

## Generation of Embryonic Stem Cell-derived Transgenic Mice by Using Tetraploid Complementation

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**ABSTRACT :** The objective of this study was to generate transgenic mice expressing human resistin gene by using the tetraploid-embryonic stem (ES) cell complementation method. Human resistin gene was amplified from human fetal liver cDNA library by PCR, cloned into pCR<sup>®</sup> 2.1 TOPO<sup>®</sup> vector and constructed in pCMV-Tag4C vector. Mammalian expression plasmid containing human resistin was transfected into D3-GL ES cells by Lipofectamine 2,000, and then after 10-12 days of transfection, the human resistin-expressing cells were selected with G418. In order to produce tetraploid embryos, blastomeres of diploid embryos at the two-cell stage were fused with two times of electric pulse using 60 V 30  $\mu$ sec (fusion rate: 2,114/2,256, 93.5%) and cultured up to the blastocyst stage (development rate: 1,862/2,114, 94.6%). The selected 15-20 ES cells were injected into tetraploid blastocysts, and then transferred into the uteri of E 2.5 d pseudopregnant recipient mice. To investigate the gestation progress, two E 19.5 mused fetuses were recovered by Cesarean section of which one fetus was confirmed to contain human resistin gene by genomic DNA-PCR. Therefore, our findings demonstrate that tetraploid-ES mouse technology can be considered as a useful tool to produce transgenic mice for the rapid analysis of gene function *in vivo*. (*Asian-Aust. J. Anim. Sci.* 2004, Vol 17, No. 12 : 1641-1646)

**Key Words :** Human Resistin, Tetraploid, Embryonic Stem Cell, Mouse

### INTRODUCTION

Mouse embryonic stem (ES) cells derived from the inner cell mass (ICM) of blastocyst are the most totipotent cells that can develop to all cell lineages, when introduced into the embryonic environment by diploid blastocyst (Bradley, 1987; Nagy et al., 1990; Lee and Piedrahita, 2003). Since tetraploid cells have only a limited potential for post-implantation development (Kubiak and Tarkowski, 1985; Kaufman, 1990; Nagy et al., 1990), the application of tetraploid embryos as host cells is one of the most important advances in this direction. When tetraploid embryos are aggregated with diploid embryos, the differentiation of tetraploid cells is mostly restricted to primitive endoderm and trophectoderm that forms extraembryonic tissues, whereas the diploid cells can form the embryo proper (James and West, 1994; James et al., 1995). Most tetraploid mouse blastocysts are not capable of completing normal development independently, but when complemented by introduction of diploid ES cells injection or morula aggregation develops into conceptus wherein the embryo proper (epiblast) is derived entirely from the ES cells and the extraembryonic lineages arise largely from the tetraploid host cells. Thus, introduction of ES cells into tetraploid embryo is a novel method for generation of mice that is completely derived from ES cells (Nagy et al., 1993; Ueda et al., 1995; Wang et al., 1997). This method allows

the immediate generation of targeted murine mutants from genetically modified ES cell clones in contrast to the standard protocol which involves the production of chimeras and several breeding steps.

Several studies demonstrated the successful generations of viable fertile ES mice derived exclusively from ES cells when early passage wild-type R1 (Nagy et al., 1993) and TT2 (Ueda et al., 1995) cells were used in aggregation with tetraploid morula. Successful production of viable and fertile ES mice directly from genetically modified ES cells using tetraploid blastocyst injection was first reported by Wang et al. (1997). Recently, tetraploid-ES method has been significantly improved through the discovery that ES cells derived from hybrid strains supported the development of viable ES mice more efficiently than inbred ES cells did (Eggan, 2001; Schwenk, 2003). In addition, it has been demonstrated that hybrid embryonic stem cell-derived mice showed apparently normal morphological, physiological, and neurological characteristics (Schwenk, 2003).

Resistin is a recently identified adipocytokine that, based on animal studies, has been proposed to be a link between obesity and type II diabetes (Steppan, 2001). It is predominantly expressed in adipocytes of rodents while in human peripheral blood mononuclear cells (PBMC) seem to be a major source. However, the role of resistin is poorly understood in humans. Based on the murine study, the literatures of human resistin is divided with reports concerning about both for and against a role of resistin in human obesity and diabetes. Answers to these doubts await the development and analysis of animal model systems designed to alter resistin levels.

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Therefore, this study was designed to generate the transgenic mice expressing human resistin gene by using tetraploid-ES cell complementation method.

## MATERIALS AND METHODS

### Construction of human resistin expression vector

The 476 bp of human resistin cDNA was amplified from human fetal liver cDNA library by PCR and cloned into pCR<sup>®</sup>2.1 TOPO<sup>®</sup> vector (Invitrogen, USA) and successfully confirmed by enzyme digestion. In order to construct human resistin mammalian expression vector, the recombinant TOPO-human/resistin was digested with EcoRI and then ligated with the pCMV-Tag4C vector (Stratagene, USA) digested with EcoR I.

### ES cell culture and human resistin gene introduction into ES cells

D3-GL mouse embryonic stem cell lines were obtained from American Type Culture Collection (ATCC, USA, No. SCRC-1003, passage 10). The clonal embryonic stem cell line ES-D3 was derived from blastocysts of a 129/SV+cp/+p mouse. They can be injected back into blastocysts and contribute to the germline. Growth medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM glutamine, 0.1 mM  $\beta$ -mercaptoethanol, 0.1 mM MEM nonessential amino acids, 20% ES qualified FBS (Hyclone, USA) and 1,000 U/ml LIF (Chemicon, UK) for additional support of the undifferentiated state. ES cells were characterized by analysis of chromosome, staining of alkaline phosphatase and expression of transcription factor Oct 4 and Nanog. For transfection, ES cells were resuspended in 50  $\mu$ l of Lipofectamine 2,000 (Invitrogen, USA) transfection reagent mixture and left at room temperature for 10 min. After 48 h, the cells were transferred to growth medium supplemented with 200  $\mu$ g/ml G418. During 10-12 days of selection period, resistant colonies were confirmed for human resistin mRNA expression by RT-PCR and protein expression by Western blot. Transfected ES cells were lysated in lysis buffer containing 1% triton and 0.35 mg/ml phenylmethylsulfonyl fluoride, after which the lysates were centrifuged for 20 min at 15,000 $\times$ g and 4°C to remove insoluble materials. The supernatant was boiled for 5 min and loaded into 15% SDS-PAGE. Blots were probed with anti-flag M2 monoclonal mouse antibody (1:1,000), and appropriate secondary antibodies (ECL anti-mouse IgG peroxidase-linked species-specific whole antibody). The membranes were washed and then developed using enhanced chemiluminescence detection system (Pierce, USA).

### Production of tetraploid embryos

Six weeks old B6D2F1 females were superovulated by intraperitoneal injection of 5 units of PMSG followed, 46-48 h later, by 5 units of hCG and mated with 8 weeks old B6D2F1 males. For electrofusion, the collected 2-cell stage embryos were pre-equilibrated in fusion medium consisting of 0.3 M mannitol solution, 0.1 M MgCl<sub>2</sub> and 0.1 mM CaCl<sub>2</sub>. Embryos were placed between the electrodes of a fusion chamber in fusion medium. An alternating current field (8 V, 10 sec) was used and two direct current pulses (60V, 30  $\mu$ sec) were applied between the pulses. After fusion, embryos were returned to G1 media (Vitrolife, Sweden) at 37°C and cultured up to blastocyst. Embryos that had not undergone fusion within 1 h were discarded. Fused embryos were assessed for cell number by Hoechst 33342 staining and karyotyping by air drying technique at blastocyst stage.

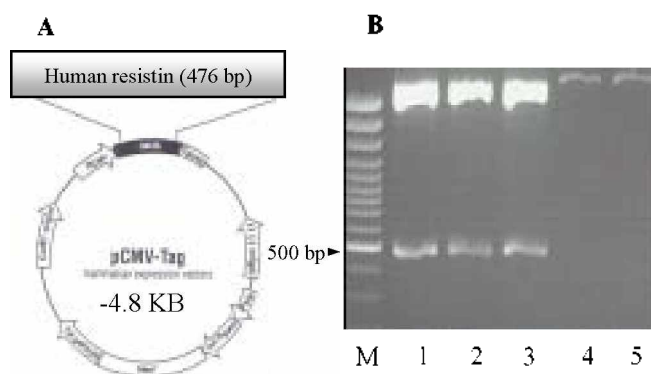
### Production of tetraploid complementation

For microinjection, 5 blastocysts were placed in a drop of culture medium under mineral oil. Injection pipette with an internal diameter of 12-15  $\mu$ m was used for ES cell injection. 15-20 ES cells (passage 14-17) were picked up in the end of injection pipette and the blastocyst to be injected was held in the vicinity of the inner cell mass. About 20 injected blastocysts were transferred to the uterine horn of 2.5 days postcoitum pseudopregnant female. Recipient mothers were killed at 19.5 days postcoitum, and pups were quickly removed from the uterus.

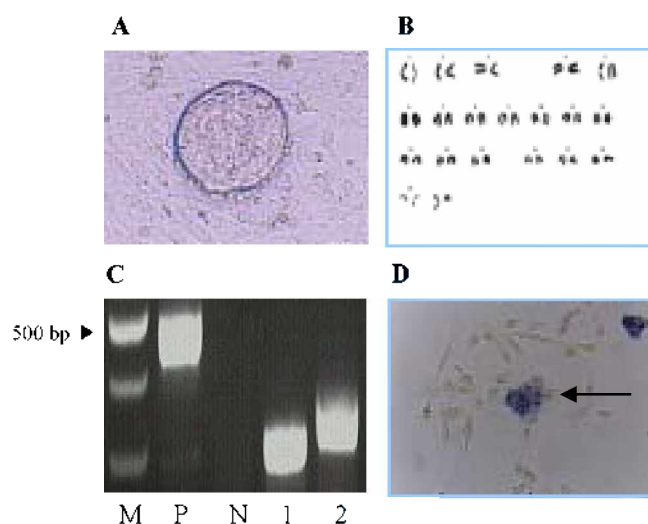
### Tetraploid derived fetus analysis

Tetraploid-ES derived fetus was analyzed by genomic DNA-PCR, microsatellite marker and glucose phosphate isomerase (GPI). Genomic DNA was extracted from the tissue samples and PCR was performed using human resistin primers. PCR amplification of the microsatellite marker (D4Mit204) was performed. The Genomic DNA was extracted from the tail biopsy of the fetus, oocyte donor mouse, recipient, and D3-GL ES cell pellets. Reaction mixtures were subjected to denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min for 35 cycles and amplicons were separated on a 3% agarose gel and visualized after staining with ethidium bromide. Primer set was as followed (D4Mit204: forward: 5'-TGCTGCAGCGATTCTCTC-3', reverse: 5'-TCAGGCACTAAGTACATGTGC-3').

To confirm that fetuses were generated from the injected ES cells, GPI analysis was performed. Various tissues of ES cell-derived fetus and control mice were homogenized in sample buffer and centrifuged. Aliquots of the supernatants were applied to Titan III cellulose acetate gel kit and run for 15 min at 120 V in a zip zone chamber (Helena Laboratories Inc, USA).



**Figure 1.** Construction of human resistin expression vector. (A) Map of human resistin expression vector. (B) Confirmation of human resistin gene insertion by enzyme digestion. M; 100 bp DNA ladder, lane 1, 2, 3; insertion of human resistin gene (476 bp), lane 4, 5; no insertion of human resistin gene.



**Figure 2.** Characterization of mouse ES cells (D3-GL). (A) *In vitro* proliferation of ES cells (400 $\times$ ). (B) Normal karyotype by Giemsa stain (40, XY). (C) Expression of transcription factor Oct4 and Nanog by RT-PCR (M; 100 bp DNA ladder, P;  $\beta$ -actin 491 bp, N; Negative control, 1; Oct4 303 bp, 2; Nanog 364 bp). (D) Positive alkaline phosphatase activity (arrow).

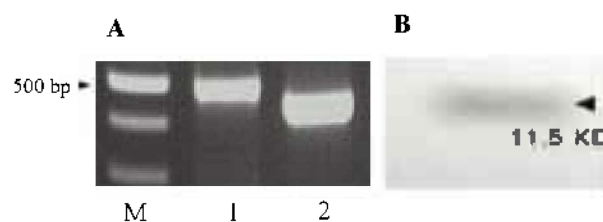
## RESULTS

### Construction of human resistin expression vector

Human resistin expression vector was confirmed by enzyme digestion and DNA sequencing. Figure 1 shows the expression vector map and the confirmation of human resistin insertion by enzyme digestion.

### ES cell culture and human resistin gene introduction into ES cells

Mouse embryonic stem cell lines (D3-GL) were characterized by analysis of chromosome, staining of alkaline phosphatase and detection of transcripts of



**Figure 3.** Expression of human resistin mRNA and protein in ES cells. (A) Expression of human resistin mRNA in transfected ES cells. M; 100 bp DNA ladder, lane 1;  $\beta$ -actin (491 bp), lane 2. human resistin (476 bp). (B) Expression of human resistin protein from transfected ES cells.

**Table 1.** Number of cells in blastocysts from fused and control embryos in mouse

	No. of blastocysts	Cell number of blastocyst (Mean $\pm$ SEM)
Control	139	76 $\pm$ 5.9 <sup>a</sup>
Fused embryos	182	36 $\pm$ 4.6 <sup>b</sup>

<sup>a,b</sup> Differ significantly,  $p < 0.05$ .

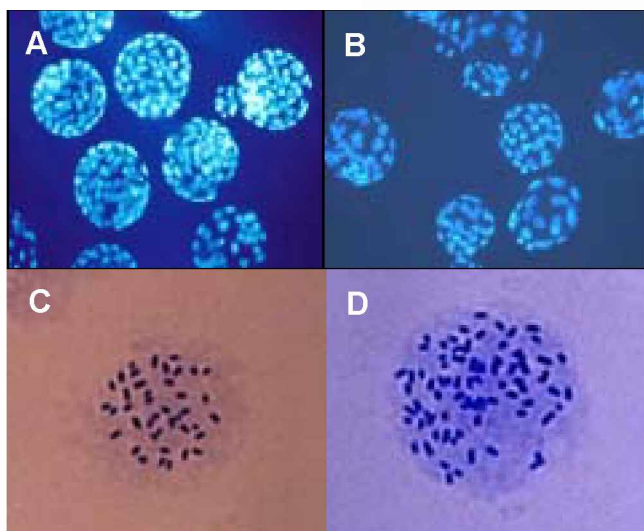
transcription factor Oct 4 and Nanog. Characterization studies showed expression of Oct4 and Nanog by RT-PCR (Figure 2C), displayed normal karyotype (Figure 2B), and tested positive for alkaline phosphatase activity (Figure 2D). After 10-12 days of selection period, human resistin transfected ES cells were confirmed for the expression of human resistin mRNA by RT-PCR (Figure 3A) and 11.5 kD resistin protein by Western blot (Figure 3B).

### Production of tetraploid embryos

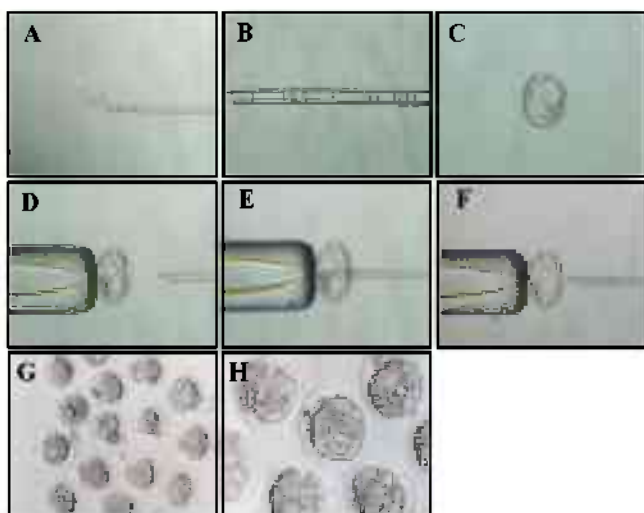
Tetraploid embryos were produced by electrofusion of 2 cell stage embryos and were cultured up to the blastocyst stage. Fused and control embryos were assessed for cell number and karyotype at the blastocyst stage. Fusion and blastocyst development rate in electrofusion group were 93.5% (2,114/2,256) and 94.6% (1,862/2,114) respectively. The number of cells in blastocysts from fused embryos was significantly fewer than that in the control (Table 1). Statistical analysis was performed by t-test. Chromosome was counted in 30 well-spread metaphase plates from control and fused blastocysts (Figure 4). Fused blastocysts exhibited a tetraploid karyotype (4 N), while control exhibited diploid karyotype (2 N).

### Tetraploid-ES cell complementation

With the tip of injection pipette having an internal diameter of 12-15  $\mu$ m, 15-20 ES cells (passage 14-17) were picked up and injected into each tetraploid blastocysts (Figure 5). After injection of the entire group, blastocysts were returned to culture medium and kept at 37 $^{\circ}$ C until transfer to recipient females. About 20 injected blastocysts were transferred into uterine horn of 2.5 days postcoitum pseudopregnant female.



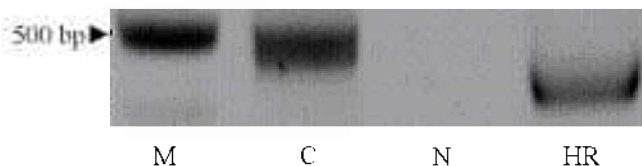
**Figure 4.** Cell counts and karyotypes of control and tetraploid blastocyst in mouse. (A) Cell counting by Hoechst 33342 staining of control blastocysts. (B) Cell counting by Hoechst 33342 staining of tetraploid blastocysts. (C) Giemsa staining of control blastocyst-2 N. (D) Giemsa staining of tetraploid blastocyst-4 N.



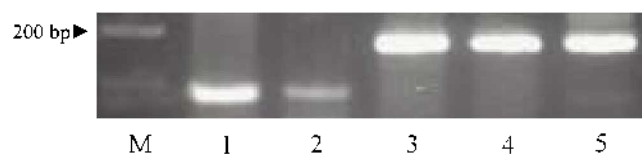
**Figure 5.** Injection of ES cells into tetraploid blastocyst in mouse. (A-B) picking up 15-20 ES cells, (C-F) injection into tetraploid blastocyst, (G-H) blastocysts after ES cell injection.

#### Embryo transfer and fetus analysis

Using tetraploid-ES two fetuses were obtained by Cesarean section at E 19.5 d and analysis was performed by genomic DNA-PCR, microsatellite marker, GPI. Genomic DNA was extracted from the tissue samples and then PCR was performed with human resistin primers (Figure 6). Only dead fetus showed integration of human resistin gene. To confirm that fetus generated using D3-GL cells by injection into tetraploid embryos were indeed exclusively of ES cell origin, GPI analysis was performed as described previously. When compared with donor and recipient mice, the lysate of the fetus was separated by another



**Figure 6.** Analysis of genomic DNA of tetraploid-ES mouse. M; 100 bp DNA ladder, C;  $\beta$ -actin (491 bp), N; Negative control, HR; human resistin (476 bp).



**Figure 7.** Analysis of microsatellite DNA with polymorphic marker on chromosome 4 (D4Mit204). M; 100 bp DNA ladder, 1; Recipient-ICR mouse, 2; Oocyte donor BDF1 mouse, 3; ES cell line (D3 GL), 4, 5; fetus from tetraploid-ES.

electrophoresis band (Table 3). Results from microsatellite DNA analysis results indicated that polymorphic pattern of tetraploid-ES derived mice was different from donor and recipient mice (Figure 7).

#### DISCUSSION

To date, ES technique has already begun to revolutionize the study of mammalian developmental genetics and the use of mice as models for human genetic diseases. In this study, mutant ES mice were produced directly from genetically manipulated ES cells by tetraploid complementation. This approach may improve the conventional transgenic methodology by providing a flexible and rapid approach to generate mutant mouse fetus. Schwenk (2003) suggested the biological characteristics of ES-tetraploid mice showed apparently normal morphological, physiological, and neurological characteristics and the comparison of various behavioral parameters revealed the normal phenotype. In addition, the histological, physiological, and neurological parameters reported in this work provide a phenotypic baseline for adult hybrid ES mice and confirms their general suitability for the analysis of mutant phenotypes. This remarkable result strongly encourages the future use of this technology for the rapid production of targeted mouse mutants.

Inbred 129 ES cell lines have been used for traditional ES cell researches. Recently, tetraploid-ES complementation technology was significantly improved through the discovery that ES cell lines derived from hybrid mouse strains support the development of viable ES mice 50-fold higher than inbred ES cells (Eggan, 2001). However, there is very little agreement that the hybrid cell line is a good result in this matter. Wilmut et al. (2001) demonstrated that the inbred ES cell line, HM-1, could be used to produce

**Table 2.** Production of offspring from different ploid blastocysts with ES cell injection in mouse

	No. of blastocyst injected	No. of recipient mice	No. of pregnancy (%)	No. of fetus by C-section	No. of fetus survived (%)	No. of fetus showing integration of human resistin
Diploid blastocyst	620	32	7(21.8)	-	31(5.0)	2
Tetraploid blastocyst	560	30	1(3.3)	2	-	1

**Table 3.** Glucose phosphatase isomerase (GPI) analysis of ES mice

	Age	Es cell contribution (%)				
		Liver	Leg	Brain	Spleen	Kidney
D3-GL injection	E 19.5	100	100	100	100	100
Donor	Adult	-	-	-	-	-

viable cloned mice, although it was less efficiently than most heterozygous ES cell lines, and that the developmental potential of reconstructed embryos was determined by both cell confluence and cell passage.

In our preliminary trial, diploid-ES mice, that could produce germ line chimera derived ES cell (D3-GL), was identified with agouti coat color and was confirmed for human resistin integration with PCR (Table 2). Subsequently in later trials using approach of tetraploid-ES technique, two fetuses were obtained by Cesarean section at E 19.5 d and one dead fetus was found to contain human resistin gene. It is likely that this observation is due to defection of clonal selection or unstable vector system (Chung, 2002). It is questionable whether fetal death was caused by loss of cell potency during consecutive culture or affection of gene function. Since selection was difficult to perform during 4-6 days of transfection, it seems that resistin is regulated by signal transducing molecule and is related to signal pathway (Song, 2002; Banerjee, 2003). There are some doubts that it could affect offspring to selection of promoter system. According to recent report in stem cells (Christopher, 2002), the CMV promoter remains a useful system for transgene expression in undifferentiated ES cells.

For the efficient introduction of foreign gene in this methodology, several critical factors, e.g., ES cell line, target gene, choice of vector, transfection system, remain as a matter to be discussed further. Furthermore, the inefficient post-natal development of ES mice (Wang, 1997; Eggen, 2001), thereby requiring intervention such as Cesarean section and fostering, make the tetraploid blastocyst injection procedure may be the only way to produce mutant mice directly from genetically altered ES cells (Schwenk, 2003). Finally, the practical advantage of this technique should be emphasized as it may save time and money for generating mutant mouse strains from cultured ES cells (Wang, 1997). Furthermore, this valuable technique will allow a rapid access to mutant fetuses and offsprings, and provide the powerful tool for many investigators in the field of mouse genetic studies.

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