行政院國家科學委員會專題研究計畫　成果報告

雞之視覺剝奪性近視的基因表現及蛋白體研究(3/3)

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Upregulation of a Metastasis Associated 37 LRP/p40 Gene in the Sclera of Chick with Form Deprivation Myopia

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Significance of myopia

- The prevention and treatment of myopia are important global public health issue.
- Especially in Asian countries where the prevalence rate of myopia is extremely high.
- The most important complication of myopia is retinal degeneration affecting the posterior pole that is associated with elongation of ocular axial length.
- Unfortunately, the actual mechanism of the development of myopia is still unknown.

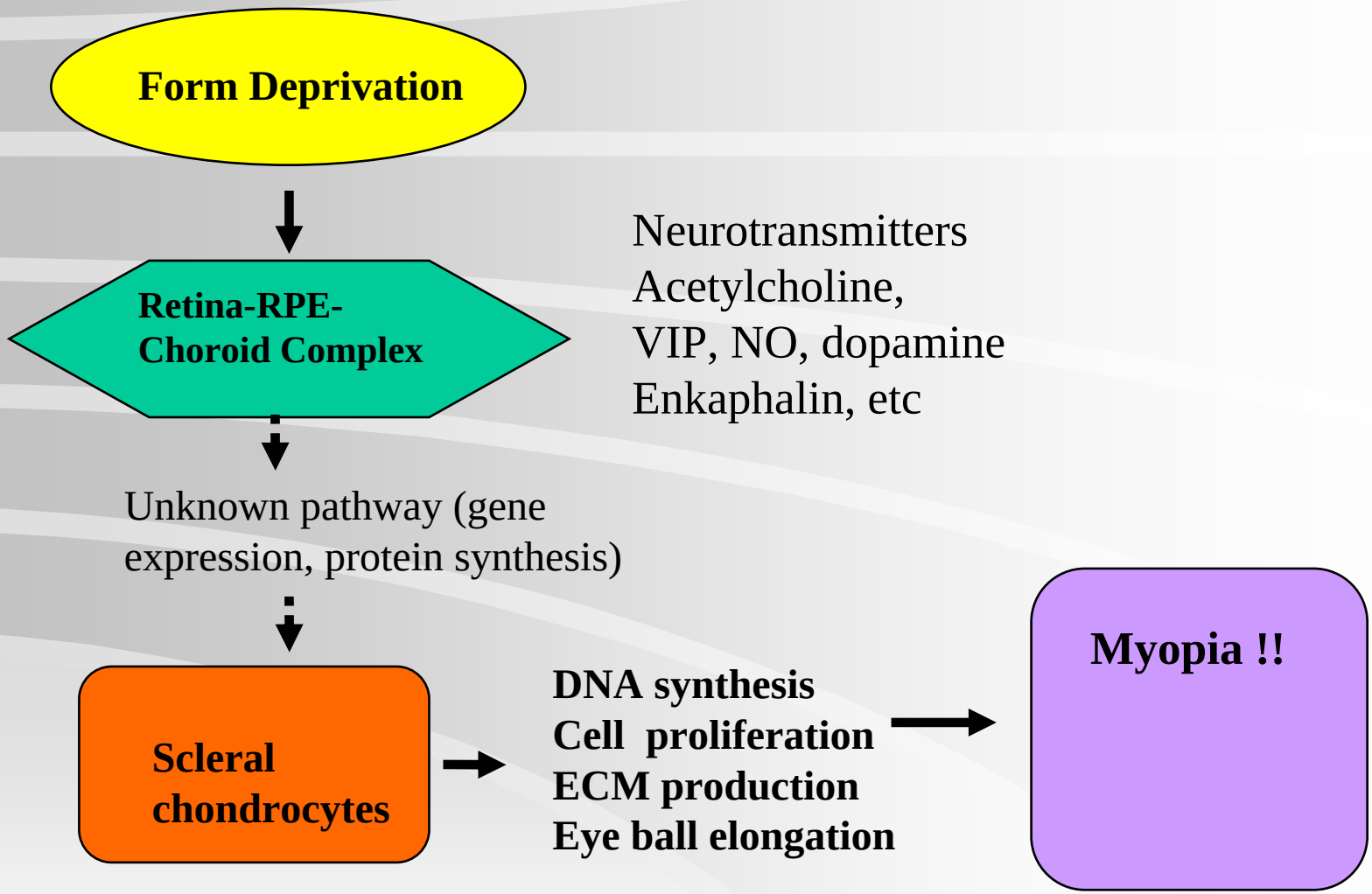
Animal models of myopia

- Depriving the retina from vision
- Lid suture, translucent goggles
- Chicks, cats, tree shrews and monkeys
- Excessive lengthening of the ocular globe associated with remodeling of extracellular matrix in the posterior sclera.
- Mechanism is unknown.

Form deprivation myopia in chicks

- Widely accepted model to study visually-regulated postnatal ocular growth.
- Morphological and biochemical studies
- Proliferation of chondrocytes, synthesis of extracellular matrix
- Increased proteoglycan synthesis
- Ocular enlargement in the chick is due to active growth of the sclera instead of passive stretch

Possible mechanism of form deprivation myopia in chick



Gene study in myopia

Isolation and identification of genes expressed in ocular tissue of myopic eyes may aid in the molecular evaluation and understanding of possible mechanisms involved in form deprivation myopia.

Global expression profiles

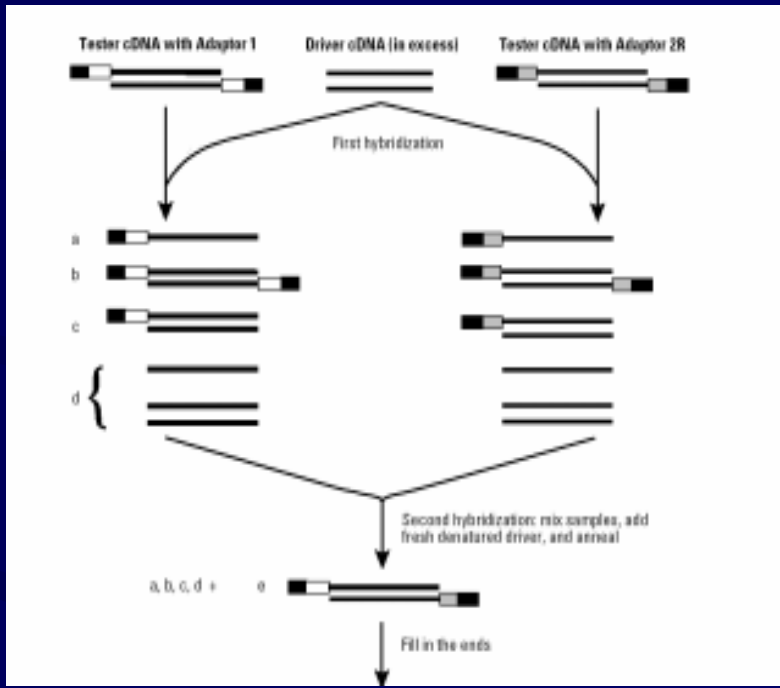
- Transcriptome
  - cDNA microarray
  - Differential display
  - Subtractive hybridization
  - Serial analysis of gene expression (SAGE)
- Proteome
  - Two dimensional gel electrophoresis
  - Mass spectrometry
  - Protein array

Suppression subtractive hybridization

- Developed by Diatchenko L, et al. *(PNAS, 1996,93:6025-6030)*
- Suppression subtraction-hybridization PCR method is used to selectively amplify target cDNA fragments and simultaneously suppress nontarget cDNA amplification.
- It can achieve greater than 1000-fold enrichment of differentially expressed cDNAs (ie. cDNA from myopic and control eyes).

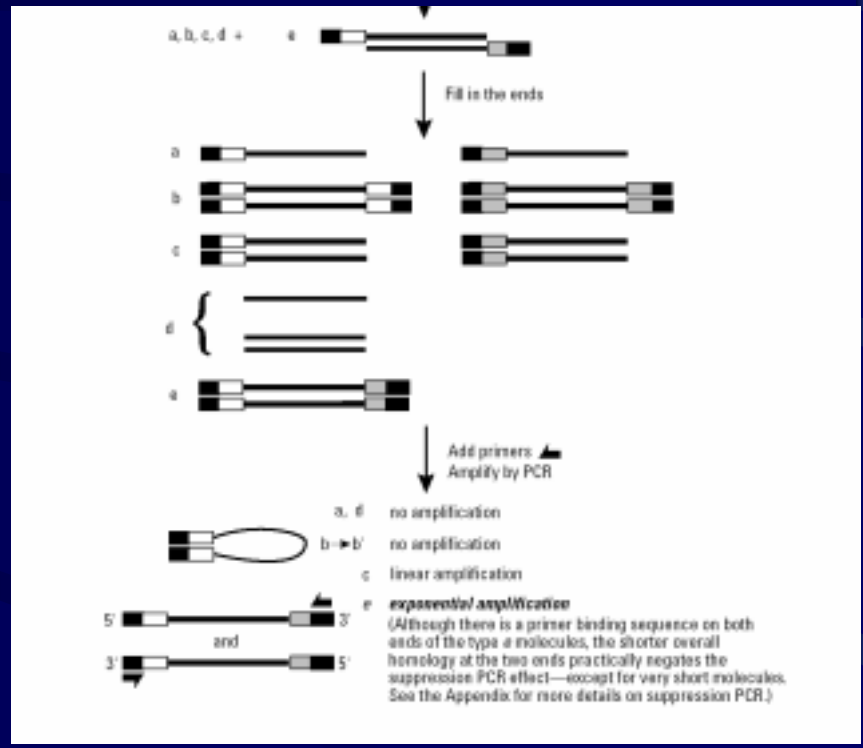
Principle of SSH

The basic idea of SSH PCR is those testers cDNA (in our experiment, the cDNA from myopic eyes) will primarily reassociate with excess driver cDNA (in our experiment, the cDNA from control eyes) while target sequences having no counterparts in driver will inevitably reassociate with each other, or remain single-stranded.



Principle of subtractive hybridization

The re-associated fragments common for driver and tester are discarded, and the remaining cDNA enriched in target sequences is cloned and analyzed.



Subtractive hybridization in eye research

- Upreglation of crystalline mRNAs in form-deprived chick eyes
  - *Ishibashi K, et al., Exp Eye Res 2000 70:153-158*
- A novel gene (retinovin) expressed selectively in the earlt stage of chick retinal development.
  - *Itami M et al., BBRC 2000 276:12-15*
- Identification of novel bovine RPE and retinal genes by subtractive hybridization
  - *Sharma S, et al., Mol Vis 2002 8:251-8*
- Identification of ciliary epithelial-specific genes using subtractive libraries and cDNA arrays in the avian eye
  - *Kubota R et al., Dev Dynamic 2004 229:529-40*

Purpose

- It is proven that subtraction-hybridization PCR is a powerful tool to study gene expression.
- In this experiment, suppression subtractive hybridization technique was used to evaluated the differential gene expression in the chick sclera of myopic and control eyes.

1. Induction of form deprivation myopia

- One day old male white Leghorn chicks.
- The right eye was covered with a semi-spherical translucent plastic goggle, and the left eye was uncovered for control.
- The chicks were maintained on 12 hours light-dark cycle for 2-14 days during the study, then they were sacrificed and both eyes enucleated.

2. Sample preparation

- Using a surgical trephine, tissue buttons with diameter of 8.5 mm was excised from part of each posterior hemisphere located temporal to the exit site of the optic nerve.
- Specimen of sclera was separated from these tissue samples.
- Specimen from 5 eyes were pooled together for further experiments.

3. RNA extraction

The samples were frozen by liquid nitrogen and ground into powder. RNA was extracted and stored frozen at -80°C.

4. Reverse transcription

Double-stranded cDNA was synthesized from 1μg of poly(A)+ RNA of the control eyes using the Great Length cDNA synthesis kit .

5. Subtraction hybridization PCR method

Subtraction-hybridization was performed using the PCR-Selected cDNA Subtraction Kit (Clontech) and procedures were carried out according to the manufacturers protocol.

6. Cloning and Sequencing of Products

The hybridized cDNA fragments was amplified by primary and nested PCR using the Advantage cDNA PCR Kit (Clontecch). The PCR products were cloned into pGEM-T vector (Promega) using T4 DNA ligase. Sequencing was performed with ABI PRISM dye terminator cycle sequencing core kit (Perkin Elmer). The resulting sequences were compared with the Genebank, EMBL and DDBJ DNA databases.

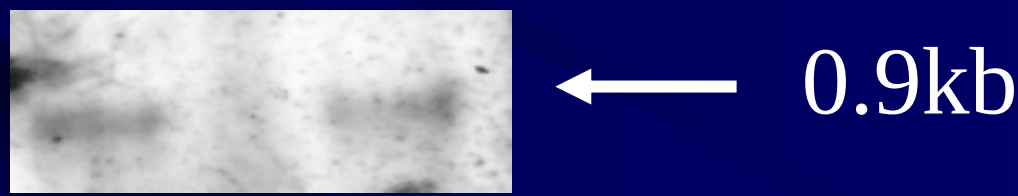
Results

1. Isolation of up-regulated gene in form deprivation myopic eyes

One out of 200 clones was isolated as a cDNA fragment of an up-regulated gene in the myopic eye samples. The cDNA was sequenced and subjected to homology search with the Genebank database. The size of insert was 306 bp and the sequence was 100% homologous to a fragment of the chick 37LRP/p40 cDNA sequence (X94369).

2. Northern hybridization

Northern hybridization using the cDNA as probe revealed a single band with size of 0.9 kb



Semi-quantitative RT-PCR

Primer sequences and amplification condition were as follows:

37 LRP/p40:

Sense: ATT TCT TCC AGG AAT ACC GGA

Antisense: TCC GGT ATT CCT GGA AGA AAT

Product size: 705bp

Expression level of GAPDH, a housekeeping gene, was examined separately and used as control for each DNA sample concentration.

GAPDH:

Sense:ACC ACT GTC CAT GCC ATC AC

Antisense:TCC ACA ACA CGG TTG CTG TA

Product size: 533 bp

PCR products were separated on a 1.5% agarose gel and photographed.

3. Results of PCR

7 day NS	7 day MS	14 day NS	14 day MS

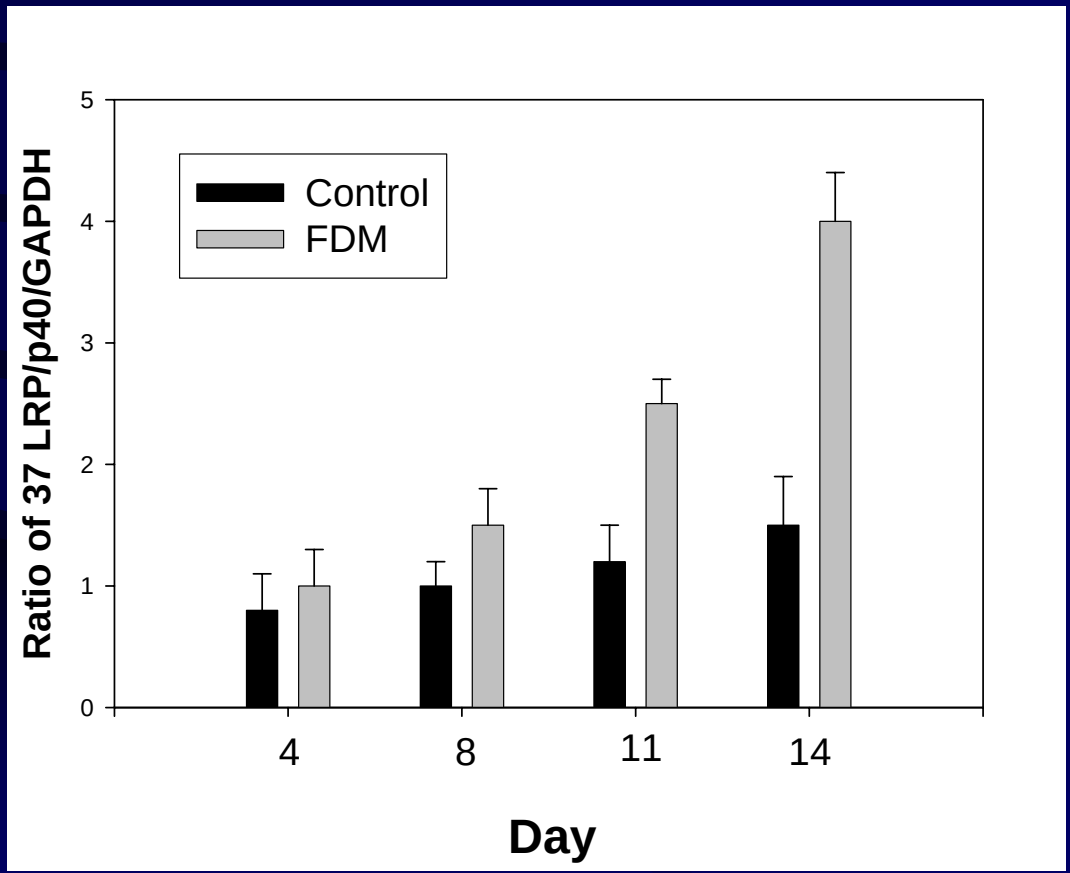
NS: normal sclera

MS: myopia sclera

GAPDH

37 LRP/p40

4. Increased expression of 37 LPR/p40 gene in FDM revealed by semi-quantitative PCR



37LRP/p40 gene

- **Identification of the active gene coding for the metastasis-associated 37LRP/p40 multifunctional protein.**
  - ***Clausse N, et al. DNA Cell Biol 1996, 15:1009-1023.***
- **Isolation from a multigene family of the active human gene of the metastasis-associated multifunctional protein 37LRP/p40 at chromosome 3p21.3.**
  - ***Jackers P, et al. Oncogene 1996, 13:495-503***

Functional of 37LRP/p40 gene

- **Precursor of the membrane-associated 67-kD laminin receptor**
- **Cytoplasmic ribosomal-associated protein (p40)**
- **Multifunction protein**
  - **Extracellularly: interaction with ECM**
  - **Intracellularly : cell translation machinery**
- **Similar to collagen binding protein HSP 47**
  - ***Natsume T et al. J Biol Chem 1994 269:31224-8***

Functional of 37LRP/p40 gene

- **Consistently up-regulated in cancer cells in correlation with their invasive and metastatic phenotype.**
- **Up-modulation in tumor cells:**
  - **metastasis potential :laminin binding activity**
  - **New growth needs: Increased protein synthesis**

Modulation of 37LRP/p40 gene

- **TNF-α and IFN-γ down regulate the expression 37 LRP/p40 gene and protein in transformed keratinocyte**
  - ***Clausse N et al. BBRC 1998 251:564-9***

Role of 37LRP/p40 gene in FDM

- **Although ribosomal proteins are considered housekeeping gene, their expression are subject to precise control mechanism and closely related to cell proliferation**
- **Active growth of sclera in FDM**
- **37 LRP/p40 may play an active role in the cellular proliferation and ECM synthesis of chondrocytes in chick sclera of FDM**
- **Modulated by growth factor: TGF-β, bFGF ?**

Further research of 37LRP/p40 gene function in FDM

- **Effect of visual recovery on the expression of 37 LRP/p40 gene**
- **Modulated by growth factor: TGF-β, bFGF, and others ?**
  - **Promoter evaluation**
  - ***In vivo* study**
  - **Tissue culture system**

Conclusion

- Subtraction hybridization technique is a valuable tool to evaluate the differential gene expression in the chick sclera of myopic and control eyes.
- Active role of 37 LRP/p40 gene in the proliferation and ECM synthesis of chondrocytes in chick sclera of FDM
- The regulatory mechanism of 37LRP/p40 in myopic eyes needs further investigation.

Thank you for your attention !