RESEARCH ARTICLE

Dense cataract and microphthalmia (*dcm*) in BALB/c mice is caused by mutations in the *GJA8* locus

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Abstract

A spontaneous mutation in BALB/c mice that causes congenital dense cataract and microphthalmia (*dcm*) was reported previously. This abnormality was found to be inheritable and the mode of inheritance indicated that this phenotype is due to mutation of an autosomal recessive gene. We performed genetic screen to identify the underlying mutations through linkage analysis with the *dcm* progenies of F_1 intercross. We identified the region of mutation on chromosome 3 and further mapping and sequence analysis identified the mutation in the *GJA*8 gene that encodes for connexin 50. The mutation represents a single nucleotide change at position 64 (G to C) that results in a change in the amino acid glycine to arginine at position 22 (G22R) and is identical to the mutation previously characterized as *lop10*. However, the phenotype of these mice differ from that of *lop10* mice and since it is one of the very few genetic models with recessive pattern of inheritance, we propose that *dcm* mice can serve as a useful model for studying the dynamics and interaction of the gap junction formation in mouse eye development.

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Introduction

Cataract can be defined as lens opacity and it can be due to multiple causes. Cataract in mice can be age-related, induced or genetic cataract. According to WHO, age-related cataract accounts for 18 million cases of blindness and 59 million cases of reduced vision worldwide. Homozygous deletion of *Epha2* in two independent strains of mice led to the development of cataract that progressed with age (Jun *et al*. 2009). Cataract is also known to be induced in mice by several factors, like galactose (Ai *et al*. 2000), UV-B (Varma *et al*. 2008), diabetes (Olofsson *et al*. 2009) and naphthoquinone (Martynkina *et al*. 2002). Studies on genetic cataracts have shown the involvement of various genes on different chromosomes, summarized in table 1. Several transcription factors, including PAX6 and SOX proteins, have been suggested as candidates for crystallin gene regulation, and their mutations can cause cataract. Congenital cataract can impair visual development resulting in blindness and is clinically and genetically highly heterogeneous disorder (Wirth *et al*. 2002). Cataract formation is frequently observed in human and animal models. Hereditary mouse cataract models have great relevance to humans because it is estimated that congenital cataracts comprise ~10% of visual loss in humans (The National Advisory Eye Council Vision Research 1987). Various surveys show that cataract is the leading treatable cause of blindness in children (Foster *et al*. 1997). Congenital cataracts are rare and occur in developed countries with a frequency of 30 cases among 100,000 births with

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Table 1. Types of known genetic cataract in mice.

Gene	Chromosome	Phenotype description	Inheritance pattern	References
lop10	3	Homozygous mice are microphthalmic with dense white cataracts but heterozygous mice have consistent variable expressivity dependent on background	Autosomal semi-dominant	Chang *et al*. 2002
lop11	8	Vacuolated cataract	Autosomal recessive	Talamas *et al*. 2006
lop12	1	Nuclear lumpy irregular cataract, heterogygotes have intermediate sized eyes	Autosomal dominant	Smith *et al*. 2000
nm1853	1	*Crygc* mutation	Autosomal dominant	Graw *et al*. 2002
lop13	15	Early white cataract (former name *nuc*)	Autosomal recessive	Varnum 1981
lop18	17	Cataract mapped to alpha-crystalline region	Autosomal recessive	Chang *et al*. 1999
corn1	2	Corneal epithelial dystrophy and white cataract by 42 days	Autosomal recessive	Wang *et al*. 2001
bs	2	Microphthalmia, cataracts and ptyergia	Autosomal recessive	Spence *et al*. 1992
No2	3	*GJA8* point mutation A47D	Autosomal semi-dominant	Xu and Ebihara 1999

further 10 cases per 100,000 being diagnosed during childhood (Gilbert and Muhit 2008). Many strains of mice with hereditary cataracts have been reported including *blind-sterile, bs* (Spence *et al*. 1992); *fidget, fi* (Konyukhov and Vakhrusheva 1969); *lens opacity, lop10* (Runge *et al*. 1992); *lens opacity, lop13; lens rupture, lr* (Fraser and Herer 1948), *dysgenetic lens, dyl* (Sanyal *et al*. 1986); and *vacuolated lens, v* (Korstanje *et al*. 2008).

The mammalian eye lens is made up of mainly two classes of proteins; crystallins that form the structural lens and the transmembrane proteins such as connexins. Since the lens lacks any vasculature, the crystallins are retained in soluble form through the maintenance of ionic balance by the actions of gap junction proteins which allow the metabolically active epithelium to regulate the precise inter-cellular communication and transport between the lens periphery and its interior. Connexins are the transmembrane proteins that form these inter-cellular channels via the formation of the gap junctions (Kumar and Gilula 1996; Saez *et al*. 2003; Olk *et al*. 2009). About 20 connexins have been identified and mutations in these have been associated with sensoneural deafness, charcot-marie-tooth neuropathy, visceroatrial heterotaxia and cataract. Three connexins, connexin 43 (Cx43) encoded by *GJA1*, connexin 46 (Cx46) encoded by *GJA3* and connexin 50 (Cx50) encoded by *GJA8*, belonging to the alpha connexin family are expressed in eye lens (Paul *et al*. 1991; White *et al*. 1992; Calera *et al*. 2006). Lens epithelial cells show a predominant expression of Cx43, during differentiation into fibres, Cx43 expression is replaced with Cx46 and Cx50 expressions (White *et al*. 1998). Distinct role for each connexin has been proposed due to differences in molecular signalling mediated by their structure, and any alteration in the interaction between the connexins could compromise inter-cellular transport, thus a number of mutations in connexins and crystallins have been reported to cause cataract (Graw 2004) .

We had previously reported a new spontaneous mutation in BALB/c mice (*dcm*) observed in a mouse colony maintained in our in-house experimental animal facility (Kohale *et al*. 2004). The mouse exhibited congenital cataract and microphthalmia at postnatal day 14 (PND 14) with no other observable abnormality. The *dcm* mice showed normal differentiation and formation of the optic vesicle and lens vesicle at an early developmental stage. However, abnormalities in developing lens fibers were noticed around gestational day 13 (GD13), with progressive deterioration thereafter. Genetic crosses characterized the mutation to be autosomal recessive. Previously reported cataract mutations on mouse chromosome 3 were predominantly dominant or semi-dominant phenotypes and phenotypically different from the *dcm* mice. Thus, the *dcm* mice are one of the very few genetic cataract models with recessive pattern of inheritance. We set out to identify the underlying genetic cause for the *dcm* phenotype. In the present study, we report the identification of the mutation by using a simple linkage analysis. We have mapped the mutation to mouse chromosome 3 and identified the specific mutation by sequencing this chromosomal region.

Materials and methods

Animals

The BALB/c mice with the dense cataract and microphthalmia (*dcm*) abnormality, of both sexes and of various ages were maintained at the Experimental Animal Facility, National Centre for Cell Science, Pune, India. Mice were kept in a barrier-maintained animal facility and were housed in standard, autoclavable cages maintained at 22 ± 2°C and 55 ± 5% relative humidity, and fed on pelleted diet with filtered water available *ad libitum*. All study protocols were approved by the Institutional Animal Care and Use Committee (IACUC). Humane animal care and use was performed under the guidelines of Committee for the Purpose of Control-

ling and Supervising Experiments on Animals (CPCSEA), Government of India.

Genetic crosses

Homozygous *dcm* male and female mice were used to initiate a breeding colony and establish a separate mutant line by use of full-sib mating. A pair of *dcm* BALB/c female mice was mated with a male wild-type C57BL/6J mice, and four such breedings were set up. The F_1 were allowed to interbreed and 16 breeding pairs were set up. The F_2 animals were analysed for the cataract phenotype and the mice with the cataract phenotype were analysed for chromosomal linkage analysis using MIT primers that amplify distinct sizes of product in the C57BL/6J and BALB/c derived chromosomes.

The rationale for using the C57BL/6J was that we had previously observed that they mate well with the *dcm* mice (Kohale *et al*. 2004). Further, a large number of polymorphic microsatellite loci with size differences that could be analysed by agarose gel electrophoresis have been reported between the BALB/c (*dcm*) and C57BL/6J strains, which is essential for the present linkage analysis.

Morphological examination

The mice obtained from various mating pairs were examined for cataract on PND 14 when their eyes were open. The lenses from homozygous *dcm* mice were obtained for examination.

SSLP-PCR method for genotyping

Tail DNA was isolated from the F_2 mice that exhibited *dcm* phenotype, and was used for the linkage analysis and genotyping. The linkage analysis was performed using simple sequence length polymorphism (SSLP) at the microsatellite loci, and a simplistic schematic representation of the strategy is shown in figure 1. The specific primers that were used in this study are derived from the ones reported by the Whitehead Institute/MIT Center for Genome Research.

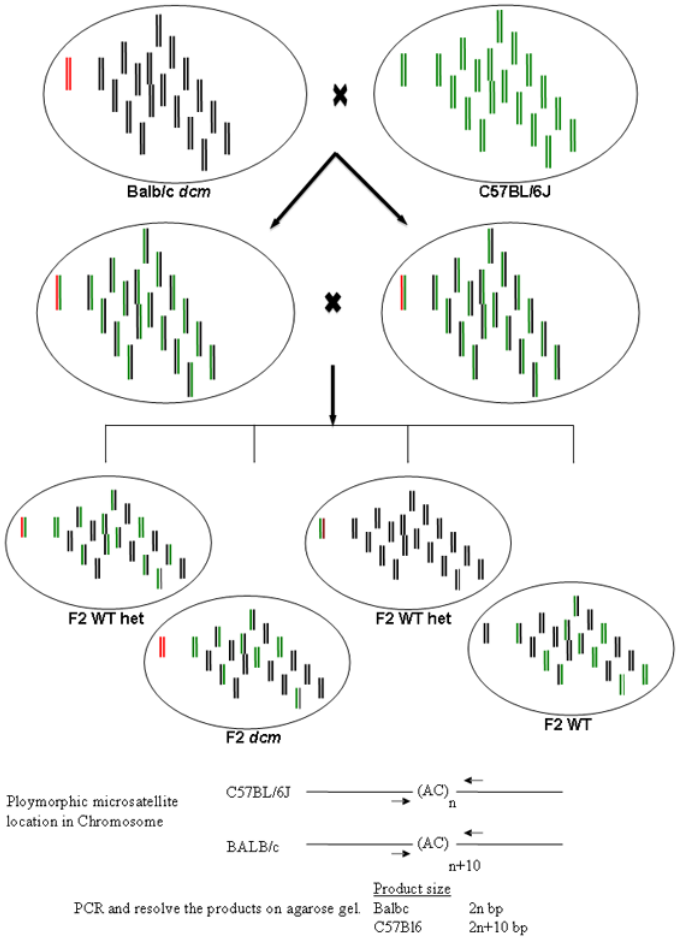

Figure 1. Strategy for SSLP analysis using PCR based polymorphic microsatellite marker assisted linkage analysis. The red lines indicate the chromosome carrying the mutations in the *dcm* mice while the remaining chromosomes are indicated by black lines. Green lines are chromosomes for C57BL/6J.

Primer name, sequence, chromosome, and the expected size of the PCR fragment for the strain are given in table 1 of the electronic supplementary material at http://www.ias.ernet/jgenet/. Standard PCR conditions were used (94°C, 30 s; 55°C 60 s; 72°C, 30 s; for 40 cycles) and the PCR products were resolved on 2.5% agarose gel. Specific region in chromosome 3 was PCR amplified and sequenced to identify the mutation in the *dcm* mice.

Results

To determine the chromosomal location of the *dcm* gene, we crossed the BALB/c (*dcm*) homozygous with C57BL/6J mice. All F_1 animals were phenotypically normal with respect to the eye and with agouti coat colour (figure 2). These F_1 mice were intercrossed, and about 25% of the F_2 progeny showed the *dcm* phenotype of cataract with microphthalmia. The coat colour of the *dcm* as well as the normal mice was variable ranging from albino to black. F_2 mice showing the *dcm* phenotype were used for the linkage analysis. Tail DNA was isolated from these F_2 animals showing the *dcm* phenotype and were analysed for chromosomal linkage with the *dcm* phenotype using PCR amplification. The primer pairs were chosen such that the PCR products have significant size difference so that they can be analysed by standard agarose gel electrophoresis. The initial screening was done using 24 animals, and for final fine mapping, a total of 48 F_2-*dcm* animals were used. Analysis of the chromosomal linkage with the *dcm* phenotype is summarized in table 2. A close linkage of a specific polymorphic marker with the mutant phenotype will result in very little product from the non-mutant parental allele (C57BL/6J). Very significant linkage of the chromosome 3 specific polymorphic markers with the *dcm* phenotype was observed. We further narrowed the putative region of mutations using a number of primers spanning the region D3Mit14 which shows greater than 70% association with the *dcm* phenotype. The results from these are summarized in table 3. The mutation was mapped to the region between D3Mit40 and D3Mit14, with both of these markers show greater than 70% association with the *dcm* phenotype. The mutation is mapped to a region about 45 cM from the centromere of the mouse chromosome 3.

Many genes involved in eye development have been identified from this region of the chromosome including genes coding for LeneP and Cx50 (figure 3). This region also has another gene coding for Cx40 which is not known to be expressed in eye lens. To identify the mutation, we PCR amplified each of these genes and the surrounding genomic region followed by sequencing. We mapped the mutation to *GJA8* gene coding for Cx50 and the mutation was a G to C conversion at nucleotide position 64 that resulted in the change of

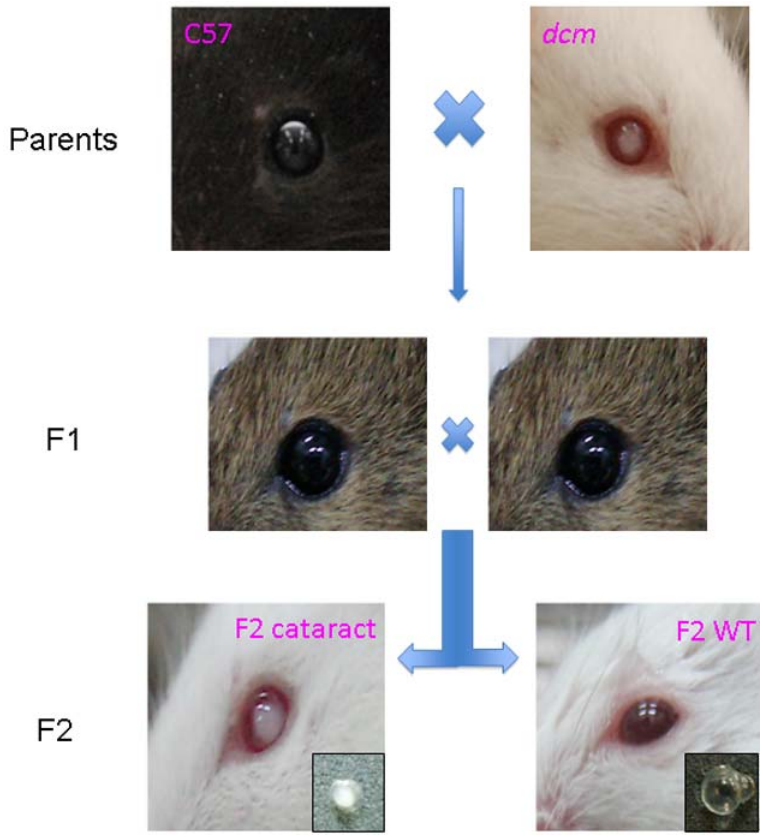

Figure 2. Representative pictures of the eyes of mice used in breeding experiments. The inserts show the dissected eye lens of the mice.

Table 2. Linkage analysis SSLP markers with *dcm* (BALB/c).

Primer name	% BALB/c	% C57BL/6J	% Heterozygous
D1mit14	32	32	36
D1mit65	29	29	42
D1mit155	21	37	42
D1mit94	27	30	43
D2mit48	17	29	54
D2mit102	44	11	44
D3mit14	**70**	13	17
D3mit65	**58**	4	38
D4mit12	5	39	56
D4mit97	20	10	70
D4mit54	8	42	50
D5mit24	22	30	48
D5mit95	17	25	58
D5mit79	13	25	62
D6mit25	25	17	58
D6mit39	36	9	55
D6mit8	33	4	63
D7mit96	22	22	56
D7mit68	24	19	57
D8mit14	18	36	46
D8mit84	10	52	38
D8mit94	18	44	38
D8mit65	30	46	24
D9mit4	12.5	30	57.5
D9mit18	25	25	50
D9mit67	17	33	50
D9mit11	10	60	30
D10mit80	24	20	56
D10mit95	25	21	54
D11mit20	21	33	46
D11mit50	14	28	58
D11mit41	25	21	54
D12mit34	37	33	30
D12mit83	25	33	42
D12mit101	35	39	26
D13mit16	33	33	44
D14mit37	37	8	55
D14mit60	37	13	50
D15mit63	8	33	59
D16mit4	37	20	43
D16mit5	26	17	57
D16mit34	16	13	71
D17mit39	25	20	55
D17mit57	39	0.5	60.5
D17mit66	57	-	43
D17mit22	41	0.5	58.5
D18mit49	30	20	50
D18mit68	16	20	64
D18mit51	12	29	59
D19mit19	16	16	68
D19mit34	17	21	62
D19mit10	16	16	68

Characters in bold are SSLP markers showing the linkage.

glycine at amino acid position 22 to arginine (G22R) (see figure 1A in electronic supplementary material). The mutation is identical to the previously reported mutation in the *Lop10* mutant mice (Chang *et al*. 2002). However, in addition to this mutation, few silent mutations were also observed in Cx40 gene (see figures 1, B&C in electronic supplementary material; table 4).

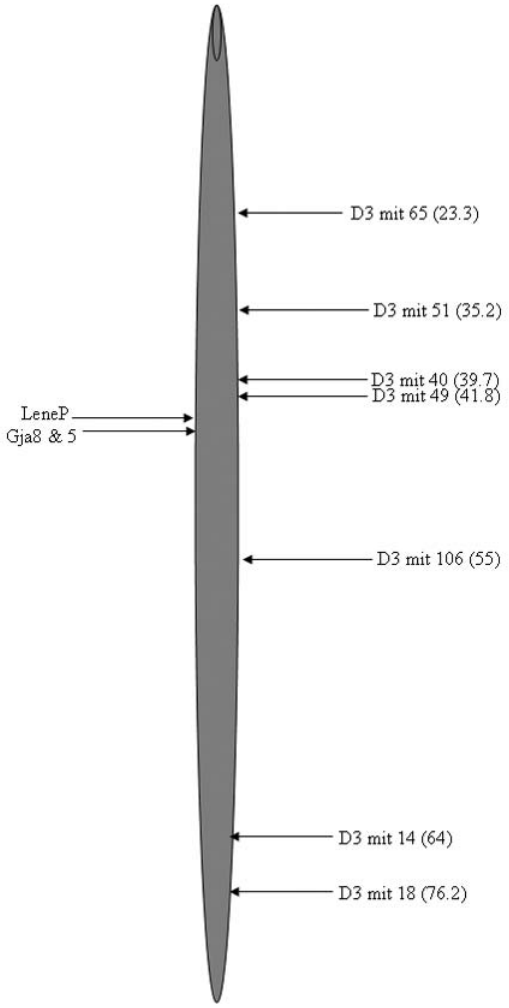

Figure 3. Physical map of mouse chromosome 3 indicating the relative positions of the various SSLP markers used to map the *dcm* locus.

Discussion

Large number of studies have identified number of genes for isolated congenital cataract (Graw 2004). Most cataracts have been associated with a small group of proteins necessary for maintaining lens transparency. Lens transparency is due to an unique arrangements of lens cells and the proteins therein. Lens consists of an anterior layer of cuboidal epithelial cells covering the fiber cells that make up the lens. The organelle free fiber cells make the lens nucleus. The cellular architecture of the fiber cells and the intercellular communication through the gap junctions are critical for maintaining the lens transparency. The lens is very rich in proteins (highest of any tissue at about 60% wet

weight), and about 90% of the cytoplasmic proteins comprise of crystallins. Short range ordered packing of the lens crystallins and the regular arrangement of lens fibers are important for the lens transparency and is disrupted in *dcm* mutants. The *dcm* mouse was described previously to have congenital (dense) cataract and microphthalmia.

Table 3. Fine mapping of *dcm* (BALB/c) in chromosome 3.

Primers	% BALB/c	% C57BL/6	% Heterozygous
D3mit18	68	10	22
D3mit40	**91.6**	0	8.4
D3mit49	**83**	0	17
D3mit51	**81**	0	19
D3mit106	54	9	37

Characters in bold are SSLP markers showing the linkage.

Table 4. Genetic alterations in the *GJA8* and *GJA5* loci in *dcm* (BALB/c) mice.

Gene	Mutation
GJA8	G64C
GJA5	C393T
GJA5	A411G
GJA5	T420C
GJA5	C1011T

The mutation led to the degeneration of the primary lens fibers during early eye development, with appearance of small vacuoles in later stages. The mouse also showed an underdeveloped iris, optic stalk, cornea and retina. The defective ocular structures, along with abnormal developing lens in *dcm* mice, lead to severe microphthalmia. Previously genetic studies have indicated that these abnormalities were caused by a single gene defect inherited in autosomal recessive manner. We have identified the mutation responsible for the *dcm* phenotype in the BALB/c mouse using linkage analysis. The mutation was mapped to the Cx50 gene and was identical to the mutation observed in another cataract mouse model *lop10*.

Cx50, a gap junction protein is expressed in the lens vesicle and is encoded by the *GJA8* gene. Several different mutations in this gene both in mice and humans have been reported to lead to cataract (Berry *et al*. 1999; Vanita *et al*. 2008; Yan *et al*. 2008). Many of the Cx50 mutations associated with cataract in mouse or humans affect the transmembrane domain or the extracellular loops, and are characterized by dominant/semi-dominant mode of inheritance. In mouse, all the three reported mutations in *GJA8* are dominant, while the knockout of *GJA8* shows a recessive mode of inheritance with respect to cataract phenotype (White *et al*. 1998). Knockout of *GJA8* gene results in hyperphosphorylation of Cx46, but the cause for the dominant phenotype of specific mutations is not clear. One reason could be due to the formation of nonfunctional heteromeric gap junction in case of heterozygous condition. In support of this argument an interaction between Cx46 and Cx50 in eye lens has been shown (Chang *et al*. 2002; Rong *et al*. 2002). Interestingly, although the *dcm* mice carry the same mutation as the *lop10* mice, the inheritance pattern for the gene is completely recessive.

Furthermore, although the mutations in *lop10* and *dcm* are identical, significant differences in the phenotype were observed between both animals. The *lop10* mutant is a semi-dominant mutation that occurred in a cross between BALB/c and AKR/J mice (Chang *et al*. 2002). In *lop10*, lens opacity was detected at PND 14, and the histologic abnormalities were evident at GD18. Herniation of the lens through the posterior capsule was a prominent feature. However, *dcm* is a recessive, nuclear cataract mutant mouse, and the histologic changes were observed at GD13, along with other severe underdeveloped ocular parts. These differences could be due to the genetic background of the two animals. We analysed the expression levels of the Cx46 in these mice by immunoblot analysis and found that although the levels are similar in the two wild-type strains, the heterozygous F_1 showed the levels similar to the wild-type. In contrast to what was reported for the *lop10* mutant the *dcm* mice had reduced levels of phosphorylated and non-phosphorylated Cx46 (figure 4). This is in sharp contrast to the *lop10* mutant where only the phospho-Cx46 levels were affected.

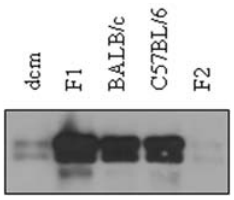

Figure 4. Western blot analysis of the Cx46 expression in the *dcm* mutant, heterozygous and wild-type mice. Ten μg of the total lens protein was resolved on SDS-PAGE and probed with Cx46 specific antibody (Santacruz Biotechnology, Santa Cruz, USA) and the lanes are as indicated.

Apart from the mutations in the *GJA8*, we also observed a few silent mutations in the adjoining gene encoding Cx40. Cx40 is not known to be expressed in eye lens and a role for these mutations in the *dcm* phenotype is not expected, but it is possible that these alterations may affect the chromosomal architecture and can affect the expression of the Cx50 in the eye lens. Our results show that *lop10* and *dcm* have the same underlying mutation, but the phenotype is different possibly due to the interaction of the mutant protein with other cellular components which may be different in these two animals.

A mouse model with cataract and microphthalmia would be highly useful in studying the pathologic processes involved in the mechanism of cataractogenesis and to improve understanding of the process of normal eye development. While the pathophysiology of the hereditary cataracts may differ from the age related cataract but understanding the biochemical and genetic basis of hereditary cataract can provide

insights into the pathways that are necessary for maintaining lens transparency and to how they are altered during the ageing process. Thus, the *dcm* mice present a very attractive model for studying the dynamics and interaction of the gap junction formation in mouse eye development, as it is one of the very few genetic models with recessive pattern of inheritance.

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