日本財団補助金による
1998年度日中医学協力事業報告書

－在留中国人研究者研究助成－

H 11 年 2 月 8 日

財団法人 日中医学協会
理事長 中島 章 殿

研究室で撮影した本人のスナップ写真、及び発表論文のコピーを添付

1．研究者氏名 劉 驍

研究機関 京都大学大学院医学研究科 研究指導者 本田孔士 教授 職名 大学院生

所在地 〒606京都市左京区吉田近衛町 電話 0757534337 内線 4337

研究テーマ マウス網膜発生におけるβ-カテニンの局在変化について

2．本年度の研究業績

(1) 学会・研究会等においての口頭発表 (有) ・ 無 (学会名・内容)

南カリフォルニア大学 Doheny 眼科研究所学術集会（平成１０年１０月９日）
マウス網膜発生におけるβ-カテニンの局在変化について

(2) 学会誌等に発表した論文 (有) ・ 無 (雑誌名・論文名)

Developmental changes in the subcellular localization of R-cadherin in chick retinal pigment epithelium
(鶏胚網膜色素上皮発生におけるR-カドヘリンの局在変化)
Xiao Liu, Akira Mizoguchi, Masatoshi Takeichi, Yoshihito Honda, Chizuka Ide
(劉 驍、溝口 明、竹市 雅俊、本田 孔士、井出 千束)
平成１０年４月１１日発行 Histochemistry and Cell Biology 雑誌 第１０８巻 第１号 ３５頁～４３頁掲載

3．今後の研究計画

軸索と成熟グリア細胞との接着は限られているが、発生における幼若グリア細胞は軸索と高い親和性をもって接着するので、軸索伸長に働く接着分子が関与していると考えられる。我々が視神経及び網膜における再生軸索の接着分子としては R-カドヘリン、およびβ-カテニンなど有効に機能するを明らかにした。今度は接着分子の具体的な応用として、有効と分かった接着分子の遺伝子をウイルスベクターに組み込んで強制的に接着分子を発現させた細胞を作成し、培養系および発生を用いて神経成長円錐とグリア細胞に発現される接着分子を明らかにし、そのアミノ酸配列、DNA 配列を明らかにする。また再生軸索が本来の中枢神経組織内を伸びる可能性を追求し、再生軸索がグリア細胞をはじめとする組織成分といかなる関係を保ちながら伸びるかについて形態学的な基礎を確立し、軸索の伸長を阻害するとされる髄鞘を除去することによって、網膜を含む中枢神経系の neuron の再生促進がどの程度図れるかを調べる。

4．研究指導者の意見

劉君は、院入学後、優秀な成績で大学院の単位順調に取得し、電子顕微鏡、免疫組織化学、細胞培養、遺伝子操作などの研究技術をよく習得し、神経網膜再生および接着分子についての論文が一つ、既に出版されており、更に新しい論文が投稿中である。研究内容は、失明予防を目的とするもので、アジアの人々に貢献するところが大きいものである。難治眼疾患の基礎的な病因を地道に追求して、重要な発見をしかかっている。彼の研究生活は極めて真面目であり、学問に対する態度も極めて精勤で、模範的な留学生活である。彼は健康に恵まれ、人格的にも円満で、よく他の研究者達とも和し、教室員からも尊敬を受けている。チームワークの保持がとてもよい。今まで研究成果は網膜の形態形成におけるＲ－カドヘリンの局在と機能の解明に貢献し、細胞生物学、発生形態学及び眼科学の発展に寄与するところが多い。彼の明晰な頭脳と真摯な生活態度から見て、帰国後は中国眼科を背負う優秀な人材になるものと確信される。

研究指導者氏名 本田孔士 印

5．研究報告

別紙形式を参考に、報告本文4000字以上で報告して下さい（枚数自由・ワープロ使用）

タイトル・要旨等は日本語で、KEY WORDS以下は日本語或いは英語で記入して下さい。

研究成果の発表予定がある場合は発表原稿・抄録集等を添付して下さい。

論文発表に当っては、日中医学協会－日本財団補助金による旨を明記して下さい。

マウス網膜発生におけるβ-カテニンの局在変化について

劉　曉
京都大学大学院医学研究科　大学院生
指導教官：本田　孔士　京都大学大学院医学研究科　教授

論文要旨

【目的】近年、神経系で発現している細胞接着分子が次々と同定されている。その中でカテニンは、カドヘリンの接着機能を細胞質領域に結合して、制御されていると考えられ、これらを介して細胞骨格系と連結している。本研究は、網膜の形態形成におけるβ-カテニンの役割を明らかにするために、マウス網膜発生におけるβ-カテニンの局在を免疫組織化学的及び分子生物学的に調べた。

【方法】マウス（C57/BL6）の12、16日の胎仔と出産後胎仔１、２、５、１０、１６、日及び１が月後のアダルトから網膜を摘出して、3.5%パラホルムアルデヒトで固定し、20μｍのクリオスタット切片を作成して、β-カテニンの免疫染色を行った。一次抗体として抗β-カテニンモノクローナル抗体、また二次抗体としてTRITC (tetramethylrhodamine isothiocyanate)標識抗IgGを用いて免疫蛍光法で染色し、蛍光顕微鏡及び共焦点レーザー顕微鏡で観察を行った。次に同じ時期のものをTrizolでtotal RNAを取って、遺伝子レベルでの発見を見るためにReverse transcription-polymerase chain reaction (RT-PCR)法によって網膜全体でのβ-カテニンの発見を調べた。

【結果】β-カテニンは胎生６日目から網膜神経節細胞に局在していた。出産後1-５日目ピークに達し、１０日目から減少し、１６日目には主に神経節細胞及び神経繊維層、外、内網状層に集積がみられた。アダルトには消失した。定量的RT-PCR法によって、mRNAレベル上以上の局在を確認した。また、Western blotで蛋白質レベル上以上の局在を確認した。

【結論】以上により、β-カテニンは、マウス網膜の形態形成に伴って網膜に限局して発現されることが分かった。β-カテニンが発生初期には網膜神経節細胞から発現され、発生後期主に神経節細胞及び神経繊維層、外、内網状層に限局して発現することは興味深い事実である。β-カテニンは、組織が分化していく際の形態変化と相関しており、発生段階に神経突起の伸長及びシナプス結合の形成と維持に重要な役割を果たしていると考えられる。

Key Words 網膜　β-カテニン　発生　マウス　RT-PCR　免疫蛍光法

Developmental Expression of Beta-catenin in Mouse Retina

Xiao Liu[1,2,3], Vijay P. Sarthy[2], Yoshihito Honda[3], Chizuka Ide[1]

From [1]Department of Anatomy and Neurobiology, Graduate School of Medicine, Kyoto University, Kyoto, Japan; [2]Department of Ophthalmology, Northwestern University Medical School, Chicago, Illinois 60611-3008; [3]Department of Ophthalmology and Visual Science, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Short title: β-catenin in mouse retina

Corresponding author: Xiao Liu M.D., Ph.D.

Department of Anatomy and Neurobiology, Graduate School of Medicine, Kyoto University, Yoshida-konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

Tel: 81-75-753-4337; Fax: 81-75-751-7286.

email: sliu@med.kyoto-u.ac.jp

Section code: BI, RC

Proprietary Interest: None

Key Words: Retina; β-catenin; Development; Mouse; RT-PCR; Immunocytochemistry.

Abbreviations: RGC: retinal ganglion cell; OPL: outer plexiform layer; IPL: inner plexiform layer

Abstract

PURPOSE. To examine the expression of β-catenin in the developing mouse retina.

METHODS. Cryostat sections of retinas from E12, E16, E20, P1, P5, P10, P16 and adult mice were prepared for immunocytochemistry and viewing by light microscopy. Localization of β-catenin was determined by immunocytochemistry. The reverse transcription-polymerase chain reaction (RT-PCR) method was used to detect β-catenin mRNA expression of embryonic eyeball and of postnatal retina stages. Total RNA was isolated and purified.

RESULTS. β-catenin mRNA was detected clearly in embryonic day (E) 12, E16, postnatal day (P) 1 and P5, Thereafter, the expression was markedly decreased, and showed weak expression in adult mouse. Immunoreactivity for β-catenin was demonstrated in ganglion cells of retina at E12, and was strongest from E16 through P5. stages. During these stages, β-catenin was expressed uniformly around the site of the plasma membranes of the retinal cells in contact with each other. Thereafter, the immunoreactivity was still positively distributed throughout the retina cells, and gradually become weaker with development and localized in inner plexiform layer and ganglion cell layer including neurofiber layer in P10. By P16, only weak but clear immunoreactivity was obtained on the outer plexiform layer, inner plexiform layer and ganglion cell. No immunoreactivity was detected in adult mouse retina except in blood vessels. And no expression was found in RPE cells in postnatal stages.

CONCLUSIONS. These findings indicate that β-catenin may play an important role in the morphogenesis of the mouse retina , especially in development of retinal ganglion cells and synapses.

Introduction

In the recent years, there is accumulating evidence that the cell adhesion molecules play a critical role in regulating differetiation and growth of the vertebrate retina. During development of the mouse retina, growth of ganglion cell axon is dependent on the presence of many factors including adhesion molecules such as NCAM, L1, N- and R-cadherin. These molecules are localized temporally and spatially along the developing optic pathway (Matsunaga, et al. 1988; Doherty et al. 1991), and in both inner and outer plexiform layers (Hankin, et al. 1994), and on many retinal cells (Grunwald GB, 1993, Cunningham BA, 1995) including retinal pigment epithelium cells (Liu, et al. 1997).

Catenins were first characterized as linking the cytoplasmic domains of cadherins to the cortical actin cytoskeleton. They are intracellular polypeptides that part of a complex sub-membraneous network which modulates the adhesive ability of cells associated with classical cadherins. (Kemler, 1993). Recent studies proved that the function of cadherin family of cell adhesion molecules is mediated by catenins (Ranscht B, 1994). In addition to their essential role in modulating cadherin adhesivity, catenins have more recently been implicated in cell and developmental signaling pathways controlling morphogenesis (Huber, et al. 1996). β-catenin was first identified as a cytoplasmic ligand required for cadherin-mediated extracellular adhesion molecule (Nagafuchi and Takeichi, 1988; Ozawa, et al. 1990). Its function was dependent on its association with cytoskeleton. Catenins can bind to actin filaments at adherens junction (Haftek, et al. 1996). Their complex pattern of temporal and spatial expression suggest the potential involvement in morphogenetic processes (Miller et al. 1997). Subsequently, β-catenin was also found to be involved in the neurocan-GalNAcPTase connection for extracellular signals controlling cadherin function. (Lilien et al. 1997). Further studies have shown that β-catenin's ability to

regulate body axis determination was associated with its signal transduction function. Recently, β-catenin has also been found to form a complex with APC, a tumor suppressor gene product which suggests a role in regulating cell proliferation (Su et al. 1993) and neuronal apotosis (Ahmed Y et al 1998). The APC-catenin complex in the Wnt signal transduction pathway may affect the transmission of contact inhibition signals and/ or the regulation of cell adhesion (Barth, et al. 1997). β-catenin also participates in modulating cytoskeletal dynamics in association with the microfilament-bundling protein fascin and tublin (Tao, et al. 1996).

Although cadherins and some catenins have been localized in the mammalian developing retina, no studies of β-catenin localization have been reported. In the present study, we have examined the β-catenin expression during development. β-catenin localization was examined by immunocytochemistry and β-catenin mRNA expression level was analyzed by RT-PCR.

Materials and Methods

The all experiments, the procedures used conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research . Time-pregnant C57/BL6 (pigmented) or CD-1 (albino) mice were used. Pups from a single litter were killed at different ages, ranging from birth to the 30th postnatal, and eyes were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2-7.4) overnight at 4℃. Next, eyes were immersed increasing concentrations of sucrose (10, 15, 20 and 25%) in 0.1 M phosphate buffer saline (PBS) at pH7.4, embedded in Tissue Tek OCT compound (Miles, Elkhart, In., USA), and quick-frozen in liquid nitrogen. Eight-micrometer sections transverse were cut on a cryostat, and mounted on gelatinized glass slides. Sections were air-dried, stored at -20℃ and processed for histochemistry. The retina of P10, P16, and adult were dissected from the posterior part of the eyes. For RT-PCR, the whole eyeball of E12, E16 and the retinas of P1, P5, P10, P16, adult were dissected rapidly and flash frozen in liquid nitrogen until use. Mice were anesthetized with metofane before fixation. Five litters were studied by immunocytochemistry.

Immunohistochemistry. A monoclonal antibody against β-catenin was used as the primary antibody for β-catenin (Transduction Laboratories, Lexington, KY., USA). Its specificity has been tested by Western blot analysis and also well documented (Su L, 1993). Tetramethyl-rhodamine-isothiocyanate-conjugated (TRITC) goat anti-rat IgG antibody (Chemicon, Ca., USA) was used as a secondary antibody to detect and visualize antigen-binding.

Sections were washed in PBS for 30 minutes and then incubated for 30 minutes with PBS containing 5% bovine serum albumin (BSA) and 0.1% Triton X-100 to block non-

specific antibody bindings. Sections were incubated with monoclonal antibody against β -catenin at 1:200 for 2 h at room temperature. After washing three times with PBS, sections were incubated for 1 h at 37℃ with TRITC-conjugated anti-mouse IgG. After rinsing with PBS, sections were mounted in DABCO mounting medium. Sections were examined with a epifluorescence microscope (Akioskope, Zeiss, Germany) and photographed using a digital CCD camera (Sony, Japan). Control sections were processed the same way except that the primary antibody was substituted with non-immune serum. Sections incubated without primary antibody were used as an additional controls.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated using Trizol reagent (Life Technologies, Gaithersburg, MD), according to the manufacturer's instructions. With embryos, RNA was isolated from the entire eyeball while at postnatal stages, RNA was isolated from isolated retina.
Reverse transcription-polymerase chain reactions (RT-PCR) were carried out in a programmable thermal cycler (model PTC-100, MJ Research, Watertown, MA). cDNA synthesis was performed with 2 ug total RNA in 80 ul reaction buffer comprised of 5 mM $MgCl_2$ solution, 1 mM deoxyribonucleoside triphosphates (dNTP), 2.5 uM Oligo d(T)16, 20 U RNase inhibitor, and 50U MuLV reverse transcriptase (Perkin-Elmer, Foster City, CA). Sample was reverse transcribed for 35 minutes at 42℃, and denatured for 5 minutes at 99℃ followed by cooling to 4℃. cDNA samples were subjected to PCR using specific primers for β -catenin and for glyceraldehyde-3-phosphate dehydrogense (GAPDH) which served as an internal control. Primers were designed using a software RightPrimer program(BioDisk, San Francisco, CA) and their specificity was confirmed by a GenBank database. Oligonucleotide primers for mouse β -catenin and GAPDH

were synthesized by Integrated DNA Technologies, Inc. (Coralville,IA). Polymerase chain reaction primers used for β-catenin were sense 5'-GAG GCT CTT GTA CGC ACC GTC-3'. anti-sense 5'-GGT TGT GAA CGT CCC GAG CA-3'. These primers correspond to sequences in whole mouse cDNA bank and given an amplified fragment of 427bp from mature β-catenin RNA. As an internal control, cDNA samples also were amplified with primers corresponding to a GAPDH. Sense 5'-GTG AAG GTC GGT GTG TGA ACG G-3'. Anti-sense 5'-ACG TCA GAT CCA CGA CGG ACA C-3'. The predicted sizes of the amplified β-catenin and GAPDH DNA products were 427 and 730 base pairs, respectively.

The PCR (final volume, 30 ul) contained 1 ul of the RT reaction, 3 ul of 10X PCR buffer, 0.5 ul $MgCl_2$ (0.8mM), 1 ul each of the sense primer and antisense primer(50 uM) of GAPDH and β-catenin, and 0.5 ul Taq DNA polymerase (Promega, Madison, WI, 2.5 U/ml)

The PCR program consisted of an initial step at 95℃ for 5 minutes, followed by 35 cycles of denaturation at 95℃ for 1 minutes, annealing at 62℃ for 1 minutes, extension at 72℃ for 1 minutes, and a final step at 72℃ for 10 minutes. Routine RT-PCR controls were done without reverse transcriptase (water control) . The total RNA from mouse testis were used as a positive control (Lin, et al. 1996) PCR products (15 ul) were analyzed by electrophoresis on 1.2% agarose gels containing 5 ug of ethidium bromide per 100 ml. The DNA bands were visualized and photographed under ultraviolet transillumination light.

Semi-Quantitative Analysis

For quantification of RT-PCR data, RT-PCR were performed in triplicate using GAPDH as an endogenous internal standard, photographs of the ethidium bromide-stained gels

were scanned in Bio-Rad Model GS-700 Imaging Densitometer using Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA). The intensity of the pixels were summed in a constant window for each band and the area under each peak was determined by cutting out and weighing the peaks from densitometer tracings. The average of the ratios β-catenin to GAPDH were calculated to compare expression of β-catenin with the internal standard.

Western blot analysis

Western blot analysis was performed using a Cell II transblotting apparatus (Novex, CA) according to the instructions supplied. 25 ul of protein samples were subjected to 10% NuPage gel followed by electrotransfer onto PVDF membranes. Membranes were treated with monoclonal antibody against β-catenin at a dilution of 1:1000. Using a ABC-Kit, antigen band was visualized. Finally, the membrane was scanned into a PhotoShop, and the image was processed.

Results

Immunohistochemical study

Cadherins have been found to be expressed early in developing retina. To campare time course of appearance with that of cadherins, localization of β-catenin was studied by immunofluorescence cytochemistry at the light microscopic level in mice aged ranging from E12 to adult. Two series of animals from each stage were examined. The intensity and distribution of β-catenin staining in both cases were similar. β-catenin immunoreactivity was first detected in ganglion cell layer at E12 (Fig. 1A). Staining gradually increased in intensity, and was distributed between cells in all layers of retina. This pattern was most prominent in E16 eyes (Fig. 1B). After birth, the pattern and intensity of β-catenin labeling remained unchanged to P5 (Fig 2A-B). At this stage, there was strong labeling in ganglion cell layer including neurofiber layer and inner plexiform layers (Fig. 2C, Fig. 3C). Thereafter, β-catenin immunoactivity was decreased and at P16, only strong staining was localized in the ganglion cell layer, and weak but quite clear staining in inner plexiform and outer plexiform layers (Fig. 2D, Fig. 3D). Finally, this labeling pattern disappeared in the 3 month-adult-retina. In the adult retina, only blood vessels and cells in choroid remained weak β-catenin-positive (Fig. 2E). No expression was examined in RPE cells at postnatal stages.

Developmental mRNA expression of β-catenin

β-catenin mRNA levels were determined by RT-PCR in total RNA samples extracted from E12 to adult mice. PCR amplification were a 427-bp band corresponding to β-

catenin mRNA and a 730 bp band for GAPDH as an internal control (Fig. 4A). At all stages tested, there was a decrease in the intesity of catenin band after P16. There were no obvious differences in the intensity of the band in E12 to P1 stages, when ratio of β-catenin to GAPDH was examined and has a decrease in RNA control from P1 to P16 and into adult retina. Furthermore, positive controls from mouse testis tissue were yielded the predicted PCR products. Additionally, samples run without reverse transcriptase generated no PCR products (Fig.4B).

β-catenin localization in Muller cell line

In order to figure characterize β-catenin mAb, we performed two kinds of experiments. In one study, we examined cellular localization of antibody binding sites and in another study we used immunoblotting to characteriz the antigen. As shown in Fig. 5, β-catenin immunoreactivity was predominantly localized to the cell surface. Indeed, the most intense staining was seen at sites that juxtaposition neighboring cells (Fig. 5A). Western blot and Coomassie protein stain showed a apperant band on 92 kDa which was corresponding to β-catenin (Fig 6).

Discussion

The present study has demonstrated that, β-catenin expression was at first localized in retinal ganglion cells beginning around E12, and that the staining was increased steadily to its maximal intensity at the birth. Thereafter, the immunoreactivity was confined to the outer plexiform layer, inner plexiform layer and ganglion cell layer including nervefiber layer at P16. Finally the reactivity disappeared from retina in adulthood. The changable complex pattern of temporal and spatial expression of β-catenin suggest the potenial role of controlling retinal morphogenesis in the development.

The major partners for the cadherins at cell-cell junctions are catenins (Nagafuchi and Takeichi. 1988). In cells without any cadherins, catenins are never concentrated in cell-cell contac sites (Kemler, 1993). Thus, it is most likely that the observed catenin distribution represents that of cadherin/catenin complexes. Molecular mechanisms at the synapses are little known. Previous studies showed the localiztion of N-cadherin was abundant within both inner and outer plexiform layer (Kljavin, et al. 1994). The similar pattern is also observed in rat retina (Doherty, et al. 1991) and chick development (Matsunaga, et al. 1988). Recent studies showed that two classes of cadherin-associated proteins, alpha N- and β-catenin colocalized with synaptophysin were associated with the N-cadherin for its localization in synaptic junctions in mouse and chick brains (Uchida, et al. 1996). Synapses are thought to be dynamic adhesion structures, as implied by their functional plasticity. Our recent observation showed that the peak level of β-catenin expression in the developing retina coincides with the synapse formation in the outer and inner plexiform layer. And β-catenin localized in these synaptic junctions are likely associated with certain cadherin molecules including N-cadherin, indicating cadherin/catenin complex may contribute a critical role in the formation and maintenance

of synaptic junctions throughout development, at least in certain retinal neurons.

Moreover, it has been known that the prevalence of β-catenin colocaliztion with N-cadherin in the outer limiting membrane, i.e., zonula adheres junctions (Romon y Cajal S, 1973) between photoreceptors and Muller cells, suggest the cadherin/catenin complex is also more important for keeping the normal histotypic morphology of the photoreceptor cell layer.

Cadherins/catenins are localized not only in synaptic junctions but also along axonal fibers, especially at embryonic stages suggesting the cadherins/catenins play multiple roles in neuronal connections (Redies et al., 1993). Changes in the distribution of β-catenin are considered to reflect, for the most part, the developmental processes of neuritis outgrowth. It has been known that during retinal development, the first ganglion axons emerge at E13, and by P1 the majority of axons have left the eye (Kuwabara, 1975). Recent studies showed N-cadherin and other adhesion molecules such as L1, N-CAM promoting retinal neurite outgrowth in vitro (Neugebauer KM et al. 1988; Hankin MH et al. 1994; Kijavin IJ et al. 1994). To date, stimulation of neurite outgrowth by N-cadherin or N-CAM-mediated binding triggers changes in intracellular Ca^{2+} by activating Ca^{2+} channels and requires the activity of a tyrosine kinase (Bixby JL et al., 1987; 1990), and recent evidence suggests that the fibroblast growth factor (FGF) receptor family members FGF-2 may be in this pathway mediate intraretinal axon guidance (Williams EJ et al 1995, Brittis PA et al 1996). Since the neurocan/GalNAaPTase pair is one of several ligand/receptor interactions that can trigger neurite outgrowth process. And β-catenin has the potential to play an important role in this process as its tyrosine phosphorylation/dephosphorylation can rapidly switch cadherin between functional and nonfunctional states. The very similar pattern as chick may suggest β-catenin is

associated with axonal processes, and may consistent with the neurocan/GalNAaPTase interactive pair regulation of neurite outgrowth in mouse (Balsamo J et al. 1995; Lilien J et al, 1997). On the other hand, retinal neuron outgrowth was ihibited by dephosphorylation of β-catenin which means cadherins function was mediated by catenins (Balsamo J et al. 1996). And in vivo studies showed N- and R-cadherin plays guidance function of optic nerve fibers in early development (Matsunaga M et al. 1988; Redies C and Takeichi M, 1993). The latest data suggested cadherin/catenin complex regulating the retinal neurite outgrowth in vivo (Wohrn JC et al. 1998). Although the mechanism of β-catenin related to these molecules is unknown, the current study indicates a temporal course similar to these molecules that may reflect a significant role for this adhesion molecule in retinal development and synapgenesis. Our results indicate that β-catenin initially has an important role in axon outgrowth of retinal ganglion cells throught the development.

In the present study, the progressive expression of β-catenin also was seen at the RNA level. suggesting that β-catenin is regulated primarily at the transcriptional level. Because of the retina isolation technique is difficult, the band seen after PCR amplification only represent the whole eyeball. But in postnatal stages, all the samples were from isolated retina. The decrease in β-catenin in the adult may reflect the function role is temperary and unique.

Acknowledgments

The author thanks Drs. M. Takeichi, A. Mizoguchi, N. Uchida, C. Podlasek for helpful discussion, T. Bervig, J. Dudley, X. He for excellent technical assistance, Y. Wang for providing the eye tissue. This research was supported by the Grants-in Aid from the Japanese Ministry of Education, Science and Culture (C.I.&Y.H), by NIH grant EY-1819 (V.S), and by grants from the Japan China Medical Association-Nippon Foundation and Murota International Foundation (X.L.).

References

Ahmed Y, Hayashi S, Levine A, Wieschaus E. Regulation of armadillo by a Drosophila APC inhibits neuronal apotosis during retinal development. Cell 1998;93:1171-1182.

Balsamo J, Ernst H, Zanin MK, Hoffman S, Lilien J. The interaction of the retina cell surface N-acetylgalactosaminylphosphotransferase with an endogenous proteoglycan ligand results in inhibition of cadherin-mediated adhesion. J. Cell Biol. 1995;129:1391-1401.

Balsamo J, Leung T, Ernst H, Zanin MK, Hoffman S, Lilien J. Regulated binding of PTP1B-like phosphatase to N-cadherin: control of cadherin-mediated adhesion by dephosphorylation of beta-catenin. J. Cell Biol. 1996;134:801-813.

Barth AI, Nathke IS, Nelson WJ. Cadherins, catenins and APC protein : interplay between cytoskeletal complex and signaling pathway. Curr. Opin. Cell Biol. 1997;9:683-690.

Bixby JL, Pratt RS, Lilien J, Reichardt. Neurite outgrowth on muscle cell surface involves extracellular receptors as well as Ca2+-dependent and independent cell adhesion molecules. Proc. Natl. Acad. Sci. USA 1987;84:2555-2559.

Bixby JL, Zhang R. Purified N-cadherin is a potent substrate for the rapid induction of neurite outgrowth. J. Cell Biol. 1990;110:1253-1260.

Brittis PA, Silver J, Walsh FS, Doherty P. Fibroblast growth facyor receptor function is required for the orderly projection of ganglion cell axons in the developing mammalian retina. Mol. Cell Neurosci. 1996;8:120-128.

Butz S, Larue L. Expression of catenins during mouse embryonic development and in adult tissues. Cell Adhesion Communication 1995;3:337-352.

Cunningham BA. Cell adhesion molecules as morphoregulators. Curr. Opin. Cell Biol. 1995;7:628-633.

Doherty P, Rowett LH, Moore SH, Mann DA, Walsh FS. Neurite outgrowth in response

to transfected N-CAM and N-cadherin reveals fundamental differences in neuronal responsiveness to CAMs. Neuron 1991;6:247-258.

Grunwald GB. The structural and functional analysis of cadherin calcium-dependent cell adhesion molecules. Curr. Opin. Cell Biol. 1993;5:797-805.

Gumbiner BM. Signal transduction by beta-catenin. Curr. Opin. Cell Biol. 1995;7:634-640.

Haegel H, Larue L, Ohsugi M, Fedorov L, Herrenknecht K, Kemler R. Lack of beta-catenin affects mouse development at gastrulation. Development 1995;121:3529-3537.

Haftek M, Hansen MU, Kaiser HW, Kreysel HW, Schmitt D. Interkeratinocyte adherens junctions: immunocytochemical visualization of cell-cell junctional structures, distinct from desmosomes, in human epidermis. J. Invest. Dermatol. 1996;106:498-504.

Hankin MH, Lagenaur CF. Cell adhesion molecules in the early developing mouse retina: retinal neurons show preferential outgrowth in vitro on L1 but not N-CAM. J. Neurobiol. 1994;25:472-487.

Huber O. Bierkamp C, Kemler R. Cadherins and catenins in development. Curr. Opin. Cell Biol. 1996;8:685-691.

Hoschuetzky H, Aberle H, Kemler R. Beta-catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. J. Cell Biol. 1994;127:1375-1380.

Kemler R From cadherins to catenins: Cytoplasmic protein interactions and regulation of cell adhesion. Trends Genet. 1993;9:317-321.

Kijavin IJ, Lagenaur C, Bixby JL, Reh TA. Cell adhesion molecule regulating neurite growth from amacrine and rod photoreceptor cells. J. Neurosci. 1994;14:5035-5049.

Kuwabata T. Development of the optic nerve of the rat. Invest. Ophthalmol. 1975;14:732-745.

Lilien J., Hoffman S, Eisenberg C, Balsamo J. Beta-catenin is a target for extracellular signals controlling cadherin function: the neurocan-GalNAcPTase connection. In: Pedersen RA, Schatten G, eds. Current Topics in Developmental Biology. San Diego: Academic Press; 1997;35:161-189.

Lin LH, DePhilip RM. Differential expression of placental(P)-cadherin in sertoli cells and peritubular myoid cells during postnatal development of the mouse testis. 1996;224:155-164.

Liu X, Mizoguchi A, Takeichi M, Honda Y, Ide C. Developmental changes in the subcellular localization of R-cadherin in chick retinal pigment epithelium. Histochem. Cell Biol. 1997;108:35-43.

Matsunaga M, Hatta K, Nagafuchi A, Takeichi M. Guidance of optic nerve fibres by N-cadherin adhesion molecules. Nature 1988;334:62-64.

Miller JR, McClay DR. Changes in the pattern of adherens junction-associated beta-catenin accompany morphogenesis in the sea urchin embryo. Development Biol. 1997;192:310-22.

Nagafuchi A, Takeichi M. Cell binding function of E-cadherin is regulated by the cytoplasmic domain. EMBO. J. 1988;7:3679-3684.

Neugebauer KM, Tomaselli KJ, Lilien J, Reichardt LF. N-cadherin, NCAM, and interrins promote retinal neurite outgrowth on astrocytes in vitro. J. Cell Biol. 1988;107:1177-1187.

Oda H, Uemura T, Shiomi K, Nagafuchi A, Tsukita S, Takeichi M. Identification of a Drosophila homologue of alpha-catenin and its association with the armadillo protein. J. Cell Biol. 1993;121:1133-1140.

Ozawa M, Ringwald M, Kemler R. Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. Proc. Natl. Acad. Sci. USA 1990;87: 4246-4250.

Ramon y Cajal S. The vertebrate retina. Rodieck RW, ed; Maguire D, Rodieck RW, trans.

San Francisco: Freeman; 1973:781-904.

Ranscht B. Cadherins and catenins: interactions and functions in embryonic development. Curr. Opin. Cell Biol. 1994;6:740-746.

Redies C, Inuzuka H, Takeichi M. Restricted expression of N- and R-cadherin on neurites of the developing chicken CNS. J. Neurosci. 1992;12:3525-3534.

Redies C, Takeichi M. N-and R- cadherin expression in the optic nerve of the chicken embryo. Glia 1993;8:161-171.

Riehl R, Johnson K, Bradley R, Grunwald GB, Cornel E, Lilienbaum A, Holt CE. Cadherin function is required for axon outgrowth in retinal ganglion cells in vivo. Neuron. 1996;17:837-848.

Su L, Vogelstein B, Kinzler. Association of the APC tumor supressor protein with catenins. Science 1993;262:1734-1737.

Tao YS, Edwards RA, Tubb B, Wang S, Bryan J, McCrea PD. Beta-catenin associates with the actin-bundling protein fascin in a noncadherin complex. J. Cell Biol. 1996;134:1271-1281.

Tomaselli KJ, Neugebauer KM, Bixby JL, Lilien J, Reichardt LF. N-cadherin and Integrins: two receptor systems that mediate neuronal process outgrowth on astrocytes surfaces. Neuron 1988;1:33-43.

Uchida N. Honjo Y. Johnson KR. Wheelock MJ. Takeichi M. The catenin/cadherin adhesion system is localized in synaptic junctions bordering transmitter release zones. J. Cell Biol. 1996;135:767-779.

Williams EJ, Furness I, Walsh FS, Doherty P. Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. Neuron 1995;13:583-594.

Wohrn JC, Puelles L, Nakagawa S, Takeichi M, Redies C. Cadherin expression in the retina and retinofugal pathways of the chicken embryo. J. Comp. Neurol. 1998;396:20-38.

Figure Legends

FIGURE 1. Reverse transcription-polymerase chain reaction (RT-PCR) products obtained from developing mouse retina. using primers derived from Amplification of beta-catenin cDNA . RNA was extracted from retina of different stage show and converted into cDNA. Samples were amplified using specific beta-catenin primers and GAPDH primers as an internal control. Lane M : 1 kb ladder Maker, Lane 1-2 : total RNA from mouse eyeball in embryonic stages, Lane 3-6 : total RNA from mouse neuro-retina in postnatal stages. Lane 7 : A positive control reaction of amplification of-adult mouse testis using the same primers, Lane 8 : a negative control reaction of P1 retina cDNA without RT. The predicted size of beta-catenin (427 bp) and GAPDH (730 bp) was obtained in lane 1-7, Note

FIGURE 2. Quantitative analysis of RT-PCR products showed in Figure 1. The ratio of the beta-catenin to GAPDH product was plotted according to the intensity of the amplified bands. The results show a progressive decrease in expression between P1 and adulthood, and may reflect a unique time course of role of beta-catenin in retinal cell morphogenesis.

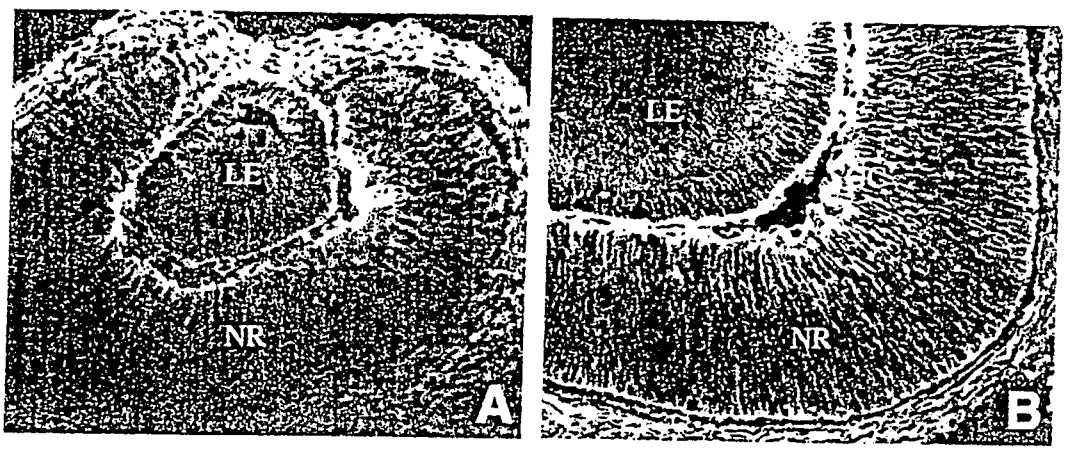
FIGURE 3. Immunocytochemical localization of beta-catenin in the embryonic mouse eye. (A) E12 eye; (B) E16 eye. (NR) neuro-retina (GC) ganglion cell layer (LE) lens. Note the beta-catenin immunoactivity localized in ganglion cells (A) in E12 and widespread the lateral circumference of all retina cells (B) in E16.

FIGURE 4. Changes in immunocytochemical localization of beta-catenin in the postnatal mouse retina. (A) P1 retina; (B) P5 retina; (C) P10 retina; (D) P16 retina;. (E) adult retina; (GCL) ganglion cell layer (IPL) inner plexiform layer; (OPL) outer plexiform layer; (RPE) retinal pigment epithelium.

FIGURE 5. Magnification microscopy of beta-catenin immunostaining in mouse retina. (A) P1 retina; (B) P5 retina; (C) P10 retina; (D) P16 retina; (GCL) ganglion cell layer (IPL) inner plexiform layer; (OPL) outer plexiform layer; Note the prominent staining can been seen of the ganglion cell layer and ganglion cell processes in two embryonic stages and localized in inner plexiform layer and outer plexiform layer in postnatal stages.

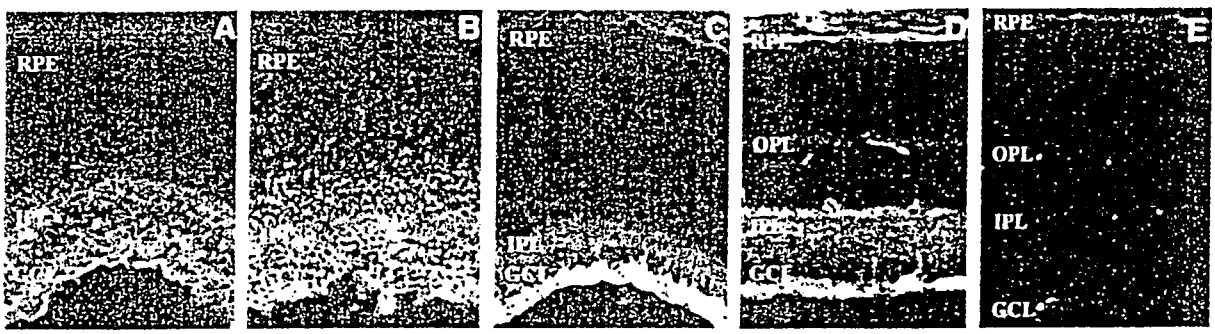
FIGURE 6. Immunofluorescence microscopy of Muller cells labeled with antibodies to beta-catenin(A) Note the strong label between the cell-cell contact site. (B) Control. Bar

FIGURE 7. Western blot analysis of beta-catenin in Muller cell line . Arrow show the recognized band at 92 kDa corresponding to beta-catenin. Lane A: Marker, Lane B: Muller cell line.



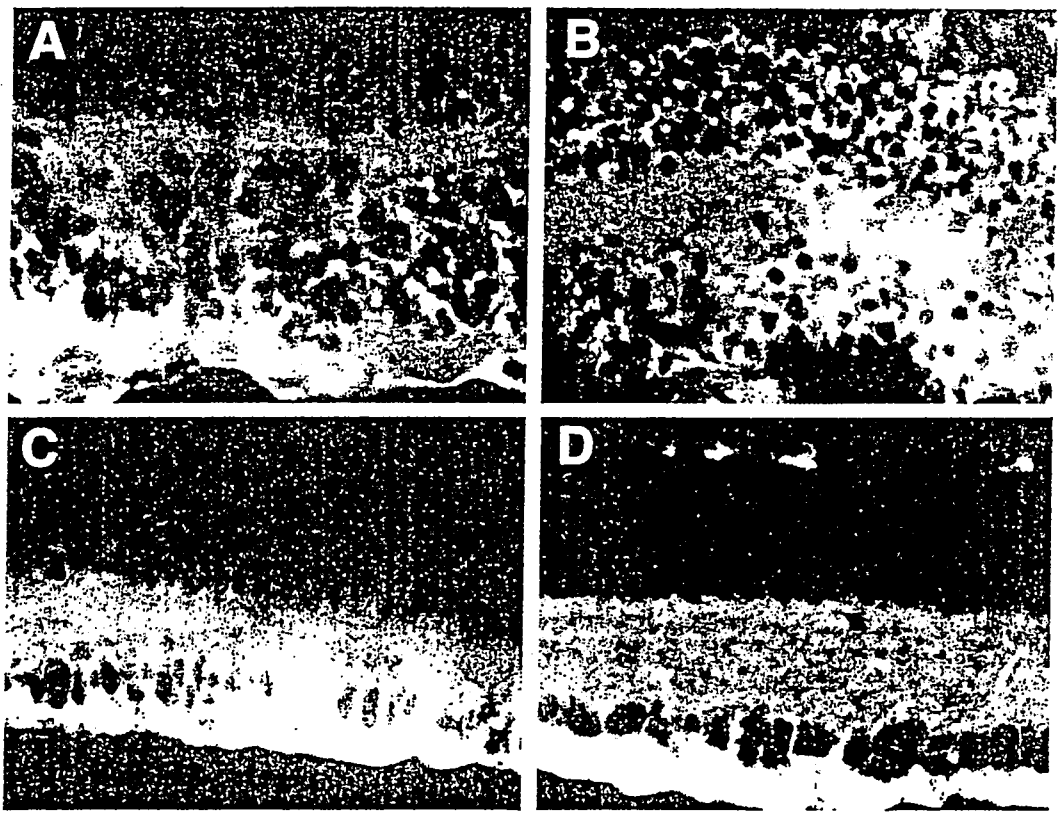
Liu et al.

Fig. 1



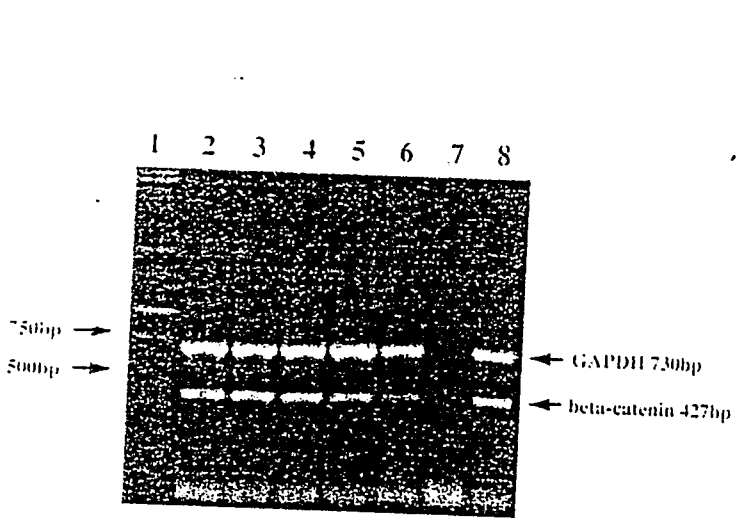
Liu et al.

Fig.2

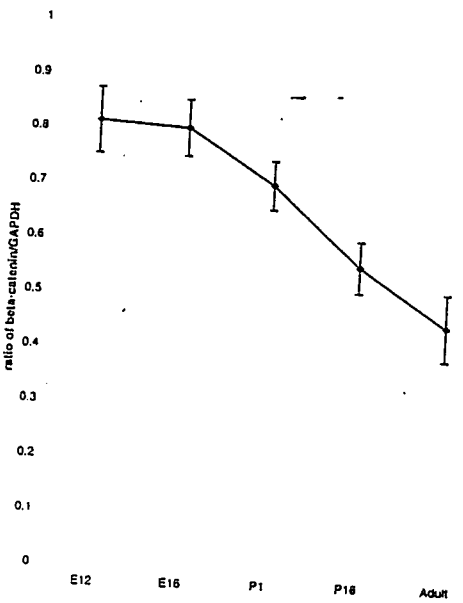


Liu et al.

Fig.3



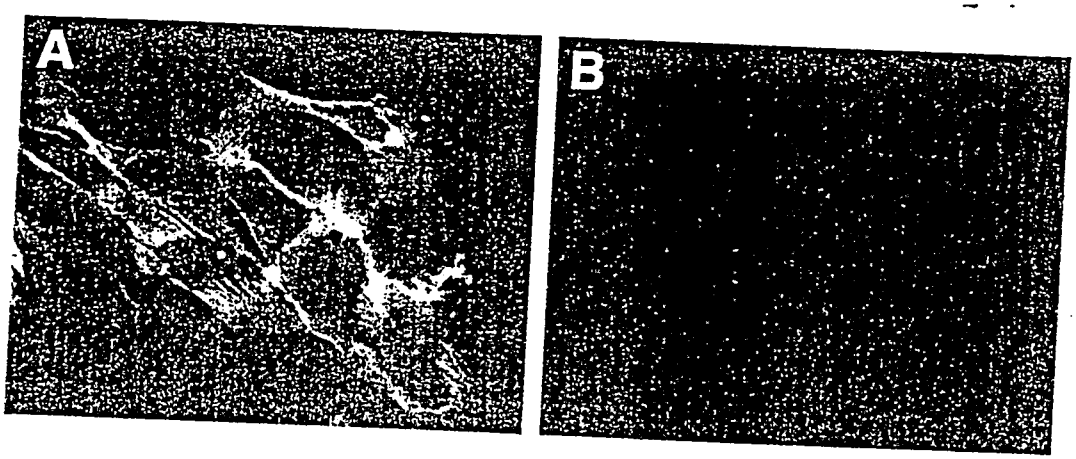
A



B

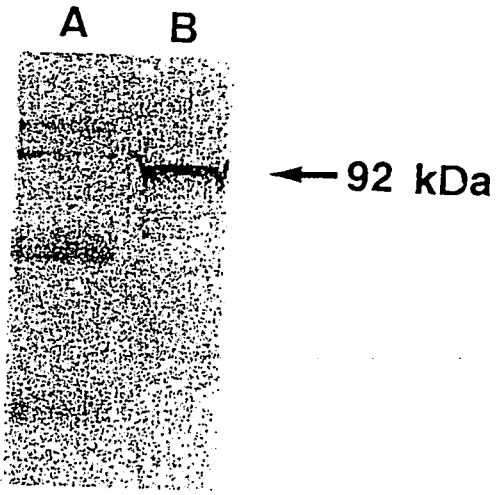
Liu et al.

Fig.4



Liu et al.

Fig.5



Liu et al.

Fig.6