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석사학위청구논문

In Vitro Developmental Characteristics of Preimplantation Embryos in WBN/Kob Rat

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성신여자대학교 대학원

생 물 학 과

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이 논문을 석사학위논문으로 제출함.

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Submitted in partial fulfillment of the

requirements for the degree of master.

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논 문 개 요

임신율과 착상 후 배 발달의 유지율이 당뇨소견 산모에서 감소되는 것으로 잘 알려져 있다. 이와 같은 문제의 기작을 이해하고 극복하기 위하여 다양한 당뇨 실험동물 모델이 이용되어져 왔다. 이중 WBN/Kob 는 자발적으로 당뇨증상이 유발되는 동물 모델로 알려져 있으며 특정 Quantitative Trait Locus(QTL)이 당뇨와 관여됨이 밝혀졌다. 따라서 본 연구에서는 자발적 당뇨증상이 있는 WBN/Kob 의 초기 배아의 발생 특성을 분석하였고, QTL 에 속한 특정 유전자의 발현 변화와 초기 배아 발생에서 보이는 특성이 관련 되어 있을 가능성을 규명하고자 하였다. WBN/Kob 의 한 배 새끼 수는 성비의 차이 없이 통계적으로 유의하게 대조군 Wistar 에 비해 감소하였다. 암컷 WBN/Kob 는 에스트로겐(estrogen)에 의해 당뇨유발이 억제되기 때문에 혈중 포도당 농도가 정상 수준으로 유지된다. 따라서 이러한 새끼 수 감소가 포도당 농도에 의한 것이 아님을 알 수 있다. 이와 관련하여 한 배 새끼 수가 감소한 원인을 초기 배아에서 찾아보고자 2-세포기의 배아를 수란관으로부터 얻고 mR1ECM 배양액에서 체외배양을 하였다. WBN/Kob 2-세포기 배아의 포배 발생율은 대조군에 비하여 유의하게 감소하였고, 2-/ 4-세포기에서 8-세포기로, 8-세포기에서 상실배까지의 발생에서 WBN/Kob 배아의 발생은 각각 지연되었다. 그러나 상실배로 발생한 배아는 대부분 포배로 발생하였다. 두 그룹간의 차이를 분석하고자 배양 96 시간 후 포배기로 발달한 배아의 할구 수를 계수하여 그 특성을 분석하였다. 포배기의 할구 수는 WBN/Kob 에서 대조군 Wistar 에 비해 유의하게 감소하는 것을 관찰할 수 있었다. 다른 한편으로 체외발생에서 관찰되는 결함이 당뇨의 원인으로 규명된 QTL 내

유전자들에 의한 가능성을 알아보고자 배아에서 발현되는 것으로 알려진 krt8, krt18, bex2, elatase1 등의 유전자 발현 변화를 Q-PCR 방법으로 분석하였다. krt8 은 2-세포기에 발현하기 시작하여 상실기 단계인 hCG 주사 후 120 시간에 가장 많은 발현 양상을 보였고, 포배로 발생하는 시기인 144 시간 이후에는 감소하였다. 그러나 WBN/Kob 배아의 경우 4-/ 8-세포기로 발달한 hCG 주사 후 96 시간에 가장 많은 발현을 하였고 이후 급격히 감소하였다. krt18 은 4-세포기에 발현하기 시작하여 8 세포기에서 상실기 단계인 hCG 주사 후 96 시간부터 급격한 증가를 하여 120 시간에 가장 많은 발현 양상을 보였다. 이때 WBN/Kob 에서는 대조군에 비해 발현 양이 증가하는 것을 관찰했고 포배기단계인 hCG 주사 후 144 시간에는 대조군에 비해 감소하는 것을 관찰하였다. 그리고 bex2 는 2-세포기에서 발현하기 시작하여 포배기 단계까지 모두 발현 하였고 8-세포시기인 hCG 주사 후 96 시간에서 가장 많은 발현 양상을 나타내었다. WBN/Kob 에서도 8-세포기인 hCG 주사 후 96 시간에 발현 양이 크게 증가하였으나 대조군에 비해서는 2 배 이상 적게 발현하였고 상실기 단계인 120 시간에서는 대조군 보다 높게 발현하였다. 또한 포배기 단계인 hCG 후 144 시간에서는 WBN/Kob 배아의 발현 양이 Wistar 배아의 발현 양보다 감소하였다. Elastase1 은 착상전 시기의 배아에서 모두 발현하지 않았다. 종합해보면 당뇨특이 QTL 이 밝혀진 WBN/Kob 는 대조군과 비교하여 착상 전 시기의 배아발달이 배아 단계 특이적 현상을 보이며 난할이 지연되나 상실배로 발달한 배아는 대부분 포배로 발생하였다. 그러나 포배를 구성하는 할구의 수는 WBN/Kob 에서 유의하게 감소하는 것을 알 수 있었다. 또한 WBN/Kob 에서 자발적 2 형 당뇨 관련 QTL 내 특정 유전자의 발현 양상이 WBN/Kob 에서 발달이 지연되는 시기인 hCG 주사 후

72 시간에서 120 시간에서 대조군과 현격한 차이를 보였다. 따라서 이러한 결과를 바탕으로 2 형 당뇨 관련 QTL 내 특정 유전자들이 초기 배아 발생에 영향을 미치는 요인중의 하나가 될 수 있음을 제안한다.

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INTRODUCTION

Type Ⅱ diabetes mellitus (DM) is a metabolic syndrome caused by an imbalance of insulin secretion from β-cells and the insulin sensitivity of target tissues. DM is usually secondary to insulitis in most type Ⅱ DM model. The pathological characteristics have been carried with animal models in which DM develops spontaneously (Akimoto et al., 2008; Tsuchitani et al., 1985; Mori et al., 1995). WBN/Kob rat is well known type Ⅱ DM model, and is secondary to chronic pan-pancreatitis (CP) (Mori et al., 1990; Nakama et al., 1985; Tsuchitani et al., 1985). All of the male rats of this strain commonly develop CP by the age of 3 months (Mori et al., 1990), and DM (characterized by hyperglycemia, hypoinsulinemia, and glycosuria) occurs at 9 months (Nakama et al., 1985). These male develops the diabetes without obesity caused by CP disease developed spontaneously. DM develops in some male rats at about 36 weeks of age, and in all the age of about 68 weeks, spontaneously. However DM is not develop spontaneously in the case of female, because female hormone suppress the onset of hyperglycemia and glycosuria (Saegusa et al., 1992). Therefore, the hyperglycemia is not detected in female WBN/Kob rat.

Genome-wide association mapping of Quantitative trait loci (QTL) has been used to know phenotype and genotype variation (Kathrin et al., 2008). These QTL exist for a wide variety of phenotypes—the Rat Genome Database (RGD) has curated records for 563 unique QTLs representing unique phenotypes (Twigger et al., 2002). QTLs represent regions of the genome cosegregating with the trait of interest and hence are highly likely to contain candidate gene(s) important to the expression of the phenotype (Rapp, 2000).

In WBN/Kob rat, two QTLs are known to be related to spontaneous induction of DM. Pancreatitis and DM in WBN/Kob locus land {*Pdwk1* (DWRat103) and *Pdwk2* (DXGot62), respectively} are identified to DM related QTL (Tsuji et al., 2001). Pancreatic morphologic changes in the WBN/Kob precede the onset of DM. Similar changes are commonly observed in the relatively advanced stage in human (Meisterfeld et al., 2008; Sjoberg et al., 1989). *Pdwk1* corresponds to human chromosome 22q12-q13.33 and 12q12-q13.2. On the other hand, *Pdwk2* corresponds to human chromosome Xp11.21-q21.2 and Xq22-q27.1. Thus, WBN/Kob rats would be a useful model for studies in both CP and CP-induced DM. DM is a common disease with pancreatic cancer and CP, disorders of the exocrine pancreas. Hyperinsulinemia and peripheral insulin resistance are the general diabetic traits in pancreatic cancer, whereas reduced islet cell mass and impaired insulin secretion are typically observed in CP. *Pdwk1* was strongly associated with the development of CP and *Pdwk2* was strongly linked to hyperinsulinemia and an increased body weight (Watanabe et al., 1999; Tsuji et al., 2001). Keratin 8 (krt8), keratin 18 (krt18), bex2 and elastase1 are belong to the WBN/Kob QTL genes, *Pdwk1* and *Pdwk2*. krt8, krt18 and elastase1 belong to these QTL genes. krt 8 and krt 18, which are the first intermediate filament proteins and diagnostic of the first epithelial cells, are expressed during embryogenesis (Brulet et al., 1980; Jackson et al., 1980). krt8 and krt18 are expressed in trophoblast derivatives, embryonic and extraembryonic endoderm (Jackson et al., 1981; Oshima, 1981), and simple epithelia of adult organs, such as liver, lung, kidney, pancreas, gastrointestinal tract, and mammary gland (Oshima et al., 1996). bex2, brain expressed X-

linked 2, is a human gene. bex2 is important for apoptosis in response to ER modulation by E2 (Alvarez, 2005). bex2 gene was expressed in preimplantation stage embryo and related to ovarian granulose cell carcinoma (Brown et al., 1999). In the case of neutrophil elastase, it is expressed in preimplantation stage embryo and is suspected as a controller of embryonic development in mouse (unpublished).

Diabetes is characterized by high concentrations of glucose in the blood, and it is associated with a range of other metabolic perturbations. Hyperglycemia is considered as a main reason of miscarriage and birth defects in pregnant women. In vivo it is very difficult to isolate factors causing embryonic lesions because of the range of abnormal factors presenting simultaneously. The embryo-culture system provides a unique opportunity for investigating effects of single factors associated with diabetes at precise stages of development (Eljington et al., 1997).

So far, the mechanisms DM affect the preimplantation stage embryos are not well established. And the relation of preimplantation embryo development and QTL genes has not been studied in preimplantation embryo. Based on them, development of early stage embryo was analyzed and made a hypothesis that. We can make a hypothesis that DM related QTL genes may affect the embryo development. The study was designed to evaluate the developmental patterns of early stage embryo of WBN/Kob and how QTL genes of a type Ⅱ DM may alter the developmental potency of preimplantation embryo.

Table 1. Litter size and sex ratio of Wistar and WBN/Kob pubs

Colony	N	Litter size	sex ratio
Wistar	6	12.1 ± 1.722	1 : 0.9
WBN/Kob	6	6.2 ± 1.169*	1 : 0.9

Average ± SD

Statistical analysis : Student *t*-test * $p < 0.05$

MATERIALS AND METHODS

Experimental animals

WBN/Kob and Wistar rat were purchased from Japan SLC. Wistar was used as control in this experiment, because WBN/Kob is originated from Wistar. 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 animal house in Sungshin Women's University with diurnal rhythm kept under the 14L:10D schedule with light-on at 06:00 hr and clean room system. Animals were fed ad libitum a standard rodent diet and water.

Superovulaion induction and embryo culture

Female rats 50-70 g body weight were superovulated by injected to 10 IU of pregnant mares serum gonadotrophin (PMSG) and followed by injection of 10 IU of human chronic gonadotrophin (hCG) after 48 hr. Superovulation induced female rat were put in the cage of stud male. The next morning of finding a vaginal plug was defined day 1 of pregnancy. After 48 hr post hCG, embryos were collected from the oviducts and then placed in culture medium, mR1ECM. The mR1ECM composition is shown in Table 2. Embryos (10 per each group) were cultured in 10 μl droplet of mR1ECM at 37℃ under 5% CO_2 in 95% air.

Table 2. Composition of mR1ECM

Ingredient	mM
NaCl	76.7
KCl	3.2
$CaCl_2$	2.0
$MgCl_2$	0.5
$NaHCO_3$	25.0
Na-pyruvate	0.5
Na-lactate	10.0
glucose	7.5
glutamine	0.1
EAA[a]	2% v/v
NEAA[b]	1% v/v
polyvinyl alcohol	1.0 mg/ml

[a] Minimal essential medium (MEM) amino acid solution (GIBCO BRL).

[b] MEM nonessential amino acid solution (GIBCO BRL).

The osmolarity of mR1ECM was about 246 mOsm.

Cell number count of preimplantation embryos using Nuclear Staining

1% trisodium citrate was treated for 4 minutes or Tyrode's solution treated about 1 hr and did next steps. The nuclei were stained with of freshly 50 mg/100 ul hoechest 33342 (Sigma) prepared in fixative mixture (acetic acid/ethanol: 1/9 volume/volume or 100% absolute alcohol). Blastocysts were washed in absolute ethanol and mounted in glycerol on glass slides under light pressure. Stained nuclei were counted using inverted microscope (Olympus BH/2, Japan).

Total RNA extraction and cDNA synthesis

Total RNAs were extracted using SideStep^{TM}Lysis & Stabilization buffer (Strategene) according to the manufacturer's instruction. Total RNA measured OD value and stored at -80℃ until used. First strand cDNA was synthesized using First-strand synthesis system (Invitrogen) Following mixture for first-strand cDNA synthesis; reaction regent 1.0 μl oligo (dT) primer (0.5 μg/ul), 0.5 μl random primers (0.1 μg/ul), 1.0 μl dNTP mix, 7 μl total RNA (5 μg/μl), 2.0 μl standard buffer (10X), 4.0 μl $MgCl_2$ (25 mM), 2.0 μl DTT (0.1 M), 1.0 μl RNase Inhibitor, 1.0 μl SuperScript^{TM} Ⅱ RT and 0.5 μl DEPC treated water. The mixtures were incubated at 65℃ for 5 minutes and pace tube at room temperature for 10 minutes for the primers to anneal to the RNA. And incubated at 42℃ for 50 minutes and incubate at 70℃ for 15 minutes to terminate cDNA synthesis.

Screening the mRNA expression for QTL gene

Transcripts of target genes were detected using RT-PCR (Table 4) using the specific primers QTL genes (Bionics, Table 5). The primer parameters were 50% GC contents, avoiding repeat base pair and lengthening 20-24 mer.

For quantitative PCR (qPCR), all PCR reactions were performed in a 20 µl reaction volume on the 7300 PCR Detection System (Applied biosystems) using the Power SYBR® Green PCR Master Mix (Applied biosystems) and 10 µM of each specific primer. PCR was performed using 2 µl RT product, produced with 1 µM of each primer pairs listed in the Table 4 in triplicate with SYBR Green PCR Master Mix (Applied Biosystems). Reactions were analyzed using Applied Biosystems 7300 System. Dissociation curves were run on all reactions to ensure amplification of a single product with the appropriate melting temperature.

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 significantly different.

Table 3. Thermal cycler schedule

Step	Temperature (℃)	Time	Cycles
Initial cycle	94	5 min	1
	59	30 sec	
	72	1 min	
Denaturation	94	1 min	37
Annealing	59	30 sec	
Extension	72	1 min	
Final cycle	94	1 min	1
	59	30 sec	
	72	7 min	
Hold	4	indefinitely	

Table 4. Sequences of primers for QTL genes

Gene		Primer sequence (5'-3')	Amplified length (bp)
Bex2	S	AAGCCGCAGGATGCTAACAA	316bp
	AS	GGACTCAGGGCATAAGGCAAAA	
krt8	S	CGCATCAGCTCTTCCAGCTTT	288bp
	AS	TGCAGCAAGCTCCATTTGGT	
krt18	S	ACTGAGAAGGAGACCATGCAAGAC	331bp
	AS	CCTTGCGGAGTCCATGAATGT	
elastase1	S	GTTCGCTTCCCTGGTCCTGTAT	337bp
	AS	CATAGCCTGCAGCCACATTGTT	
ß-actin	S	CAGGGTGTGATGGTGGGAAT	287bp
	AS	TGTGGTACGACCAGAGGCATACA	

RESULTS

The Litter size and sex ratio

To know whether there is difference in litter size between WBN/Kob and Wistar, litter size was recorded at neonatal day 1. The litter size was significantly smaller in WBN/Kob (6.2±1.16) compared with the Wistar rats (12.1±1.72) (Table 1). There was no significant difference in sex ratio of Wistar and WBN/Kob pubs.

In vitro development of Wistar and WBN/Kob embryos after induced superovulation

To evaluate the reason of decreased size of litter, preimplantation stage embryos were collected and cultured. 2-cell stage embryos were collected after superovulation induction. Developmental rate of early stage embryo, in WBN/Kob at given times were different from Wistar (Table 5, Fig. 1). The developmental rate to blastocyst was significantly lower in WBN/Kob embryos (62.6%) compared to Wistar embryos (86.5%) (Fig. 2). In addition developmental retardation in WBN/Kob embryos was observed between 4-cell and blastocyst stage compared to Wistar embryos. At 96 hr post hCG injection, developmental rate of WBN/Kob embryos from 2- or 4-cell to 8-cell (40.0%) was significantly lower than Wistar embryos (72.7%). Developmental rate of WBN/Kob embryos from 4- or 8-cell to morula (73.0%) was also lower than Wistar embryos (95.0%) at 120 hr post hCG. And developmental rate of WBN/Kob embryos from morula to blastcyst (69.9%) was lower than Wistar embryos (83.9%) at 120 hr post hCG (Fig. 3).

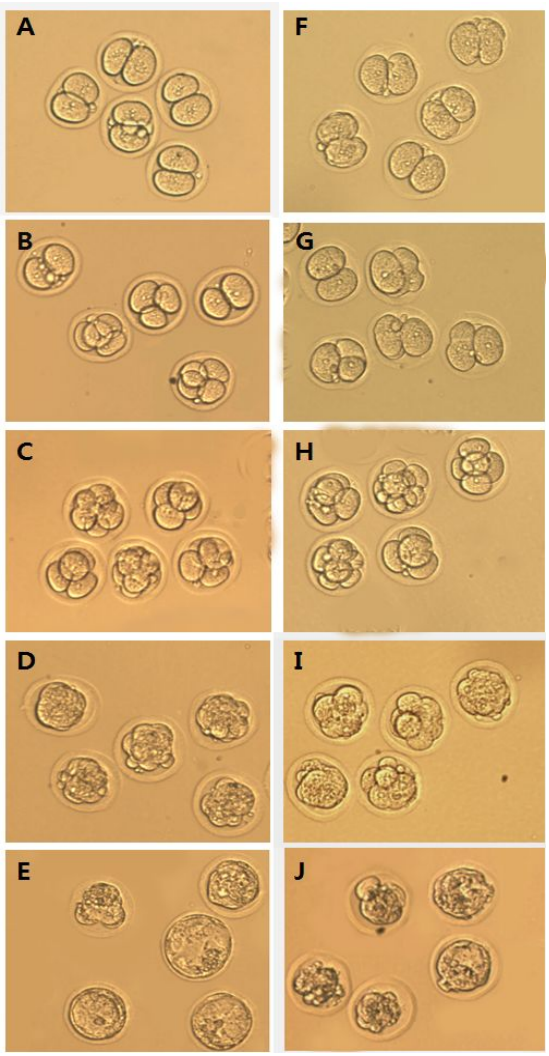

Figure 1. Photomicrograph of preimplantation stage: embryos of Wistar and WBN/Kob rats.

Developmental patterns were recorded using photomicrograph at the times. A - E were the pictures for the Wistar, and F - J were for the WBN/Kob.

A, F: embryos at 48 hr after hCG injection, B, G: embryos at 72 hr, C, H: 96 hr, D, I: 120 hr and E, J: 144 hr after hCG injection (Magnification X100)

Table 5. In vitro development of preimplantation embryos

After hCG injection	Wistar	WBN/Kob
48 hr	2-cell	2-cell
72 hr	2-cell, 4-cell	2-cell, 4-cell
96 hr	4-cell < 8-cell	4-cell ≒ 8-cell
120 hr	Morula, Blastocyst	Morula
144 hr	Blastocyst	Blastocyst

2-cell stage embryos were collected at 48 hr after hCG injection and cultured for 96 hr. At given times, the embryonic stages were analyzed using inverted microscope (Olympus X20).

Figure 2. Reduction of the rate of blastocyst development in WBN/Kob.

The developmental stage of embryos were analyzed after 96 hr after cultere. Ther rate of blastocyst was significantly low in WBN/Kob.

Average ± SD, Statistical analysis : Student *t*-test * $p < 0.001$ Wistar vs. WBN/Kob

Total embryo N = 137, 91 (Wistar, WBN/Kob, respectively)

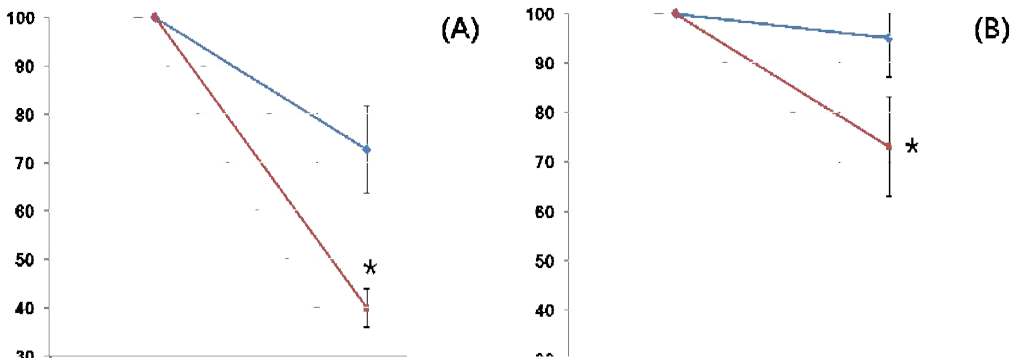

Figure 3. Reduction of develpment in preimplantation stage embryo of WBN/Kob in vitro.

It was analyzed retardation of development was observed during 2- or 4-cell (72 hr) to 8-cell (96 hr) (A), 8-cell (96 hr) to morula (120 hr) (B) and during morula (120 hr) to blastocyst (144 hr) (C). Average ± SD.

Statistical analysis : Student *t*-test $p < 0.05$. Wistar vs. WBN/Kob

Cell number count of preimplantation embryos

Most of morula developed to blastocyst in WBN/Kob, although the rate of development to blastocyst was significantly low. Therefore, we supposed the possibility that the retardation was the reason of developmental rate and litter number in WBN/Kob rats. In blastocyst stage, we checked the cell number. Cell number of blastocyst was counted at 144 hr after hCG injection. Cell number was 35.8 ± 5.17 in Wistar embryos and 21.8 ± 5.62 in WBN/Kob embryos (Fig. 4). Cell number of WBN/Kob blastocysts was significantly lower compared to that of Wistar blastocysts (Fig.5).

QTL genes profile in embryo development

It is well known that there is no DM and CP in female WBN/Kob rat. DM related QTL genes were screened in given time point of embryo after hCG injection (Fig. 6). krt8 gene was expressed in 2-cell stage of Wistar embryo but not in WBN/Kob embryos. krt8 gene was dramatically increased in WBN/Kob embryos compared to Wistar embryos at 96 hr after hCG injection. However, it was decreased at 120 hr and 144 hr after hCG injection compared to Wistar embryos. krt8 in WBN/Kob embryos decreased 6 fold compared to Wistar embryos at 144 hr after hCG injection. Krt8 gene was suspected to have a role in 96 hr time point after hCG injection (Fig. 6A). krt18 gene was expressed at 96 hr, 120 hr, 144 hr after hCG injection in two groups. At 48 hr after hCG injection, krt18 gene was expressed in WBN/Kob embryos but not in Wistar embryos. krt18 gene was only expressed in Wistar embryos at 72 hr after hCG injection. krt18 gene expression of WBN/Kob embryos were higher than

Wistar embryos except at 144 hr. Gene of WBN/Kob embryos was highly expressed at 96 and 120 hr after hCG injection compared to Wistar embryos (Fig. 6B). bex2 gene was not detected in WBN/Kob embryos at 48 hr after hCG injection of 2-cell stage. The expression level of bex2 mRNA was strongly lower in WBN/Kob embryos at 96 hr after hCG injection compared to Wistar embryos. But it is higher than Wistar embryos in 120 hr after hCG injection. The peak was appeared at 96 hr both groups (Fig. 6C). However, in the case of elastase1, we could not detect in preimplantation embryos of rat (Fig. 6D). These results mean that the QTL genes may one of the cause of decrease the developmental rate to blastocyst.

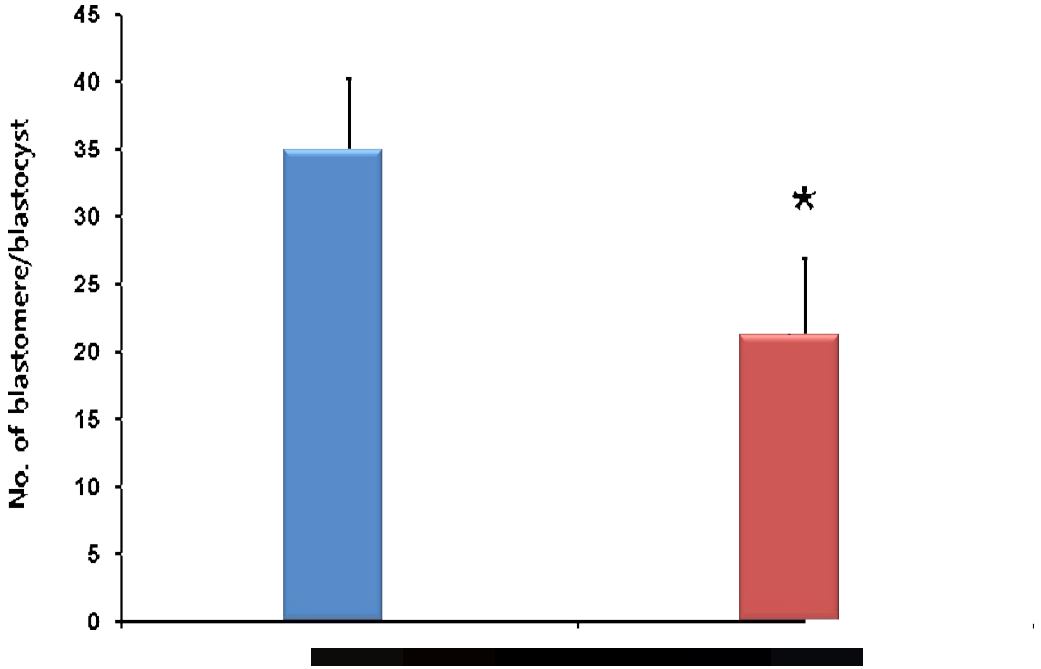

Figure 4. Total cell number of blastocyst of Wistar and WBN/Kob after 96 hr culture

Average ± SD, Statistical analysis : Student *t*-test * $p < 0.05$

The numbers of examined embryos were 10 in each groups.

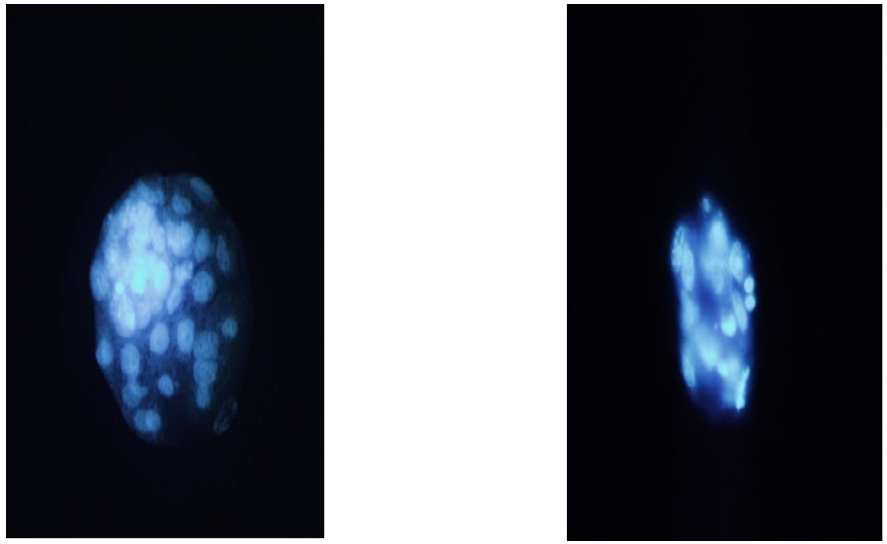

Figure 5. Fluorophotomicrograph of blastocyst Which was stained with Hoechest 33342

Blastocyst stage embryos were fixed and stained as mentioned in Materials and Methods. (A) control, (B) WBN/Kob. (Magnification X400)

A

B

C

D

Figure 5. Expression profiles of DM QTL specific genes in preimplantation embryos of Wistar and WBN/Kob.

qRT-PCR performed as mentioned at Materials and Methods. The level of krt8 (A), krt18 (B), bex2 (C) and elastase1 (D) showed that big difference between WBN/Kob and Wistar embryos.

DISCUSSION

WBN/Kob rat have smaller litter size than Wistar, although the female is not suffer DM. In type Ⅰ DM, it is clear that defects of folliculogenesis are present. And blastocyst is lower rate in type Ⅰ DM rat (Vercheval et al., 1990; Kim et al., 2007). Based on them, it is possible that both type Ⅰ DM and type Ⅱ DM can affect the embryonic development. Preimplantation stage embryos of WBN/Kob develop to the blastocyst in vitro, but the developmental rate is lower than that of the wild-type Wistar embryos. Developmental delay was critically appeared from 4-cell stage to morula stage in WBN/Kob embryos. Those interesting results may be one of the reason of decreased litter size in WBN/Kob embryos.

Glucose is an essential nutrient for most mammalian cells. Glucose is employed as an energy source via either glycolysis or the tricarboxylic acid (TCA) cycle. Additionally, it can be metabolized via the pentose phosphate pathway to produce NADPH and ribose-5-phospate, which can then either re-enter the glycolytic pathway or be employed for nucleotide biosynthesis (Zheng et al., 2007). Glucose plays at this early stage in development. Preimplantation embryos from a number of species are unable to utilize glucose as an energy substrate prior to compaction (Pantaleon et al., 2007). Glycogen synthesis occurs during this period. Glycogen levels rise 10-fold between the one- and two-cell stages in mouse embryos. Adequate glycogen stores may be necessary to meet the embryos' energy needs later during compaction or implantation.

A vast number of studies have been conducted on glucose metabolism in mammalian preimplantation embryos. Most of the reports have shown that the presence of glucose in early preimplantation embryos up to the 8-cell stage is detrimental to further embryo development in vitro in several species, including the hamster, mouse, rat, and human. Glucose-utilizing pathways are active throughout embryonic development. PI3-K may promote embryo survival is through the maintenance of glucose uptake by the regulation of glucose transporter expression at the cell surface. PI3-K/Akt pathway is critical for glucose metabolism in the preimplantation embryo via its ability to regulate GLUT1 expression at the plasma membrane and thus glucose uptake by blastocysts as well as the activity of a key glycolytic enzyme (Riley et al., 2006). Pre-implantation embryo studies using an *in vivo* mouse model have shown that maternal hyperglycemia resulted in downregulation of the embryonic facilitative glucose transporters GLUT1, 2 and 3 at the blastocyst stage of development (Moley, 2001).

An exposure of bovine embryos to high concentrations of glucose (10–30 mM) during development from the one-cell to the blastocyst stage resulted in a decrease in the total number of cells in the blastocysts and an increase in the frequency of apoptotic cells (Karja et al., 2006). Preimplantation period in mammalian development is a critical stage and that a hyperglycemic insult incurred during this period alone can have long lasting detrimental effects on embryo survival and development (Riley et al., 2006). Embryos from 2-cell to blastocyst grown in a maternal diabetic environment in vivo and in vitro have displayed marked apoptosis and disturbed development. Embryos in diabetic

conditions have shown to express decreased gene activity and impacted embryonic growth (Cederberg et al., 1997; Sivan et al., 1997). Although hyperglycemia is not observed in our laboratory and also it has been reported (Pani et al., 2002), the total cell number was decreased in WBN/Kob at blastocyst stage, significantly. It means that the dramatically decrease of total blastomere number is caused by different factors from type Ⅰ and type Ⅱ DM.

Therefore, we investigated gene as a positional candidate gene for DM related QTL. In recent genetic analyses, a number of QTL controlling susceptibility to these diseases have been identified. Some QTL overlap the diseases, raising the possibility that there may be disease genes common to diabetes. Given the large number of QTL identified and the large QTL support interval sizes attained in the genetic segregation analyses used, we investigated krt8, krt18, bex2 and elastase1 gene, in which are located in those QTLs. Keratins are epithelia-specific intermediate-filament protein. krt8 and krt18 are partner, it is found in simple or single-layed epithelial tissue (Moll et al., 1982). These are the first intermediate-filament protein and expressed during murine embryo genesis and are found in trophectoderm at blastocyst stage (Brulet et al., 1980). krt8 and krt18 strongly expressed in pancreas of krt8 and transforming growth factor-ß type Ⅱ receptor transgenic mice. Alterations in differentiation and increased keratin expression also disturb the processes of proliferation and apotosis (Casanova, 1999). krt8 and krt18 has consideration of the possible functions of these proteins in both normal development and in tumorigenesis (Oshima et al., 1996). bex1, for brain expressed X-linked gene, is localized X chromosome. bex2 and bex3 has

highly similar tanscripts to bex1. bex 3 is most similar in sequence to pHGR74. pHGR74 is human homologue of bex3. It was found that is associated with spontaneous juvenile ovarian granulose cell carcinoma (Brown et al., 1999). bex1 gene expressed parthenogenetic, normal fertilized, and preimplantation stage mouse embryos. The bex1 gene appears to be expressed from the maternal X chromosome in blastocyst (Williams, 2002). bex2 modulates apoptosis of breast cancer cells in response to E2. NGF/BEX2/NF-KB pathway is involved in regulating apoptosis in breast cancer cells and in modulating response to tamoxifen in primary tumors (Naderi et al., 2007). This is an initial survey of variation across the DM related gene in WBN/Kob rat during preimplantation period. The expression profile of DM related QTL genes were screened in various stages of WBN/Kob and Wistar embryos after hCG injection. krt8 expression showed peak at 96 hr in WBN/Kob embryos. But its expression was decreased at 120 hr and 144 hr after hCG injection in WBN/Kob embryos compared to Wistar embryos. krt18 was expressed in WBN/Kob embryos at 48 hr after hCG injection but not in Wistar embryos. In 72 hr after hCG injection, krt18 was only expressed in Wistar embryos. krt18 expression of WBN/Kob embryos were higher than Wistar embryos at 96 hr, 120 hr after hCG injection compared to Wistar embryos. But its expression was decreased compared to Wistar embryos at 144 hr after hCG injection. The level of bex2 was significantly decreased in WBN/Kob embryos compared to Wistar embryos at 96 hr. But bex2 of WBN/Kob embryos was higher than Wistar embryos at 120 hr after hCG injection. In summary, krt8 and krt18 expression in WBN/Kob embryos is highr than Wistar embryos at 96 hr after

hCG. bex2 gene is lower than Wistar embryos. But elastase1 was not detected in all stage of embryos in both groups. We found increased the different profiles in gene expression in WBN/Kob embryos from its control, Wistar. These data suggest that the QTL genes may regurate the development of preimplantation embryo.

On summary, in inheritable DM model WBN/Kob rat, the developmental retardation was observed at early embryonic stage and decreases the cell number of blastocyst, although the blood glucose level is normal. On the other hand, DM related QTLs of WBN/Kob has highly corresponds the specific locus in human chromosomes. Some genes located in these QTLs showed genetic patterns for WBN/Kob specific expression. Therefore it is suggested that the development of preimplantation embryo may regulate by the genes of type Ⅱ DM related QTL.

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ABSTRACT

In Vitro Developmental Characteristics of Preimplantation Embryos in WBN/Kob Rat

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Pre-existing diabetes is caused the reduced fertility in female. To solve those problems, the various animal models were used. WBN/Kob is spontaneously develop diabetes in male and it is depend on Quantitative Trait Locus (QTL). However characterization of the preimplantation embryos and the effect QTL genes have not been evaluated in type Ⅱ diabetes mellitus (DM) models including WBN/Kob rat. We hypothesized that a type Ⅱ DM may alter preimplantation embryo's ability of differentiation, relative to QTL. Litter size of WBN/Kob embryos was significantly smaller than the Wistar embryos without no different in sex ratio. Usually hyperglycemia is a cause of defect in embryo development but WBN/Kob female is not suffering the DM by estrogen. Therefore it is cleared that the decrease of litter size did not result of hyperglycemia. To evaluate the reason, two-cell embryos were harvested from

the oviducts and cultured in vitro in mR1ECM medium (rat 1-cell embryo culture medium). We observed developmental rate from 2-cell to blastocyst for 144 hr after hCG injection between Wistar and WBN/Kob. Liter size in WBN/Kob rats (6.2 ± 1.16) was significantly less than Wistar rats (12.1 ± 1.72). The rate of blastocyst development from 2-cell stage was significantly decreased in WBN/Kob embryos compare to Wistar embryos. Developmental retardation was observed between 4-cell and morula stage. However, after morula stage, there was no difference in developmental rate to blastocyst. To evaluate the difference between two groups, the blastomere number was counted using differential staining method. The total cell number was significantly less in WBN/Kob blastocyst than that of the control blastocyst. Also, to know the possibility, whether DM specific QTL genes affect the development of early stage embryos, the expression patterns analyzed using QPCR of a few genes. krt8 gene in WBN/Kob embryo were increased twofold compared to Wistar embryos at 96 hr after hCG injection but decreased at 120 hr and 144 hr after hCG injection. krt18 gene in WBN/Kob embryos was increased twofold compared to Wistar embryos after 96 hr and 120 hr after hCG injection. But at 144 hr after hCG injection, WBN/Kob embryos were decreased compare to Wistar embryos. bex2 gene in two groups was strongly increased at 96 hr after hCG injection and WBN/Kob embryos was smaller than Wistar embryos in this stage. WBN/Kob embryos were increased compared to Wistar embryos at 120 hr after hCG injection but decreased at 144 hr after hCG injection. On summary, the early stage embryo of WBN/Kob was showed developmental delay in vitro. In addition the total number of

blastocyst was significantly less than control. On the other hand, DM specific QTL genes expressed in preimplantation stage embryo were showed the different expression patterns from control. Based on that it is suggest that the retardation of early embryonic development and the reduced rate of blastocyst development were caused by genes where are the members of Type Ⅱ DM QTL.

감사의 글

인쇄본을 넘기지 못할 것 같았는데…… 어느덧 인쇄본을 넘기려고 감사의 글을 쓰고 있는 것이 믿기지 않습니다. 때론 힘들기도 보람되기도 즐겁기도 한 그 동안의 시간들이 주마등처럼 지나갑니다. 처음엔 2 년이라는 시간이 길게만 느껴졌는데 벌써 어느덧 학위과정을 마치고 다시 새로운 출발을 하고자 하니 설렘 반 두려움 반 입니다. 하지만 저에게 도움을 주셨던 많은 분들을 생각하면 힘차게 다시 나갈 수 있을 것이란 희망이 생겨납니다.

먼저 아무것도 모르는 제가 부족하지만 논문을 쓸 수 있도록 이끌어 주신 지도교수이신 전용필 교수님께 깊은 감사 드립니다. 학부 때부터 2 년 반이라는 시간을 먼저 솔선수범하시는 모습과 깊은 배려로 이끌어주신 교수님의 모습은 저에게 꿈과 희망을 키워주셨습니다. 교수님의 가르침대로 앞으로 목표를 향해 최선을 다하는 모습으로 보답하겠습니다. 그리고 학부 때부터 실험에 도움을 주시고 언제든지 반겨주시면서 심사위원장을 해주신 김해권 교수님과 바쁘신 가운데도 논문심사를 위해 시간을 내어주시고 많은 조언을 해주신 윤산현 박사님께도 깊은 감사의 마음을 전합니다. 학부 때부터 큰 가르침을 주시고 대학원에 와서 학과조교를 하면서 많이 부족한 저에게 애정 어린 격려를 주셨던 생물학과 배인하 교수님, 오용자 교수님, 박경숙 교수님, 강혜순 교수님, 윤진호 교수님, 김인순 교수님께도 감사의 말씀을 전합니다.

2 년 반이라는 시간 동안 없어서는 안될 우리 cheons lab 식구들 에게도 감사의 마음을 전합니다. 만삭의 몸이셨어도 열정적인 모습으로 완벽히 일을 하시던 혜영 언니, 대학원생들 중 홀로 미국학회에 가게 돼서 외로운 저를 오셔서 응원해주시고 돌아와서 논문관련 실험에 절대적인 조언을 아낌없이 해주셨던 성은언니, 학업과

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