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—Original—

Histological Study of the Hypertrophic Placentas and Open Eyelids Observed in Cloned Fetuses

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Abstract. Mice cloned from somatic or ES cells showed signs of phenotypically various abnormalities. These abnormalities are now considered to result from aberrant gene expressions by epigenetic reprogramming errors but it is still unclear when these abnormalities occur and what histological changes occur during the gestation period. To address these issues, we histologically examined the hypertrophic placentas and open eyelids at 12.5, 17.5 and 19.5 days of the gestation period in ES-derived cloned mice that we have previously reported. In the placentas, the histology revealed that the hypertrophy had already occurred at 12.5 dpc and that the main change was the proliferation of trophoblast cells in the labyrinth layer. In the fetuses and placentas at 17.5 and 19.5 dpc, extensive proliferation of spongiotrophoblast and glycogen cells in the spongiotrophoblast layer and enlarged trophoblast giant cells were observed. Open eyelids in cloned mice were observed from 17.5 dpc, whereas the eyelids of the control mice had already been closed. The histology showed the malformation of eyelids where the formation of the stratum corneum and stratum granulosum in the epidermis was insufficient. Based on the histology described here, further comparative studies of the gene expression and histology of abnormalities seen in cloned mice and in gene-targeted and spontaneously mutated mice with similar phenotypic abnormalities could help illuminate these abnormalities and could contribute to the development of somatic cloning technology.

Key words: Cloned fetus, Abnormality, Hypertrophic placenta, Open eyelid, Histology

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In mice cloned from somatic or embryonic stem (ES) cells, various abnormalities are often observed. The most common morphological abnormality is the remarkable hypertrophy of placentas. Other abnormalities, including increased body weight, open eyelids, and umbilical hernia, were also observed [1–10]. It is now considered that these abnormalities, including their occurrence in cloned livestock, might result from aberrant gene expressions or methylations due to epigenetic reprogramming errors [7, 11–16]. In fact, the phenotypic abnormalities of the cloned parents from an ES cell line were not transmitted to the progeny [10]. The phenotypic abnormalities may result in the extremely low efficiency in the production rate of cloned animals [5, 17–21]. In other words, the stable production of cloned animals may result from the control of gene expression or DNA methylation and the suppression of the phenotypic abnormalities. With

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regard to the latter, it may be important to determine when the phenotypic abnormalities occur and what histological changes occur during the gestation period. Based on the histology of the abnormalities in cloned mice, comparative studies of the histology and gene expression in gene-targeted or spontaneously mutated mice with similar phenotypic abnormalities will help illuminate the abnormalities seen in cloned animals and will contribute to the development of somatic cloning technology.

We previously reported multiple abnormalities: the remarkable hypertrophy of placentas, increased body weight, and open eyelids in mice cloned from an ES cell line [10]. To investigate these abnormalities, we carried out histological analysis about the hypertrophic placentas and open eyelids of these cloned fetuses at 12.5, 17.5 and 19.5 days of gestation.

Materials and Methods

Production and recovery of cloned mice

Cloned and control mice were produced as previously described [22]. In short, we conducted nuclear transfer (NT) with oviduct-specific glycoprotein gene-targeted ES cells (TT2 line) arrested at the metaphase as donor cells. NT embryos developed to the morula and blastocyst stage after *in vitro* culture were then transferred to the uteri of Jcl: MCH mice (Japan CLEA CO. Ltd., Japan) at 2.5 days of pseudopregnancy. Control pups that have the same genetic and cytoplasmic constitution as the cloned embryos were produced by means of pronuclei transfer by the same micromanipulation method. We recovered the fetuses and placentas at 12.5, 17.5 and 19.5 days post coitum (dpc). Gene-targeted mice derived from chimeric mice using these ES cells appeared normal.

Macroscopy and histological analysis

After the fetuses and placentas were observed by macroscopy, they were fixed in 10% buffered formalin and embedded in paraffin. Samples were cut into 3–4 μm sections. Serial sections were mounted on slides and stained with hematoxylin-eosin. The specimens were compared with the controls by microscopy.

Results

Macroscopy

In cloned fetuses, hypertrophic placentas were observed in all cases at each developmental stage (12.5, 17.5 and 19.5 dpc). In addition, the eyelids remained open in all cloned fetuses recovered at 17.5 and 19.5 dpc (Fig. 1), although the eyelids of mice generally close at 15.5 to 16.5 dpc.

Histology

Eye (Fig. 2A): At 12.5, 17.5 and 19.5 dpc, cloned fetuses did not show any abnormalities in the tissues, such as the cornea and retina, in comparison with control fetuses.

Eyelid (Fig. 2A and B): At 12.5 dpc, no differences between the eyelids of the clones and the controls were observed. In the epidermis of the clones at 17.5 and 19.5 dpc, the thickness of the stratum corneum was insufficient and that of the stratum granulosum was entirely or mostly deficient. In the dermal layer, the fibrous structures were coarser than in the controls.

Body skin: At 12.5 dpc, no relevant differences were observed between the skin of the clones and that of the controls. Waving in the clones appeared to be gentler than in the controls but hair follicle formation and the thickness of the epidermis and dermal layers were the same as in the controls. In the dermis, the connective tissue was rougher than in the controls, and the development of the extracellular matrix appeared to be insufficient but those differences were not great in comparison with the differences observed in the eyelids.

Placenta (Fig. 3): The histology showed that the proliferation of trophoblast cells in the labyrinth layer cells had already occurred in the placentas of the clones at 12.5 dpc but it had not occurred in the placentas of the controls. At 17.5 dpc, the remarkable histological changes had occurred in the clones and were essentially the same at 19.5 dpc [5, 10]. In short, the hypertrophic placenta of the clones was characterized as an extensive proliferation of spongiotrophoblast and glycogen cells in the spongiotrophoblast layer and enlargement of trophoblast giant cells in comparison with the placentas of the controls. The borders of the spongiotrophoblast layer and the labyrinthine layer were ambiguous.

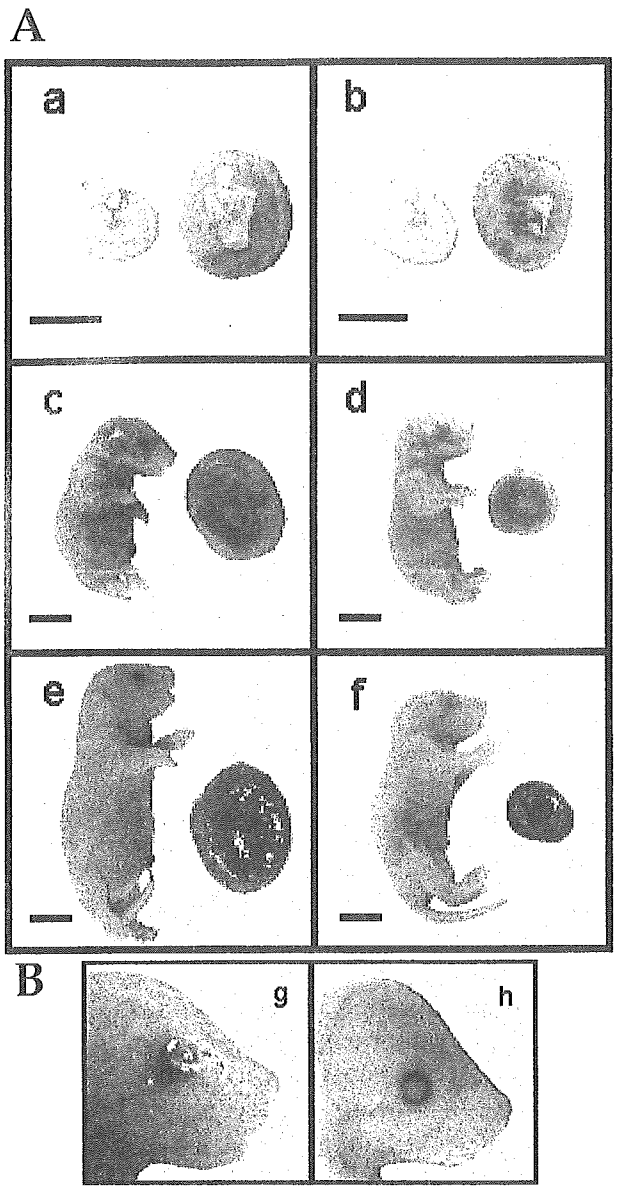


Fig. 1. A: ES-derived cloned (left) and control (right) fetuses and their placentas at 12.5 (a, b), 17.5 (c, d) and 19.5 dpc (e, f). Bar = 500 μm. B: Eye of ES-derived cloned (g) and control (h) fetuses at 17.5 dpc. The eyelid of the cloned fetus is open, but the control's has closed.

Discussion

The technology of producing cloned individuals has been a greatly hoped-for application in medical science and biology but almost all clones have shown various phenotypic abnormalities that are not present in animals produced by natural mating. These abnormalities represent a barrier to the medical use of clones [23, 24] and to the cloning of excellent livestock [18]. An accumulation of studies on the phenotypic abnormalities combined with analyses of gene expression or DNA methylation could overcome this barrier to the development of somatic cloning technology. In this report, we investigated the histological changes in the hypertrophic placentas and open eyelids of cloned mice with multiple abnormalities to reveal when phenotypic abnormalities occurred and what symptoms were displayed.

Eyelid fusion in mice normally occurs between 15.5 and 16.5 dpc, and the later separation starts by about 12 days after birth but in all 20 cloned pups that we produced from an ES cell line, the eyelids remained open at 19.5 dpc [10]. Amano [9] also reported open eyelids in 2 of 19 cloned pups. Furthermore, Tamashiro [4] reported that eye-opening was significantly late in mice cloned from cumulus cells, when compared with normal mice. This syndrome is often seen in chimeric pups produced by combining ES cells with normal embryos, although the details of the syndrome are not clear. These results indicate that the eyelids of mice may be easily influenced by aberrant gene expression. As shown by macroscopy, the eyelids of clones at 17.5 dpc remained open, whereas those of the phenotypes of cloned fetuses at 12.5 dpc were not different from those of the controls. Further histological examination revealed that in clones at 17.5 and 19.5 dpc, keratinization in the epidermis of the eyelids was insufficient, and in the dermal layer the connective tissue was rough, but in cloned fetuses at 12.5 dpc the eyelids and their surrounding tissues were normal. In contrast, the histology of body skin in clones was the same as that in controls. These findings indicate that abnormal keratinization in clones may be caused by the non-fusion of eyelids.

Open eyelids were also reported in NC-eob [25], GP/Bc [26], TGFα KO [27] and MEKK1 KO [28] mice. Comparative studies of the gene expression and histology of these mice and cloned mice will elucidate in more detail the causes of the abnormalities seen in cloned mice.

Mice cloned from somatic and ES cells commonly had hypertrophy of the placenta [1, 5, 6, 8–10] but there is still little information about when the hypertrophy occurs. Ogura [3] reported the hypertrophy by macroscopy at 12.5 dpc of fetuses

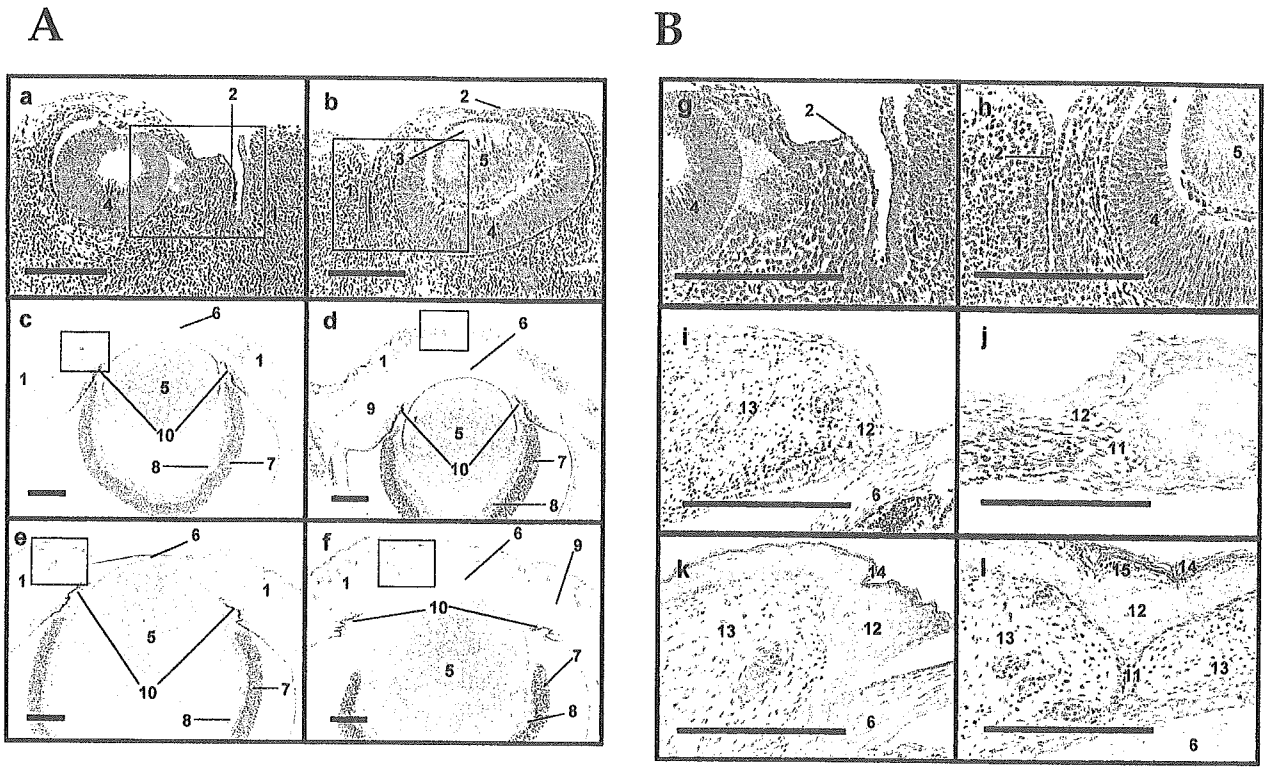
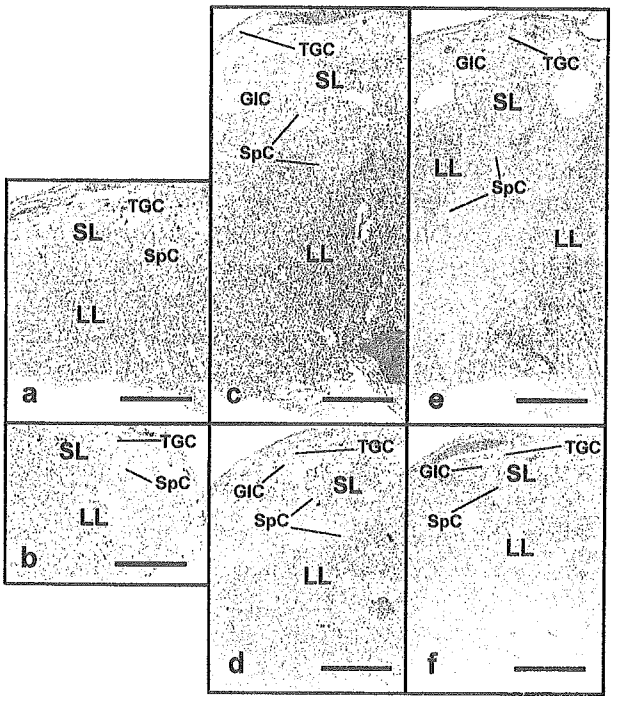


Fig. 2. A: Histology of the eye region in ES-derived cloned (left) and control (right) fetuses at 12.5 (a, b), 17.5 (c, d) and 19.5 dpc (e, f). B: Precise histological sections indicated by rectangles in Fig 2A with high power magnification. 1. Eyelid, 2. Cornea ectoderm, 3. Cavity of lens vesicle, 4. Optic cup, 5. Lens, 6. Cornea, 7. Pigment layer of retina, 8. Neural layer of retina, 9. Conjunctival sac, 10. Iris, 11. Fused eyelid margins, 12. Epidermis, 13. Dermis, 14. Stratum corneum, 15. Stratum granulosum. Bar = 100 μm.



cloned from immature sertoli cells. In the present study, we also observed that the placentas of clones from ES cells were hypertrophic at 12.5 dpc. The histology of the clones' placentas at 12.5 dpc showed aberrant proliferation of trophoblast cells in the labyrinth layer in comparison with that of the controls'. The weight of the cloned placenta at 17.5 dpc was 3.6 times that at 12.5 dpc, but only 1.6 times in control placenta (unpublished data). This suggests that the distinct disorder of the proliferation, orientation and arrangement of cells forming the placenta of a cloned fetus occurred after 12.5 dpc. The volume of the labyrinth layer usually increases from about 12 to 17.5 dpc [29]. Factors related to this increase may influence the

Fig. 3. Histology of placentas in ES-derived cloned (upper) and control (lower) fetuses at 12.5 (a, b), 17.5 (c, d) and 19.5 dpc (e, f). SL: Spongiotrophoblast Layer, LL: Labyrinthine Layer, TGC: Trophoblast giant cell, SpC: Spongiotrophoblast cell, GlC: Glycogen cell. Bar = 100 μm.

appearance of hypertrophic placentas. H19 and Igf2r genes were cited as candidates for these factors, since the placentas of their gene-targeted mice also showed signs of hypertrophy [30].

Approaching the study of epigenetic modification or reprogramming in cloned animals has been extensively done by gene expression analysis. When the expression in 10 genes was examined in clones from somatic cells at 12.5 and 19.5 dpc, 4 out of 10 genes, namely Peg1/Mest, Meg1/Grb10, Igfbp2 and Esx1, were lower than in the controls [13]. Our study on the expression of nine imprinting genes in ES-derived cloned fetuses at 9.5, 12.5 and 17.5 dpc showed extensive diversity (unpublished data). It still remains unclear whether the abnormal expressions of these genes are directly or indirectly related to the abnormalities in cloned pups and placentas. Eggenschwiler [30] reported that placental weights in H19 and/or Igf2r gene-targeted mice were heavier than in wild-type mice. In addition, each placenta examined showed different gene expressions [7, 13, 14], although hypertrophy of the placenta is commonly seen in all cloned mice regardless of the condition of the original cells. These researchers' work has not shown that hypertrophic placentas in cloned mice are closely related to the abnormal expressions of H19 or Igf2. These results may indicate that other genes are responsible for hypertrophic placentas as has been recently reported by microarray analysis of hypertrophic placentas [16].

In the present study, we investigated the successive histological changes in the placentas and eyelids of cloned mice with multiple abnormalities. The phenotypic abnormalities may result from aberrant gene expressions. Based on the histology described here, comparative studies of gene expression and the histology of cloned mice with gene-targeted and spontaneously mutated mice with similar phenotypic abnormalities will help illuminate the abnormalities seen in cloned animals and will contribute to the development of somatic cloning technology.

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Mutations of the *PAX6* Gene Detected in Patients with Congenital Optic Nerve Anomalies

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Summary

The *PAX6* gene is involved in ocular morphogenesis, The gene is expressed in developing CNS and eye tissues through developmental stages, and *PAX6* mutations have been detected in various types of ocular anomalies including aniridia, Peters' anomaly, corneal dystrophy, congenital cataracts and foveal hypoplasia. However, *PAX6* mutations have not been identified so far in patients with optic nerve anomalies.

Here we ascertained novel mutations in eight pedigrees with congenital optic nerve anomalies, including coloboma, morning glory disc anomaly, and optic nerve hypoplasia/aplasia. These mutations provide useful information for elucidating the *PAX6* protein function in the eye morphogenesis.

Introduction

The *Pax6* gene encodes a transcription factor that recognizes target genes through DNA binding of its paired domain, and is involved in eye morphogenesis (1). The gene was first isolated as a candidate gene for aniridia in humans and for small eye in the mouse and rat, respectively, by positional cloning (2-4). The *eyeless* gene, a *Pax6* homologue of *Drosophila melenogaster*, subsequently was identified and found to induce ectopic formation of eyes on the wings, legs and antennae by target expression (5, 6). The *Pax6* homologue of *Xenopus larvae* also is able to induce ectopic eyes in the tadpole body; thus, the gene is a master control gene for eye morphogenesis (7). The expression pattern of the *Pax6/*

PAX6 gene also indicates its multiple functions in mammals: The gene is expressed in the developing central nervous system (CNS), as well as in ocular tissues derived from the ectoderm and neuroectoderm including the corneal epithelium, lens and retina (8, 9).

Genetic analysis has detected numerous mutations of the *PAX6* gene in human aniridia patients, which are summarized in a database at http://www.hgu.mrc.ac.uk/Softdata/PAX6. Since most of these mutations result in premature translational termination on one of the alleles, haploinsufficiency of the gene has been suggested to cause the aniridia phenotype, in which the eye tissues are totally affected (10, 11). However, missense mutations have been rarely found to date. With some exceptional missense mutations found in aniridia patients, most missense mutations generate a distinctive non-aniridia phenotype, including anterior segment anomalies, congenital cataracts and foveal hypoplasia, in which restricted eye tissue was affected. (12-19). Missense mutations causing less dynamic loss of function or dominant-negative effect may underline subtle eye defects. Because the majority of amino acid residues have been conserved, distinct missense mutations alter the degree and specificity of DNA-binding of PAX6 protein in different ways and generate a wide spectrum of phenotypes.

In situ hybridization and immunohistochemistry indicated expression of *PAX6* in development of the optic nerve: *PAX6* is expressed in the optic stalk at an early stage, and later in the ganglion cells of the retina, whose nerve fibers project through the optic nerve to the CNS (8, 9). Hypoplasia of the optic nerve is often found in aniridia patients, (20), and the *small eye* (*Sey*^{H}) mouse mutant carrying a deletion of *Pax6* locus shows coloboma, a defect of the initial invagination in the optic stalk and eye vesicle (21). However, *PAX6* mutations have not been identified so far in patients with optic nerve anomalies. Here we present eight novel mutations of the *PAX6* gene related to optic nerve anomalies.

Methods

We collected DNA samples form 155 individuals with optic nerve anomalies. When a mutation was detected, DNA samples of the family members were also collected. After obtaining informed consent, blood samples were collected from peripheral veins into lithium heparin tubes. Genomic DNA was prepared from isolated leukocytes using a standard phenol/chloroform procedure. The DNA samples for normal controls was also prepared from more than 100 normal individuals.

PCR primers used for amplification of *PAX6* exons were synthesized using a DNA/RNA synthesizer (Applied Biosystems, model 392) based on the reported sequence (17, 19). The PCR conditions we used were previously described (17, 19), and adjusted to an annealing temperature of 55°C for exon 5a and 13, and 60°C for others with Mg 2+ concentrations at 1.5mM. SSCP analyses were carried out after radio-labeling with a-^{32}P-

dATP in the PCR reaction, using conventional sized gels of 5% polyacrylamide under at least 3 different conditions in concentrations of glycerol and at a running temperature. Nucleotide sequences were determined directly or after cloning on pUC18 using a Sequenase version 2 kit (Amersham) with PCR primers or universal primers in pUC18. Each sequence variation was confirmed in at least 6 independent clones.

Results

Patient 1, a 5-year-old girl, had bilateral morning glory disc anomaly. Mutation analysis identified 619C→T nucleotide substitution (according to accession no. M93650), which is expected to result in P68S. Patient 2, a 21-year-old male, had bilateral optic nerve hypoplasia and 1030C→T (Q205X). Patient 3, a 1-year-old boy, had an iris anomaly, large coloboma of the retina and choroids, a remnant of hyaloid vessel proliferation (persistent hyperplastic primary vitreous) bilaterally, and growth and mental retardation; mutation analysis identified 1190T→C (F258S). Patient 4, a 2-year-old boy, had bilateral optic nerve hypoplasia, growth and mental retardation, an enlarged ventricle by computed tomography (CT) study, multiple spike and wave bursts by electroencephalogram, and vesicoureteral reflux; mutation analysis identified 1292G→T (S292I). Patient 5, a 1-year-old girl, had Peters' anomaly and slight corneal opacity and deep excavation of the optic nerve head in the left eye, and 1504T→C (S363P). Patient 6, a 2-month-old girl, had microphthalmos in the right eye, and iris dysplasia and optic nerve aplasia with a remnant of the hyaloid vessels in the left eye, and 1550A→G (Q378R). Patient 7, a 19-year-old male, had bilateral optic nerve hypoplasia and 1558A→G (M381V). Patient 8, a 4-month-old girl, had bilateral optic nerve aplasia and 1588A→G (T391A).

All patients except patients 3 and 4 had normal growth, intelligence, and physical examinations and appearance on CT. Each had a normal karyotype. The mutations detected here occurred on one of the alleles, were thus heterozygous, and were not detected in unaffected immediate family members or in more than 100 normal individuals, indicating sporadic occurrence. Since mutations of the *PAX2* gene were detected in patients with optic nerve anomalies associated with renal anomaly (papillorenal syndrome) (22, 23), we screened for *PAX2* but failed to detect any mutation in these patients (data not shown).

Discussion

In the present study, we found *PAX6* mutations in patients with various types of optic nerve anomalies. Since numerous tissues, including retinal ganglion cells, glial cells, mesenchymes, contribute to form the architecture the optic nerve, a variety of clinical entities of developmental anomalies have been established to date by ophthalmoscopy and medical imaging

examinations (24). The optic nerve first arises as the optic stalk between the forebrain and optic vesicle at 3 weeks' human gestation. At 5 to 6 weeks, vessels invade through the embryonic fissure, a transiently appearing ventral cleft of the optic stalk and optic cup, into the vitreous cavity. Then, nerve fibers of retinal ganglion cells begin to project into the CNS at 8-10 weeks. In the middle stage, the optic nerve is coated with a collagen sheath and propped up by glial cells, and the nerve head is transiently covered with glial cells and hyaloid vessels. In this series of events, developmental failure of embryonic fissure (resulting in coloboma), ganglion cells of the retina (optic nerve hypoplasia/aplasia), and hyaloid vessels (persistent hyperplastic primary vitreous) is well known to be disease-causative (24). Most patients with these anomalies are sporadic, but an autosomal-dominant trait has been reported in families with coloboma and with optic nerve hypoplasia. Because *Pax6/PAX6* is expressed in numerous eye tissues throughout developmental stage (concerning optic nerve development, in the optic stalk and retinal progenitors at an early stage, and retinal ganglion cells at a late stage), it is not surprising that a variety of phenotypes are caused by *PAX6* mutations.

Seven of eight novel mutations found in our patients were missense, one of which was positioned in the paired domain (PD), one in the homeodomain (HD), and other five in the proline-serine-threonine-rich transactivating domain (PST). The proline residue at 68 in the PD, and the phenylalanine residue at 258 in the HD are conserved throughout all the Pax family members identified to date. These amino acid residues may play an important role in its reaction or maintenance of the protein structure. In contrast, amino acids of the PST are considerably diversified in the animals: The variegated eye phylae from the compound eye and the camera eye may depend on diversification of the PST function.

Although few missense mutations in these DNA binding or transactivating domains have been detected in *PAX6* so far, most mutations are predicted to alter the protein function (19, 25, 26). Some missense mutations have been suggested to be recurrently associated with a specific phenotype in eye anomalies. Two patients who had an R128C mutation in the C-terminal subdomain of the PD, independently identified in Japan and Europe, showed the same phenotype, with hypoplasia of the fovea. Mutations associated with Peters' anomaly identified to date are positioned in the N-terminal subdomain of the PD. In contrast, a missese mutation in the alternatively spliced exon is associated with a variety of eye anomaly phenotypes. No distinct positional effect of the missense mutations on phenotypic manifestation was found in the present study. Four patients with hypoplasia/aplasia of the optic nerve carried mutations in the PST. However, Patients 1, 3 and 5, who had colomoma-related anomalies, carried a respective mutation in PD, HD, and PST. Patients 3 and 6 showed a variant of aniridia, and patient 5 showed Peters' anomaly in addition to optic nerve anomalies. Because the *Pax6/PAX6* gene is expressed repeat-

edly throughout eye tissues, these missense mutations probably disturb PAX6 protein function by altering DNA-binding specificity in different ways, and resulting in various phenotypes.

Recent investigations attached importance to *Pax6* interactions including upstream regulators and downstream targets (27, 28). The *Pax2* gene, expressed in the ventral half of the early developing eye, optic stalk, CNS and urogenital tract (29, 30), is considered to mutually interact with *Pax6*. *Pax2* mutant mice showed defects in the kidney, CNS, optic nerve and retinal layer of the eye, and *PAX2* mutations of the gene have been reported in human renal-coloboma syndrome (22, 23). In the optic stalk and ventral forebrain of the zebrafish embryo, expression of *Pax2* and *Pax6* is negatively correlated under the control of Sonic hedgehog signaling (31). Because numerous processes are needed for optic nerve formation, the two genes probably share the work, for example in opening and closing the embryonic fissure. *PAX6* mutants may modify *PAX2* function and cause optic nerve anomalies. Functional analyses are under way using *PAX6* constructs with mutations, and should validate the significance of the mutation we detected.

(Details of this study will be published in the June version of journal The American Journal of Human Genetics.)

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眼組織

東　範行

眼はきわめて複雑な構造をもつ器官である．ヒトを含む高等動物の眼はカメラに類似しておりカメラ眼と呼ばれるが(図1)，この全体を再生，交換することはできない．しかし，多くの疾患では部分的な組織に障害がおこるので，従来からその組織を代用品で交換する治療が行われてきた．代表的なものが混濁した水晶体(白内障)に対する眼内レンズの移植である．再生医療も，個々の組織レベルで修復を行うべく研究が行われている．眼球組織は，その機能的特質から，網膜より前方の透明組織と網膜以後の神経組織に大別される．

透明組織

眼球の中で，前から角膜・水晶体・硝子体の3つは透明組織で，網膜までに光を到達させる役割がある．さらに角膜と水晶体は光を屈折して網膜に結像させるレンズの働きももつ．これらはいずれも角膜混濁・白内障・硝子体出血など，濁ることによって機能を失うので，透明性をもつ組織を再生させることが目的となる．このうち，硝子体は99%が水分で，残りの大部分もコラーゲン網であり，コラーゲンは手術によって取り除いても差し支えがないので，代用硝子体に対する研究はほとんど行われていない．

▣ 角　膜

角膜は古くより移植が行われてきたが，ドナー角膜が不足しているため再生の研究は盛んに行われている．角膜は上皮・実質・内皮の3層からなるが，これらを兼ね備えた移植可能な再生角膜はいまだ作られていない．個々の組織としては，上皮は培養シートとして皮膚のように欠損部を覆う治療がすでに行われている[1]．実質はコラーゲン塊で細胞成分が少ないので代用材料が多く検討されているが，まだ実用的ではない[2]．ヒト角膜の内皮は，能動輸送によって角膜実質内の水分量をコントロールし透明性を保つ重要な機能をもつが，発生以後は分裂しないので，ひとたび障害されれば角膜が混濁する．これに対して自己ないしは他種動物の培養細胞シートの移植が試みられている[3]．角膜の形態形成遺伝子はいまだ発見されていない．

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0289-0585/03/¥500/論文/JCLS

▣ 水晶体

水晶体の透明性は，これを構成する水晶体線維(細胞体)内のクリスタリン蛋白に依存する．これが変性すると透明性を失うので，水晶体はひとたび混濁すると回復させることができない．現在は，手術で濁った水晶体を除去して合成樹脂でできた眼内レンズを移植する治療が行われている(図2)．これによって満足度の高い視覚が得られるので，水晶体の再生の研究もほとんど行われていない．しかし，合成樹脂の眼内レンズでは，厚みを変えて遠近に焦点を合わせる調節力が欠如しているので，これを得るためには再生水晶体を使うのが最も適切である．近年，ニワトリで水晶体を形成する遺伝子L-Mafが発見された．これを鶏胚に異所性に導入すると，導入部位は水晶体に分化転換しクリスタリンが産生される[4]．クリスタリンをもつ細胞でも混濁していれば意味がないが，L-Maf導入によって透明組織を作ることは可能である(図3)．

神経網膜

角膜・水晶体のような透明組織の再生医療は混濁したものを置換することを目的とするのに対し，網膜・視神経は構造が複雑な上に，網膜での光・画像刺激受容-情報処理から視神経での伝達，中枢への投射までの機能を回復させることが必要なので，はるかに難しい．しかし，これらの神経組織がひとたび傷害されると，重篤で回復不能な視覚障害を来たす(図4)．神経保護や欠損物質を補う遺伝子治療なども検討されているが，根本的な治療は再生医療である．

▣ 網膜全体の再生と移植

網膜は複雑な構造を持っており，これを完全に再生させる試みはほとんど成功していない．脊椎動物の成獣では唯一サンショウウオにおいて，外科的に神経網膜を除去すると網膜色素上皮からほぼ完全な層構造をもった神経網膜が再生し，視覚が復元する[5]．しかし，他の両生類では網膜細胞が再生するものの層構造の復元ができない．さらに高等動物では，ニワトリやウズラの初期胚の網膜色素上皮原基にfibriblast growth factor(FGF)，ことにFGF2やFGF8を投与すると，神経網膜に分化転換がおこる(図5)[6,7]．しかし，発生初期(受精後4.5日まで)に限られる．

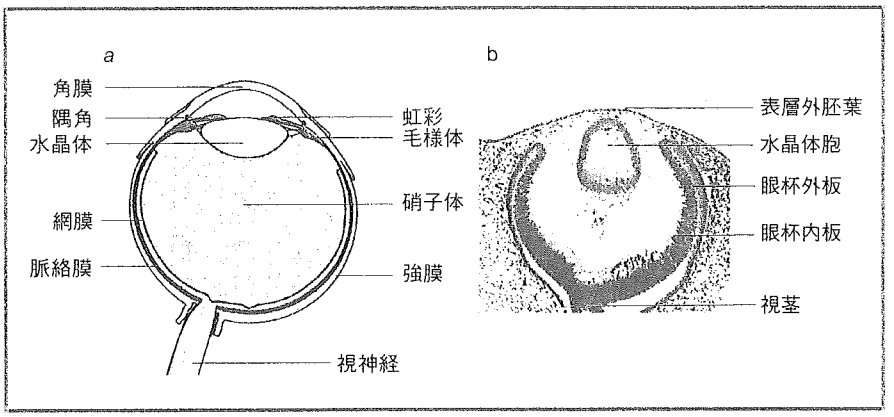

図 1　a）眼球の構造　b）発生初期の眼球（ヒト胎齢 5 週）

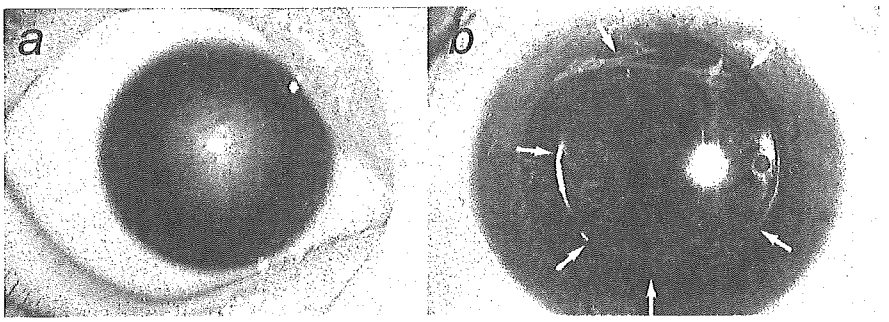

図 2　a）白内障　b）眼内レンズ挿入眼

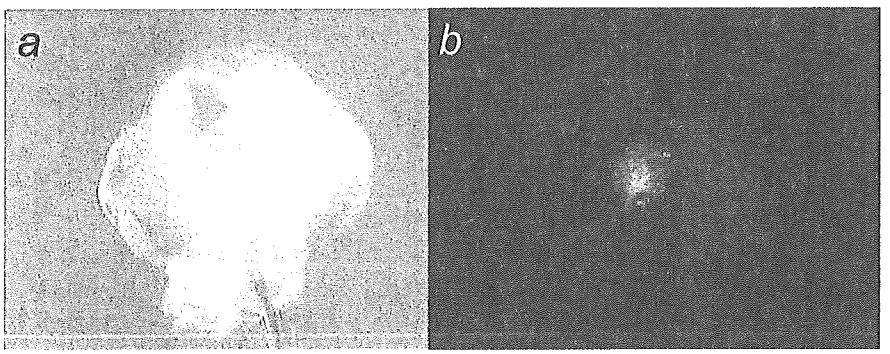

図 3　L-Maf 導入による異所性水晶体形成（stage 14 に導入，stage 28 の所見）
a）頭部の透明な異所性水晶体組織　b）GFP 所見

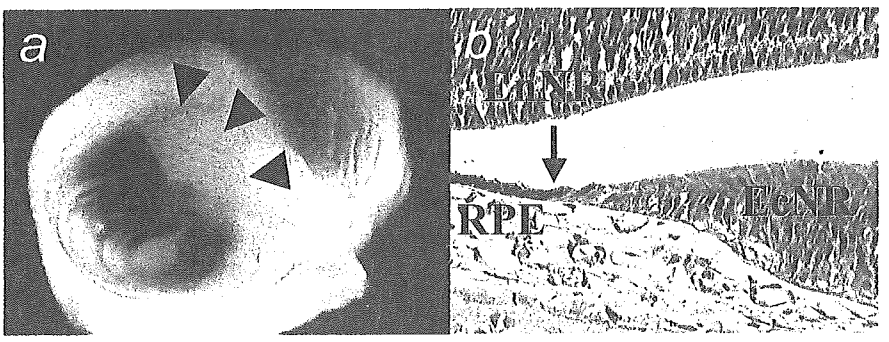

図 5　FGF 8 導入による鶏胚網膜色素上皮の神経網膜への分化転換
（stage 16 に導入，stage 30 の所見）
a）実体顕微鏡所見（矢頭，色素を失った色素上皮）
b）組織所見（HE 染色．EnNR：本来の網膜，EcNR：異所性網膜，RPE：網膜色素上皮，矢印：RPE から EcNR への移行部）

ラットでも初期眼球原器に FGF を作用させると，色素上皮から神経網膜の分化転換がおこるが，層構造は不完全でやはり発生初期（E15）に限られる．したがって，胎生期の組織を使っても，機能する網膜を再生させることはいまだ難しい．たとえそれが可能になって移植したとしても，神経線維を視神経から中枢のしかるべき部位へ投射させ，形態を認知する視覚を得ることはきわめて困難である．

▣ 細胞移植

これに対して，障害された網膜の中に不足した細胞を補うことは，その周囲にシナ

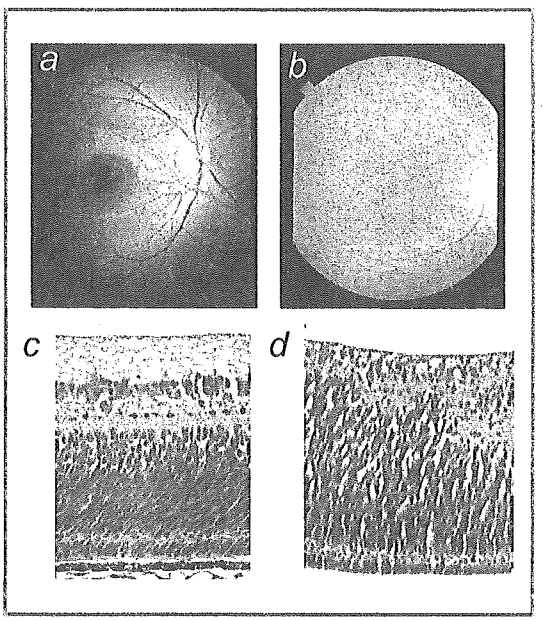

図 4　正常網膜（a，c）と変性網膜（b，d）の眼底（a，b）と組織所見（c，d）

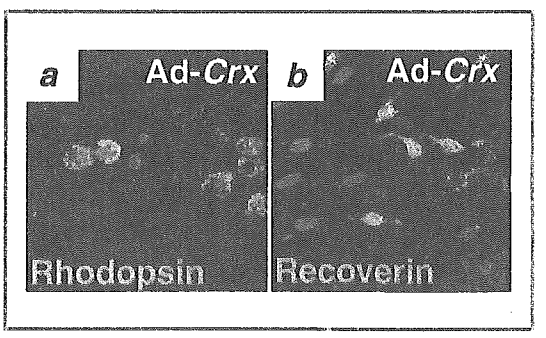

図 6　アデノウイルスベクターを用いて Crx 遺伝子を導入した培養虹彩細胞
a）赤：ロドプシン陽性細胞．青：核染色．
b）赤：リカバリン陽性細胞．青：核染色．
（京都大学，高橋政代先生より供与）

プスを作れば既存の機能を使うことができ，やや現実的である．

まず *in vitro* の実験では，孵化後のニワトリ網膜細胞に急性化学的障害を与えると，網膜内ブリアのミューラー細胞が脱分化してニューロンになることが見出されている[8]．また，マウス成獣の毛様体前縁の色素細胞は培養によってsphere colonyを形成し，視細胞・双極細胞・ミューラー細胞などの網膜特異細胞に分化する[9]．この部位は初期眼杯における神経網膜と色素上皮の境界部にあたり，発生的にみても未熟であるので，成獣になっても多分化能を残していると思われる．さらに，ラット成獣の虹彩細胞に視細胞の形成遺伝子Crxを導入し，杆体に特異的なロドプシンを発現する細胞が高率に得られている(図6)[10]．

In vivo では，網膜色素変性症患者で胎児網膜を試験的に移植した報告があるが，有効な結果は得ていない[11]．動物実験では，中枢神経の幹細胞として知られている海馬歯状回由来の幹細胞の網膜内移植実験がある[12]．この細胞をFGF 2添加無血清培地で培養すると，未分化なまま増殖し，血清添加培地ではニューロン・アストロサイト・オリゴデンドログリアに分化する．この細胞をラット網膜内に移植すると生着はみられたが，網膜固有の細胞に分化することは確認されていない(図7)．同じく，ES細胞や骨髄間葉細胞が *in vivo* 網膜や網膜培養ペレットに移植されて生着は確認されているが，網膜特異的細胞への分化は確認されていない(図8)．

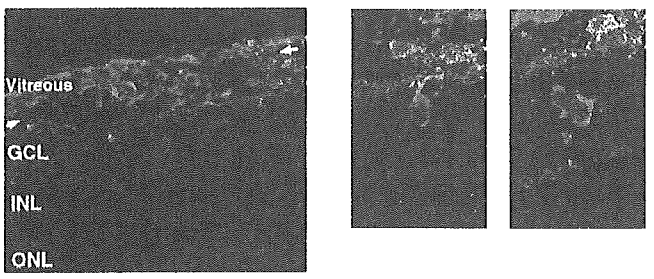

図 7　ラット成体脳由来神経幹細胞を生後3日目のラット網膜に移植し4週間後
緑：移植細胞．赤：網状層．ONL：外顆粒層．INL：内顆粒層．GCL：神経節細胞層．
(京都大学，高橋政代先生より供与)

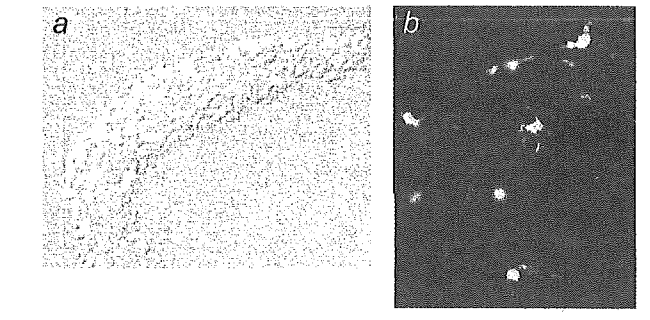

図 8　ラット網膜ペレットにおけるGFP発現マウス骨髄間葉細胞の生着
a）E 15網膜ペレット，移植0日
b）移植後5日
(杏林大学，渡邊　卓先生より供与)

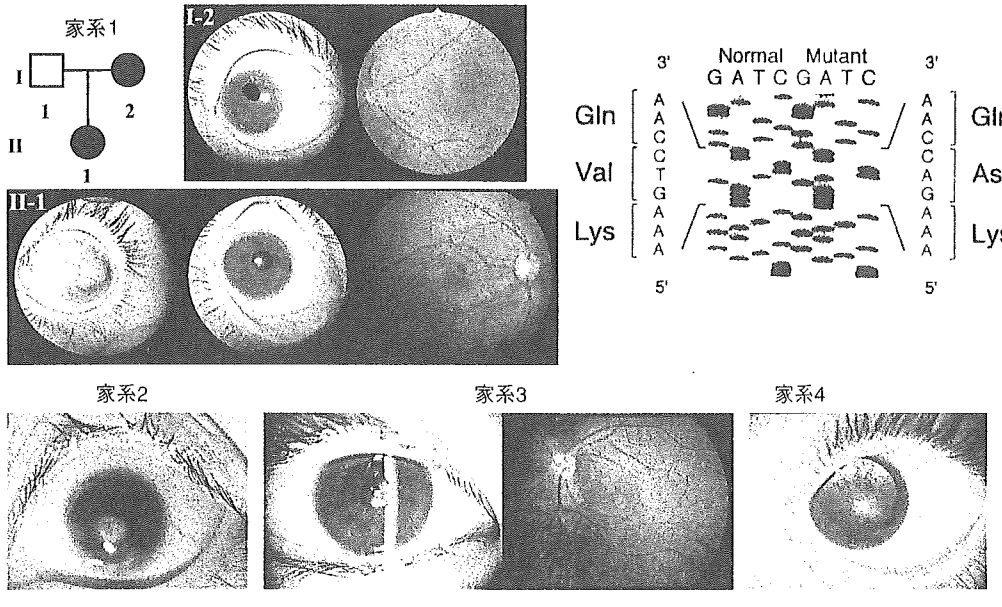

図 9　Pax 6遺伝子の変異(V 54 Dミスセンス変異)による多彩な眼先天異常

しかし，先に述べた *in vitro* 実験で得られた網膜特異細胞を網膜内に移植させ，生着して周囲とシナプスを作ることができれば，障害され減少した細胞の機能を補うことができる．細胞の網膜内移植は，網膜内あるいは網膜外(色素上皮との間)に直接注入するとこれを損傷するので，主に眼球内(硝子体内)に細胞を注入し，網膜上に落下して中に侵入させることをめざす．この方法では発生期網膜には侵入するが，発生が終了した網膜では侵入がみられない．これは発生が進むにつれて網膜表面に厚い基底膜が形成されるためと考えられる．視覚を障害する網膜疾患のほとんどが成年発症であるので，成熟網膜への移植を行わなければならないが，動物実験では成獣でも網膜に機械的損傷や虚血障害を与えたり，変性モデル動物であれば網膜内侵入がみられるので[13,14]，ヒトでも疾患によって障害された網膜では可能であるかもしれない．いずれにせよ，網膜内の情報伝達ネットワークは厳密に作られているので，これら細胞移植を行っても適切な形態を認知する視覚の復元は難しく，物が歪んだり，一部が欠けて見えるなどの不備が予測される．しかし，失明に至るような重症な障害において，光覚を復元したり視野を拡げるなどの治療には大いに期待できる．

網膜色素上皮

網膜色素上皮は発生学的には眼杯外板に由来するが，神経網膜の機能維持に重要な役割を果たす．その1つは網膜視細胞内の視物質 turn over であり，古くなった視細胞外節を貪食するとともに，視物質代謝においてレチノール再利用に関与する．あと1つは，能動輸送において網膜を含む眼球の水分調整を行っている．これによって網膜の内(硝子体側)と外(脈絡膜側)には水圧差が生じ，網膜は色素上皮に吸引されるようにして付着している．これが障害されて網膜と色素上皮間に水が溜まるのが網膜剝離である．近年，網膜色素上皮の障害によって視力が障害する疾患が増加している．ことに加齢黄斑変性は脈絡膜に新生血管が発生し，色素上皮を破って網膜下に侵入しこれを障害する疾患で，欧米では成人の失明の第一原因となっており，わが国でも高齢化に伴って急増している．これに対して新生血管を抜去する手術が行われるが，色素上皮が損傷されている限りは視力が回復しない．したがって，色素上皮の移植ないしは再生が必要となる．

色素上皮の移植は，サルで実験が多く行われ，ヒトの臨床でもすでに網膜色素変性症や加齢黄斑変性に対して試験的に行われている[15,16]．移植する組織としては，眼底において視覚として使われていない部位から採られた網膜色素上皮，虹彩の色素細胞(採取後培養してシート状になったものを使用)を用いた自己移植，胎児組織を用いた他家移植がある．いずれの方法でも視力の予後はやや向上ないしは不変とする報告が多い．他家移植は拒絶反応が問題となる．さらに，網膜色素上皮細胞は神経網膜に向かって細胞形態に方向性があるが，培養細胞ではこれが得られない．自己の網膜色素層を移植するのがよいが，手術手技が高度で，術後に出血などの合併症がおこりえる．

最近，サル ES 細胞をマウス頭蓋骨由来 PA 6 ストローマ細胞と共培養することによって，網膜色素上皮細胞シートを作ることに成功したとの報告がなされた[17]．この細胞シートは培養皿上で生体と同様に方向性をもち，十分な大きさが得られるので，網膜色素上皮移植に利用できる可能性が高い．

視神経の再生

視神経は外側膝状体へ向かう網膜神経節細胞のニューロン束と，これを囲むオリゴデンドロサイト・アストロサイトから構成される．末梢神経は切断しても再生するが，視神経のニューロンはこれとは異なり，中枢神経のように再生しないと考えられてきた．しかし，ラットの視神経を切断し，これを末梢神経につなげると，その中をニューロンが再生して伸びていくことが明らかになった[18]．この現象にはシュワン細胞の役割が関与していることが示唆されている．将来は緑内障や視神経炎による視神経障害の治療に応用されることが期待されている．

電気刺激による視覚復元

組織の再生と並行して，電子工学によって失われた機能を復元する試みも行われている．これは本来の再生医学ではないが，将来相補う形で進んでいくと思われるので，少し触れておく．網膜から視神経・視路のどこかが傷害されても，他の部位では機能が温存されているので，微小電極で残存する部位を刺激することによって代用の視覚を伝えることは可能である．このためには，光センサーあるいは外部画像獲得システム・イメージプロセッサー・刺激電極が必要で，その電気信号を与える刺激電極は網膜・視神経あるいは中枢に設置される．多くの網膜変性では視細胞が障害されることが多いので，双極細胞以降の2次細胞が温存されており，網膜に刺激電極を設置する場合はこれを刺激する．これまでにヒトのボランティアで行われたものでは，網膜の上あるいは下にチップを縫着し，光ないしは文字を認知できたとの報告がある[19]．網膜では像が写る場所に応じてマッピングがまだ容易であるが，視神経・中枢でははるかに複雑であり，いまだ光の認知にとどまっている[20,21]．

眼の形態形成遺伝子

眼は複雑な構造であるにもかかわらず，形態形成のしくみは急速に明らかになってきている．これはmaster control遺伝子Pax 6，あるいはその下流で眼の形成を担う遺伝子群が早期から発見されたためである．Pax 6はほぼすべての動物で眼の形成に関わっており[22]，ショウジョウバエ胚で予定眼領域以外の場所に導入すると，触覚・肢・胸などに異所性複眼が形成され，光に反応する[23]．Pax 6の下流にある遺伝子，Eyes absent，Dachshund，Sine oculisもみつかり，これらでもPax 6ほどではないが小さい複眼を異所性に作ることができるので，準master control遺伝子ということができる[24]．ヒトでも角膜・虹彩・水晶体・網膜・視神経に至る先天異常でPax 6を始めとする形態形成遺伝子の変異がみつかっており，ほぼすべての眼組織の形成に関わっていることが推測される(図9)[25,26]．Pax 6による異所眼の形成は，高等動物ではアフリカツメガエルのオタマジャクシでも可能だが，眼を構成する組織の多くは存在するものの構築は不完全である[27]．しかし，部分的な組織を作ることは可能と思われる．先に述べたL-Mafによる水晶体，Crxによる網膜視細胞の形成実験はこれに相当し，今後さまざまな幹細胞に導入する研究が進められると推測される．これら形態形成遺伝子は，ShhやFGFなどの細胞外シグナルによって発現誘導されることも明らかになりつつある[28,29]．導入ベクターの問題や遺伝子の発現量から考えると，誘導条件決定を基礎的に明らかにして，外的導入より至適環境下で内因性に発現させる方法の開発が好ましい．

むすび

眼の再生医学においては，個々の組織において代用組織と再生組織の両方で研究が進められている．再生の研究はまだ緒についたばかりであるが，一部の組織では臨床応用へ向かいつつある．再生における基礎的裏づけとして，master control遺伝子を中心とする形態形成遺伝子カスケードと，その発現誘導を明らかにすることが重要である．

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ORIGINAL ARTICLE

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Identification of a novel polymorphism involving a CGG repeat in the *PTCH* gene and a genome-wide screening of CGG-containing genes

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Abstract Mutations in the human homologue of the *Drosophila patched* gene (*PTCH*) are responsible for the hereditary disorder called nevoid basal cell carcinoma syndrome (NBCCS). *PTCH* has a CGG triplet repeat located 4 bp upstream of the first methionine codon. Here we report a novel polymorphism involving the number of the CGG-repeat. The major allele (86.3%) contained a repeat size of seven, whereas the minor allele contained eight. No significant difference in the distributions of genotypes was observed between normal and NBCCS individuals. However, when the repeat was inserted between a heterologous promoter and the luciferase gene, the longer repeats tended to induce higher luciferase activities, suggesting that the repeat length potentially affects the levels of gene expression. A genome-wide screening revealed that 68 and 146 genes contained a CGG/CCG repeat in the coding region and in the 5'-untranslated region (5'-UTR), respectively. None of the genes had this repeat in 3'-UTR. Interestingly, the number of genes with a CGG repeat in the 5'-UTR was significantly higher than that with a CCG repeat in the 5'-UTR. The localization of a CGG/CCG repeat in *PTCH* is quite unique in that only four other genes have been found in which the repeat is localized up to 4 bp upstream of the first methionine.

Keywords PTCH · Nevoid basal cell carcinoma syndrome · Gorlin syndrome · Polymorphism · Triplet repeat

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Introduction

Mutations in the *PTCH* gene are responsible for the hereditary disorder called nevoid basal cell carcinoma syndrome (NBCCS; MIM# 109400) (Hahn et al., 1996; Johnson et al., 1996). NBCCS, also called Gorlin syndrome, is an autosomal dominant neurocutaneous disorder characterized by developmental abnormalities and tumorigenesis such as palmar and plantar pits, jaw cysts, calcification of the falx cerebri, skeletal anomalies, basal cell carcinoma, ovarian fibroma, and medulloblastoma (Gorlin, 1987). *PTCH* (MIM # 601309) is a human homologue of the *Drosophila* segment polarity gene *patched*. It has been mapped to 9q22.3-q31 and consists of 23 exons encoding a protein with 1,447 amino acid residues. The PTCH protein is a receptor for a secreted molecule Sonic hedgehog and has twelve transmembrane domains. At least two forms of PTCH protein are known to exist, reflecting the use of alternative exon 1a versus 1b (Hahn et al., 1996; Wicking et al. 1997a). Mutations in exon 1b have not been investigated so far due to, at least in part, the extreme GC-rich sequence (Wicking et al. 1997a; Fujii et al. 2003a). In the course of analyzing mutations in exon 1b, using a new set of primers and a PCR condition, we discovered a novel polymorphism involving a CGG trinucleotide repeat immediately upstream of the first in-frame methionine codon. We compared allele frequencies between healthy individuals and NBCCS patients. We also investigated the effect of the repeat length on the gene expression using a heterologous reporter gene. In addition, the results of a genome-wide screening of CGG/CCG-containing genes are demonstrated.

Materials and methods

DNA samples

After informed consent was obtained from 51 healthy, unrelated individuals and 14 patients with NBCCS, total genomic DNAs were isolated from peripheral leukocytes by the standard phenol/

chloroform extraction method. Patients were diagnosed as having NBCCS according to the clinical criteria (Kimonis et al. 1997). All studies were approved by the local ethnic committee. Among 14 patients with NBCCS, *PTCH* mutations were found in 11. Some of the mutations have already been reported (Fujii et al. 1999, Fujii et al. 2003a, Fujii et al. 2003b) and some will be reported elsewhere.

Polymerase chain reaction and sequencing

The genomic region of *PTCH* including the 5'-untranslated region (5'-UTR) and exon 1b was amplified by using the forward primer, 5'-CGCGCAATGTGGCAATGGAA-3', and the reverse primer, 5'-AGAGGAGGGAAGAGAAAGTG-3'. The polymerase chain reaction (PCR) was carried out in a 20 μl reaction volume by using LA Taq with GC Buffer (TaKaRa) according to the manufacturer's instruction. PCR was run for 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 3 min on a Program Temp Control System PC-800 (ASTEC, Fukuoka, Japan). Both the sense and antisense strands of the PCR products were directly sequenced by using the same primers as described above. PCR products purified by a QIAquick PCR Purification Kit (QIAGEN) were used as the template DNA for cycle sequencing with a CEQ DTCS Quick-Start Kit (Beckman Coulter). Sequencing analysis was performed on a CEQ 8000 Genetic Analysis System (Beckman Coulter) according to the manufacturer's instructions.

Plasmid construction

Luciferase constructs containing the sequence of *PTCH* 5'-UTR were generated by a PCR-mediated method described previously (Imai et al. 1991) using pGV-P2 (Wako Chemicals, Osaka, Japan) as a template. The authenticity of all constructs was confirmed by sequencing.

Luciferase assay

The human embryonic kidney cell line 293 growing on six-well culture plates were cotransfected using Effectene reagent (QIAGEN) with 0.5 μg of luciferase plasmid and 0.5 μg of pCMVβGal. The cells were harvested at 24 h after the transfection and used for a luciferase assay. Luciferase activities were measured as described previously and normalized for transfection efficiency based on β-galactosidase activities (Shikama et al. 2001).

Real-time quantitative RT-PCR

Total RNA was extracted from the transfected cells described above using TRIzol reagent (Invitrogen). One-step RT-PCR was performed with a 7700 ABI PRISM Sequence Detector System (Perkin Elmer-Applied Biosystems) using primers 5'-TCTGGATC-TACTGGTCTGCCTAA-3' and 5'-GCGCACTTTGAATCTTG-TAATCCTG-3'. To normalize the expression of luciferase, the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) housekeeping gene was also amplified, using primers 5'-GAAGGTGAAGGT-CGGAGT-3' and 5'-GAAGATGGTGATGGGATTTC-3'. Fluorogenic probes 5'-CAAATCATTCCGGATACTGC-3' and 5'-CAAGCTTCCCGTTCTCAGCC-3' carrying 5' 6-carboxy-fluorescein as a reporter dye and 3' 6-carboxy-tetramethyl-rhodamine as a quencher dye were used to detect the PCR product of luciferase and *GAPDH*, respectively. In every experiment, *GAPDH* was amplified using a series of dilutions of a known amount of the standard RNA supplied by Perkin Elmer to prepare a standard curve.

Computational screen

Human mRNA sequences that contain more than seven repeats of CGG were downloaded from NCBI nucleotide databases using the search program termed as "Search for short, nearly exact sequences" with $(CGG)_7$ as a query (http://www.ncbi.nlm.nih.gov/BLAST/). A full screening of the genes of interest was confirmed because genes with exact matches (bit score 42) were followed by the genes with partial matches (bit score less than 42). Genes for unidentified coding sequences were excluded from further study.

Statistical analysis

Genotype distributions and allele frequencies of CGG repeat numbers were compared between cases and controls by means of the χ^2 test. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated by Wolf's method.

Results and discussion

The *PTCH* gene has two alternative first exons—exon 1a and exon 1b (Fig. 1A). Exon 1b contains the first in-frame methionine codon, while exon 1a is a noncoding exon. We noticed a CGG trinucleotide repeat located 4 bp upstream of the first methionine codon. Although exon 1b is a coding exon, mutations in this exon have not been reported. In the course of analyzing mutations in exon 1b using samples from NBCCS individuals that do not have a mutation elsewhere in *PTCH*, we discovered a novel polymorphism involving the CGG trinucleotide repeat (Fig. 1B). The major allele contained

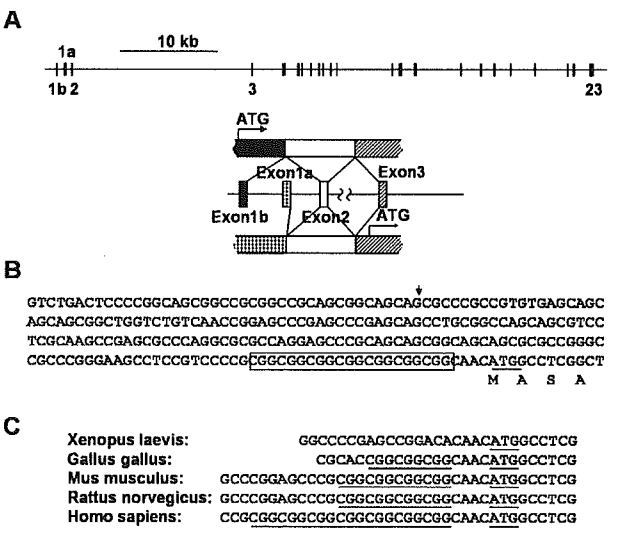

Fig. 1 **A** Genomic organization of human *PTCH*. The *PTCH* locus based on the sequence AL161729 is shown at the *top*. Two cDNA sequences, GenBank U43148 and U59464, are generated by alternative splicing using exon 1a and 1b, respectively, as schematically depicted at the *bottom*. **B** Nucleotide sequence of the human *PTCH* gene including 5'-UTR and exon 1b. The first methionine codon is *underlined*. Polymorphic CGG repeat is *boxed*. The putative transcription start site is indicated by an *arrowhead*. **C** Nucleotide sequence alignment of the *PTCH* genes. CGG repeat and the first methionine codon are underlined