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Distribution of fibronectin, laminin, tenascin, chondroitin sulfate proteoglycan and HNK-1 epitope in the head region of homozygous rat small eye (*rSey)* embryos

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ABSTRACT Rat small eye (*rSey*) involves a mutation in *Pax-6* gene. The midbrain crest cells of *rSey* homozygote fail to migrate normally towards the frontonasal region resulting in absence of eyes and nose. In the present study, comparison of the distribution patterns of fibronectin, laminin, tenascin, chondroitin sulfate proteoglycan and HNK-1 epitope in craniofacial region between that of *rSey* homozygous and the wild type embryos at the day 11 of gestation were investigated. The results showed alteration of distribution pattern of HNK-1 epitope at the mesenchyme adjacent to the frontonasal ectoderm where the *Pax-6* gene was strongly expressed. However, the other molecules did not show any obviously different distribution.

KEYWORDS: *rSey*, HNK-1, *Pax-6*, extracellular matrix, neural crest cell.

INTRODUCTION

Rat small eye (*rSey*) is a spontaneously arising mutation in the rat Sprague-Dawley (SD) strain.[1-2] The mutation has been found in *Pax-6* gene which is one of the members of paired box-containing genes.[1-2] The *Pax-6* gene expresses early in regions of the pre-sumptive lens, corneal ectoderm and mostly in the developing nervous system, including the forebrain, hindbrain and spinal cord.[3-6] Mutations in *Pax-6* gene have been demonstrated to result in different degrees of eye disorder in many species, including eyeless in *Dorsophila*, *vab-3* mutant in *C. elegans*, small eye (*Sey*) in mouse, and aniridia in human.[7-14] Closely resembling *Sey* in mouse, *rSey* heterozygous adults have small eyes. In homozygotes, the lens and the nasal placodes fail to develop resulting in lack of eyes, lack of nose and perinatal death.[1-2] Except for the lens and the nasal placodes, the *rSey* mutation does not affect any other cranial regions such as maxilla, mandible and hyoid arch.

Cranial neural crest cells are fundamentally involved in the formation of craniofacial structures including eyes and noses. The nasal placodes in frontonasal region and eye rudiments are derived from neural crest cells that migrated from both the forebrain and the anterior midbrain.[15-18] Osumi-Yamashita et al[19] labeled premigratory neural crest cells of the *rSey* homozygotes with dye and demonstrated that the anterior midbrain crest cells migrated to the eye rudiments, but did not migrate further to the frontonasal region. However, the forebrain crest cells, which also contributed to the frontonasal region, migrated normally.[2] When premigratory midbrain crest cells obtained from wild type embryos were injected into corresponding area of the *rSey* homozygotes, the injected cells could not migrate further from the eye rudiments into frontonasal region while they successfully migrated in the wild type hosts.[19] This evidence suggests that the impair ed migration does not originate from the crest cells themselves but rather from the environment that they encounter.

It was demonstrated that some extracellular matrix and adhesion molecules are environmental factors related to the migration of the cranial crest cells.[20-21] We propose two hypotheses for the effects of the environmental factors on the impaired migration of the midbrain crest cells. One is loss of permissive molecules for migration, the other is the pathway has been blocked by some molecules. In order to clarify these hypotheses, fibronectin and laminin were investigated for the former hypothesis since they are extracellular matrix molecules that promote migration of neural crest cells *in vitro*, as well as being found along the migration pathway.[22-28] Tenascin, chondroitin sulfate proteoglycan and a carbohydrate epitope recognized by HNK-1 antibody were used to clarify the latter hypothesis. Tenascin has been suggested to play a role in migration of neural crest cells because of its distribution pattern and since it causes causing abnormal migration of neural crest

cells.[29-33] Moreover, chondroitin sulfate proteoglycan has been found to inhibit migration of neural crest cells.[34-37] HNK-1 antibody recognizes an carbohydrate epitope which is shared by several adhesion molecules as well as avian neural crest cells.[38] It has been demonstrated both *in vitro* and *in vivo* that HNK-1 carbohydrate epitopes perturbed cranial crest cell migration in avian development.[38-40]

Materials and Methods

Preparation of embryos

rSey mutant was originally found in the Sprague-Dawley (SD) rat colony in the Safety Research Laboratories, Yamanouchi Pharmaceutical Co Ltd.[1-2]

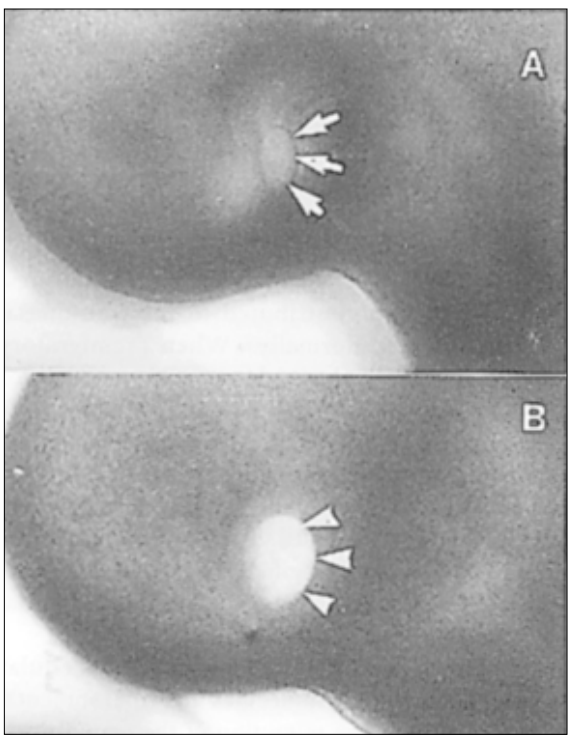

Fig 1. Lateral view of whole-mount bright-field images of day 11 wild type (A) and *rSey* homozygous embryos (B). Arrows indicate the developing eyes of the wild type embryo. Note the transparent developing eyes of the *rSey* homozygous embryo (arrowheads).

About one-fourth *rSey* homozygous embryos were obtained from intercrosses of *rSey* heterozygotes parents. Wild type embryos at the same stage were obtained from SD parents. Day of vaginal plug observed was designated as day 0 of gestation. On day 11 of gestation the embryos were dissected out from the uterus in phosphate-buffered saline (PBS, pH 7.4). The day 11 homozygous embryos (Figure 1B) were distinguished from the wild type ones (Figure 1A) by the abnormal transparent eyes. The embryos were collected, fixed for 2-4 hours at room temperature in 4% paraformaldehyde in PBS, washed in PBS, transferred to series of sucrose in PBS and quickly frozen in OCT compound (Tissue Tek Products), or dehydrated through graded series of ethanol and embedded in paraffin (paraplast). Serial horizontal sections of the head region were made at 12-14 micrometer thick using a cryostat (Reichert) and then the sections were placed on poly-L-lysine (Sigma) coated glass slides. For paraffin embedded samples, the serial sections were made at 8 micrometer thickness using a microtome (Spencer) and placed on glass slides. For each antibody studied, 6-14 samples of both the wild type and the *rSey* homozygous embryos were used for confirmation (Table1).

Immunohistochemistry

The distributions of fibronectin, laminin, tenascin, chondroitin sulfate proteoglycan and HNK-1 were determined using a biotin-streptavidin complex system (Vector Laboratories). Briefly, the sections were pretreated with 0.3% hydrogen peroxide (H_2O_2) in methanol to remove internal oxidation. Then the primary antibodies, diluted in 2% horse serum in PBS, at each optimal concentration as shown in Table1 were applied on the sections and then incubated overnight. For negative controls, the primary antibodies were replaced with 2% horse serum in PBS. After being washed in PBS three times, the sections were incubated in appropriate biotinylated second antibodies for 30 minutes and

Table 1 Number of samples, dilution of primary antibodies, and types of secondary antibodies used in the study.

Primary antibody	No. of samples		Dilution	Secondary antibody
	SD	rSey		
Anti Rat Fibronectin (Chemicon)	12	7	1:2000	Anti-Rabbit IgG (Vector)
Anti Rat Laminin (Chemicon)	8	6	1:4000	Anti-Rabbit IgG (Vector)
Anti Tenascin (Gift from Dr. Osumi- Yamashita)	10	6	1:1000	Anti-Rabbit IgG (Vector)
Anti Chondroitin sulfate proteoglycan (Seikagaku)	10	6	1:200	Anti-Mouse IGM (Vector)
HNK-1 (anti-leu-7; Becton-Dickinson)	14	9	1:50	Anti-Mouse IGM (Vector)

the biotin-avidin complex conjugated with peroxidase for 1 hour. Bound antibody was visualized with 0.2mg/ml diaminobenzidine and 0.03 % H_2O_2 in 0.05 M TRIS buffer (pH 7.4). Intense dark brown staining was designated as positive staining when all samples showed similar staining patterns. The sections were counterstained with methyl green, observed and photographed under an optical microscope (New Vanox AH2, Olympus).

Results

Distributions of studied molecules were observed in the head regions especially in the developing eyes and noses, which were defective in homozygous *rSey* embryos. On day 11 of gestation, the nasal placodes appeared as thickening of epithelium in the frontonasal region. Sections were cut coronally so that the optic vesicles and nasal placodes were seen in the same sections. Parts of the midbrain and the forebrain with bulb of the optic vesicles were observed in the sections (Figures 2A, 2C, 3A, 3C, 4A and 4C). The sections of the *rSey* homozygous embryos showed no epithelial thickening of the nasal placodes, as well as slight changes in the optic vesicles (figures 2B, 2D, 3B, 3D, 4B and 4D).

Distribution of HNK-1 epitope

In wild type embryo, the positive reactivity of HNK-1 antibody staining was observed in some neuroepithelial cells of the optic vesicles (Fig. 2 A, 2C). The rest of the mesenchymal tissue as well as the epithelium including the nasal placodes were negatively stained (Fig. 2 C). In *rSey* homozygous embryo, there was no positive staining observed at the optic vesicles. In contrast, intense positive staining was observed in the frontonasal epithelium and in the small area of the mesenchyme just underneath the stained epithelium (Fig. 2 B, D). It is interesting that the stained area in the *rSey* homozygous embryo was distinctly different from that of the wild type one.

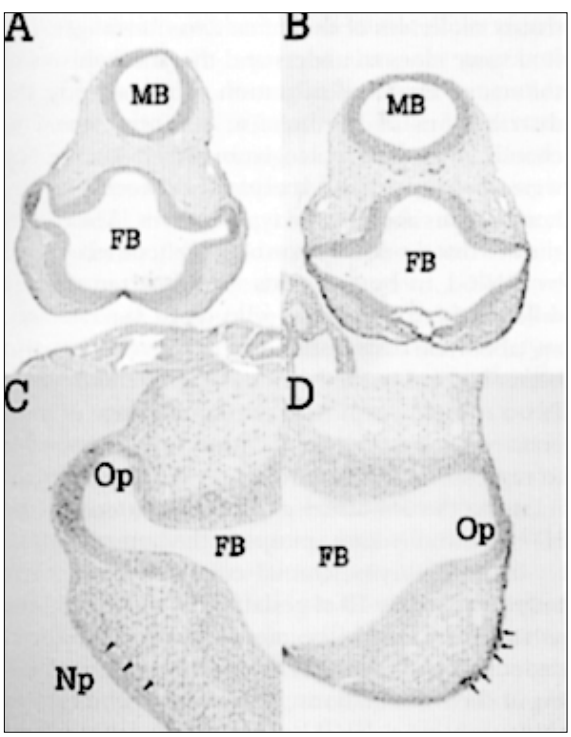

Fig 2. Coronal paraffin sections of heads of 25-somite wild type (A, C) and 24-somite *rSey* homozygous embryos (B, D) staining with HNK-1 antibody. Arrows indicate the intense staining in the frontonasal epithelium and the mesenchyme underneath in *rSey* homozygous embryos, whereas no staining is seen in wild type embryos. Arrowheads indicate the epithelial thickening of the nasal placode. Op, optic vesicle; NP, nasal placode; FB, forebrain; MB, midbrain. (A and B) x160, (C and D) x 500.

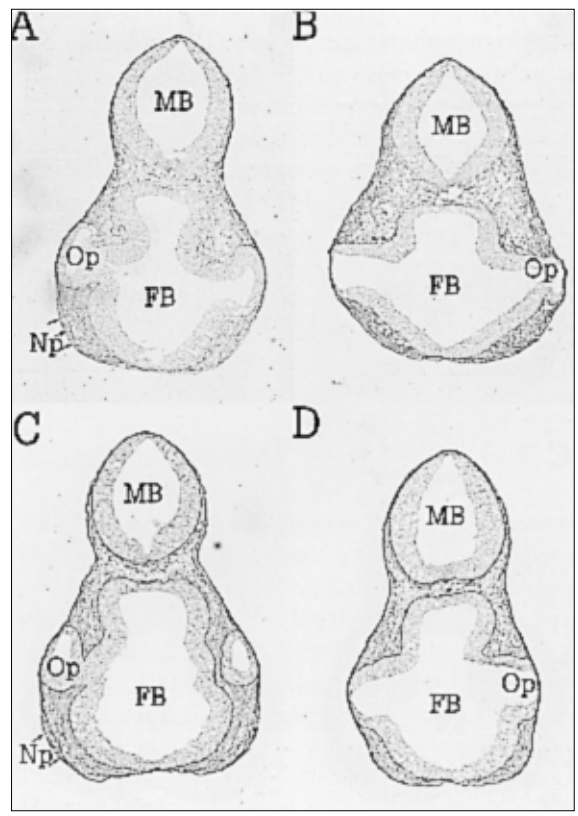

Fig 3. Coronal cryo-sections of heads of day 11 embryos staining with anti-fibronectin (A, B) and anti-laminin (C, D). A and C: 24-somite wild type, B and D: 24-somite and 22-somite *rSey* homozygous embryos, respectively. More intense staining of fibronectin in B was caused by longer peroxidase reaction time. Arrows indicate the epithelial thickening of the nasal placode. Op, optic vesicle; NP, nasal placode; FB, forebrain; MB, midbrain. (A, B, C and D) x160.

Distributions of fibronectin and laminin

In the head region, intense positive staining with anti-fibronectin was generally observed in the mesenchyme of both the wild type and the *rSey* homozygous embryos. The neuroepithelium, as well as the lens and the nasal placodes were negative (Figure 3 A, B). Staining with anti-laminin showed nearly the same pattern to that with anti-fibronectin, but more intense positive staining could be observed at the basement membrane of the epithelium (Figure 3 C, D).

Distribution of tenascin and chondroitin sulfate proteoglycan

Positive staining of tenascin antiserum was observed in the mesenchyme located centrally between the midbrain and forebrain neuroepithelium of both the wild type and the *rSey* homozygous embryos (Figure 4 A, B), but not in the mesenchyme of the frontonasal region. In contrast to tenascin, positive staining of chondroitin sulfate proteoglycan antibody was observed in the mesenchyme located peripherally between the midbrain and forebrain epithelium, in the frontonasal region and in some neuroepithelial cells (Figure 4 C, D). Both the positive staining of tenascin antiserum and chondroitin sulfate proteoglycan antibody were not observed in the lens and nasal placodes. The distribution of these positive stains showed no obvious difference between the *rSey* homozygous and wild type embryos.

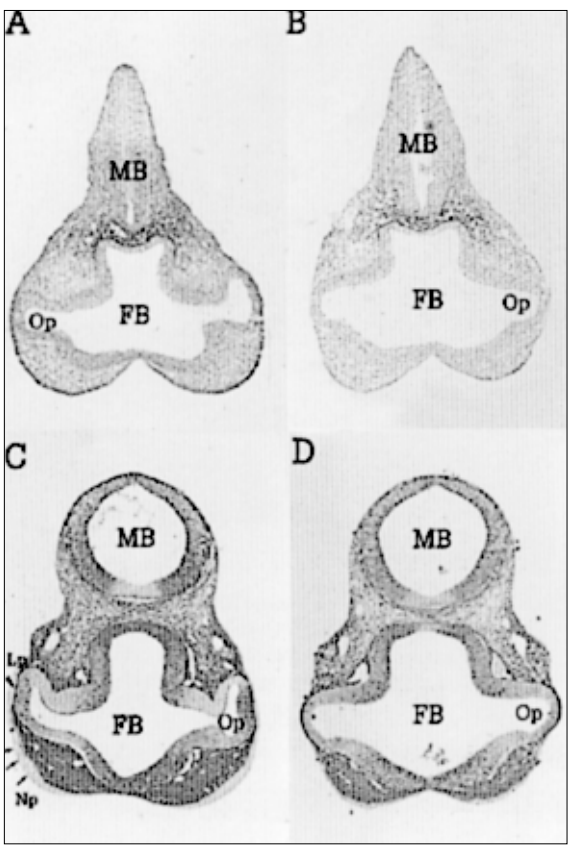

Fig 4. Coronal cryo-sections of heads of day 11 embryos staining with tenascin antiserum (A, B) and paraffin sections staining with anti-chondroitin sulfate proteoglycan (C, D). A and C: 24-somite and 25-somite wild type, B and D: 24-somite *rSey* homozygous embryos. Arrows and arrowheads indicate the epithelial thickening of the nasal and lens placodes, respectively. Op, optic vesicle; NP, nasal placode; FB, forebrain; MB, midbrain. (A, B, C and D) x160.

Discussion

Neural crest cells encounter various tissues and the surrounding extracellular matrix molecules during their migration. It has been suggested that interactions between the cell surface and the extracellular matrix may be permissive for the migration.[20-21] In the *rSey* homozygous embryos, it has been shown that some environmental factors were not permissive for the migration of the particular groups of midbrain crest cells.[2,19] Therefore, the distribution of extracellular matrix molecules of the mutant was investigated to find some clues to understand the mechanisms of the neural crest cell migration. In this study, the distributions of fibronectin, laminin, tenascin, chondroitin sulfate proteoglycan and HNK-1 epitope were investigated and compared between the *rSey* homozygous and the wild type embryos. The results showed that the distribution of the epitope recognized by HNK-1 in homozygous *rSey* was remarkably different from that of the wild type. Interestingly, an abnormal distribution of this epitope has also been observed in the frontonasal epithelium where *Pax-6* is highly expressed during the stage of midbrain crest cell migration.[2,3,8] Therefore, it is reasonable to suggest that the mutation of the *Pax-6* gene might relate to the abnormal expression pattern of the HNK-1 carbohydrate epitope in the homozygotes.

In rat embryos, cranial crest cells start their migration on day 10 of gestation.[15-18] They migrate as streams through the mesenchyme of the head region, avoiding the mesenchymal core, until reaching at their destinations. The stage of embryos in this study was day 11 when the forebrain and midbrain crest cells are propagating on the extracellular matrix and on the surface of cells located near the surface epithelium of the nasal placodes of the frontonasal region. It is obvious that the alteration of the distribution of the HNK-1 epitope in the *rSey* homozygous embryos was observed at the time and the place where the midbrain crest cells

should normally be propagating. In order to clarify whether the epitope is distributed throughout the onset of midbrain crest cells migration, we further investigated its distribution in day-10 wild type embryos from the stage prior to migration at presomite stage to 12-somite stage. It has been found that HNK-1 epitope was generally expressed prior to the onset of migration at the presomite stage and gradually disappeared during migration progression. Moreover, about one-fourth (14 out of 64 samples) of day-10 embryos obtained from intercrossed of 4 heterozygous *rSey* parents showed excessive distribution of the epitope at 3-somite to 8-somite stage. Although the genotype should be further confirmed, we inferred that the embryos were homozygotes. It is possible that the excessive distribution of HNK-1 epitope at early stage of migration (3-somite to 8-somite) prevented *rSey* homozygous midbrain crest cells from migrating towards frontonasal region. The presence of HNK-1 epitope at areas corresponding to nasal placodes also supports the hypothesis that HNK-1 epitope acts as a barrier to midbrain crest cell migration.

Fibronectin and laminin are extracellular matrix molecules that are expressed along the migration pathway as well as distinctively promote migration of neural crest cells *in vitro*.[22-28] Similar to previous studies, the distributions of fibronectin and laminin were observed not only in the migration pathway of the cranial crest cells, but generally in the head mesenchyme.[24, 27] Besides the intense positive staining due to the thickness of the sections, no obvious difference in fibronectin and laminin distributions between the *rSey* homozygotes and wild type embryos could be observed. From previous studies both *in vitro* and *in vivo*, many researchers proposed that the HNK-1 antibody causes abnormalities in cranial crest migration by perturbing interactions between neural crest cells and laminin substrates.[38-40] The results in the present study revealed that the expression of the *Pax-6* mutation during midbrain crest cell migration[2, 3, 8] did not affect the distribution of laminin, which was generally found in the extracellular matrix of various tissues, but particularly affected the epitope recognized by the HNK-1 antibody. It is possible that HNK-1 epitope might particularly relate to the migration of the midbrain crest cells.

Tenascin (cytotactin, GMEM, J1) is an extracellular matrix glycoprotein that has been suggested to play an inductive role on cell mobility in neural crest migration, wound healing and metastasis of mammary tumors.[30-33, 41-43] Most of the previous studies indicated that the distribution of tenascin coincided with the pathways of trunk neural crest cell migration *in vivo*.[29-31] When coated on the culture plates, tenascin substrates inhibited migration and spreading of the cells.[32-33] From the present study, the distribution of tenascin in the head region is limited to the core mesenchyme, which is devoid of cranial crest cells. This finding agrees with the study of Stern et al[44] that J1/tenascin-related molecules seem not to have a permissive effect in head region but rather have an inhibitory effect on the migration of neural crest cells.

Chondroitin sulfate proteoglycan was found to bind to tenascin (cytotactin) therefore it is sometimes called cytotactin- binding proteoglycan (CTB proteoglycan).[45-46] Tenascin and chondroitin sulfate proteoglycan are differentially distributed in the head mesenchyme during neural crest cell migration.[29, 34-37] In the present study, chondroitin sulfate proteoglycan was observed generally in the mesenchyme of the head of day-11 embryos of both wild type and *rSey* homozygous embryos. Therefore, the distributions of these two molecules in head regions seem not to relate to the migration pathway of the cranial crest cells.

Our results demonstrate that the distribution of HNK-1 epitope is remarkably different between the homozygous *rSey* and the wild type embryos, whereas those of fibronectin, laminin, tenascin and chondroitin sulfate proteoglycan are not distinctly different. We still must speculate as to whether, in fact, HNK-1 epitope influences the migration of cranial crest cells, especially the midbrain ones in normal development.

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