돌연변이가 야기된 돼지 로돕신 유전자를 지닌 형질전환동물의 생산

김도형 · 김진회 · 이훈택 · 정길생

건국대학교 동물자원연구센터

The Production of Transgenic Mouse Harboring Mutated Pig Rhodopsin Gene

Kim, D.H, J.H. Kim, H.T. Lee and K.S. Chung

Animal Resources Research Center, Kon-Kuk University

요 약

광수용기에서 특이적 발현을 유도하는 로돕신 유전자에 존재하는 다양한 점 돌연변이는 의해서 일반적으로 색소성 망막염을 초래한다. 이 질병의 특징은 광수용기 세포의 퇴화로 인한 광수용기의 기능상실을 초래하고, 결과적으로 시각상실을 유발한다. 로돕신 유전자는 광수용기의 바깥분절에 존재하며, 빛 에너지를 흡수하여, 시각반응을 야기한다. 본 실험에 사용된 로돕신 유전자는 총 12.5 kb의 돼지 로돕신 게놈 유전자로서, 망막에서 특이발현을 유도하는 4 kb의 프로모터, 돌연변이를 지닌 5.6 kb의 유전자, 그리고 poly A site 로 구성되어 있다. 호르몬 투여에 의해 과배란이 유기된 C57BL /6J 생쥐 난자의 웅성전핵에 미세주입법에 의해 도입한 후, 위임신한 생쥐의 난관에 이식하였다. 그 결과 태어난 산자로 부터 돌연변이 로돕신 유전자를 지닌 6마리의 형질전환동물을 PCR과 Southern blot analysis를 통하여 확인할 수 있었다. 또한, 이 형질전환 동물들은 로돕신 유전자를 그 자손에게 안정적으로 전달하는 것을 확인하였다. 본 연구는 광수용기 퇴화의 원인규명 및 치료방법을 연구하기 위하여 돌연변이를 지닌 질환모델동물을 생산하는데 있다. 이들 형질전환 동물은 로돕신 유전자의 돌연변이에 의해 야기되는 시각상실의 기전에 관한 연구와 사람의 장님을 치료하기 위한 질환모델동물로서 유용하게 이용될 것이다.

I. INTRODUCTION

The vertebrate photoreceptor is a specialized neuron consisting of a cell body and an outer segment attached to a connecting cilium. The outer segment is a membrane rich structure, densely packed with flattened disk containing rhodopsin visual pigment and other proteins involved in visual transduction. The visual pigment stimulated by light undergoes a conformational change and activate a G-protein mediated enzyme cascade that culminates a change in membrane conductance (Van Nie et al., 1978; Cohen, 1983; Sanyal, 1987).

Rhodopsin is composed of an opsin molecule bound to the chromophore 11-cis retinal and is a transmembrane component of the discs. The mammalian opsin gene is composed of 5-exon and 4-intron with highly homologous exon sequences and with precise conservation of intron positions (Al-Ubaidi et al., 1990). Opsin molecules are normally synthesized and glycosylated in the rough endoplasmic reticulum, furth-

er modified in the Golgi complex, and transported across the inner segment in post-Golgi vesicles toward the base of the outer segment. Opsin combines with vitamin A tinal at the base of the outer segment, and probably in the inner segment as well, to form rhodopsin (Berson et al., 1991). Its major function is to capture photons and initiate visual transduction (Stryer, 1991).

In human, a mutation in gene encoding photoreceptor-specific proteins have been shown to cause retinitis pigmentosa (RP), a group of inherited disorders characterized by degeneration of photoreceptors. Mouse models of RP include the spontaneous mutant *rds* (retinal degeneration slow) (LaVail, 1981) and *rd* (retinal degeneration) (Sanyal et al., 1988).

At early stage of RP, even though these abnormalities did not lost vision, patients show the reduced and delayed electrical response or electro rectinogram (ERG) (Berson et al., 1968). By the conditions of disease progress, patients gradually lose their peripheral vision and eventually lost their central vision. In addition to, the autosomal dominant retinitis pigmentosa (ADRP) is less prevalent and severe than that of the recessive form (Drija et al., 1990).

Over 70 distinct mutations that co-segregated with the disease have been reported so far. Among the these mutations, about 25% of all ADRP cases appear to be caused by various mutations in the gene encoding rhodopsin (Drija et al., 1990; Sung et al., 1991; Gal et al., 1991; Kajiwara et al., 1991), the light sensitive pigment in rod photoreceptors, and to a much less extent *peripherin (rds/peripherin)* (Kajiwara et al., 1991; Farrar et al., 1991), a rod specific protein. It is likely to be involved in maintaining the structural integrity of the outer segment discs. Each of these mutations altered the open reading frame and would be expected to result in an abnormal

function of rhodopsin (Olsson et al., 1992).

The two most frequently observed mutations of the rhodopsin gene in ADRP patients were proline 23 and proline 347. Clinical studies have indicated that patients with a mutation in proline 23 generally may have a less severe disease than those with a mutation in proline 347 (Berson et al., 1991).

In this study, we will design to produce the transgenic mouse harboring a mutated rhodopsin gene on the purpose of providing a useful model to study condition of the disease caused by the photoreceptor degeneration.

II. MATERIALS AND METHODS

1. Mice

C57BL/6J mice in this study were used. All the mouse were kept under a 12 hr light : 12 hr dark cycle.

2. Recovery and culture of embryos

C57BL/6J females were superovulated by injection of 5 international unit (IU) of pregnant mare serum gonadotropin (Sigma Chemical Co. USA) and then by subsequent injection of 5 IU human chorionic gonadotropin (Sigma Chemical Co. USA) after 48 hr interval. Females were placed with fertile C57BL/6J males. One cell eggs were collected at 20 hr after hCG injection by the flushing the oviduct. Obtained embryos were cultured in incubator at 37℃, 5% CO_2, 95% O_2 in modified whitten's medium until predominant male pronuclei are observed.

3. Construction of transgene

The transgene (Fig. 1) consists of a 12.5 kb porcine genomic fragment that contained 4 kb of retina specific rhodopsin promoter, 5.6 kb of mutated rhodopsin genomic DNA, and 2.9 kb of poly A site. The mutated rhodopsin gene was
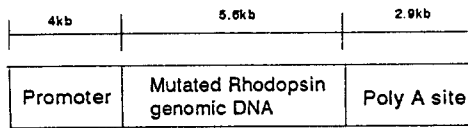
4kb	5.6kb	2.9kb
Promoter	Mutated Rhodopsin genomic DNA	Poly A site

Fig. 1. Structure of expression vector with porcine genomic DNA habouring mutated rhodopsin.

constructed by oligonucleotide directed mutagenesis and insertion of a mutated fragment into pBluescript sk(-) vector (Stratagene, USA) as described by Kunkel (Kunkel, 1985).

4. DNA purification

DNA for microinjection was prepared according to previous report (Sambrook et al., 1989) and purified on cesium chloride gradient. Briefly, plasmid DNA for microinjection was digested with Sal I and Eag I (0.1 unit /μl). The 12.5 kb fragment was subjected by agarose gel electrophoresis (1%), and then purified with Geneclean II ™ (Bio 101, Japan). DNA was finally diluted 2 μg /ml in 10 mM Tris (pH 7.4) and 0.1 mM EDTA for microinjection (Sambrook et al., 1989).

5. Production of transgenic mouse

Purified DNA was microinjected into the pronucleus of fertilized mouse one-cell eggs. Microinjected eggs were transferred the same day into the oviducts of pseudopregnant mice that had been randomly mated to vasectomized male mice (C57BL /6J) (Hogan et al, 1986).

6. Screening of transgenic mouse

For the identification of transgenic mouse, DNA of each samples was extracted from tail tissue of mice according to previous report (Hogan et al., 1986). Briefly, tail tissue was digested overnight in 700 μl of 10 mM Tris (pH 7.5), 100 mM NaCl, 20 mM EDTA, and 35 μl of proteinase K (10mg /ml) at 55℃. Aliquots of the supernatants were extracted with phenol /chloroform (1:1), and treated with 13 μl of DNase-free RNase per ml for 1hr at 37℃. The DNA was reextracted with phenol /chloroform, precipitated with ethanol, and suspended in TE buffer.

The integration of mutated pig rhodopsin gene was identified by PCR (polymerase chain reaction) and /or Southern blot analysis. For PCR analyses, 5′primer (TTCCGGAACTGCATGCTCAC) and 3′primer (ACAGGGTCCTGAGTTTCAAC) were used. Thirty PCR cycles were performed in a SingleBlock™ System (Ericomp, USA) with an initial denaturation of 5 min at 95℃ and final extension of 10 min at 72℃. For amplification of genomic DNA, PCR was performed at 95℃ /30 sec, annealed 55℃ /1 min, and extended 72℃ /2 min for 30 cycles. The PCR products were elctrophoresesed at 1.2% agarose gel and photographed under ultraviolet light.

Southern blotting was performed with DIG™, DNA Detection Nonradioactive Kit (Boehringer Mannheim, Germany) as according to methods recommended by company. Amplified DNAs were run on a 1.2% agarose gel (IBI, USA), transferred onto high bond nylon membrane and baked dry for 30 min at 80℃. Blots were done in hybridization solution (5x SSC, 0.1% N-laurylsarcosine, Na-salt (Sigma, USA), 0.02% SDS, 1% blocking reagent) and probed with 0.9 kb rhodopsin DNA labeled with digoxigenin-11-dUTP. After overnight hybridization, membranes were washed two times at high stringency with 2x SSC /0.1% SDS at room temperature for 5 min and 0.1% SSC /0.1% SDS at 68℃ for 15 min under each wash condition. Color precipition was developed after incubation in the dark with

color solution (45μl NBT solution, 35μl X-phosphate solution in 10 ml of 100 mM Tris-HCl; 100 mM NaCl; 50 mM $MgCl_2$; pH 9.5).

Ⅲ. RESULTS AND DISCUSSION

Transgene used for this study was 12.5 kb of porcine genomic fragment that encompass a mutated rhodopsin gene as well as 4.0 kb of promoter and 2.9 kb of poly A site. A mutation of rhodopsin gene is substition proline to serine in codon 347 (Fig. 1).

To develop transgenic mice with a point mutation (Pro-347-Ser), the mutated rhodopsin gene was microinjected into pronuclei of fertilized mouse embryos following the removal of vector sequence. Mice used for this experiment were inbred strain (C57BL/6J) because some mouse strains were well-known to have nonsense mutations (Pittler and Baehr, 1991).

Transgenic animals carrying the mutated rhodopsin gene were screened by PCR and/or Southern blot analysis. We produced total 126 mice following microinjection of mutated rhodopsin gene and then, 6 of 126 mice were identified as transgenic animals (Table 1).

The specific primers for the screening of mutant porcine rhodopsin DNA were made by mutated sequence introduced into exon IV of the transgene. The tail tissues of mouse were digested with protease K, purified, and used for PCR reaction. Amplified porcine rhodopsin gene give products of about 240 bp on agarose gel. Figure 2 shows the results of analysis of two

transgenic mice carrying a mutated porcine rhodopsin gene. In Fig. 2A, lane 3 and 4 show about 240 bp. It means that two mice are transgenic. Fig. 2B also depicts identification of F1 transgenic animal (lane 3 and 8).

For the confirmation of transgenic mice, Southern blot analysis was re-performed using the same samples of Fig. 2. The PCR products were denatured, electrophoresesed through an agarose gel, transferred onto a nylon membrane, dried, hybridized with a transgenic specific rhodopsin probe, and detected by color precipitation with DIG™ Kit. As shown in Fig. 3, lane 3 and 4 show positive signals. This result is consistent with the PCR results (Fig. 2A). By these analysis, we concluded that we have successfully developed the transgenic mice as shown in Fig. 2B. On the contrary to the results of the PCR analysis, lane 6 was transgenic when re-analysized by Southern blotting. This result suggest that lane 6 was mosaic mouse. This mosaic may be caused by integration of foreign gene at later stages than the one cell stage of embryos (Palmitter et al., 1984; Wilkie et al., 1986).

To investigate effects of mutant pig rhodopsin gene expression in the transgenic mice, a control experiment may be necessary. Previous data showed that transgenic mouse line, Pro-347-Ser, showed the expression of pig rhodopsin gene comparable to that of endogenous mouse rhodopsin gene. In these mice, retina developed normally but degeneration was noticeable at 3 weeks of age. By 7 weeks, about 5% of the photoreceptors had degenerated. Althoug-

Table 1. Production of transgenic mouse by PCR and Southern blot analysis

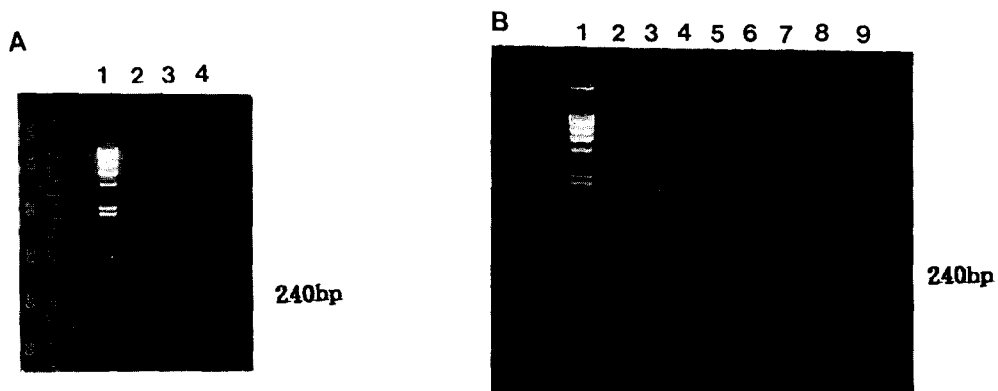| Genes injected	No. of embryos injected	No. of embryos survived	No. of embryos transferred	No. of neonates born	No. of transgenic offspring
Pro-347-Ser	673	612(90.9%)	574(85.3%)	126(18.7%)	6(0.9%)

Fig. 2. Screening of transgenic mice harbouring mutated rhodopsin gene by the PCR. (A) Analysis of founder mice. Lane 1: lamda/Hind Ⅲ digest; Lane 2-4: F0 mice (B) Identification of F1 offspring of transgenic mice. Lane 1: lamda/Hind Ⅲ digest; Lane 2-9: F1 mice.
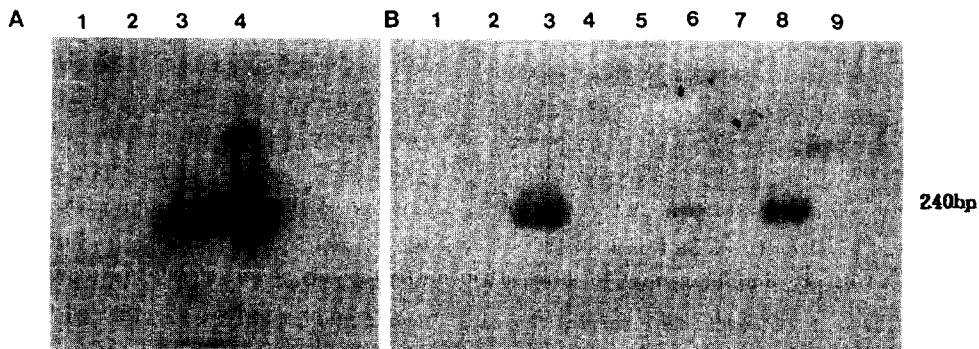

Fig. 3. Southern blot analysis of tail DNA amplified by the PCR to detect transgenic mice. (A) Analysis of founder mice. Lane. 1: lamda/Hind III digest; Lane 2-4: F0 mice (B) Identification of F1 offspring of transgenic mouse. Lane 1: lamda/Hind III digest; Lane 2-9: F0 mice.

h this progress was slow but virtually completed by 12 month (Wong and Hao, 1992).

At present, mechanisms that related to the phenotype of photoreceptor degeneration remain to be unknown. However, it is well-known that Royal College of Surgeons (RCS) rat undergoes photoreceptor degeneration because of a genetic defect in the retinal pigment epithelial cells (Faktorovich et al., 1990). Furthermore, subret-

inal or intravitreal injection of basic fibroblast growth factor into the diseased eye of RCS rat showed the delayed degeneration of photoreceptors in the retina for at last 2 months after a single injection. Thus, it means that photoreceptor degeneration in RCS rats by bFGF treatment was ameliorated even though the treatment of bFGF completely was not cured the genetic defect. The degenerative pathway

of photoreceptor by bFGF treatment was red-
uced the speed of degeneration at later stages
of RP, it may have important implications for
the design of therapeutic strategies for human
RP and related blinding disorders.

In conclusion, our developed transgenic mice
will be provided as a model for human disease
even though the pathogeny of retina has not
been identified. The study to rescue photorecep-
tor degeneration in this inherited disorder will
be addressed in further future.

IV. ACKNOWLEDGEMENT

V. SUMMARY

It is generally known that mutations in any of
several genes encoding photoreceptor-specific
proteins have resulted in retinitis pigmentosa
(RP), a disease characterized by losing photo-
receptor function with progressive degeneration
of photoreceptor cells and eventually leading to
blindness. To study the procure and cure of
photoreceptor degeneration, we produced tran-
sgenic mice. Transgene consisted of a 12.5kb
genomic DNA fragment that contains mutated
pig rhodopsin gene (Pro-347-Ser) including both
the 5'-franking (4.0 kb) and the 3'-franking (2.9
kb) sequences. This gene was used for the pro-
duction of transgenic mouse. The mutated rhod-
opsin DNA was microinjected into male pron-
uclei of fertilized mouse (C57BL/6J) embryos.
We detected transgenic animals harboring muta-
ted rhodopsin gene by PCR and Southern blot
analysis. These transgenic mice showed stable
transmission of microinjected rhodopsin gene
into their offspring. Therefore these animals
will provide a novel approach to study the mec-
hanism of the photoreceptor degeneration and
be provided as a disease model for the treat-
ment of the blind in human.

VI. REFERENCES

1. Al-Ubaidi M. R., S. J. Pitter, M. S. Cham-
   pagne, J. T. Triantafyllos, J. F. McGinnis ,
   and W. Baehr. 1990. Mouse opsin - gene struc-
   ture and molecular basis of multiple transcrip-
   ts. J. Biol. Chem., 265: 20563-20569.
2. Berson E. L., P. Couras, and R. D. Gunkel.
   1968. Rod response in retinitis pigmentosa,
   dominantly inherited. Arch. Ophthal., 80:
   58-67.
3. Berson, E. L., B. Rosner, M. A. Sandberg,
   and T. P. Drija. 1991. Ocular findings in pat-
   ients with autosomal dominant retinitis pig-
   mentosa and a rhodopsin gene defect
   (pro-23-his). Arch. Ophthalmol., 109: 92-101.
4. Blanton, S H., J R. Heckenlively, A. W. Cot-
   tingham, J. Friedman, L. A. Sadle, M. Wag-
   ner, L. H. Friedman, and S. P. Daiger. 1991.
   Linking mapping of autosomal dominant reti-
   nitis pigmentosa (Rp1) to the pericentric re-
   gion of human chromosome 8. Genomics,
   11:857-869.
5. Cohen, A. I. 1983 . Some cytological and in-
   itial biochemical observations on photorec-
   eptors in retinas of rds mice. Invest. Opthal-
   mol. Vis. Sci., 24: 832-843.
6. Drija, T. P., T. L. McGee, E. Reichel, L. B.
   Hahn, G. S. Cowley, D. W. Yandell, M. A.
   Sandderg and E. L. Berson. 1990. A point
   mutation of the rhodopsin gene in one form of
   retinitis pigmentosa. Nature., 343: 364-366.
7. Faktorovich E. G., R. H. Steinberg, D. Yas-
   umura, M. T. Matthes, and M. M. Lavail.
   1990. Photoreceptor degeneration in inherited
   retinal dystrophy delayed by basic fibroblast

growth factor. Nature, 347: 83-87.

8. Farrar G. J., P. Kenna, S. A. Jordan, R. Kumar-Singh, M. M. Humphries, E. M. Sharp, D. M. Sheils and P. Hempries. 1991. A three-base-pair deletion in the peripherin-RDS gene in one form of retinitis pigmentosa. Science, 354: 478-480.

9. Gal, A., A. Artlich, M. Ludwig, G. Niemeyer, K. Olek, and A. Schinzel. 1991. Pro-347-Arg mutation of the rhodopsin gene in autosomal dominant retinitis pigmentosa. Genomics, 11: 468-470.

10. Hogan B., F. Constantini, and E. Lacy. 1986. Manipulating the Mouse Embryo: A Laboratory Manual (Cold Spring Harbor Lab., Cold Spring Harbor, NY).

11. Kajiwara K., L. B. Hahn, S. Mukai, G. H. Travis, E. L. Berson, and T. P. Drija. 1991. Mutations in the human retinal degeneration slow gene in autosomal dominant retinitis pigmentosa. Nature, 354: 480-483.

12. Kunkel T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA, 82: 477-492.

13. LaVail M. M. 1981. Invest. Ophthalmol. Visual. Sci., 21: 630-657.

14. Olsson J. E., J. W. Gordon, B. S. Pawlyk, D. Roof, A. Hayes, R. S. Molday, S. Mukai, G. S. Cowley, E. L. Berson, and T. P. Drija. 1992. Transgenic mice with a rhodopsin mutation (Pro23His): A mouse model of autosomal dominant retinitis pigmentosa. Neuron, 9: 815-830.

15. Palmitter R. D., T. M. Wilkie, H. Y. Chen, and R. L. Brinster. 1984. Transmission distortion and mosaicism in an unusual transgenic animal pedigree. Cell, 36: 869-867.

16. Pittler S. J. and W. Baehr. 1991. Identification of a nonsense mutation in the rod photoreceptor cGMP phosphodiesterase beta-subunit gene of the rd mouse. Proc. Natl. Acad. Sci. USA, 88: 8322-8326.

17. Sambrook J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning - A labolatory manual (Cold Spring Harbor Lab., Cold Spring Harbor, NY).

18. Sanyal S., A. DeReuiter, and R. K. Hawkins. 1980. Development and degeneration of retina in rds mutant mice: light microscopy. J. Comp. Neurol., 194: 193-198.

19. Sanyal S., R. K. Hawkins, and G. H. Zilmaker. 1988. Curr. Eye. Res., 7: 1183-1190.

20. Stryer L. 1991. Visual excitation and recovery. J. Biol. Chem., 266: 10711-10714.

21. Sung, C. -H., C. M. Davenport, J. C. Hennessey, I. H. Maumenee, S. G. Jaconson, J. R. Heckenlively, R. Nowakowski, G. Fishman, P. Gouras, and J. Nathans. 1991. Rhodopsin mutations in autosomal dominant retinitis pigmentosa. Proc. Natl. Acad. Sci. USA, 88: 6481-6485.

22. Van Nie R., D. Ivanyi, and P. Demant. 1978. A new H-2 linked mutation, rds, causing retinal degeneration in the mouse. Tissue Antigens, 12: 106-108.

23. Wilkie T. M., R. L. Brinster, and R. D. Palmitter. 1986. Germline and somatic mosaicism in transgenic mice. Dev. Biol., 118: 9-18.

24. Wong F. and Y. Hao. 1992. Transgenic mouse of retinal degeneration caused by mutations in proline 347 of rhodopsin. Invest. Ophthal. Vis. Sci. [Suppl.], abstract. p944.