

# Overexpression of Na<sup>+</sup>-Dependent Myo-inositol Transporter Gene in Mouse Lens Led to Congenital Cataract

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**PURPOSE.** Maintaining appropriate osmotic pressure is essential for maintaining lens transparency. This study was performed to investigate whether high levels of myo-inositol, one of the major organic osmolytes in the lens, would lead to cataract development.

**METHODS.** Transgenic mouse lines carrying the bovine Na<sup>+</sup>-dependent myo-inositol transporter (bSMIT) cDNA under the control of the mouse  $\alpha$ A-crystallin promoter were generated.

**RESULTS.** Increased bSMIT expression was accompanied by increased myo-inositol level in the lens and increased uptake of (<sup>3</sup>H) myo-inositol by the lens in culture. The transgenic mice developed observable cataract under normal rearing conditions beginning at 2 to 8 weeks of age, and the severity of cataract development was correlated to the level of bSMIT gene expression and lens myo-inositol accumulation. For transgenic mouse line 3352, heterozygous mice did not develop cataract, whereas homozygous ones did. Prenatal feeding of heterozygous 3352 mice with high myo-inositol diet led to cataract development, indicating that cataract development was not merely due to a nonspecific effect of SMIT overexpression. Introducing aldose reductase overexpressing transgene into heterozygous 3352 mice also led to cataract development, indicating that this type of cataract is primarily due to osmotic stress.

**CONCLUSIONS.** The present results indicate that high levels of myo-inositol and sorbitol in the lens contribute to cataract development. This is a useful model to study the role of osmotic stress in cataractogenesis during lens development. (*Invest Ophthalmol Vis Sci.* 2000;41:1467-1472)

Cataract is the most important cause of blindness in the world. Nearly 16 million people are estimated to be blind because of cataract.<sup>1</sup> There are a number of causes for cataract, including congenital cataract, cataract from infection, cataract from UV and X-ray irradiation and oxidation damage, and cataract associated with several diseases, particularly diabetes. The transparency in mammalian lenses is due to the presence of crystallin structures formed by highly ordered association of several proteins. Changes in ionic environment, a reduction in the level of antioxidants such as reduced glutathione and ascorbic acid, and changes in the level of other solutes may lead to random protein aggregation and disruption of the crystallin structures, resulting in lens opacity and cataract. Therefore, the lens needs to stabilize the intracellular osmotic pressure by regulating the influx and efflux of water, osmolytes, and other solutes.

Osmotic stress due to the accumulation of sorbitol in the lens is most likely the cause of diabetic cataract. This is based on the fact that sorbitol accumulates to high levels in the lenses of diabetic animals<sup>2,3</sup> and that the administration of an inhibitor to aldose reductase (AR), the enzyme that reduces glucose to sorbitol, prevents the formation of diabetic cataracts.<sup>4,5</sup> Lenses cultured in high glucose medium also developed opacity as in diabetic cataract.<sup>6</sup> Development of lens opacity in vitro can be prevented by AR inhibitors or if the medium is made hypertonic to balance the increased sorbitol accumulation in the lens, indicating that osmotic stress is the cause of diabetic cataract.<sup>7,8</sup> Further support of this model is provided by the fact that mice, which normally do not develop diabetic cataract, became susceptible to development of this type of cataract when their lens AR level was increased and that the rate of cataract development was accelerated when sorbitol dehydrogenase-deficient mutation was introduced into these mice to block the conversion of sorbitol to fructose.<sup>9</sup>

To determine whether cataract caused by sorbitol accumulation is a consequence of osmotic stress rather than the toxic effect of sorbitol, we wanted to increase the lens myo-inositol (MI) level to see if that also causes cataract. Myo-inositol is one of the three major osmolytes in the lens besides sorbitol and taurine.<sup>10</sup> Influx of MI into lens is dependent on the Na<sup>+</sup>-dependent MI transporter (SMIT).<sup>11</sup> In this study, we produced transgenic mice that overexpress SMIT constitutively in lens cells and found that they developed congenital cataract under normal rearing condition beginning at 2 to 8 weeks of age. These results provide strong evidence that a high level of

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osmolytes in the lens is the main factor contributing to osmotic cataract.

## METHODS

### Generation of Transgenic Mice

A DNA fragment containing the  $\alpha$ Acry-bSMIT hybrid gene (3.7 kb) was released from the vector by *NotI* digestions and microinjected into oocytes from CBA egg donors fertilized by C57BL males. Identification of transgenic mice was performed by polymerase chain reaction on genomic DNA extracted from the tail and then confirmed by Southern blot hybridization using SV40 polyA fragment as probe (data not shown). All animals in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### RNA Isolation and Northern Blot Analysis

Poly(A)<sup>+</sup> RNA was prepared from brain, heart, kidney, lens, liver, and muscle of 2-week-old bSMIT transgenic mice and normal C57BL mice with oligo (dT) columns (Qiagen, Hilden, Germany). Three micrograms of mRNA was separated on a 1.5% agarose gel containing 3% formaldehyde and blotted onto a Hybond N<sup>+</sup> membrane according to the manufacturer's protocol. Probes were labeled with <sup>32</sup>P-dCTP with random priming, using a commercially available kit (Amersham, Buckinghamshire, England). A 1.8-kb bSMIT cDNA fragment released from *EcoRI* and *PstI* digest of  $\alpha$ Acry-bSMIT was used to hybridize specific transgene expression, and GAPDH cDNA probe was used as control.

### In Situ Hybridization

Embryos, 15.5 (E15.5) and 17.5 (E17.5) days old, and adult mice from transgenic line 3388 and normal C57 were killed, and their eyeballs isolated for cryosectioning. Cryostated sections (10- $\mu$ m thick) were mounted on siliconized slides. The slides were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and then dehydrated in increasing concentrations of ethanol. A 1.0-kb segment of bSMIT cDNA, released by *EcoRI* and *SphI* digest and subcloned into pBluescript II SK (+) vector, was used as hybridization probe. Sense and antisense riboprobes were generated with T7 and T3 RNA polymerase, respectively, in the presence of <sup>35</sup>S-CTP (Amersham). In situ hybridization to mouse eyeball sections was performed as previously described.<sup>12</sup>

### Assay of SMIT Activity

Mouse lens Na<sup>+</sup>-dependent MI uptake was measured essentially as described previously with slight modifications.<sup>13,14</sup> Lenses from 4-week-old transgenic mice and nontransgenic mice were isolated and cultured in Medium 199 (GIBCO, Gaithersburg, MD) for 24 hours. Lenses that remained clear were then switched to an incubation solution consisting of basic M199, 10  $\mu$ M MI, and 0.5  $\mu$ Ci/ml (<sup>3</sup>H)myo-inositol (Amersham). After 4 hours' incubation, lenses were removed, washed 2 times in saline, gently blotted, and transferred to a scintillation vial containing 0.2 ml of 2.5% sodium dodecyl sulfate solution and kept overnight at 50°C to dissolve the lens. Then 3 ml of scintillation fluid was added, and the contents of the vials were counted in a liquid scintillation counter (model

LS 6800; Beckman, Los Angeles, CA). In addition, 2 sets of standards were prepared using 10  $\mu$ l aliquots of the incubation medium A and B containing <sup>3</sup>H to determine the specific activity (counts per minute per nanomole of MI) of the incubation medium. Myo-inositol uptake was calculated as nanomoles per lens per hour from the specific activity of the incubation solution and <sup>3</sup>H counts of sample. In parallel experiments, 1 mM ouabain was added to the medium to measure incorporation of MI in the absence of ATPase.

### Measurement of MI Level in Mouse Lenses

Myo-inositol levels in mouse lenses were determined by high-performance liquid chromatography (HPLC).<sup>15</sup> Mice were killed by cervical dislocation, and lenses were quickly removed and frozen in liquid nitrogen. They were stored at -80°C until processed. After weighing, each pair of lenses was homogenized in 1 ml of 5% trichloroacetic acid (TCA), and the slurry was centrifuged at 14000g for 10 minutes. The supernatant was saved, and TCA was removed by fourfold extraction with diethyl ether. The resultant solution was subsequently concentrated to remove the ether and lyophilized. The dried sample powder was reconstituted in 500  $\mu$ l of filtered dH<sub>2</sub>O, and 50  $\mu$ l of 0.25 M trans-1,2-diaminocyclohexane-N,N,N',N'-tetra-acetic acid (CDTA) was added to remove metal ions. After deionization with an anion/cation exchange resin (Bio-Rad, Hercules, CA), the extracts were filtered through a 0.45- $\mu$ m polysulphon cartridge filter (Alltech, Deerfield, IL) before injection into the HPLC system. A 5.5  $\mu$ l aliquot of a 1 mM xylitol was added as an internal standard. The concentrations of MI were determined by a DX500 HPLC system (Dionex, Sunnyvale, CA) using a CarboPac (MA-1) anion exchange column (Dionex) with isocratic elution by 420 mM NaOH. The peak areas were normalized to xylitol, and concentrations were quantified against known standard curves run on the same day.

### Monitoring Cataract

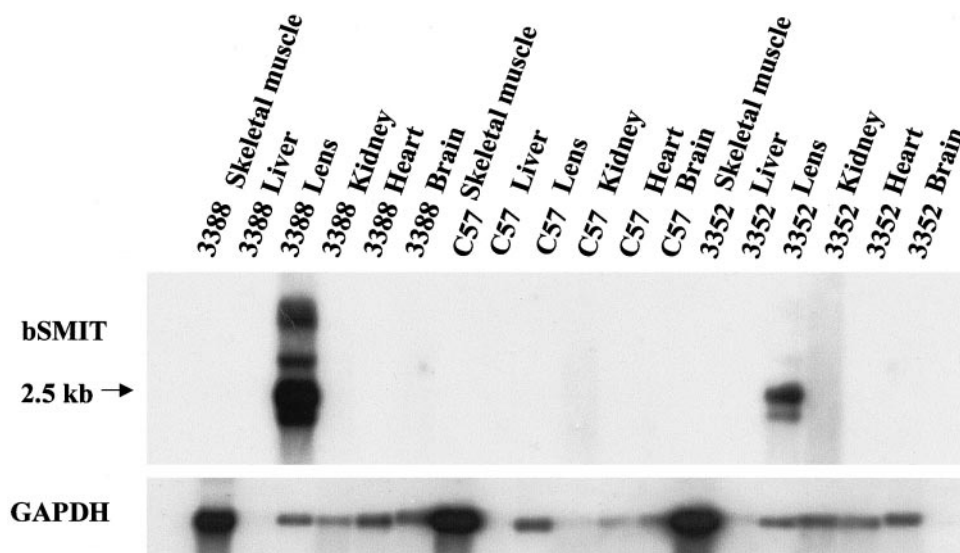
Mouse pupil was dilated with 1% tropicamide (Alcon, Puurs, Belgium), and the lenses were monitored with a slit-lamp microscope and recorded by photography.

### High MI Diet

A 3% MI (wt/wt) was added to standard mouse chow.<sup>16</sup> After mating with a line 3352 homozygous male, C57BL female mice were divided into three groups: group 1, immediately started feeding with 3% MI diet after mating and continued after birth; group 2, started 3% MI diet after giving birth; and group 3, immediately started feeding with 3% MI diet after mating and stopped after birth. The cataracts of transgenic and nontransgenic offspring in each group were checked weekly from 2 to 10 weeks of age. Four 4-week-old heterozygous pups from each group were killed for lens MI quantitation by HPLC.

### Crossing of bSMIT Transgenic Mice with hAR Transgenic Mice

Mice homozygous for bSMIT transgene (bSMIT +/+) from line 3352 were mated with homozygous hAR transgenic mice (hAR +/+) line CAR222<sup>9</sup> to introduce AR overexpressing transgene into line 3352 mice. These double heterozygous transgenic offspring were monitored for cataract development weekly from 2 to 10 weeks of age. Six of them were killed at 4 weeks



**FIGURE 1.** Northern blot analysis of bSMIT mRNA in various tissues of transgenic and normal mice. The 2.5 kb bSMIT and GAPDH transcripts are indicated.

of age, and their lens MI and sorbitol levels were determined by HPLC.

## RESULTS

### Development of Transgenic Mice Overexpressing bSMIT in Lenses

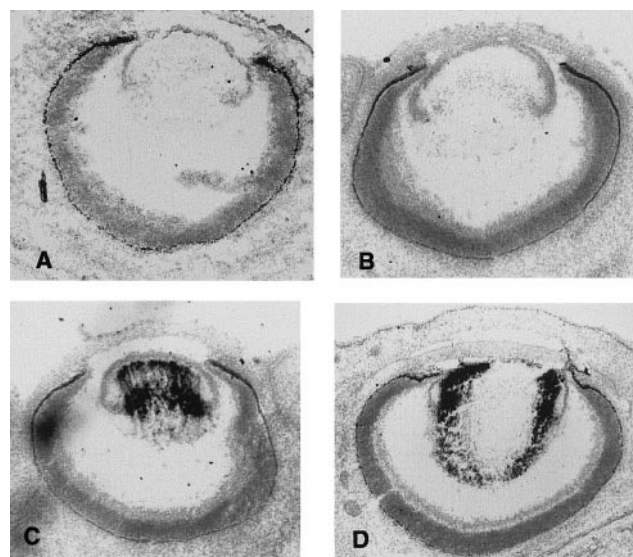
Myo-inositol is one of the major organic osmolytes in the lens. Its accumulation in the lens is mainly through a specific  $\text{Na}^+$ -dependent myo-inositol transporter (SMIT) on the cell membrane. To increase the level of MI in the lens we developed transgenic mice that express high levels of SMIT in their lens cells. We wanted to determine whether a high level of MI would make them susceptible to development of cataract. The bovine SMIT cDNA<sup>17</sup> containing the entire SMIT coding region was fused to mouse  $\alpha$ A-crystallin promoter that directs the expression of heterologous gene in the mouse lens, generating the plasmid  $\alpha$ Acry-bSMIT. The fragment containing crystallin promoter-bSMIT hybrid gene was released from  $\alpha$ Acry-bSMIT and injected into mouse oocytes. The oocytes were then allowed to develop to term in foster mothers. Five transgenic lines were developed, and two of them, lines 3352 and 3388, were characterized in greater detail, and they were also bred to homozygous state to double the level of expression of their transgenes. Northern blot analysis hybridization showed that bSMIT transgene is only expressed in the lens and not in other tissues such as brain, heart, liver, kidney, and skeletal muscle of the transgenic mice (Fig. 1). The transgene expression level was higher in line 3388 than in line 3352. In situ hybridization results showed that bSMIT mRNA was detected in the nuclear and cortical regions of the lens at E15.5 stage (15.5 days postcoital), but at E17.5 expression was restricted to the fiber cells at the bow region (Fig. 2).

To determine whether the expression of the bSMIT transgene leads to higher SMIT activity, lenses from 4-week-old transgenic and nontransgenic mice were cultured *in vitro*, and the rate of uptake of radioactively labeled MI from the medium was measured. As shown in Table 1, line 3388, which has a higher level of bSMIT mRNA than line 3352, has higher SMIT

activity in their lenses than 3352, and homozygous transgenic mice have higher lens SMIT activity than their heterozygous counterparts. Furthermore, the transgenic mice accumulated more MI in their lenses, proportional to the level of their lens SMIT activity. The level of SMIT expression does not seem to correlate with the copy numbers of the bSMIT transgene (data not shown). The level of transgene expressed is largely influenced by the chromosomal location of their integration sites.

### Congenital Cataract in Transgenic Mice

Under normal rearing conditions, both heterozygous and homozygous 3388 mice developed cataract. For 3352 mice, only



**FIGURE 2.** Bright field photomicrographs demonstrating the location of bSMIT gene expression during embryonic development. (A) E15.5 nontransgenic mouse with antisense probe; (B) E15.5 3388 homozygous mouse with sense probe; (C) E15.5 3388 homozygous mouse with antisense probe; (D) E17.5 3388 homozygous mouse with antisense probe.

TABLE 1. Lens SMIT Activity, MI Level, and the Rate of Congenital Cataract Development

Mouse Line Genotype	Lens SMIT Activity, nmol/h/lens	Lens Myo-inositol Level, nmol/mg wwt	Age of Onset of Opacity, Weeks	% of Mice Reaching Different Stages of Cataract		
				Stage I	Stage II	Stage III
3352						
Heterozygous	5.0 ± 0.36	9.2 ± 1.16	N	0% (0/84)	0% (0/84)	0% (0/84)
Homozygous	7.4 ± 1.13	14.4 ± 1.10	4-8	65.2% (43/66)	0% (0/66)	0% (0/66)
3388						
Heterozygous	5.7 ± 0.70	11.9 ± 4.16	4-8	59.7% (37/62)	9.7% (6/62)	0% (0/62)
Homozygous	11.1 ± 3.32	23.6 ± 3.90	2-6	15.0% (9/60)	61.7% (37/60)	8.0% (5/60)
Normal mice	4.1 ± 0.38	4.2 ± 0.61	N	0% (0/160)	0% (0/160)	0% (0/160)

Lens SMIT activity and MI level of the transgenic and normal mice were assayed at 4 weeks of age, and data are expressed as mean ± SE from 6 mice. The cataracts of transgenic mice were monitored and recorded by slit-lamp microscope weekly from 2 to 10 weeks of age and three stages of cataract were defined as in Figure 3. N, no observable cataract during the course of experiment.

the homozygous mice developed cataract. Furthermore, the age of onset and severity of the cataract were different between the two mouse lines and different between the homozygous and heterozygous mice (Table 1). The progression of cataract development was arbitrarily divided into three stages as shown in Figure 3. The first stage is represented by the appearance of localized opacification in the lens nucleus. Stage 2 occurs when opacification covers the entire nucleus of the lens, and stage 3 is reached when the entire lens becomes opaque. Some of the homozygous 3388 mice, with the highest MI level in their lens, developed stage 3 cataract, and signs of cataract were observable as early as 2 weeks after birth. Heterozygous 3388 mice never developed stage 3 cataract, and earliest observable cataract occurred 4 weeks after birth. None of the heterozygous 3352 mice developed cataract, whereas approximately 65% of the homozygous mice from this line developed stage 1 cataract. Cataract deteriorated progressively from 2 to 8 weeks of age, after which time the morphology of the cataract did not change. Thus, the rate of development and

severity of the cataract correlated with the level of bSMIT expression. Therefore, it is unlikely that cataract development in these mice is due to activation or inactivation of some genes at the integration site of the transgene.

### Effect of High MI Diet on Cataract Development

To demonstrate that the cataract in transgenic mice was caused by a high level of MI in the lens and not some anomalous effect of overexpression of protein, heterozygous 3352 mice, which do not develop cataract under normal rearing conditions, were fed with 3% MI diet to increase the lens MI level. It has been reported that feeding rats a diet supplemented with 1% MI increases serum MI levels by ~4.5-fold.<sup>18</sup> As shown in Table 2, prenatal feeding (feeding the pregnant mother) of heterozygous 3352 mice with a high MI diet led to cataract (stage 1) development, indicating that cataract development is not merely due to a nonspecific effect of SMIT overexpression. Nontransgenic mice similarly fed with a high MI diet did not develop cataract (data not shown). Interestingly, postnatal

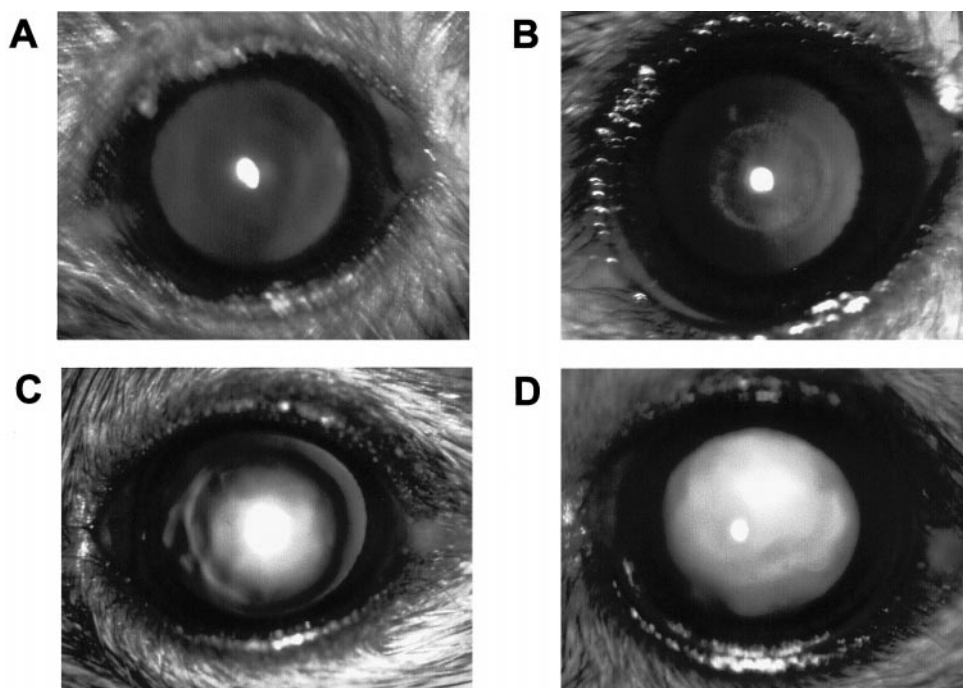


FIGURE 3. Stages of cataract development in transgenic mice. (A) Eye showing normal lens with no sign of cataract. (B) Stage 1: slight opacification appears in the lens nucleus. (C) Stage 2: opacification covers the entire lens nucleus. (D) Stage 3: complete opacification throughout the entire lens.

TABLE 2. Effect of 3% MI Diet on the Rate of Cataract Formation and Lens MI Level

Diet	Age of Starting 3% Myo-inositol Diet	Age of Ending 3% Myo-inositol Diet	Lens Myo-inositol Level, nmol/mg wwt	% of Mice Developing Cataract
Normal diet	—	—	9.2 ± 1.16	0% (0/84)
3% Myo-inositol diet	Embryo day 1	Newborn	9.2 ± 0.58	52.9% (9/17)
	Embryo day 1	10-week-old	12.1 ± 1.50	44.0% (11/25)
	Newborn	10-week-old	12.1 ± 0.58	0% (0/22)

MI level of the heterozygous 3352 mice were assayed at 4 weeks of age, and all data points are mean ± SE from 4 mice.

feeding had no effect, indicating that damage is done at the embryonic stage, even though cataract is observed 4 to 8 weeks after birth. Assay of lens MI showed that a high MI diet significantly increased the lens MI level (Table 2).

### Synergistic Effect of MI and Sorbitol on Cataract Development

The results of our experiments clearly showed that a high level of MI causes cataract. However, it is not clear whether a high level of MI causes cataract because of osmotic stress or because too much MI perturbs the phosphoinositide signal transduction system. Because MI is the precursor for various phosphoinositides, it is possible that a high level of MI may increase the levels of these important signal transducing molecules, thereby affecting various cellular functions. To see if this is true, we introduced into heterozygous 3352 mice, the AR overexpressing transgene to increase the lens osmolyte content. We had previously developed transgenic mice that overexpress AR in their lenses. When induced to become diabetic, these mice accumulated sorbitol in their lenses and developed cataract at a rate proportional to their lens AR activity.<sup>9</sup> One of these lines of AR transgenic mice, CAR222, is resistant to development of diabetic cataract unless they are made homozygous for the AR transgene. We mated homozygous 3352 with homozygous CAR222. All their offspring were heterozygous for the bSMIT and AR transgenes. Seventy-eight percent of these bSMIT-AR double transgenic mice developed stage 1 congenital cataract under normal rearing conditions beginning at 4 to 8 weeks of age (Table 3). This clearly showed that the two osmolytes MI and sorbitol acted synergistically to cause cataract, indicating that a high level of MI causes cataract because of osmotic stress.

## DISCUSSION

Myo-inositol is a major osmolyte in the lens. Its level is primarily regulated by its specific transporter SMIT. For many types of cell, including lens epithelial cells, an increase in extracellular osmotic pressure induces the transcription of SMIT, AR, and taurine transporter. Consequently, intracellular levels of MI, sorbitol, and taurine are increased to balance the increased

osmotic pressure. However, too much osmolyte is deleterious to the cells. We previously have shown that diabetic cataract is the result of a large accumulation of sorbitol beyond what the lens osmoregulatory system can handle. Transgenic mice that overexpress AR only developed cataract when they were made diabetic or when fed with a high galactose diet. These are models for osmotic cataract in mature lenses. In the present study we demonstrated that osmotic stress during embryonic lens development causes cataract as well. Overexpression of SMIT in the lens causes congenital cataract. The severity and time of onset of lens opacity are proportional to the lens SMIT activity and MI accumulation, indicating that cataract development is not due to activation or inactivation of genes at the transgene integration site.

During the preparation of this manuscript, results similar to ours were reported.<sup>19</sup> However, our experiments provide further insight on the pathogenesis of this type of cataract. Heterozygous 3352 mice overexpress SMIT but not to a level high enough to cause cataract. We showed that prenatal feeding of 3352 mice with 3% MI diet led to congenital cataract, whereas postnatal feeding has no effect. This convincingly shows that cataract in these transgenic mice is caused by excess MI and not by some anomalous effect of too much protein in the lens. This result also indicates that the developing lens is more sensitive to osmotic stress. It is likely that in the early stages of lens development, the osmoregulatory machinery in the lens is not fully operative, and consequently it cannot accommodate a modest increase in osmolyte content. When the lens is matured, its osmoregulatory machinery can accommodate a modest fluctuation in osmolyte levels. Why, then, do mice overexpressing AR in their lenses not develop congenital cataract and only develop cataract when they are made diabetic or fed with high galactose diet? This may be due to the fact that AR is not very efficient in reducing glucose to sorbitol. The  $K_m$  of AR for glucose is estimated to be  $\approx 30$  to 100 mM.<sup>20,21</sup> Thus, only under hyperglycemic conditions is AR able to convert significant amounts of glucose to sorbitol to cause osmotic stress. It will be interesting to see whether a pregnant female mouse overexpressing AR is made diabetic, and whether or not the pups will develop congenital cataract.

TABLE 3. Cataract Formation in SMIT/AR Double Transgenic Mice

Mouse Line	Genotype	Lens Myo-inositol	Lens Sorbitol	% of Mice Developing Cataract
Normal mice	—	4.3 ± 0.61	<0.08	0% (0/160)
hAR222	Heterozygous	2.3 ± 0.16	1.8 ± 0.42	0% (0/38)
bSMIT3352	Heterozygous	9.2 ± 1.16	<0.08	0% (0/84)
hAR222/bSMIT3352	Heterozygous	7.3 ± 0.34	2.4 ± 0.30	78.26% (36/46)

The cataracts developed in these mice are stage 1 cataract defined in Figure 3. The amounts of MI and sorbitol are expressed in nmol/mg wet wt, and values are mean ± SE from 6 mice.

The level of MI in the lenses of normal mice is approximately 4.2 nmol/mg wet wt. In the SMIT transgenic mice, lenses that accumulated up to 9.2 nmol/mg wet wt of MI did not develop cataract, whereas those with 11.8 nmol/mg wet wt of MI did. This indicates that the lens can accommodate up to twice as much as the normal level of MI without any deleterious effect, but not much beyond that level. Therefore, when the CAR222 AR transgene was incorporated into the 3352 SMIT transgenic mice, the AR-bSMIT double transgenic offspring developed congenital cataract even though individually these transgenes do not cause cataract development. This clearly indicates that it is the osmotic stress that causes cataract, not the toxic effect of MI or sorbitol. However, we cannot rule out the possibility that excess MI in the developing lens may sensitize the lens cells to osmotic stress because of perturbation of the phosphoinositide signal transduction systems. It is interesting to note that the total osmolyte (sorbitol and MI) level in the lenses of 4-week-old AR-bSMIT double transgenic mice is similar to that of 4-week-old bSMIT transgenic mice. In the lenses of the double transgenic mice, the MI level is reduced to compensate for the increase in sorbitol level, suggesting that in these matured lenses the total osmolyte level is maintained to an appropriate level by the lens' osmoregulatory machinery.

The SMIT gene is located in chromosome 21 in the region essential for the development of Down syndrome (DS).<sup>22</sup> Down syndrome patients, who have an extra copy of chromosome 21, often develop congenital cataract.<sup>23</sup> Osmotic stress caused by overexpression of SMIT as demonstrated in this report could explain the high incidence of congenital cataract in these patients. However, the morphology of the cataract in DS patients is quite different from that of transgenic mice overexpressing SMIT, suggesting that different mechanisms may be involved. Cataract in DS patients is characterized by localized opacity in the cortical region,<sup>24,25</sup> not the nuclear cataract observed in SMIT transgenic mice. It is possible that these morphologic differences are the consequences of subtle differences in lens development between the mouse and humans, or resulting from differences in temporal and spatial expressions between the endogenous SMIT gene and the SMIT transgene. Although SMIT activity from the expression of endogenous gene is found in lens epithelial cells, SMIT transgene is primarily expressed in the fiber cells. Clearly, further experiments are necessary to determine whether overexpression of SMIT contributes to cataract development in DS patients.

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