as a triggering event and suggested as a regulator for ovulation (Robker *et al.* 2000 Kim *et al.* 2006). Using microarray methodology, we identified ovulation time specific genes which were showed time specific and tissue specific expression (Fig. 1).

One of the interesting genes was Krox-24. Krox-24 (Egr-1, NGFI-A, zif268 and TIS8) is one of a family of zinc-finger transcription factors which includes NGFI-C, Krox-20, and Egr-3 (Slade *et al.* 2000). Protein Krox-24 have a Cys<sub>2</sub>-His<sub>2</sub> zinc finger DNA binding domain which binds to a cognate DNA element, specific GC-rich enhancer element, (consensus: GCG (G/T) GGGCG) recognized in the promoters of a variety of gene, and activates transcription (Slade *et al.* 2000). Especially Krox-24-dependent gene expression involved functional positive and/or negative between Krox-24 and a number of other

transcription factors including steroidogenic factor-1 (SF-1) (Mouillet *et al.* 2004 Lee *et al.* 1996), glucocorticoid receptor (Wong *et al.* 1998), p53 (Liu *et al.* 2001), Sp1 (Srivastava *et al.* 1998), NAB1 (Russo *et al.* 1995) and NAB2 (Svaren *et al.* 1996).

Role as a transcription factor is depending on Ras-Raf-MEK-ERK1/2 and serum-response elements (Sakamoto *et al.* 1991). The nuclear accumulation of Krox-24 appears to require interaction of Gab-1 (growth factor receptor-bound protein 1 (Grb-2) – associated binder-1) with ERK1/2 via the proline-rich MET-binding domain in Gab1 (Osawa *et al.* 2004)

Krox-24 expression is quite widespread; the endothelial system, thymus, muscle, cartilage, bone, and part of the central and peripheral nervous system (Mcmahon *et al.* 1990 Watson *et al.* 1990). Indeed Krox-24 induced in many cell types by growth, differentiation, and apoptotic stimuli (Lee *et al.* 1996). Several line of evidence indicated that Krox-24 is an important regulator of inflammation (Kim 2006). Also, Krox-24 plays the important role in the ability of IL-13 to stimulate chemokines, proteases, antiproteases, apoptosis regulators, and activate TGF $\beta$  and caspases (Cho *et al.* 2006). And it

mediates apoptosis, and is a prerequisite for tissue growth factor- $\beta$  (TGF $\beta$ )-induced fibrosis and remodeling (Lee *et al.* 2004).

Follicle stimulating hormone (FSH) induces the growth of recruited secondary follicles or early tertiary follicle, LH induces ovulation in the large follicles through both oocyte maturation induction and structural modification in the wall of large follicles. Even the gonadotropin is a primary governor in ovulation, but their action modulated at the ovarian level by tissue receptivity and by local regulators. These local regulations can operate within the follicles or between follicles.

A few of molecules are suspected as a local regulator for ovulation. Krox-24 expressed in growing follicles. And its expression is localized in the granulosa layer, and possibly in the theca internal layer of the larger antral follicles. FSH as well as LH can induce expression of Krox-24 in granulosa cells *in vivo* and *in vitro* (Russell *et al.* 2003). It has been suggested that, Krox-24 have a role in regulating the rapid reprogramming of follicular cells to luteal cells (Richard *et al.* 2004 Robker *et al.* 1998).

According to our previous microarray data Krox-24 and its

binding proteins expression after LH surge, it showed peak after LH surge (Fig. 1). In addition Krox-24 is known as an important regulator for inflammation. Based on them it is suspected that Krox-24 is involved in ovulation. Therefore, in this study, we examined the role of Krox-24 in ovulation and checked its possible downstream genes.

## **Material and Methods**

### **Animal**

All experiments involving animals were conducted according to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health. Animals were maintained under standard conditions at Sungshin Women's University diurnal rhythm kept under the 14L : 10D schedule with light-on at 06:00 hr and clean room system. Animals were fed a standard rodent diet and water *ad libitum* from weaning at 21 days of age.

Colonies of Krox-24 knockout (KO) mice were sustained by hetero female mice and KO male mice. The genotype of the mice was determined by PCR analysis of genomic DNA from tails using Extract-N-Amp™ Tissue PCR Kit (SIGMA, St. Louis, MO, USA).

### **Oocyte collection and *In Vitro* Fertilization**

To know the fertility of Krox-24 KO mice, ovulation was induced using gonadotropins. Animals were primed with 5 i.u. pregnant mare's serum gonadotropin (PMSG) by i.p. injection,

and injected 5 i.u. human chorionic gonadotropin (hCG) after 48 hr ovaries were collected according to the time schedule and then placed in culture medium, BWW contained 0.4% bovine serum albumin (BSA). The large follicles were punctured with sterile needles, and cumulus-oocyte complex were collected, washed, and transferred to medium for *in vitro* fertilization. In the case of oocyte collected from large follicle at 0, 2, 4, 6, 9 and 12 hr post hCG injection were kept in maturation medium and used for further examination.

Embryos (10 per group) were cultured at 37°C under 95% air and 5% CO<sub>2</sub> in 10µl droplet of BWW containing 0.4% BSA.

### **Chemicals and Primers**

Most of chemicals and reagents purchased from Sigma-Aldrich Corp. (St.Louis, MO, USA). Agarose purchased from SeKem Corp.(Takara, Japan). All of the target gene specific primers were synthesized at Sigma-Genosys (St. Louis, MO, USA) and Bionics (Seoul, Korea). They were adjusted to 48~53% GC content and to avoided repetition. The lengths of primer were adjusted to total 21~24mer (Table 1).

**Table 1. Primers of putative Krox-24 regulated genes**

Name	Sequence (5'-3')	Size(mer)	Tm(°C)	GC content (%)	Product size(bp)
36B4 S	CGA CCT GGA AGT CCA ACT ACT TCC T	25	68.23	52.0	
36B4 AS	GCA CCT TAT TGG CCA ACA GCA T	22	68.83	50.0	303
PR S	CAT GAC TGA GCT GCA GGC AAA	21	68.73	52.17	
PR AS	AAG CTC TGG CCC AAA GAG ACA	21	67.24	52.17	445
ADAMTS-1 S	AGG AGT TCT TCA TTC AGC CAG CG	23	69.56	52.38	
ADAMTS-1 AS	AGG TAA TGC TTT AGA CCG CTG CC	23	68.0	52.38	367
TGF beta 1 S	CGG TGC TCG CTT TGT ACA ACA	21	68.18	52.38	
TGF beta 1 AS	CCG TAC AAC TCC AGT GAC GTC AA	23	68.14	52.17	389

## **Vaginal smear**

Krox-24 KO mice show defect in LH $\beta$  subunit synthesis. In order to examine the role of Krox-24 in synthesis of sex steroid hormones indirectly, Krox-24 KO and wild-type mice were investigated to by vaginal smear for 2 weeks. Analysis was undertaken to define by the presence of epithelial cells.

## **Histological analysis**

To determine the roles of Krox-24 in ovulation regulation, histological analysis was undertaken in ovaries collected from 2, 6, 10 and 12 months old Krox-24 KO mice. Collected ovaries were fixed with 10% neutralized buffered formalin for overnight and transferred to 70% Et-OH. And then samples put in the 100% Et-OH for 60 minutes in twice, put in the mixed 100% Et-OH and xylene solution for 40 minutes 2 times, put in the 100% xylene solution for 40 minutes 2 times, put in paraffin for overnight and embedded with paraffin. We did serial sections (4 $\mu$ m), mounted on glass slides, and stained with hematoxylin and eosin.

## **Krox-24 and putative Krox-24 downstream gene expression analysis**

We examined the expression pattern of Krox-24 gene and its putative downstream target genes which are known in other tissues as a Krox-24 target gene. Wild-type female mice (3 weeks) were superovulated with 5 i.u hCG injection after 48 hrs in 5 i.u PMSG primed mice. Mice were sacrificed at exact times by experimental schedules (at 0, 2, 4, 6, 9, 12 and 15 hrs after hCG injection), and ovaries were quickly frozen with liquid nitrogen and stored at -80°C.

Total RNA was extracted with TRIzol (Invitrogen, San Diego, CA, USA), according to the manufacturer's instructions and stored at -80°C. In order to the reverse transcription (RT), seven micrograms of total RNA were used. First strand cDNAs were synthesized using First Strand cDNA synthesis kit (Stratagene, CA, USA) according to the manual of company with modification. Briefly the mixtures were incubated at 42°C for 60 minutes and incubated at 70°C for 15 minutes to terminate cDNA synthesis. PCR did under the presented condition at Table 2. The products were resolved on a 1% agarose gel run in TBE buffer (0.45M Tris-borate, 0.0001M

**Table 2. Thermal Cycler schedule**

<b>Step</b>	<b>Temperature (°C)</b>	<b>Time</b>	<b>Cycle</b>
<b>Initial cycle</b>	94	5 mins	1 cycle
	59	30 sec	
	72	1 mins	
<b>Denaturation</b>	94	1 mins	37 cycles
<b>Annealing</b>	59	30 sec	
<b>Extension</b>	72	1 mins	
<b>Final cycle</b>	94	1 mins	1 cycle
	59	30 sec	
	72	7 mins	
<b>Hold</b>	4	indefinitely	

EDTA) and visualized by ethidium bromide (Matsuoka *et al.* 2007)

### **Statistical analysis**

Data shown are an average ( $\pm$ SD) of 6 biological replicates. At *t* distribution analysis was performed on the samples within each set. A p-value less than 0.05 was considered to be a significant difference.

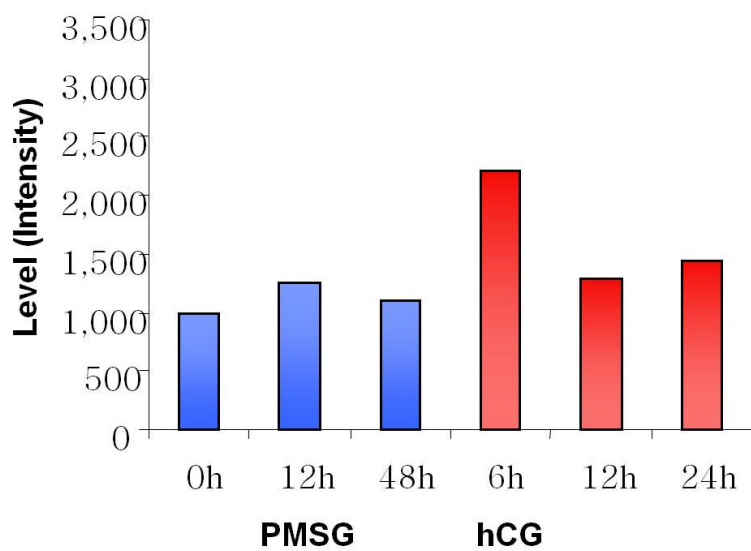
## **Result**

### **Krox-24 expression in preovulatory follicles**

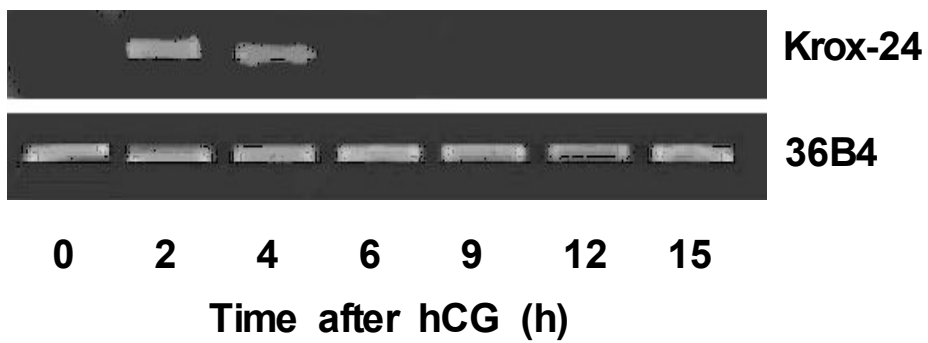
Using microarray method, we identified Krox-24 which was expressed by hCG (Fig. 1). Using RT-PCR method, we could detect the Krox-24 within 2 hr after hCG injection in wild-type mice. It showed maximal expression at 2 hr and continued until 4 hr after hCG injection (Fig. 2).

### **Detrimental effect of Krox-24 in ovary**

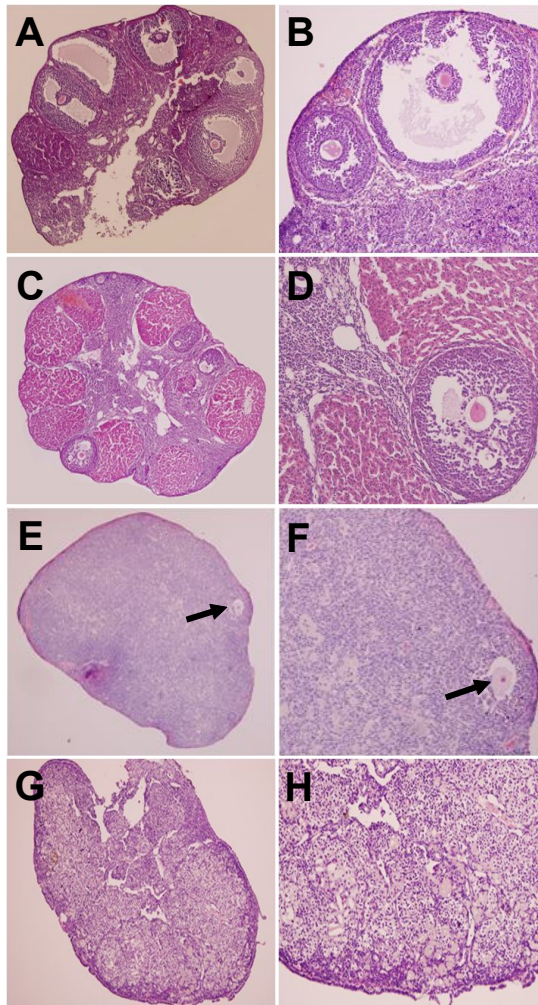
It is suspected that Krox-24 involved in folliculogenesis or ovulation, we examined the detrimental effect of Krox-24 using Krox-24 KO mice. Krox-24 KO mice were sacrificed according to the time schedule (2, 6, 10 and 12 months) and ovaries were used for histological analysis. Various stage follicles were existed until 6 months. However, the pools of follicle were dramatically reduced in 10 months old female. Large follicles or corpus lutea could not find in the ovarian samples (Fig. 3E-F). After 12 months, ovarian structures were totally changed. Follicles did not exist in those ovary (Fig. 3G-H)



**Figure 1. Expression profiles of Krox-24 mRNA in ovary during superovulation induction in microarray data-base.**



**Figure 2. Induction of Krox-24 mRNA expression by hCG. Expression of Krox-24 mRNA showed maximal expression from 2 hr until 4 hr post hCG injection.**



**Figure 3. Ovarian defect in Krox-24 KO mice. Various stages of follicles were observed in the young mice, 2 months old (A and B) or 6 months old (C and D). However the pool of follicles was dramatically reduced in 10 months old (E and F). These ovaries had few follicles. Some of small follicles were accumulated with blood (arrow showed hemorrhage follicle E and F). In the case of 12 months old, follicles did not exist in ovary. Magnification of A,C,E,G = x 40 and B,D,F,H = x200.**

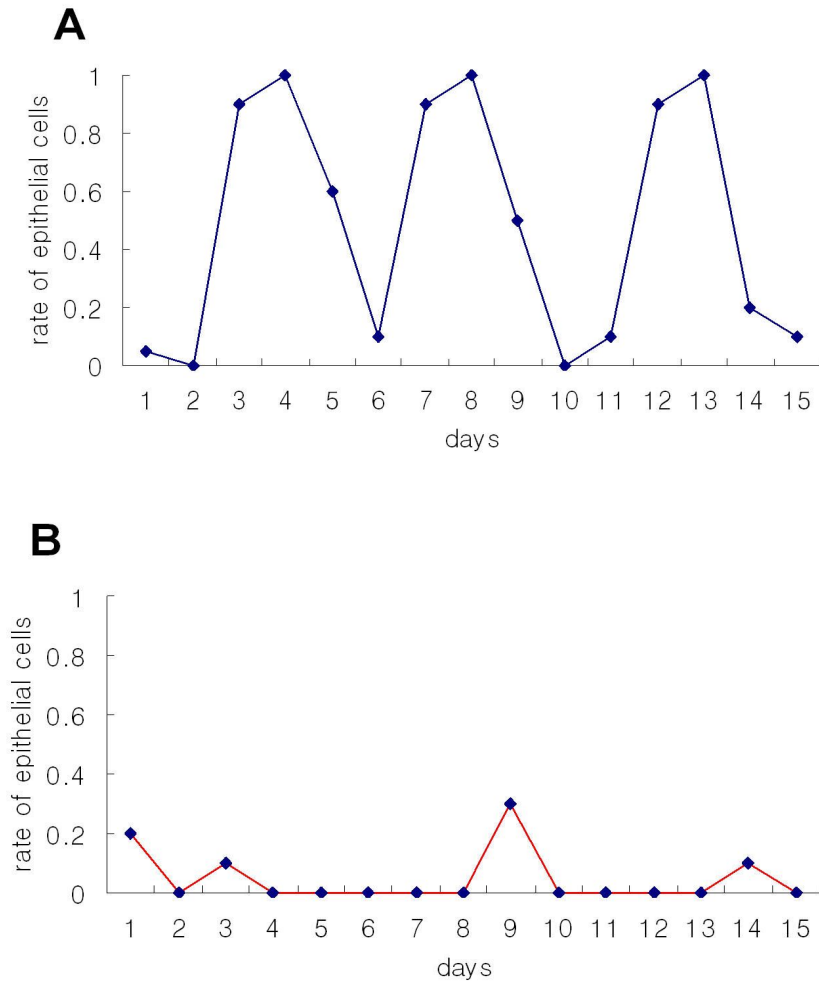
### **Disturbing estrus cycle**

Cytological analysis was performed using vaginal smear in wild-type and KO mice. Estrus cycle was analyzed as mentioned at Material and Methods. Cyclic changes of estrus cycle were observed in wild-type mice, every 4~5 day (Fig. 4A), but cyclic changes could not be detected in Krox-24 KO mice (Fig. 4B). Epithelial cells could be observed and the rate of epithelial cell, however, it was slight.

### **Decrease the number of ovulated oocyte and disturb fertility of ovulated or punctured oocyte**

In order to determine to the Krox-24 involved in follicular rupture. Krox-24 KO mice were primed with PMSG and hCG. The ovaries were collected at 0, 2, 4, 6, 9, 12 and 15 hrs after hCG injection. The numbers of punctured oocytes or ovulated oocytes in Krox-24 mice were significantly decreased (Table 3).

Large follicle was clearly appeared at 48 hr post PMSG injection. However some of them filled with blood (Fig. 5). In KO group after hCG injection, the hemorrhagic follicles observed from 0 hr and the phenotypes were not different until



**Figure 4. Abnormality of estrus cycle in Krox-24 KO mice. A, estrus cycle of 8 weeks old wild-type mice for 2 weeks. They show the regulatory cycle each 4~5 days. B, estrus cycle of the 8 weeks old Krox-24 KO mice. They have no estrus stage, usually diestrus stage persists (n=4).**

**Table 3. Numbers of ovulated or punctured oocyte in Krox-24 KO mice during superovulation induction. Punctured oocytes were collected only from large follicle in hCG administered mice with PMSG primed mice after 48 hr.**

Superovulation	Wild-type	Krox-24 KO
No. of ovulated oocyte	28.0 ± 2.22	16.0 ± 2.0*
No. of punctured oocyte	37.0 ± 6.34	30.84 ± 5.5*

**Average ± SD**

**\*Significance: P < 0.05**

4 hr. However, the follicles become more opaque after 6 hr than 4 hr KO or control 6 hr (Fig. 5).

### **Histologically unovulated oocyte could be detected in superovulation induced Krox-24 mice**

Histological analysis was done to reveal the role of Krox-24 in ovulation. Superovulation was induced in Krox-24 KO mice using gonadotropins. Ovaries were collected at 15 hr post hCG and under went examination. Unruptured follicles could be observed. Some of oocyte could not escape the follicle and trapped with in them (Fig. 6).

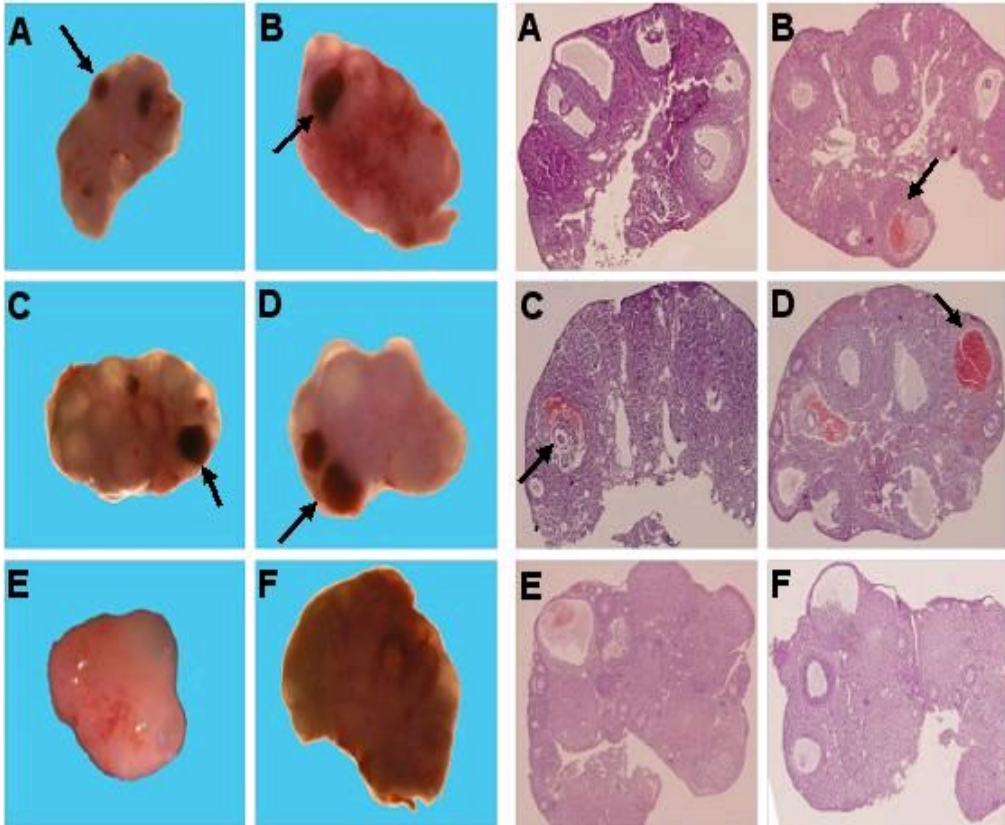
### **Cumulus-oocyte complex in superovulation-induced Krox-24 KO mice**

Maturation of oocyte is a factor for ovulation. Functional and structural changes in cumulus-oocyte are criteria to determine the quality of oocyte. Because it was explored that Krox-24 involved in ovulation.

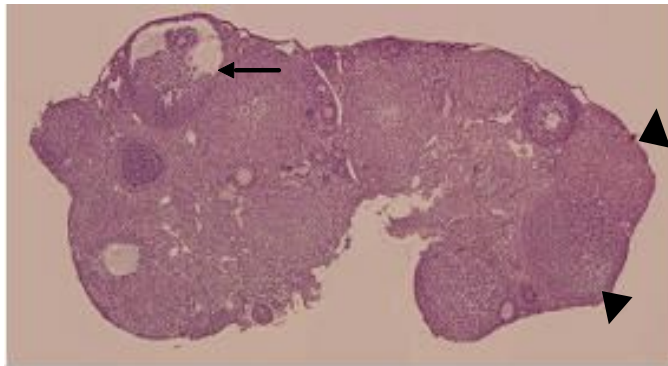
of unrupture, first the cumulus expansion was examined after hCG injection, ovary collected at various time points 0, 2, 4, 6, 9, 12and 15 hrs. Oocytes were collected after puncture of

**Panel I**

**Panel II**



**Figure 5. Photographs of various stage ovaries in Krox-24 KO mice. Ovaries were collected during superovulation induction. Panel I; photographs of various stage ovaries in Krox-24 KO mice during ovulation induction, Panel II; H-E staining of various stage ovaries in Krox-24 KO mice during ovulation induction. A, 0 hr; B, 2 hr; C, 4 hr; D, 6 hr E, 12 hr F, 15 hr post hCG injection. Some of ovaries were filled with blood (arrow in A, B, C, D)**



**Figure 6. Ovulation defect in Krox-24 KO mice. Some of oocyte was trapped within follicles (arrow), some of follicles were ovulated in superovulation induced ovary (arrow head)**

large follicle with sterilized needle in BWW medium containing 0.4% BSA. And collected oocytes were cultured in 10I medium covered with mineral oil which was equilibrated with BWW medium.

The oocyte of all stages has a problem. The oocytes at 0, 2, 4 hrs post hCG injection has shown a few normal morphology (Fig. 7A-C), also late stage (at 6, 9, 12 hrs post hCG injection) oocytes were showed abnormality. Densely accumulated GC bodies were existed and some of the oocytes existed cumulus free condition (Fig. 7). Ovulated or punctured oocytes were involved in *in vitro* fertilization. However no oocytes go to cleavage (Table 4).

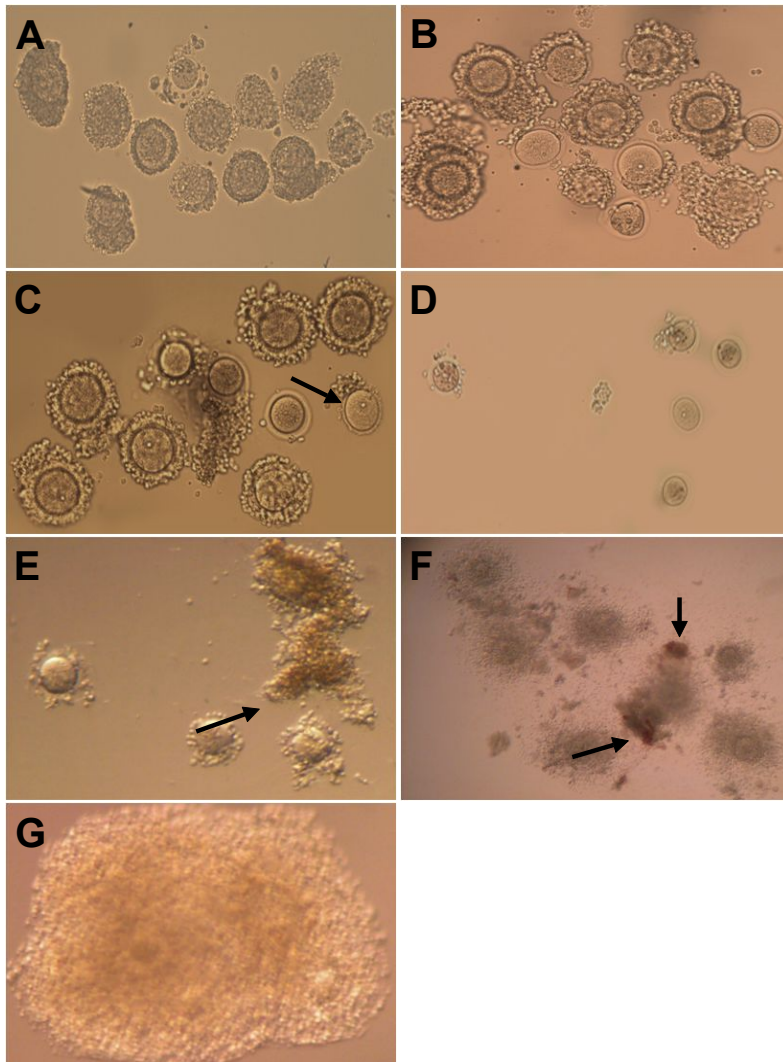
### **Expression regulation of putative Krox-24 regulated gene**

To identify the putative downstream genes of Krox-24, ovaries were collected after hCG injection in wild-type and KO mice. PR is known to be regulated by LH in ovary. PR mRNA specific bands were detected from 2 hr post hCG injection, and showed maximal expression at 4 and 6 hrs post hCG injection. ADAMTS-1 was dramatically increased at 12 hrs post hCG injection in wild-type mice. On the other hand, TGF $\beta$

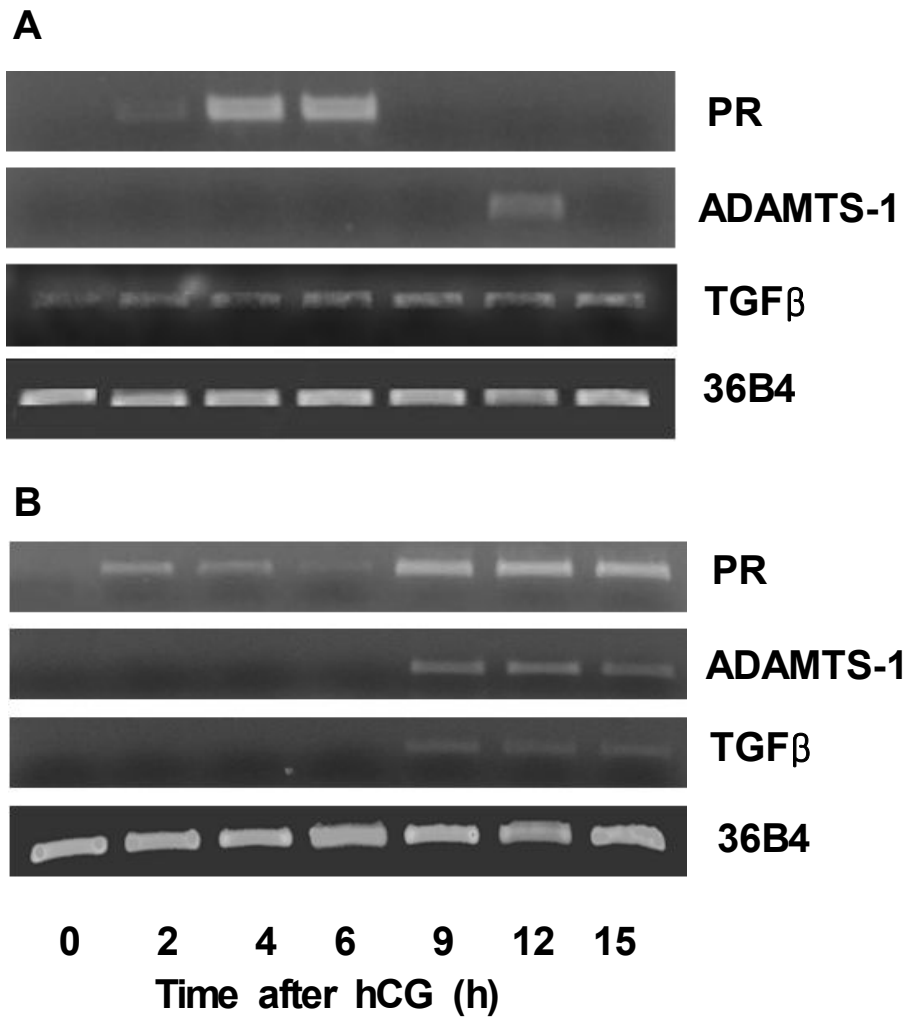
expression patterns were not show fluctuation through the time course (Fig. 8A). In Krox-24 KO mice, interestingly PR mRNA specific bands appeared every time point. ADAMTS-1 mRNA expression pattern also changed in Krox-24 KO mice. It showed maximal expression at 9 to 15 hrs post hCG injection. TGF $\beta$  expression patterns also dramatically changed as shown Fig. 8B. It was expressed from 9 hr post hCG injection.

### **Krox-24 binding domain in putative Krox-24 downstream gene**

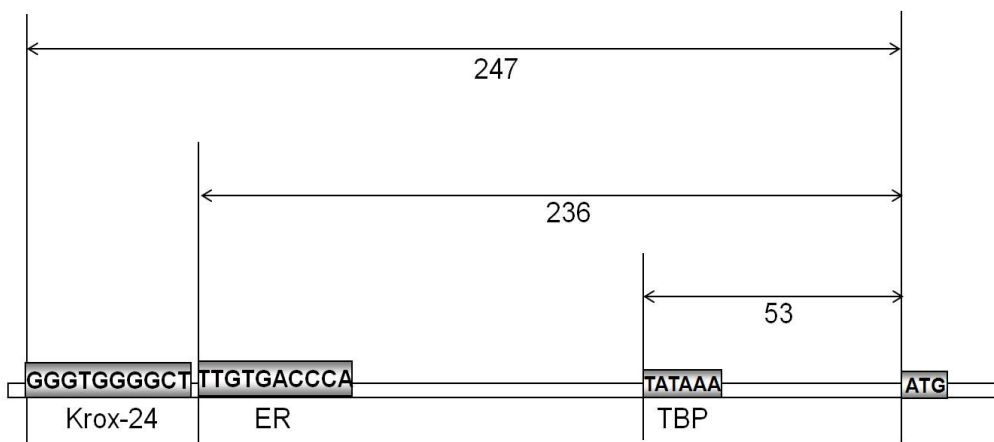
We observed expression of Krox-24 downstream genes in ovary. ADAMTS-1 is well known as a downstream gene of PR in ovary. As seen Fig. 8, PR expression is suspected that it is under the control of Krox-24. Using the web tools AliBaba 2.1, Krox-24 binding site was detected near the TATA box the RNA polymerase II binding site (TBP) (Kim *et al.* 2006). Moreover estrogen receptor (ER) placed near to Krox-24. Therefore, we assumed to that PR is a critical mediator between Krox-24 and ADAMTS-1 (Fig 9).



**Figure 7. Punctured cumulus-oocyte complex at various physiological status during superovulation induction. A, 0 hr post hCG injection. B, 2 hr C, 4 hr D, 6 hr E, 9 hr F, 12 hr and E, 15 hr post hCG injection.**



**Figure 8.** Expression of putative Krox-24 downstream genes in wild-type (A) and Krox-24 KO (B) mice. The ovaries were collected at 0 hr, 2 hr, 4hr, 6 hr, 9 hr, 12 hr, 15 hr after hCG injection.



**Figure 9. Krox-24 binding sites analysis using in silico way. Using the web tool AliBaba 2.1 a Krox-24 binding site was detected near the TATA box and the RNA polymerase II binding site (TBP).**

## Discussion

Ovulation is final step in folliculogenesis and is a triggering event in communication between reproductive tracts and oocytes to embryo development and implantation. To escape from the large follicle, oocyte has to undergo maturation processing and specific inflammatory progress has to occur in an apical part of large follicles. Late stages of folliculogenesis are mainly regulated by pituitary hormones, FSH and LH. FSH and LH levels are primarily regulated by GnRH level and negative feed back by follicle products.

Many mechanical pathways involved in the tuning regulation mechanisms of LH. Krox-24 influences female reproductive capacity through its regulation to LH- $\beta$  transcription (Lee *et al.* 1996). Interestingly Krox-24 and its related molecules were dramatically induced by hCG in ovary. Based on them, we suspected that Krox-24 involved in late steps of folliculogenesis.

Microarray result was conformed using RT-PCR. Krox-24 specific mRNA could be detected in the wild-type but it could not detect in KO mice. In rat, Krox-24 expressed in granulosa cells under the FSH and LH (Russell *et al.* 2003). Therefore it

is cleared that Krox-24 express under the gonadotropin in follicles. The induced epithelial proliferation in uterus or vagina result by estrogen which expressed in the growing follicles. In control, rhythmical changes in estrus cycle were observed, but it was not perfectly disappeared in KO mice. Vaginal fluid filled with leukocyte but not epithelium. Also external reproductive organ morphology seems to immature and very dry. This results substitute to conditions of ovary. With the results for estrus cycle confirm the previous reports (Lee *et al.* 1996). Put together it is suggested that Krox-24 involve in follicle growth.

From the histological studies using various stage older mice (2, 6, 10 and 12 months old), we know that the Krox-24 have a critical role in folliculogenesis or ovarian function. Compared with 2 month and 6 months old mice, ovary of older mice have not many follicles and various-stage follicles could not observe in their ovaries, some of early tertiary follicles filled with blood or had heterotypic cells. It is suggested that Krox-24 involve in regulation of ovarian function.

In 8 and 12 weeks old KO mice, ovary responded well to the gonadotropin and developed large follicles like in wild-type mice. But many of them filled with blood likes polycystic

ovarian syndrome (PCOS). Those results clearly illustrate that Krox-24 is a molecule which is involved in normal folliculogenesis.

We induced superovulation on 2~3 months old Krox-24 KO mice. The quality of punctured oocytes during superovulation induction was very poor as seen in the results. Expansion of cumulus was not complete (Fig 7). After induction of *in vitro* maturation, those oocytes involved *in vitro* fertilization. At 24 – 48 hr post insemination, we observed the cleavage rate, but the cleavage rate of oocyte was 0% in KO mice. These results clearly showed that Krox-24 is important for oogenesis within follicles. FSH as well as LH induced Krox-24 expression in granulosa cell in ovary between *in vivo* and *in vitro*. So, throughout the analysis of Krox-24 KO, we detected that Krox-24 plays a role of ovulation and oogenesis. Those mean that the cumulus did not work properly. Based on them it is suggested that one of the working routes of Krox-24 in ovulation is the cumulus-oocyte complex communication. Exogenous FSH and LH induce the development of large follicle but the

**Table 4. Cleavage rates of oocytes after *in vitro* fertilization which were isolated from Krox-24 KO mice. The sperms originated CD-1 male mice, 12 weeks old.**

	Wild-type	Krox-24 KO
Rate of Cleavage (%)	61.29 (38 / 62)	0* (0 / 42)

**Average  $\pm$ SD (Number of cleaved eggs / total number of oocytes)**

**\*Significance : P < 0.05**

numbers of ovulated oocyte were significantly decreased. In addition from the histological studies, a few of follicles filled with blood and trapped within postovulation stage follicles. Put together, those results clearly showed that Krox-24 has a crucial role in ovulation.

Transcription factor Krox-24 involve in expression of many factors. They are expressed in the ovary and important roles in folliculogenesis and/or ovulation such as p53 (Liu *et al*, 1998 Nair *et al*, 1997), cytokines, and growth factors TNF $\alpha$  (Guha *et al*, 2001) and TGF $\beta$ , fibronectin (Liu *et al*, 2000), or cellular adhesion factors CD-44 (Maltzman *et al*, 1996), cathepsin L, ADAMTS-1, fibronectin (Russell *et al*. 2003).

Among these genes, we choose to a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTS-1), Tissue growth factor  $\beta$  (TGF $\beta$ ), and progesterone receptor (PR). In Krox-24 KO mice, the expression patterns of PR, ADAMTS-1 and TGF $\beta$  were pretty different compared with wild-type mice. TGF $\beta$  mRNA specific bands are detected in all groups of wild. However TGF $\beta$  mRNA specific bands were detected only 9, 12 and 15 hrs post hCG injection. We used to methodology of in silico way to find promoter region. Using

in silico way the Krox-24 binding domains were identified in PR, ADAMTS-1 and TGF $\beta$ , from the results it was suggested that Krox-24 suppressed the expression of PR in ovary. In addition it is suggested that ADAMTS-1 and TGF $\beta$  is under the control of the Krox-24.

On summary, Krox-24 regulated PR expression and the next down stream genes, ADAMTS-1 and TGF $\beta$ . It is revealed that Krox-24 involve in folliculogenesis, ovulation and oogenesis. Based on those results, it is suggested that Krox-24 regulated PR expression in large follicle and ADAMTS-1 through PR. As a result all of these study, we can notice that Krox-24 is cause involving molecule in ovulation in mouse.

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## **Abstract**

The roles of KROX-24 in ovulation in mouse

: Krox-24 in ovulation and expression regulation  
of putative downstream genes

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Folliculogenesis are regulated by gonadotropins, steroid hormones and auto-/paracrine factors, selective matured oocyte was progressed the ovulation. The ovulation cause it to be expressed protease, immuno-cellular regulatory molecule and immune factors crowd specific ovulated site by luteinizing hormone (LH), however many factors unknown. Using the DNA microarray methodology to identified time specific and tissue specific expressed gene in ovary after LH surge to before ovulation. We identified to related gene with ovulation among time specific expressed gene after LH surge. Krox-24 well known to regulated formation of follicles-regulated hormone in pituitary. Through the microarray, we identified the relative gene of Krox-24 expressed at ovulation specific. In this study, we

tried to know about the actual state of Krox-24 with the various experimental methods in folliculogenesis and after ovulation. Krox-24 expression in ovary after LH surge dramatically increased, it confirmed to microarray data. In histological study, the pool of follicles was dramatically reduced in Krox-24 KO mice (8 months old). And these ovaries didn't observed growing follicles and some follicles accumulated with blood. Wild-type mice have a rhythmical change every 4~5days. But Krox-24 KO mice have irregular cycle and always have diestrus stage. We experimented to roles of the ovulation and overcome of defect of pituitary using artificially induction of LH. The numbers of ovulated oocyte or punctured oocyte were significantly reduced compared to control group in Krox-24 KO mice. Krox-24 gene highly expressed at 2~4 hrs after hCG injection in granulosa cell in ovary. We determined to expression pattern of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS-1) and tissue growth factor- $\beta$  (TGF $\beta$ ). Also, ADAMTS-1 was well known to PR downstream gene, we analyzed PR expression regulation by Krox-24 using the PCR or in silico way. ADAMTS-1 are expressed at 12 hr after hCG injection, but TGF $\beta$  was expressed all stage post hCG injection and PR was expressed at 4~6 hrs after hCG injection. While observed expression of ADAMTS-1 at 12 hr hCG injection, PR is expressed at all stage in Krox-24 KO mice. Using in silico way the ER binding

domain and Krox-24 binding domain could found in PR promoter region. From these results it is suggested that Krox-24 are work in successful ovulation and folliculogenesis in mice ovary. In addition Krox-24 may regulate ovulation through PR downstream including ADAMTS-1 or TGF $\beta$ .

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