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석사학위청구논문

Identification of Bax related genes in the pregnant uterus : roles in Decidualization

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성신여자대학교 대학원

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전윤미의 석사학위 논문으로 인준함.

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논 문 개 요

포유동물에서 자궁내막의 분화는 성공적인 배아의 착상과 모체 보호를 위해 필수적이다. 착상 동안 자궁 기질 세포는 형태적, 기능적인 변화를 겪게 되는데 이러한 과정을 '탈락막화' 라고 한다. 이는 난소 호르몬인 에스트로겐과 프로게스테론에 의해서 조절되며, 다양한 전사인자, 형태형성물질, 사이토카인, 세포주기 조절인자에 의해 영향을 받는다. 생쥐의 경우, 임신 5일차에 탈락막 반응이 시작되며 착상중인 배아 주변의 기질세포들이 증식한다. 임신 6일차에 배아 부착 부분과 가까운 기질세포는 세포 증식을 중지하고 1차 탈락막 부위를 형성하게 되며, 이 부분 바깥쪽의 세포는 계속적으로 증식하여 이후 2차 탈락막 부위를 형성하게 된다. 탈락막 세포는 배아의 발달에 필요한 성장인자나 사이토카인을 공급하는 역할을 하며, 발달중인 배아에게 충분한 영양분을 공급하기 위해 모체의 혈관 형성을 돕는다. 탈락막의 퇴화는 성공적인 태반 형성과 배아가 정상적으로 발달할 수 있는 공간 형성을 위해 중요하며, 매우 정교하게 조절되는 과정이다. 탈락막의 퇴화는 세포의 자연사에 의해 진행되며, *Bcl-2* 계인 *Bax* 등이 이 과정에서 중요한 조절인자로 알려져 왔다. 그러나 이전의 우리 연구단의 연구에서, 대조군과 비교하여 털색이 검거나(Black Coat, B.C) 또는 흰(White Coat, W.C) *Bax* 적중 생쥐에서 임신률이나 착상률에는 차이가 없었으나, 출산율은 W.C *Bax* 적중 생쥐에서 유의하게 낮았다. 이는 *Bax* 유전자는 탈락막의 퇴화에 직접적인 작용이 없

고 *Bax* 관련 유전자가 탈락막 분화에 작용할 가능성이 있음을 의미한다. 본 연구에서는 세가지 실험군의 임신 7일차의 탈락막을 Microarray 방법을 이용하여 분석하고 정량 real time PCR 방법을 이용하여 대량 분석 방법의 정확성을 증명하였고, 세포 증식과 분화, 자연사, 세포주기와 관련된 많은 수의 유전자들이 유의적인 발현변이를 나타내는 것으로 확인 하였다. 또한 세가지 실험군의 자궁 기질세포를 체외 배양하면서 스테로이드 호르몬을 이용하여 탈락막 반응을 유도한 결과 흰 털색의 *Bax* 적중 생쥐에서 불완전한 탈락막 반응이 일어남을 확인하였다. 한편 microarray 분석에서 세포 증식과 관련된 *c-Myc* 관련 유전자들의 유의한 감소가 있어 자궁 기질 세포를 체외 배양하며 탈락막화를 유도하는 동안 *c-Myc* antagonist를 처리한 결과 탈락막 반응을 억제함을 확인하였다. 이러한 결과를 바탕으로 흰 털색의 *Bax* 적중 생쥐는 *Bax* 및 *Bax*와 연관된 털색 유전자 (tyrosinase,Tyr) 부위와 눈색 pink-eyed dilution의 상실은 탈락막화 과정에서 관찰되는 세포 증식, 분화, 자연사와 관련된 유전자인 *c-Myc*과 *c-Myc*의 하위 유전자 등의 발현을 적절하게 조절하지 못하게 되고 이로 인해 자궁 기질의 탈락막 분화가 정상적으로 일어나지 않음을 추정 할 수 있다.

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INTRODUCTION

Mammalian uterus is constructed with various cell types, such as luminal epithelial cell, glandular epithelial cell, stromal cell, vascular cells, and smooth muscle cells. Synchronized development of embryo to the uterine physiology, and a crosstalk between the blastocyst and uterine luminal epithelium are essential for implantation process. Without the establishment of this molecules dialogue, the embryo can not implant to uterus. On the other hand, failure of the endometrial growth and differentiation after blastocyst attachment results in spontaneous abortion (Farrar et al., 1992; Paria et al., 2001; Xia et al., 2008).

The maternal sex steroid hormones, estrogen (E) and progesterone (P), are pivotal in early uterine decidual differentiation events. These hormones orchestrate molecular and cellular modifications in the uterine endometrium that make it competent to attach to the blastocyst and initiate the process of implantation (Lee et al., 2013; Wang et al., 2010). E stimulates proliferation of the luminal and glandular epithelium, and expression of progesterone receptors in stromal cells. Subsequently, E and P regulate a series of complex interactions at the interface between the developing embryo and the stromal cells, leading to the formation of a decidua, which supports embryo growth and pregnancy (Carson et al., 2000; Cheon et al., 2002; Huet-Hudson et al., 1989).

The mouse blastocysts arrive to the uterine lumen 4 days after fertilization and the initial attachment reaction occurs approximately midnight on Day 4 of pregnancy (Day 1 = day of vaginal plug detection). The attachment of the blastocyst to the uterine epithelium triggers the process of decidualization. This process involves the remarkable transformation of fibroblastic-like endometrial stromal cells into morphologically and functionally distinct cells. These cells are characterized increasing size, storage and the secretory functions (Wang et al., 2012). Decidualization is known as a complex interplay between transcription factors, morphogens, cytokines, cell cycle regulators and signaling pathways. Initially the undifferentiated stromal cells undergo mitotic expansion, and then they enter the differentiation program at the site of the implanting blastocyst that converts them into decidual cells (Cha et al., 2012; Paria et al., 2001; Ramathal et al., 2010; Wang et al., 2010). During these processes, substantial changes in the extracellular matrix are characterized. Extracellular matrix (ECM) materials, including type IV collagen, laminin, entactin, and fibronectin are accumulated in the outer area of decidua and reformed the cellular structures and functions (Ansell et al., 1974; Leivo et al., 1980; Wang et al., 2010; Wartiovaara et al., 1979; Wewer et al., 1986). A variety of functions have been attributed to the decidua, such as providing a source of growth factors and cytokines that support embryo development, mediate an immune regulatory role during pregnancy and regulating trophoblast invasion. Decidual cells are also support maternal blood vessel formation in order to perfuse and nourish the

developing embryo (Ramathal et al., 2010; Wang et al., 2010). Besides, many decidual cells undergo endoreduplication (polyploidy), a process by which cells undergo rounds of DNA replication without cytokinesis. Endoreduplication may serve to support embryonic growth by increasing protein synthesis through enhanced gene transcription (Cha et al., 2012).

In the case of mouse, decidualization is normally initiated at the antimesometrial area, the presumptive site of placentation. Between the day 5 afternoon and the day 6 morning, stromal cells surrounding the implanting blastocyst cease proliferating and undergo differentiation into decidual cells and form primary decidual zone (PDZ). Stromal cells surrounding PDZ continue to proliferate and differentiate into polyploid decidual cells forming a zone around the PDZ, secondary decidual zone (SDZ). The SDZ is fully developed by day 7, while the PDZ progressively degenerates by apoptosis up to day 8. After day 8, the placental and embryonic growth slowly replaces the SDZ (Das et al., 2009; Lin et al., 1997; Paria et al., 1999; Welsh et al., 1985; 1991). Placentation progress and form the functional physiological unit by the regression of decidua. The main way of that is apoptosis and remodeling of ECM. Therefore, degradation of deciduas by apoptosis should be strictly regulated for the successful placentation and the spacing for embryo. Ultimately, the timely and ordered regulation of the functional and structural changes in the endometrial tissue surrounding the implanting embryo is critical for the establishment of early pregnancy (Ramathal et al., 2010).

Apoptosis is a physiological programmed cell death that initiates an active process of cellular self-destruction without inducing an immune response of inflammatory reaction. This occurred when the cells are subjected to DNA damage or death signals. Cell shrinkage, chromatin condensation, DNA fragmentation and the formation of the apoptotic bodies are the criteria of apoptotic cells (Bilotas et al., 2007; Correia-da-Silva et al., 2005; Shooner et al., 2005). The Bcl-2 family is composed of anti-apoptotic and pro-apoptotic member and is a major regulator of mitochondria pathway (Ren et al., 2010; Youle and Strasser., 2008). *Bax* is a pro-apoptotic member of the Bcl-2 family which responsible for evoking or increasing apoptosis in many tissues. Previously it was suggested that *Bax* regulates apoptosis of decidua. However, interestingly female *Bax* knockout mice are fertile.

From previous our studies, we evaluated that regardless of coat color, there is no difference in pregnant rate and implantation rate between black coat color *Bax* KO mice and white coat color *Bax* KO mice. On the other hand, white coat color *Bax* KO mice, delivery rate was significantly decreased and did not properly respond to the decidual signaling. It suggested that *Bax* related genes which involved in coat color and eye color may have key roles in decidualization. Therefore, in this study, the functional roles of *Bax* related gene in uterine stromal cell differentiation was examined using *Bax* knockout mice and *in vitro* primary endometrial stromal cell culture model for decidualization.

MATERIALS AND METHODS

Experimental animals

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

Bax knockout (KO) female mice were generated by *Bax* heterozygote ($Bax^{+/-}$) breeding (Knudson et al., 1995). The genotypes of the mice were determined by PCR analysis of genomic DNA from ear tissue.

The black coat mice designated as $Bax^{-/-}$(B.C), and white coat as $Bax^{-/-}$(W.C).

Sampling of Uterus

Pregnant $Bax^{+/-}$, $Bax^{-/-}$(B.C) and $Bax^{-/-}$(W.C) mice were mated with fertile $Bax^{+/-}$ stud male. Vaginal plug was checked on the next day morning. The day on which the vaginal plug was confirmed was considered as day 1 of pregnancy. $Bax^{+/-}$, $Bax^{-/-}$(B.C), $Bax^{-/-}$(W.C) mice were sacrificed on day 7 of gestation and the uteri were collected, respectively. Pregnancy on day 7 was confirmed by recovering embryos

from reproductive tracts at from 10:00 to 11:00. The embryos were removed from uteri by dissecting the decidua. And then the uteri were frozen in liquid nitrogen and kept at -80℃ until RNA extraction.

Total RNA extraction

Total RNA of uteri was extracted using Trizol reagent (Invitrogen, San Diego, CA, USA) according to manufacturer's instruction with minor modification. Briefly, the uterine tissues were homogenized in Trizol reagent (1 ml/100 mg tissue) and stored for 5 min at room temperature (RT). The chloroform (0.2 ml/1 ml Trizol reagent) was added to the homogenates and shaked vigorously for 15 sec. After then, the mixture kept for 15 min at RT and centrifuged ≥10,000g (12,000 RPM) for 15 min at 4℃. After RNA in the aqueous phase was transferred into a new tube, the RNA was precipitated by mixing 0.5 ml isopropyl alcohol, mixed slowly, kept for 10 min at RT. And centrifuged 12,000 RPM for 8 min at 4℃. The supernatant was removed, the RAN pellet was washed briefly in 1 ml 75% ethanol, mixed by inverting, and centrifuged 7,500 RPM for 5 min at 4℃. The supernatant was removed, dried to remove ethanol and added 50 μl DEPC treated water. Total RNA quality and quantity were assessed by Agilent bioanalyzer™ 2100 analysis.

First strand cDNA synthesis

In order to perform reverse-transcription, 5 μg total RNAs were used. First strand cDNAs were synthesized using Accuscript first strand cDNA synthesis kit (Stratagene, CA, USA) according to manufacturer's

instruction. Briefly, reaction reagents were 5 μg total RNA, 5.0 μl Accuscript buffer (10X), 1.0 μl oligo dT primer (0.5 μg/μl), 1.0 μl random primers (0.1 μg/μl), 2 μl dNTP mix (100 mM), RNase- free water. Reaction mixture was incubated at 65℃ for 5 min, placed the tube at RT to allow the primers to anneal to the RNA for 10 min, and then added 4.0 μl DTT (100mM), 2.0 μl RNase block ribonuclease inhibitor (40 U/ml), 1.0 μl Accuscript multiple temperature RT. The mixture was incubated at 42℃ for 1 hr and 70℃ for 15 min to terminate cDNA synthesis.

Quantitative RT- PCR analysis

For quantification of expression levels, transcripts of target genes were amplified using RT-PCR (Table 1) and the specific primers (Table 2). The primer parameters were 50% GC contents, avoiding repeat base pair and lengthening 20-24 mer. Quantitative RT-PCR was performed using SYBR Premix Ex Taq™ (TaKaRa, Japan) and Thermal Cycler Dice Real Time System TP800 (TaKaRa, Japan). Each reaction was run in triplicate and consisted of 1.0 μl cDNA. Dissociation curves were run on all reactions to ensure amplification of a single product with the appropriate melting temperature. The fold change in gene expression was calculated using the ΔΔCt method with the housekeeping gene, a ribosomal protein, 36B4, as the internal control.

Microarray analysis

Microarray analysis is useful for identifying changes in gene expression which are critical for the process of decidualization during early pregnancy. Gene expression was analyzed with Genechip® Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA), which is comprised of over 45,000 probe sets representing approximately 39,000 well-characterized mouse genes. For each gene, eleven pairs of oligonucleotide probes are synthesized in situ on the array. For microarray analysis, biotinylated cRNA were prepared according to the standard Affymetrix protocol from 250 ng total RNA (Expression Analysis Technical Manual, 2001, Affymetrix). Following fragmentation, 15 μg of aRNA were hybridized for 16 hr at 45℃ on GeneChip Mouse Genome Array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450. GeneChips were scanned using the Affymetrix GeneChip Scanner 3000 7G. The arrays were analyzed using an Affymetrix scanner with its associated software. Gene expression levels were calculated with Feature Extraction v10.7.3.1 (Agilent technologies). The data were analyzed with Robust Multi-array Analysis (RMA) using Affymetrix default analysis settings and global scaling as normalization method. The normalized and log transformed intensity values were then analyzed using GeneSpring GX12.5 (Agilent technologies,CA). This normalization method aims to equalize the distribution of intensities for each array in a set of arrays. Fold change filters included the requirement that the genes be present at certain expression values, with a cut-off at a 2-fold change compared to the control.

Endometrial stromal cells culture and *in vitro* decidualization induction

Mouse endometrial stromal cells (mESCs) were isolated of *Bax*$^{+/-}$ mice, *Bax*$^{-/-}$(B.C) mice, *Bax*$^{-/-}$(W.C) mice in PMSG primed, respectively. Uteri were cut into 1 ㎜³ pieces and incubated in DMEM:F12 (Sigma-Aldrich Corp., St. Louis, MO) supplemented with 0.002 mg/ml Collagenase Type I (Gibco, Rockville, MD, USA) and 100 ㎍/ml penicillin/ streptomycin (Sigma-Aldrich Corp., St. Louis, MO) for 1 hr 30 min at 37℃ in orbital shaking incubator with 150 RPM. After incubation, the solution was passed through 100 μm and 40 μm sieve (BD Falcon, BD Biosciences, San Jose, CA) and the suspensions were centrifuged at 1,000 RPM for 5 min. The mESCs were then resuspended in DMEM:F12 supplemented with 10% charcoal dextran (cFBS; Sigma-Aldrich Corp., St.Louis, MO) - stripped FBS. The mESCs were plated in 24-well cell culture plates onto glass cover slip at 1 X 10^5 cells per well. And after 30 min, the medium was aspirated, and changed the fresh media containing 15nM 17β-estradiol (E_2) and 10% cFBS. After 24 hr, medium was changed to DMEM:F12 with 10% cFBS without steroid hormone. After two day, decidualization was induced by DMEM:F12 media containing 10% cFBS, 1nM E_2 and 1μM P_4 (Sigma-Aldrich Corp., St. Louis, MO). The cells were harvested at 24 hr, 48 hr, 72 hr and 96 hr, respectively. Control samples received no hormone supplementation.

Embryo transfer

To get the competent blastocyst, CD-1 female mice (6-8weeks) were subjected to superovulation; 5IU of PMSG was injected to

subcutaneously and followed by 5IU of hCG after 48 hr, and followed by mating with stud male mice. Reproductive cycles of the recipients *Bax*$^{+/-}$, *Bax*$^{-/-}$(B.C) and *Bax*$^{-/-}$(W.C) female mice were synchronized with 2.5IU of PMSG and hCG with 48 hr term, and pseudopregnancy were induced by vasectomized CD-1 male mice. The next day morning, these mice were examined for the presence of vaginal plug, and this was defined day 1 of pregnancy. At day 4 of pregnancy, the blastocysts were collected from the uterus by flushing into each uterine horn using BWW medium containing 0.4% BSA. Only the healthy blastocysts were incubated at 37℃ under 5% CO_2 in 95% air until transferred. After embryo preparation, recipient mice were anesthetized with ketamine (40 mg/kg) and xylazine (10 mg/kg). Upon anesthetics, mice underwent dorsal incision, and uterine horn was exteriorized. The 10 blastocysts were loaded into a glass transfer pipette and were transferred into the uterine lumen. Embryo transfers were performed between 15:00 - 16:00 PM. The embryo transferred mice were sacrificed at 96 hr after embryo transfer. And then the uteri were frozen in liquid nitrogen and kept at -80℃ until used.

Immunofluorescence

Dissected mouse uterine horns were quickly freezed with liquid nitrogen and were kept at -80℃ until sectioned. Tissues were serially sectioned at 8μm with Cryostat (LEICA CM1950 Cryostat Microtome). The frozen sections were mounted onto glass slides and fixed in 4% paraformaldehyde containing 0.15% picric acid in phosphate-buffered

saline (PBS) for 20 min. The sections were washed with PBS and subjected to immunofluorescence. For blocking and permeabilization, tissues were incubated in PBS containing 0.3% Triton X-100 and 10% goat normal serum for 1 hr. The sections were kept for 2 hr at RT with first antibody, polyclonal mouse alkaline phosphatase (ALP) (dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA) in PBS with 0.1% BSA. After washing in PBS with 0.1% BSA, tissues were incubated with Cy3 conjugated affinipure Goat Anti-Rabbit IgG diluted 1:200 in PBS with 0.1% BSA for 2 hr at room temperature. After washing in PBS with 0.1% BAS, tissues were counterstained with Hoechst 33258 for 20 min. Slides were washed and mounted. Specific signaling of alkaline phosphatase was observed under the fluorescence microscope (Olympus BX60).

Treatment of c-MYC antagonist

Mouse endometrial stromal cells (mESCs) were isolated from *Bax*$^{+/-}$ mice, and in *vitro* decidualization induction by E_2 and P_4 in E_2 primed. 0 μM, 50 μM Mycro1 (Chemical Diversity Research Inst., Khimki, Russia) was treated during *in vitro* decidual induction to block c-MYC. After induction 96 hr, the cells were harvested, respectively.

Statistical analysis

All experiments were conducted at least in triplicate. The Student's t-test was performed to evaluate the statistical significance between control and experiment groups. Results were presented as Mean ± SD. A p-value < 0.05 was considered to be significantly different.

Table 1. Thermal cycler schedule

Step		Temperature (℃)	Time
Hold	Hold	94	30 sec
3 step PCR (45 cycle)	Denaturation	95	1 min
	Annealing	59	30 sec
	Extension	72	1 min
Dissociation	Denaturation	95	15 sec
	Annealing	60	30 sec
	Extension	95	15 sec
Hold		4	Indefinitely

Table 2. Primer sequences for genes which were changed more than 2-folds in *Bax*$^{-/-}$ (W.C) decidua

Gene	Symbol	NCBI gene reference		Primer sequence (5'-3')	Amplified length (bp)
CD24a antigen	*Cd24a*	NM_009846	S	GCTCCTACCCACGCAGATTTACT	231
			AS	TTTGGGGTAGGTGTAGAAGATGGG	
Transforming growth factor, beta induced	*Tgfßi*	NM_009369	S	AACTGCTCAATGCTCTCCGCTA	173
			AS	ATGGTGGTCAGCCTTCAGCA	
Carbonic anhydrase 2	*Car2*	NM_009801	S	ATCCTTGCTCCCTTCTTCCTG	194
			AS	GTTGTCCACCATCGCTTCTTCA	
Calbindin 1	*Calb1*	NM_009788	S	AGAATCCCACCTGCAGTCATCT	245
			AS	TGGGTAAGACGTGAGCCAACTCT	
Glutathione peroxidase 3	*Gpx3*	NM_001083929	S	CATTTGGCTTGGTCATTCTGG	245
			AS	AGGGCAGGAGTTCTTCAGGA	
Lymphocyte antigen 6 complex, locus A	*Ly6a*	NM_010738	S	GCAATGTAGCAGTTCCCAATG	200
			AS	TTTCACACACTACTCCCACCTTG	

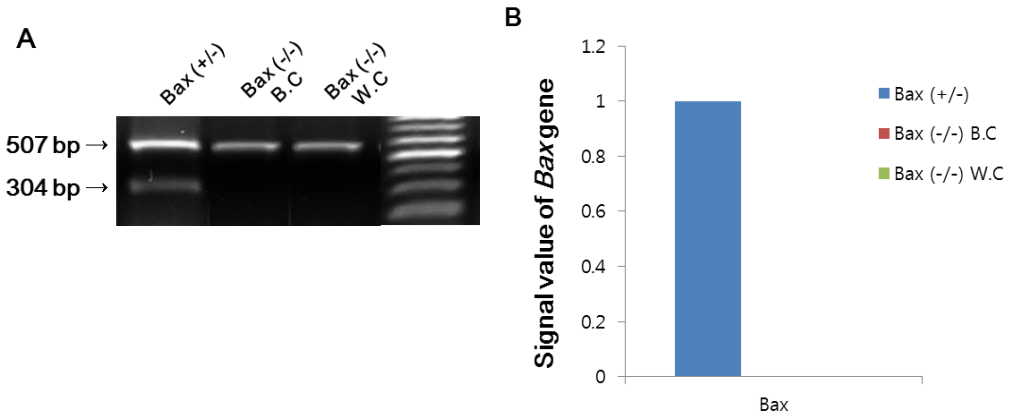
Gene	Symbol	NCBI gene reference	Primer sequence (5'-3')		Amplified length (bp)
Regulator of G-protein signaling 2	*Rgs2*	NM_009061	S	AGGATTGGAAGACCCGTTT	272
			AS	GGACAGTTTTTGGGGTGATTTGG	
Gap junction protein, alpha 1	*Gja1*	NM_010288	S	GAGAACCTACATCATCAGCATCCT	201
			AS	ACCAAGGACACCACCAGCAT	
ribosomal protein, large, P0	*36B4*	NM_007475	S	CGACCTGGAAGTCCAACTACTTCCT	303
			AS	ATGCTGTTGGCCCAAATAAGGTGC	

RESULTS

Bax gene knockout of the C57BL/6 mice.

To confirm the *Bax* gene knockout, mice were genotyping from genomic DNA with specific primer. According to Jackson laboratory, *Bax* heterozygote (+/-) resulted in 304bp and 507 bp, on the other hand, *Bax* knockout (-/-) resulted in 507bp (Fig. 1A). Before experiments, genotyping was conducted to confirm genotype.

As expected, in the microarray analysis result, *Bax* gene of $Bax^{-/-}$(B.C) and $Bax^{-/-}$(W.C) was not detected (Fig. 1B,C).



C

Gene	Symbol	Signal value		
		$Bax^{+/-}$	$Bax^{-/-}$ (B.C)	$Bax^{-/-}$ (W.C)
BCL2-associated X protein	*Bax*	7.51	0	0

Fig 1. Confirm of *Bax* gene knockout in used mice.

A. Genotyping of mice.

B. Signal value of *Bax* gene. *, Statistically significant change (p<0.05).

C. Result of microarray analysis.

Specifying the gene expression profiles in *Bax* knockout mice in decidua.

To gain insight into murine uterine genes associated with the induction of decidualization in the uterus during pregnancy, the decidua of *Bax*$^{+/-}$, *Bax*$^{-/-}$(B.C) and *Bax*$^{-/-}$(W.C) mice were analyzed in day 7 of pregnancy (NPD 7), respectively. High density cDNA microarray screening was used to evaluate global changes in gene expression. Gene expression was analyzed with Genechip® Mouse Genome 430 2.0 Arrays, which is comprised of over 45,000 probe sets representing approximately 39,000 well-characterized mouse genes.

Global expression profiles revealed and characterized the distinctive gene expression pattern of *Bax*$^{-/-}$(W.C) compared with the other groups during decidualization. The clustering dendrogram revealed that the branch tree distances minimal between *Bax*$^{-/-}$(B.C) and *Bax*$^{-/-}$(W.C). It means that *Bax*$^{-/-}$(B.C) and *Bax*$^{-/-}$(W.C) are more similar than *Bax*$^{+/-}$ (Fig. 2).

Intensity scatter plot is a graph that identifying changed genes of sample using signal value of control/experimental group. The X axis is signal value of control group and the Y axis is signal value of experimental group. Based on the middle line (2 fold line), the upper side indicates over-expression genes and the bottom side indicates down-expression genes. The intensity scattering patterns were similar between A, B and C in Fig. 3. Thus the data produced by this platform was judged to a well behaved.

Intensity ratio histogragh shows the log intensity in the expression

variation can be found by the step-by-step. The X axis is experimental / control group log2 ratio and the Y axis is the number of changed genes. Except to the middle two bars, other bars are significantly changed expression gene. As expected from the Fig. 3, the differences in each dataset were distributed as a bell-shaped curve (Fig. 4).

A total of 4582 genes were significantly changed at least 2-fold ($p<0.05$) during decidualization in *Bax*$^{-/-}$(B.C) compared with *Bax*$^{+/-}$. Among these genes, 2439 genes were up-regulated and 2143 genes were down-regulated. A total of 4783 genes were significantly changed at least 2-fold ($p<0.05$) during decidualization in *Bax*$^{-/-}$(W.C) compared with *Bax*$^{+/-}$. Among these genes, 2381 genes were up-regulated and 2412 genes were down-regulated. A total of 3300 genes were significantly changed at least 2-fold ($p<0.05$) during decidualization in *Bax*$^{-/-}$(W.C) compared with *Bax*$^{-/-}$(B.C). Among these genes, 1489 genes were up-regulated and 1811 genes were down-regulated (Fig. 2, 3, 4, Table 3). From these results, it is suggested that the large number of genes were changed and associated with decidualization. These highly over expressed or down regulated genes were thought to be involved in decidual defects.

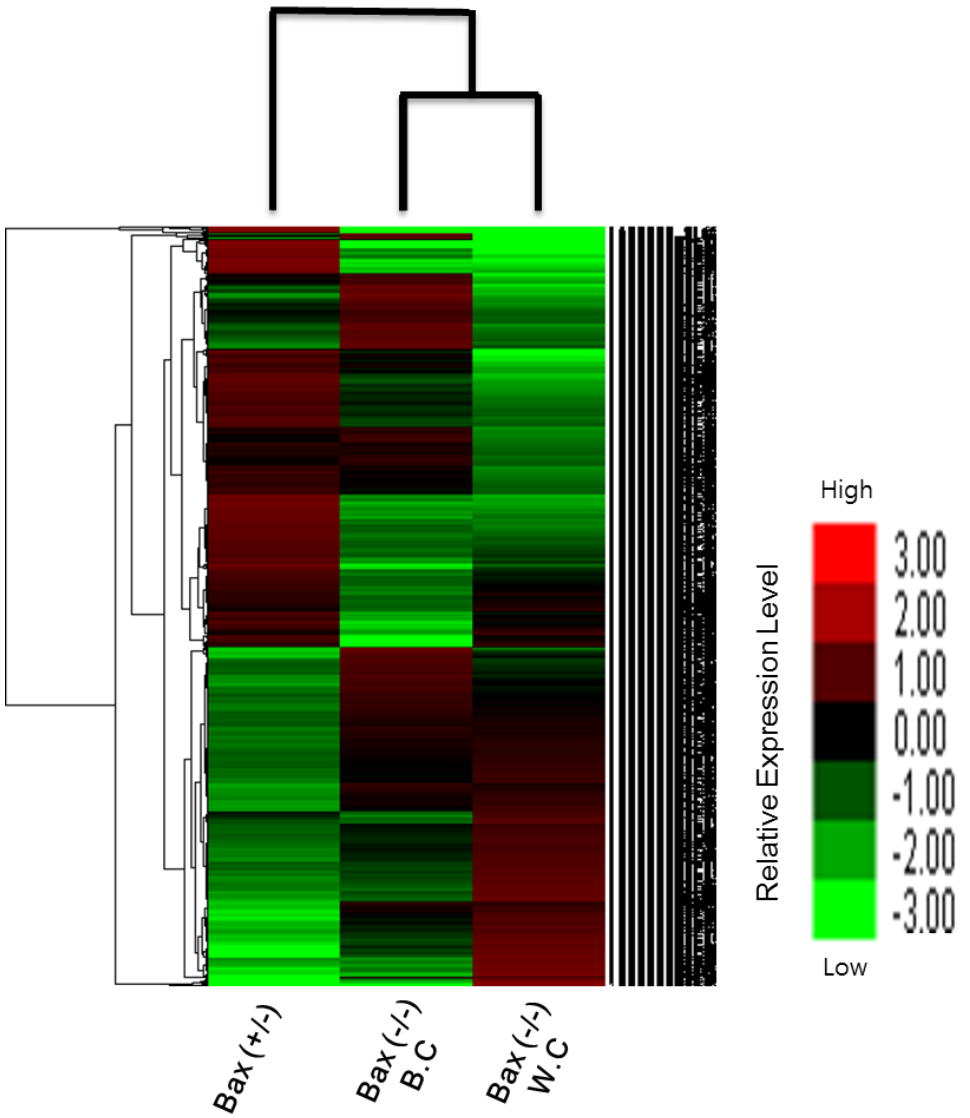


Figure 2. Hierarchical clustering analysis of mouse genes from decidua at day7 of gestation in $Bax^{+/-}$, $Bax^{-/-}$(B.C), $Bax^{-/-}$(W.C) mice.

Heatmap representing relative changes in their expression level were indicated by a color code. Red indicates that the gene in up-regulated, and green indicates that the gene is down-regulated.

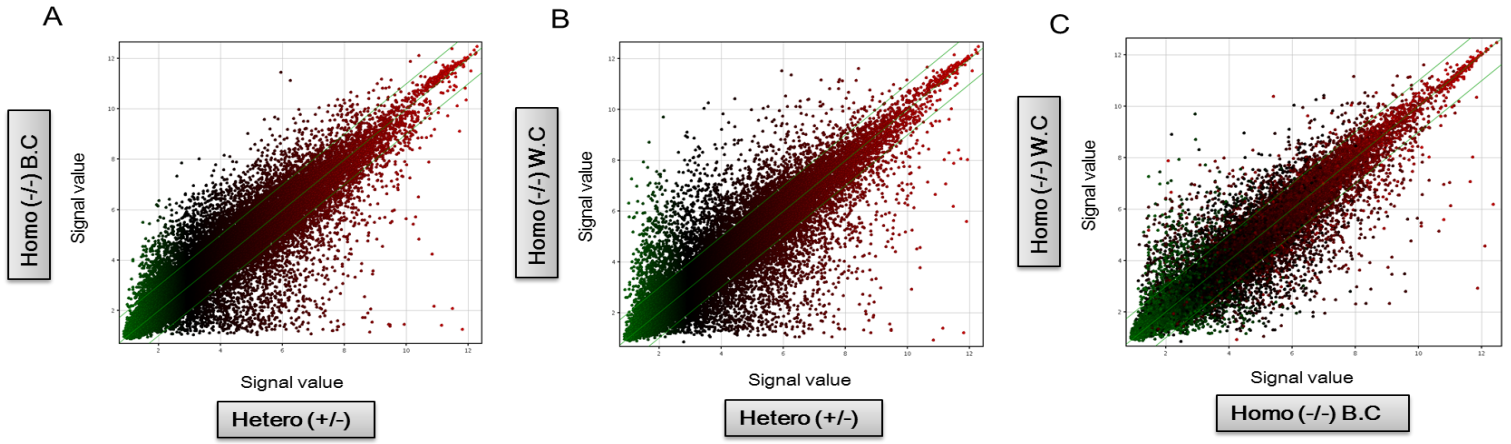


Figure 3. Intensity scatter plot analysis of mouse genes from decidua at day7 of gestation in *Bax*$^{+/-}$, *Bax*$^{-/-}$(B.C), *Bax*$^{-/-}$(W.C) mice.

The X axis is signal value of control group and the Y axis is signal value of experimental group. Based on the middle line (2 fold line), the upper side indicates over-expression genes and the bottom side indicates down expression genes.

A. *Bax*$^{-/-}$ (B.C) compare with *Bax*$^{+/-}$.

B. *Bax*$^{-/-}$ (W.C) compare with *Bax*$^{+/-}$.

C. *Bax*$^{-/-}$ (W.C) compare with *Bax*$^{-/-}$ (B.C).

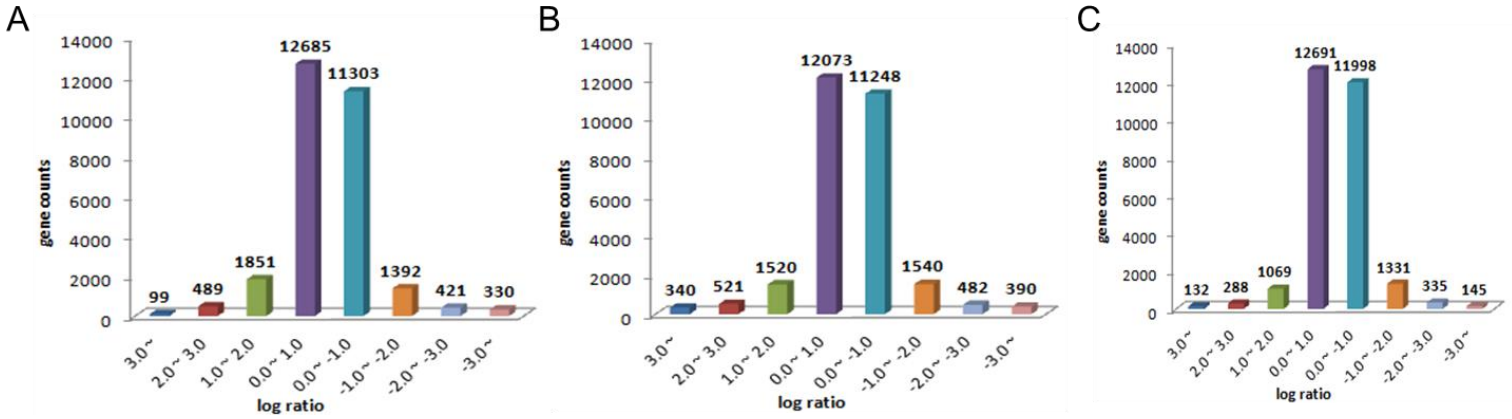


Figure 4. Intensity ratio histogram analysis of mouse genes from decidua at day7 of gestation in *Bax*$^{+/-}$, *Bax*$^{-/-}$(B.C), *Bax*$^{-/-}$(W.C) mice.

The X axis is experimental / control group log2 ratio and the Y axis is the number of changed genes. Except to the middle two line, other line are significantly changed at least 2-fold expression gene. ($p<0.05$)

A. *Bax*$^{-/-}$ (B.C) compare to *Bax*$^{+/-}$

B. *Bax*$^{-/-}$ (W.C) compare to *Bax*$^{+/-}$.

C. *Bax*$^{-/-}$ (W.C) compare to *Bax*$^{-/-}$ (B.C).

Table 3. Summary of differentially expressed genes

	The number of total changed genes	> 2 fold changed genes	
		Over-regulation	Down-regulation
Bax$^{-/-}$(B.C) compare with *Bax*$^{+/-}$	4582	2439	2143
Bax$^{-/-}$(W.C) compare with *Bax*$^{+/-}$	4783	2381	2412
Bax$^{-/-}$(W.C) compare with *Bax*$^{-/-}$(B.C)	3300	1489	1811

Classification by biological function of the genes which were changed more than 2 folds.

Up-regulated or down-regulated genes are categorized according to their known biological functions, such as apoptosis, behavior, cell adhesion, cell cycle, cell differentiation, cell migration, cell proliferation, growth, homeostasis, immune response, inflammatory response, lipid metabolism, response to stress, signal transduction, transcription, translation transport (Fig. 6).

Global expression profiles revealed that genes involving cell proliferation (Fig. 5A), cell differentiation (Fig. 5B), apoptosis (Fig. 5C), cell cycle (Fig. 5D) were down-regulated in *Bax*$^{-/-}$(W.C) decidua compared with the other groups.

The genes which are involved in cell proliferation, differentiation, apoptosis and cell cycle were major and significantly up- and down-regulated in decidua of *Bax*$^{-/-}$(B.C) mice compared with that of *Bax*$^{+/-}$ mice ($p<0.05$) (Fig. 6A). Cell proliferation related 378 genes, cell differentiation related 642 genes, apoptosis related 359 genes, and cell cycle related 238 genes were up- or down-regulated in *Bax*$^{-/-}$(W.C) mice compared with *Bax*$^{+/-}$ mice ($p<0.05$) (Fig. 6B). In comparison between *Bax*$^{-/-}$(W.C) and *Bax*$^{-/-}$(B.C), cell proliferation related 277 genes, cell differentiation related 466 genes, apoptosis related 239 genes, and cell cycle related 281 genes were significantly up- or down-regulated ($p<0.05$) (Fig. 6C).

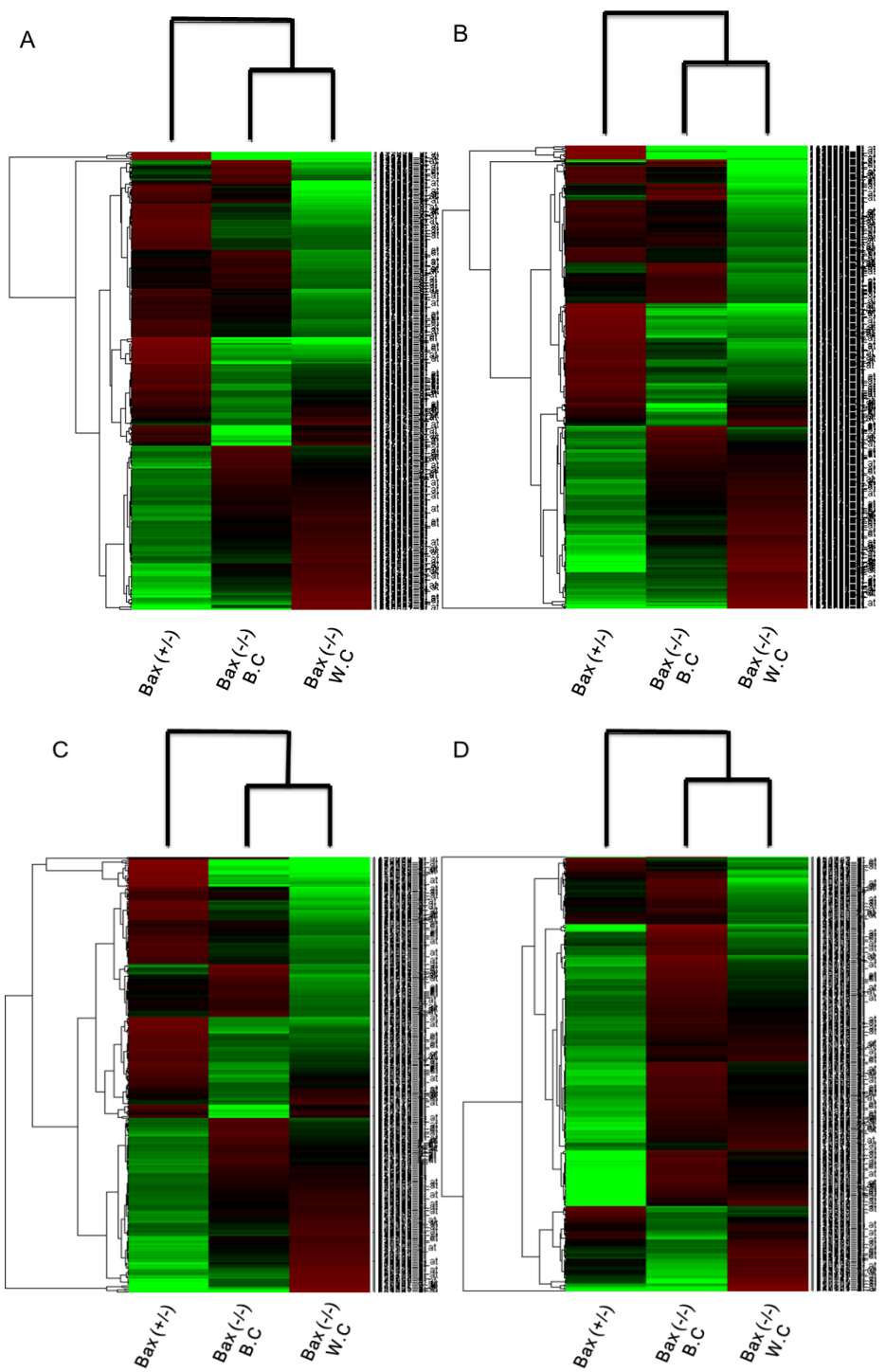
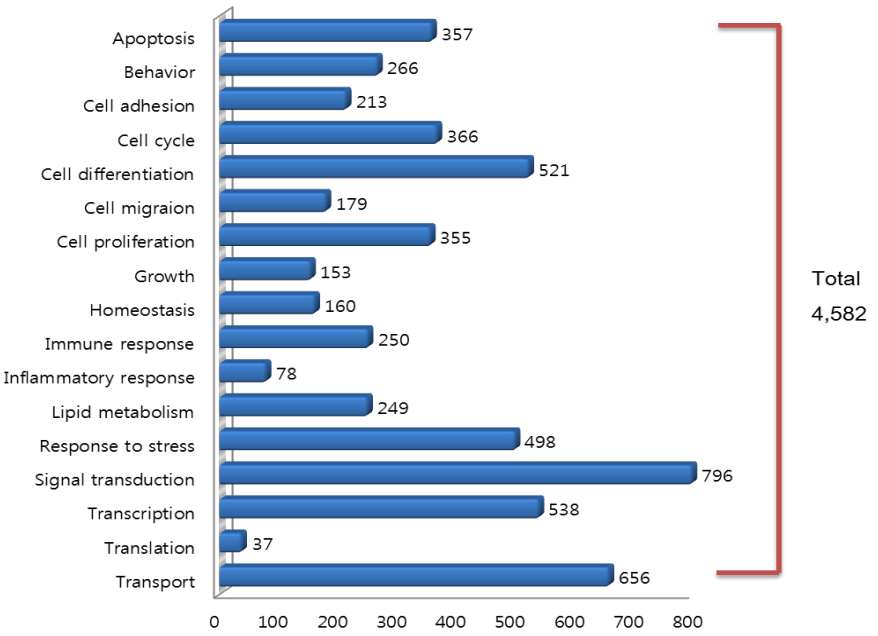

A
B
C
D
Bax (+/-)
Bax (-/-) B.C
Bax (-/-) W.C

Figure 5. Hierarchical clustering analysis according to their functions.

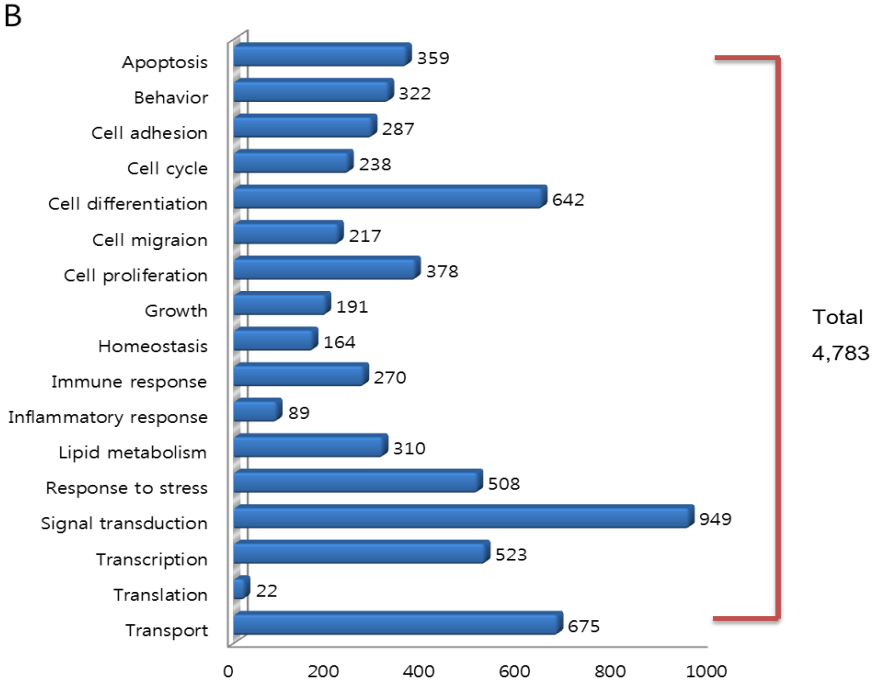
Relative changes in their expression level were indicated by a color code. Red indicates that the gene is up-regulated, and green indicates that the gene is down-regulated.

A. Relative change in gene expression belong to proliferation.
B. Relative change in gene expression belong to cell differentiation.
C. Relative change in gene expression belong to apoptosis.
D. Relative change in gene expression belong to cell cycle.

A


Apoptosis
Behavior
Cell adhesion
Cell cycle
Cell differentiation
Cell migraion
Cell proliferation
Growth
Homeostasis
Immune response
Inflammatory response
Lipid metabolism
Response to stress
Signal transduction
Transcription
Translation
Transport
357
266
213
366
521
179
355
153
160
250
78
249
498
796
538
37
656
0
100
200
300
400
500
600
700
800
Total
4,582

B


Apoptosis
Behavior
Cell adhesion
Cell cycle
Cell differentiation
Cell migraion
Cell proliferation
Growth
Homeostasis
Immune response
Inflammatory response
Lipid metabolism
Response to stress
Signal transduction
Transcription
Translation
Transport
359
322
287
238
642
217
378
191
164
270
89
310
508
949
523
22
675
0
200
400
600
800
1000
Total
4,783

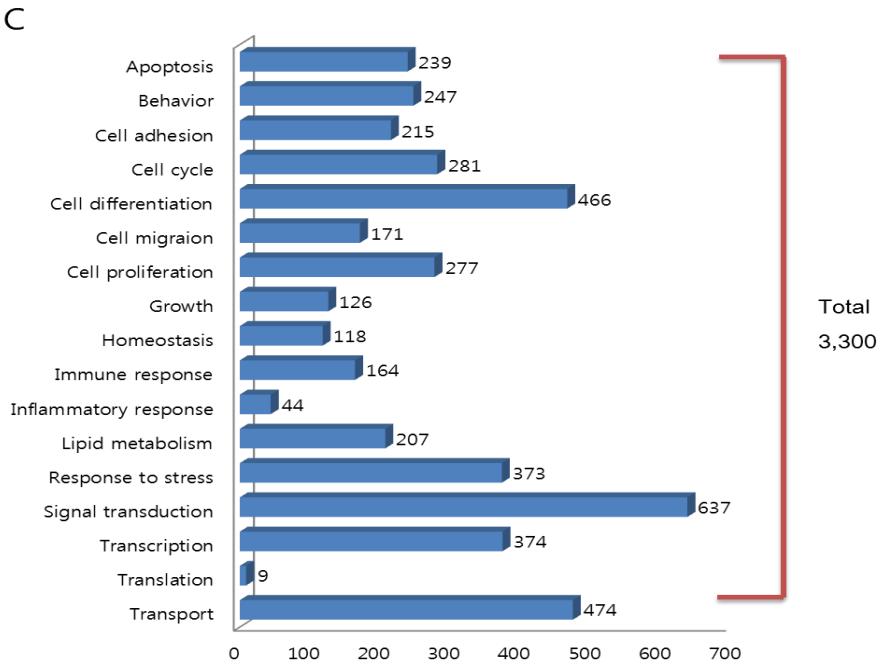


Figure 6. Distribution of changed gene expression among different functional classes.

Up-regulated or down-regulated genes are categorized according to their biological functions.

A. *Bax*$^{-/-}$(B.C) compare with *Bax*$^{+/-}$.

B. *Bax*$^{-/-}$(W.C) compare with *Bax*$^{+/-}$.

C. *Bax*$^{-/-}$(W.C) compare with *Bax*$^{-/-}$(B.C).

Expression patterns could be classified with 9 clusters.

Gene expression patterns can be clustered to nine groups. Cluster analysis was conducted into 3486 genes that significantly changed between three groups.

Cluster 1 (123 genes, 3.5%) showed increase or similar levels in the *Bax*$^{-/-}$(B.C) and a dramatic decrease in the *Bax*$^{-/-}$(W.C). Cluster 2 (547 genes, 15.7%) showed increase in the *Bax*$^{-/-}$(B.C) and the *Bax*$^{-/-}$(W.C) and showed peak at *Bax*$^{-/-}$(W.C). Cluster 3 (443 genes, 12.7%) showed decrease in the *Bax*$^{-/-}$(B.C) and a dramatic increase in the *Bax*$^{-/-}$(W.C). Cluster 4 (454 genes, 13.0%) showed a steady decrease in the *Bax*$^{-/-}$(B.C), keep constant in the *Bax*$^{-/-}$(W.C). Cluster 5 (201 genes, 5.8%) showed a dramatic increase in the *Bax*$^{-/-}$(B.C) and the *Bax*$^{-/-}$(W.C) and showed peak at *Bax*$^{-/-}$(W.C). Cluster 6 (542 genes, 15.5%) showed decrease in the *Bax*$^{-/-}$(B.C) and the *Bax*$^{-/-}$(W.C), gradually. Cluster 7 (80 genes, 2.3%) showed a dramatic decrease in the *Bax*$^{-/-}$(B.C), keep constant in the *Bax*$^{-/-}$(W.C). Cluster 8 (368 genes, 10.6%) showed a dramatic increase in the *Bax*$^{-/-}$(B.C), decrease in the *Bax*$^{-/-}$(W.C). Cluster 9 (728 genes, 20.8%) showed a steady increase in the *Bax*$^{-/-}$(B.C) and the *Bax*$^{-/-}$(W.C), gradually (Fig. 7A).

A

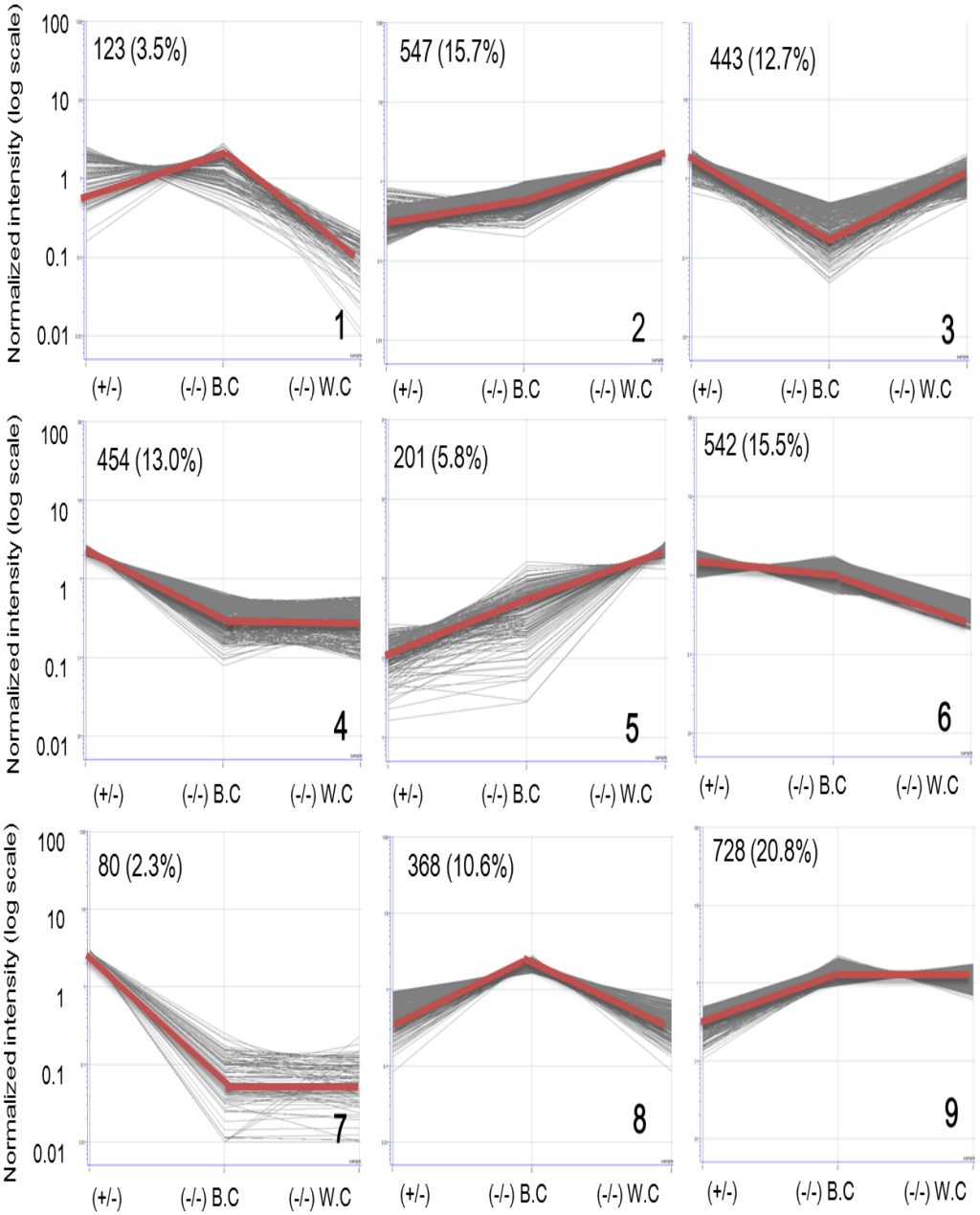

Normalized intensity (log scale)
100
10
1
0.1
0.01
123 (3.5%)
1
547 (15.7%)
2
443 (12.7%)
3
454 (13.0%)
4
201 (5.8%)
5
542 (15.5%)
6
80 (2.3%)
7
368 (10.6%)
8
728 (20.8%)
9
(+/-)
(-/-) B.C
(-/-) W.C

Figure 7. Cluster analysis of expression pattern.

Gene expression patterns were clustered to nine group.

A. Expression changes analyzed by clustering method. The X axis is three group, respectively. The Y axis is normalized intensity (log scale). Red line indicates novel patterns of gene expression.

Transcriptional validation of microarray data.

To validate the microarray data, real time RT-PCR method was employed. A panel of up-regulated genes (Table 4) and down-regulated genes (Table 5) were chosen and applied to analysis. Expression levels of up-regulated genes, *Cd24a, Tgfbi, Car2* and *Calb1* were significantly higher in *Bax*$^{-/-}$(W.C) decidua compared with *Bax*$^{+/-}$ or *Bax*$^{-/-}$(B.C) (Table 4, Fig. 8A-D). The expression levels of down-regulated genes, *Gpx3, Ly6a, Rgs2* and *Gja1* were significantly lower in *Bax*$^{-/-}$(W.C) decidua than those of *Bax*$^{+/-}$ or *Bax*$^{-/-}$(B.C) (Table 5, Fig. 8E-H).

Table 4. Microarray data summary of up-regulated genes in *Bax*$^{-/-}$(W.C) compare with the other group

Gene Symbol	Signal value			Fold change of *Bax*$^{-/-}$(B.C) compare with *Bax*$^{+/-}$	Fold change of *Bax*$^{-/-}$(W.C) compare with *Bax*$^{+/-}$
	Bax$^{+/-}$	*Bax*$^{-/-}$(B.C)	*Bax*$^{-/-}$(W.C)		
Cd24a	344.54	1145.61	3122.33	3.32	9.06
Tgfßi	326.77	849.2	2236.48	2.59	6.84
Car2	180.52	584.87	1792.47	3.23	9.92
Calb1	11.72	107.87	1209.27	9.2	103.14

Table 5. Microarray data summary of down-regulated genes in *Bax*$^{-/-}$(W.C) compare with the other group

Gene Symbol	Signal value			Fold change of *Bax*$^{-/-}$(B.C) compare with *Bax*$^{+/-}$	Fold change of *Bax*$^{-/-}$(W.C) compare with Bax$^{+/-}$
	Bax$^{+/-}$	*Bax*$^{-/-}$(B.C)	*Bax*$^{-/-}$(W.C)		
Gpx3	3853.99	1791.86	47.84	-2.15	-80.55
Ly6a	3935.87	1300.69	259.02	-3.02	-15.19
Rgs2	1076.93	929.87	215.36	-1.15	-5.0
Gja1	2608.22	834.71	313.87	-3.12	-8.3

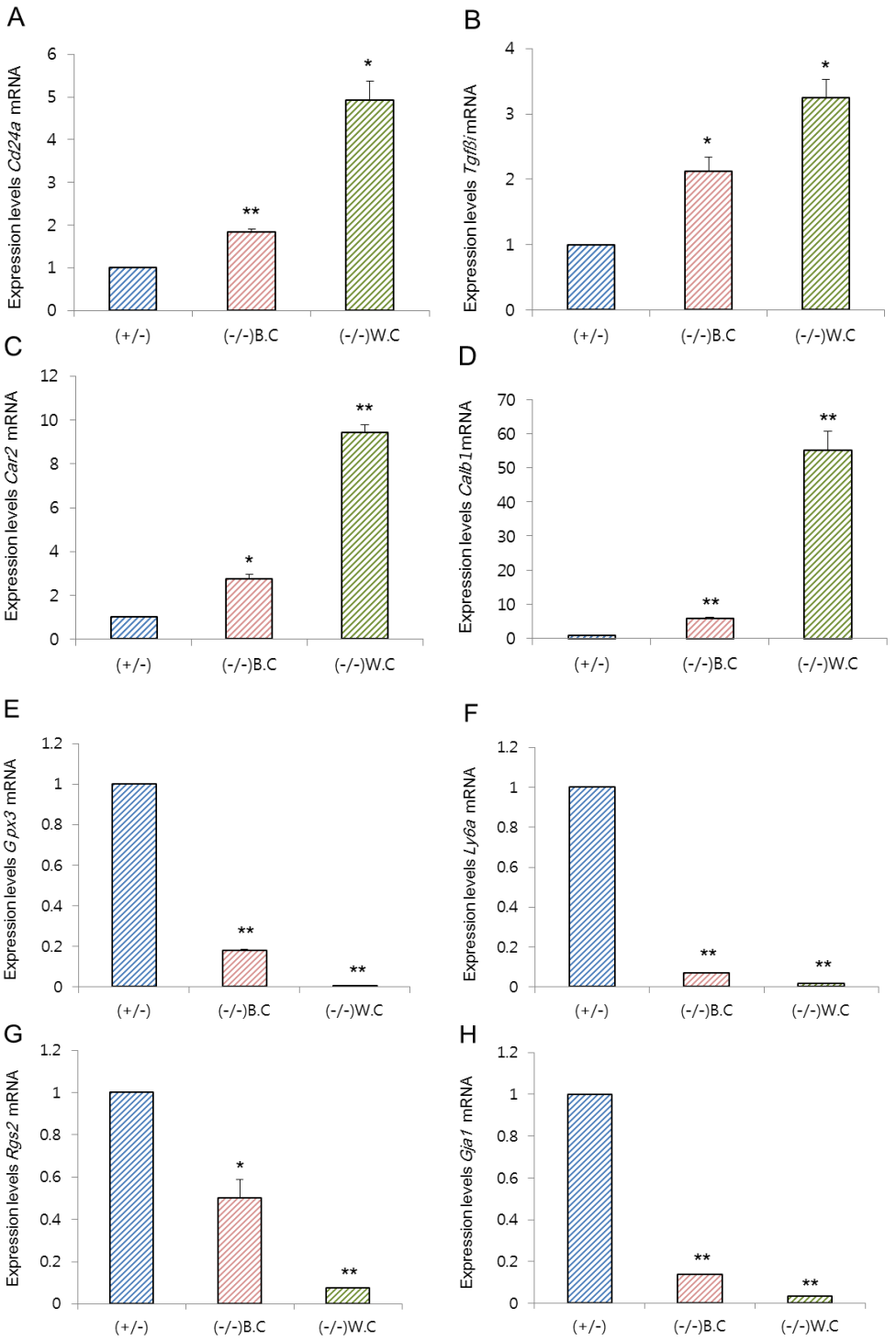

A
Expression levels Cd24a mRNA
6
5
4
3
2
1
0
*
**
(+/-)
(-/-)B.C
(-/-)W.C
B
Expression levels Tgfβi mRNA
4
3
2
1
0
*
*
(+/-)
(-/-)B.C
(-/-)W.C
C
Expression levels Car2 mRNA
12
10
8
6
4
2
0
**
*
(+/-)
(-/-)B.C
(-/-)W.C
D
Expression levels Calb1mRNA
70
60
50
40
30
20
10
0
**
**
(+/-)
(-/-)B.C
(-/-)W.C
E
Expression levels Gpx3 mRNA
1.2
1
0.8
0.6
0.4
0.2
0
**
**
(+/-)
(-/-)B.C
(-/-)W.C
F
Expression levels Ly6a mRNA
1.2
1
0.8
0.6
0.4
0.2
0
**
**
(+/-)
(-/-)B.C
(-/-)W.C
G
Expression levels Rgs2 mRNA
1.2
1
0.8
0.6
0.4
0.2
0
*
**
(+/-)
(-/-)B.C
(-/-)W.C
H
Expression levels Gja1 mRNA
1.2
1
0.8
0.6
0.4
0.2
0
**
**
(+/-)
(-/-)B.C
(-/-)W.C

Figure 8. Confirm the microarray data with quantitative RT-PCR. The selected genes were up- or down- regulated genes in decidua *Bax*$^{-/-}$.

Total RNA was extracted from the decidua at day 7 of gestation in *Bax*$^{+/-}$, *Bax*$^{-/-}$ (B.C), *Bax*$^{-/-}$(W.C) mice, respectively. qRT-PCR analysis was used to validate gene array data, which showed up- or down regulated genes in the *Bax*$^{-/-}$ (B.C) and the *Bax*$^{-/-}$ (W.C), gradually. Analysis was repeated three times, and mRNA level normalized using *36B4* mRNA level as the housekeeping control. Values represent the mean ± SD. Statistical analysis was performed by Student's t-test with hetero type. *, Statistically significant change (p<0.05). **, Statistically significant change (p< 0.01).

The expression levels of *Cd24a* (A), *Tgfßi* (B), *Car2* (C), *Calb1* (D), *Gpx3* (E), *Ly6a* (F), *Rgs2* (G), *Gja1* (H) mRNA expression at day 7 of gestation in *Bax*$^{+/-}$, *Bax*$^{-/-}$(B.C), *Bax*$^{-/-}$(W.C) mice, respectively.

Decreased Alkaline phosphatase protein levels in implantation area in *Bax*$^{-/-}$ (W.C).

To examine the formation of functional decidua, healthy embryos were transferred into the uterine lumen. The recipient mice were sacrificed at 96 hr after embryo transfer and got uterus. Using immunofluorescence method, alkaline phosphatase (ALP) protein was localization in the decidua of *Bax*$^{+/-}$, *Bax*$^{-/-}$(B.C) and *Bax*$^{-/-}$(W.C) mice, respectively. While *Bax*$^{+/-}$ and *Bax*$^{-/-}$(B.C) implantation areas around embryo were detected ALP protein, *Bax*$^{-/-}$(W.C) did not detected (Fig. 9A-C). These results indicate that *Bax*$^{-/-}$(W.C) mice are not properly respons to the decidual signaling.

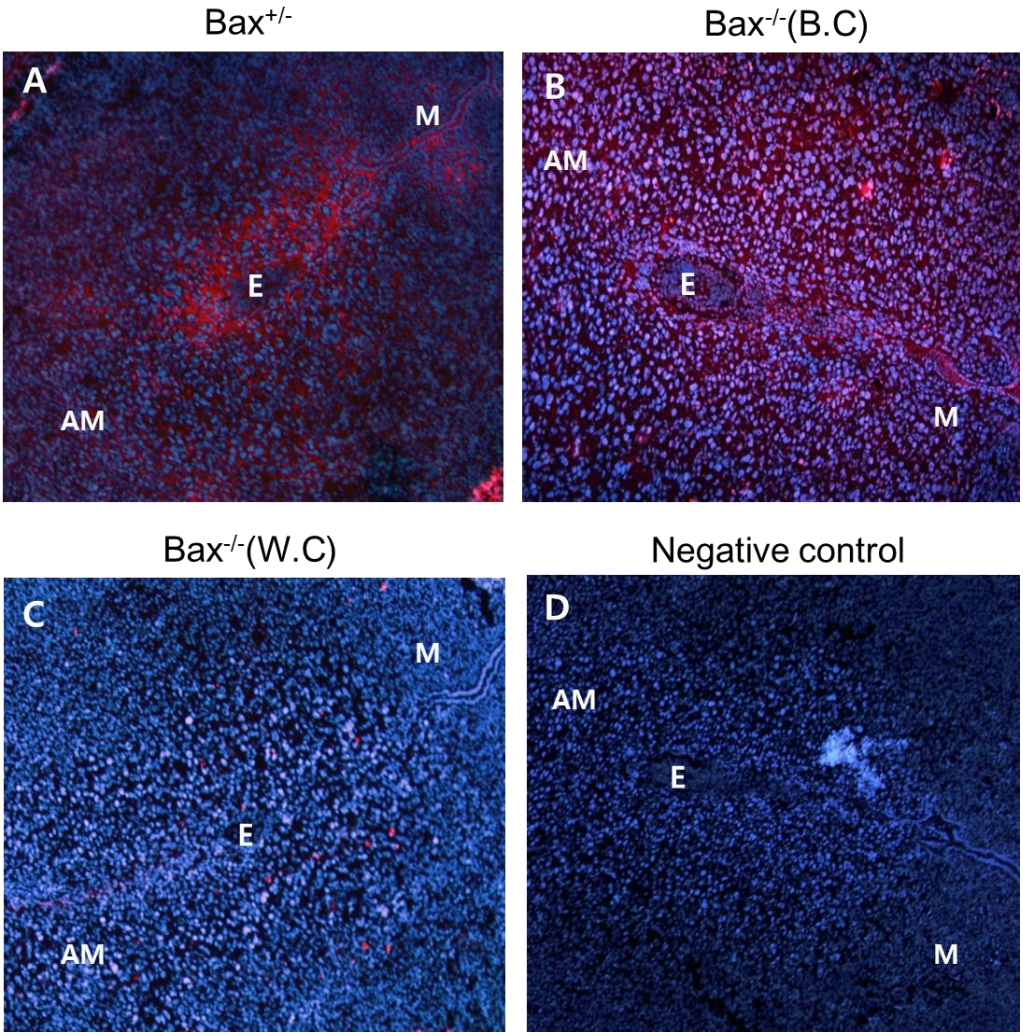


Figure 9. Immunofluorescence of ALP in decidual zone of mice.

Immunofluorescence staining of alkaline phosphatase protein in decidual zone at 96 hr after embryo transfer of (A) *Bax*$^{+/-}$, (B) *Bax*$^{-/-}$(B.C), (C) *Bax*$^{-/-}$(W.C) mice, respectively. (D) Negative control. Red color indicated that ALP protein, blue color indicated that nucleus. Abbreviation: AM, antimesometrium; E, embryo; M, mesometrium. Magnification: x100.

Inhibition of the the decidual differentiation of endometrial stromal cells by c-MYC antagonist.

The expression levels of *c-Myc* downstream genes were dramatically decreased in decidua of *Bax*$^{-/-}$ mice. Thus, to evaluate the potential role of Micro1 as a c-MYC antagonist in stromal cells during decidualization, *in vitro* decidual induction culture method was conducted. Mouse endometrial stromal cells (mESCs) were isolated of *Bax*$^{+/-}$ mice in PMSG primed. During in vitro decidualization induction, cells were treated with 50 μM Micro 1.

By the qRT-PCR result, the expression levels of *c-myc* mRNA were significantly decreased at 50 μM concentration. As the *c-myc* mRNA expression, the expression levels of *Prl*, *Igfbp1*, *Cx43*, *Doc1*, and *Ctla2ß* mRNA were significantly decreased at 50 μM concentration (Fig. 10). From these result, the Micro1 significantly suppressed *c-myc* mRNA and the level of mRNAs which are involved in decidualization, even though the cells were cultured in decidual induction media.

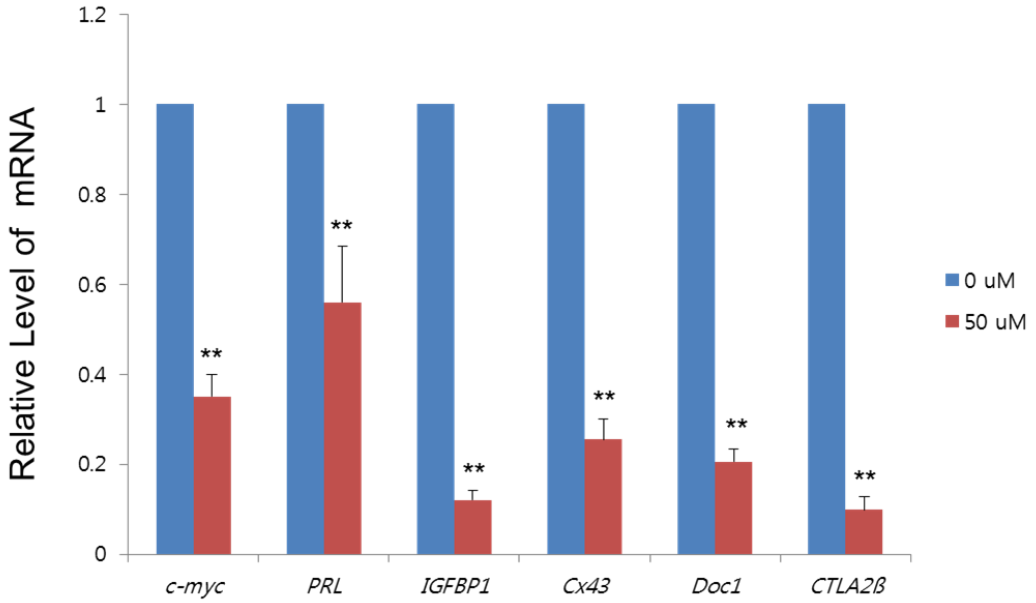


Figure 10. The level of mRNAs which are known decidualization markers during *in vitro* decidual induction.

Mouse endometrial stromal cells (mESCs) were isolated of *Bax*$^{+/-}$ mice in PMSG primed. During in vitro decidualization induction, cells were treated with 50 μM Mycro1. After 96 hr, total RNA was extracted from the cells. Analysis was repeated three times, and mRNA level normalized using *36B4* mRNA level as the housekeeping control. Values represent the mean ± SD. Statistical analysis was performed by Student's t-test with concentration matched.

*, Statistically significant change (p<0.05). **, Statistically significant change (p < 0.01).

DISCUSSION

One of the preconditions for mammalian reproduction is successful reciprocal communication between the implanting blastocyst and the receptive uterus. Implantation can just occur if the uterus is receptive to the approaching blastocyst. And there is a defined “window of receptivity”. The attachment of blastocyst to uterine epithelium triggers the process of decidualization, which involves a dramatic transformation of the fibroblastic endometrial stromal cells intrinsic the surface epithelium into morphologically and functionally different cells, this is termed decidua. After embryo implantation, the stromal differentiation to decidual and rearrangement of endometrial tissue at the implantation chamber require the apoptotic death (Chatzaki et al., 2003). Therefore, apoptosis is crucial procedure in implantation of the embryo.

Microarray analysis has established to be the most powerful technique for the global gene expression profiling (Hamatani et al., 2004; Schena et al., 1995). Many of the developmental biologists have turned to genome-scale expression analysis to expedite gene discovery. For instance, to examine the developmental regulation of gene expression, cDNA and oligonucleotide microarrays have been widely used. In this study, using Affymetrix oligonucleotide arrays containing >39,000 transcripts and variants, we report here changes in the global patterns of gene expression that occur during decidualization of *Bax*$^{+/-}$, *Bax*$^{-/-}$(B.C), *Bax*$^{-/-}$ (W.C), respectively. The branch tree distance was nearest between *Bax* knockout mice. It means that *Bax*$^{-/-}$(B.C) and *Bax*$^{-/-}$(W.C) are more similar than *Bax*$^{+/-}$. And based on extensive validation by quantitative real time polymerase chain reaction (qRT-PCR), the array results with this strict standard have been confirmed for

fundamentally genes tested (Carter et al., 2003 ; Hamatani et al., 2004). Previously, in our research group evaluated the fertility of *Bax* knockout mice. The fertility was decreased and decidual defect was suggested in *Bax*$^{-/-}$(W.C) mice. Global expression analysis using microarray reveal that the expect is correct. From the result of microarray analysis, it is suggested that the large number of genes were changed and associated with decidualization during early pregnancy. These highly over expressed or down expressed genes were thought to be involved in decidual defects.

Stromal cell proliferation is a prerequisite for differentiation during implantation and the cell cycle regulation is critical. Interestingly from the expression profiles in microarray, the cell cycle related genes were dramatically decreased in *Bax*$^{-/-}$ (W.C.). It suggested that a partial defect in cell proliferation in the stromal cells of implantation area is caused the defect the fertility of *Bax*$^{-/-}$(W.C). It means that cell cycle molecules have been suggested to be intimately involved in this process. Because of these reason, focus on changed genes of cell proliferation, differentiation, apoptosis and cell cycle. When healthy embryo transfer to the uterine horn of mice, *Bax*$^{+/-}$, *Bax*$^{-/-}$(B.C) implantation areas around embryo were highly expressed of alkaline phosphatase (ALP). On the other hand, *Bax*$^{-/-}$(W.C) did not expressed ALP. ALP is highly expressed in functional decidualized cell, so it is known as decidualization marker. These results indicate that *Bax*$^{-/-}$(W.C) mice are not properly respond to the decidual signaling during implantation period.

Coat color of C57BL/6 *Bax* mice using this experiment is quite extensive. According to backcross data from the Mouse Genome Database, *Bcl 2*-associate X protein (*Bax*) is at 29.32 cM, pink-eyed dilution (*P*) is at 33.44 cM, and tyrosinase (*Tyr*) is at 49.01 cM on chromosome 7. In other words, the coat color

loci *Tyr* and *P* are linked to the *Bax* gene. From these facts, we suggested that a series of deletions around the coat color locus on mouse chromosome 7 may be include one or more regulatory genes that control of decidualization during implantation period.

c-Myc gene is a member of the basic helix-loop-helix leucine zipper protein family, and is to be important for neural crest (Wei et al., 2007). The transcription factor *c-Myc* has a central role in multiple cellular processes important for embryonic development, including cell growth, differentiation, proliferation, and apoptosis (Amati et al., 1998; Eisenman, 2001; Grandori et al., 2000; Pelengaris et al., 2002). Its expression and posttranscriptional stabilization are tightly regulated upon induction by growth stimulatory signals. color (Wei et al., 2007). In microarray database, interestingly, *c-Myc* downstream genes were down-regulated their expression. In addition, *c-Myc* deficiency in neural crest resulted in viable adult mice that have defects in coat. In addiotion, the *Bax*$^{-/-}$ mice, used in this study, also represent a change in coat color. Interestingly, the expression levels of *c-Myc* specific mRNA were reduced in *Bax*$^{-/-}$(W.C) mice than other group mice. Using *in vitro* decidualization induction system, antagonist of *c-MYC*, a candidate of *Bax* related genes, suppressed the proper decidual reaction. It means that *Bax* regulate stromal cell proliferation and differentiation.

In conclusion, we have provided that *Bax* and *cMyc* related genes are involved in decidual response in pregnant uterus. The absence of *Bax* may don't modulate the expression of *c-Myc* and the down regulation of *c-Myc* is the cause the defect in decidual differentiation in the uterine stromal.

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ABSTRACT

Identification of Bax related genes in the pregnant uterus

: roles in Decidualization

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The process of embryo implantation consists of attachment and invasion of the uterine luminal epithelium. Successful implantation requires the rapid remodeling of the uterine stromal cells in a process termed decidualization. Decidualization is characterized by morphological and functional changes in the uterine stromal cells. This is a complex interplay of transcription factors, morphogens, cytokines, cell cycle regulators and signaling pathways. On day 5 of gestation at the beginning of decidualization, only stromal cells surrounding the implanting blastocyst proliferate. On day 6, the stromal cells in immediate proximity to blastocyst apposition cease to proliferate and form the primary decidual zone (PDZ). And the cells outside the PDZ continue to proliferate and form the secondary decidual zone (SDZ). At the same time with the organogenesis and the growth of embryo, antimesometrial and mesometrial decidua regress by apoptosis and this process is known to be mediated by products of the *Bcl-2*

gene family. Previous study, we observed that black coat color *Bax* KO mice are did not difference in pregnancy rate, delivery rate, litter size, implantation rate and decidualization responsibility compare to wild type. But, decidualization responsibility of white coat color *Bax* KO mice was abnormal. Therefore, in this study, we examined the functional roles of Bax related gene in uterine stromal cell differentiation using *Bax* knockout mice and in vitro decidual model. A total of 4582 genes were significantly changed at least 2-fold during decidualization in $Bax^{-/-}$(B.C) compared with $Bax^{+/-}$. Among these genes, 2439 genes were up-regulated and 2143 genes were down-regulated. A total of 4783 genes were significantly changed at least 2-fold during decidualization in $Bax^{-/-}$(W.C) compared with $Bax^{+/-}$. Among these genes, 2381 genes were up-regulated and 2412 genes were down-regulated. A total of 3300 genes were significantly changed at least 2-fold during decidualization in $Bax^{-/-}$(W.C) compared with $Bax^{-/-}$(B.C). Among these genes, 1489 genes were up-regulated and 1811 genes were down-regulated. From these results, it is suggested that the large number of genes were changed and is associated with decidualization during early pregnancy. Using in vitro decidualization induction system, antagonist of *c-MYC*, a candidate of *Bax* related genes, suppressed the proper decidual reaction. It is suggested that the absence of *Bax* can't modulate the expression of *c-Myc* and the down regulation of *c-Myc* is the cause of loss the decidual differentiation in the uterine stroma.