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Research Article

DNA SEQUENCING OF REGENERATED LENS UNDER THE INFLUENCE OF VITAMIN - A IN YOUNG SWISS ALBINO MICE

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ABSTRACT
The preset study supports previous finding that Vitamin A can induce and accelerates lens regeneration in pigmented epithelial cells (PECs) of dorsal iris in Swiss albino mice. In Lens regeneration, several workers have shown that Vitamin A possess the mitogenic activity which causes functional impairment of retinoid receptors and thereby inhibits the lens regeneration. The purpose of present study to understanding the DNA base pair difference between normal lens and regenerated lens DNA. The work was mainly based on histological and molecular aspects of lens regeneration. The study concludes that the base pairs of regenerated lens DNA and normal lens DNA were almost similar except the SNPs. There may be some mutation or aberration of DNA base pair alignment present in regenerated DNA base pair compare to normal DNA base pair.

***Keywords**: DNA Sequencing, Regenerated Lens, Swiss Albino Mice*

INTRODUCTION
Lens regeneration provides a clear example of trans-differentiation of one differentiated cellular type having a distinctive pattern of metabolic activities to another cellular type, which is morphologically and biochemically distinct from the original. An abundant literature exists on lens regeneration in amphibians (Reyer, 1971; Reyer, 1990; Stone, 1959; Yamada, 1967; Jangir *et al.,* 1995). Lens regeneration from non ocular tissue (dorsal iris) has been well documented in amphibians (Reyer, 1954; Reyer, 1977; Eguchi and Itoh, 1982; Eguchi, 1988). Regeneration is a developmental process which occurs during post embryonic period. It is the ability of fully developed organism to replace lost part by growth or remodeling of somatic tissues. Regeneration involves all those fundamental processes including cell proliferation, cell movement, morphogenesis, histogenesis and growth which occur during ontogenetic development in embryonic and larval stages. But lens regeneration differs from general regenerative process rather it provides a clear example of "metaplasia" During lens regeneration there is a transformation of one differentiated cellular type, having a distinctive pattern of metabolic activities to another cellular type, which is morphologically different from original and which synthesized a different array of macromolecules. The process was to as "metaplasia" Colucci (1891) had first described lens regeneration from the dorsal iris termed wolffian regeneration. Lens regeneration is considered as example of transdifferentiation. Transdifferentiation is a process by which differentiated cells alter their identity to become other distinct cell type. When the lens of a newt is removed, the process of regeneration is initiated from the dorsal iris. The pigment epithelial cells (PECs) from the dorsal iris proliferate, dedifferentiate, and then transdifferentiate into lens cells. PECs initiate DNA synthesis and eventually lose their characteristics of origin, such as pigmentation. At about 7–10 days post-lentectomy a small vesicle is formed at the tip of the dorsal iris. Cells in this vesicle then transdifferentiate into lens cells and form the lens vesicle (10–15 days). Cells from the posterior part of the lens vesicle differentiate to form the lens fibers (15–20 days). Lens regeneration is complete by 25 days post-lentectomy1. P.A. Tsonis. Regeneration in vertebrates (2000). In current study we have found the difference in DNA base pair sequence of normal lens and regenerated lens under the influence of Vitamin A. We used Specific Gene RXR alpha as a primer in PCR and DNA Sequencing technique for provide information regarding base pair similarities and difference. It's a computational method of bioinformatics.
DNA isolated from regenerated and normal lens of swiss albino mice. The first isolation of DNA was done in 1869 by Friedrich Miescher. This isolated DNA was used for DNA sequencing here we have used

Sanger's chain-termination method for determine the order of the four bases adenine, guanine, cytosine, and thymine in strand of DNA. BLAST (Basic Local Alignment Search Tool) algorithm and program were designed by Stephen Altschul, Warren Gish, Webb Miller, Eugene Myers, and David J. Lipman at the NIH and was published in the Journal of Molecular Biologyin 1990. With the help of BLAST we can find out the similarity and dissimilarity between query sequence and data base sequence. Electropherogram provide easily understandable graphically representation of both the DNA sequence and give information regarding similarities and differentiation occur in the base pair alignment of both DNA sequence. With this DNA sequence we were finding the effect of retinoic acid (A derivatives of Vitamin A) on regeneration of lens in Swiss albino mice. When any two human genomes are compared side by side, they are 99.9% identical (Cooper *et al.*, 1985). DNA base pair alignment having dissimilarities and similarities will be compared by SNP technique.

MATERIALS AND METHODS

Lens Regeneration provides a good model for the study of trans-differentiation ability of Somatic Cells. For this purpose young Swiss Albino Mice were employed as experimental animals. Nutrition and healthy environment were provided to swiss albino mice for healthy growth. The present work was designed into two parts:-

Figure 1: Photograph showing rearing of mice colonies in plastic case

The experiments were carried out on newly born young swiss albino mice (2 days to 40 days) lentectomy was carried out on 50 animals under local anesthesia (2% xylocaine). A longitudinal slit was made in the cornea of the right eye under a stereoscopic binocular microscope. The complete intact lens along with lens capsule was extracted through the incision. Following the operation, 40 IU/ml solution of vitamin A was injected intra peritoneal (I.P) on alternate days. In the case of 25 operated animals where vitamin A was not given, served as the control group. In second part or project following steps were performed:-

1. Genomic DNA was isolated from Mice Lens samples (Control and Regenerated Lens) using GeneiPureTM Mammalian genomic DNA Purification kit (# 117304)
2. Using Gene specific primers ~54bp fragment of **rxr alpha danio rerio** gene was amplified using Taq DNA Polymerase.
3. The PCR product was cloned into T vector (Instant ligation kit # 105611) and sequenced.
4. Sequence data was analyzed to detect the SNP in the gene.

RESULTS AND DISCUSSION

Result and Analysis

Group A:- Control – The animals were not given any treatment after their lentectomy. Only sham injections were given on alternate days. 5-5 each animals were preserved in Bouins Solution on days 2,7,15,20 and 40 days after operation

Group B:- Treated – The animals of this group were given treatment of Vitamin A after their lentactomy on alternate day basis. 5-5 each Animals were preserved in Bouins Solution on day 2,7,15,20 and 40 days after operation. All preserved animals were used for histological examination for find out different stage of transdifferentiation

Results and Observation of First Part of Experiment

First, the cells are cuboidal and slightly taller in shape, and then they began to elongate and enter in the lumen of vesicle. The lumen which contains the primary lens fibre nuclei began to differentiate in to the secondary lens fibers. At least, the nuclei of the secondary lens fibers progressively disappear.

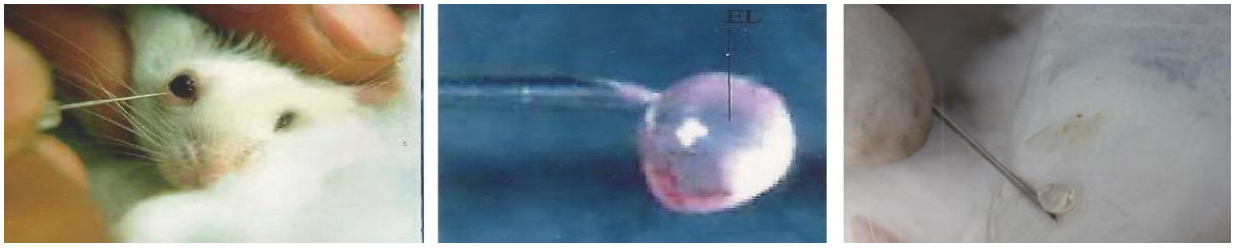
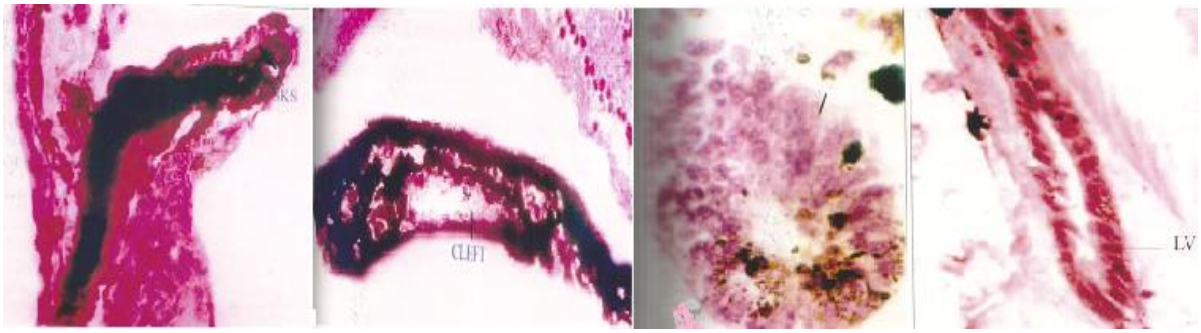
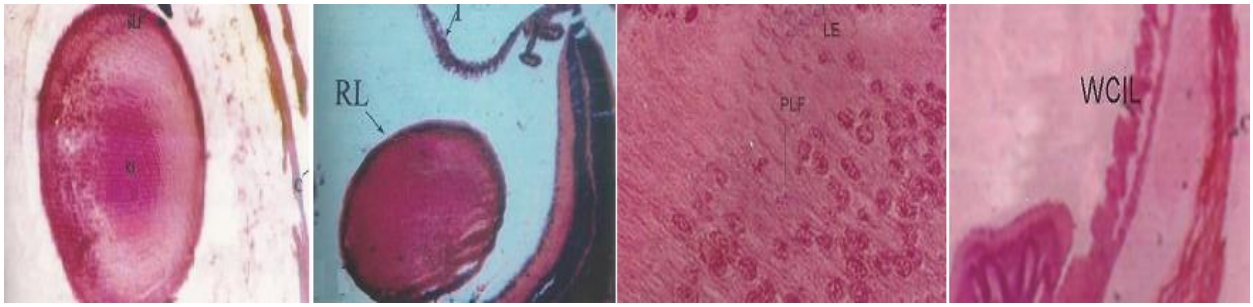


Figure 2: Microphotograph showing insertion of needle in to the right eye of young swissalbino mice and extracted lens of young swiss albino mice



A B C D

Figure 3: (A).Microphotograph of section through dorsal iris of vitamin A treated young swiss albino mice showing transdifferentiation of iris into lens cells. Pupillary margin of dorsal iris becomes swollen and knob like. (B) Section through dorsal iris showing the formation of initial lens vesicle. The central calls are transformed in to lens forming cells.(C) section through dorsal the eye showing well defined lens vesicle at the tip of dorsal iris. Mitotic figures are also visible in the epithelium. (D) Section through the dorsal iris of operated eye of vitamin A treated mice showing formation of lens vesicle at the tip of dorsal iris



A B C D

Figure 4: (A). Microphotograph of section passing through the L.S. of vitamin A treated swiss albino mice regenerated lens. Section showing well differentiated lens with secondary lens fibers. (B). Section showing detached regenerated lens and it's position aling with dorsal iris and retina (C).Section passing through the regenerated lens of vitamin A treated young swiss albino mice showing differentiation of primary lens fibers. (D). Section passing through the operated eye of untreated control group of young swiss albino mice showing lens regenerated case with wavy and thick epithelium of iris

Table 1: Showing the percentile of regeneration of new lens in Swiss albino Mice under influence of Vitamin A

Sr. no	Group	Regenerated Lens	Regenerated Lentoids	Non-Regenerated Case	Percentile of Regeneration
1	Gr. A : - Vitamin A Treated	34	4	2	85%
2	Gr. B :- Control group Non treated	Nil	7	33	17.50%

Isolated DNA was stored at -20° C in sterile vials with marking of vials. This isolated DNA sample was further used in PCR

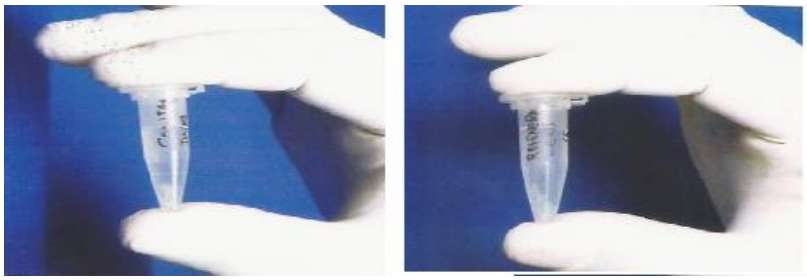


Figure 5: Microphotograph showing isolated lens DNA from control group (non-treated with vitamin A) and regenerated group (treated with vitamin A) of swiss albino mice in eppendrof

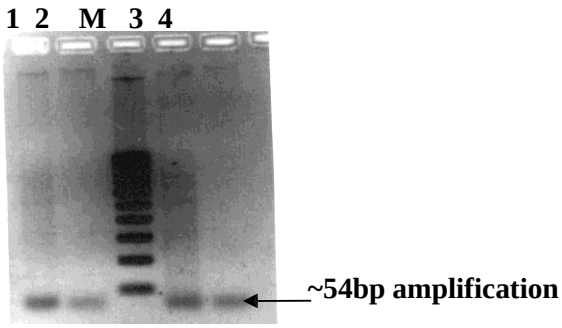
Steps Followed for this Project

1. The generxralphad aniorerio was amplified using gene specific primers.
2. The sequences of the primers are as follow.

Forwardprimer: **5'-AATGCTTCTTTCTGCTTTCC-3'**
Reverseprimer: **5'-CTGAGAGGAGAGGATGTCAC-3'**

PCR conditions:

Step1	940C–5min
Step2	940C-30sec
Step3	580C-30secfor35cycles
Step4	720C-30sec
Step5	720C-10min



PCR Amplification of rxralphadaniorerіogene
Lane1-2: PCR amplification of Normal Lens
Lane M: StepUpTM100bp Ladder(#118707)
Lane 3-4: PCR amplification of Regenerated Lens

1. The PCR products were loaded on 1.5% agarose gel.

2. The amplified PCR products were Cloned into T vector
3. Clones were confirmed digesting the plasmids with *NcoI* restriction enzyme.
4. Positives Clones were sequenced with M13F primer (Vector specific primer)
5. The sequencing data was studied for SNPs.

***Sequence Data* NormalLens#1**

GATGTAATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCG**CCAT**
GGTTAATGCTTCTTTCTGCTTTCCGCACGAGTGAGTGACATCCTCTCCTCTCAGAT**CCATGG**C
CGCGGGATATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTT
GGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAG
CTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCAT
AAAG

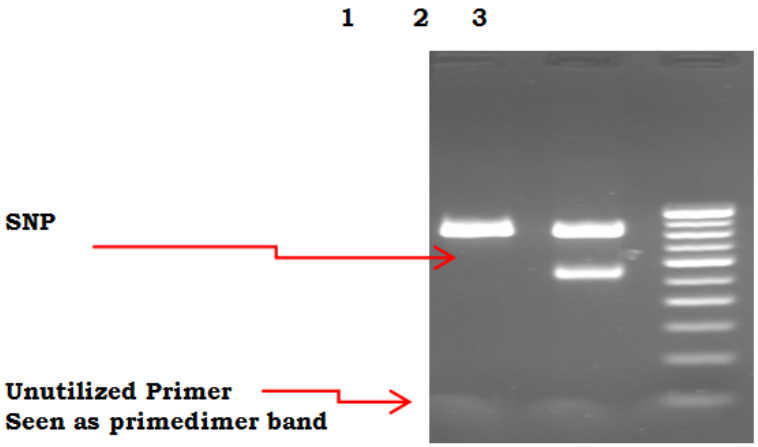
RegeneratedLens#2

CCGATGTATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCG**CCAT**
GGTTAATGCTTCTTTCTGCTTTCCTCTCAGGTGACATCCTCTCCTCTCAGAT**CCATGG**CCGCG
GGATATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGAT
GCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTT
TCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGT
GT

Blue Indicates gene of interest

Red Indicates *NcoI* Restriction enzyme site

SNP presence in 1.5 % Agarose gel (Stained with EtBr) visible on transilluminator



Lane1: Normal DNA (Normal Lens DNA)
Lane2: SNP DNA (Regenerated Lens DNA)
Lane3: 100bp DNA Ladder

Regenerated DNA gives 2 bands whereas amplification using normal DNA gives only one band. Hence it can be concluded that template #1 is normal and template # 2 is SNP type.

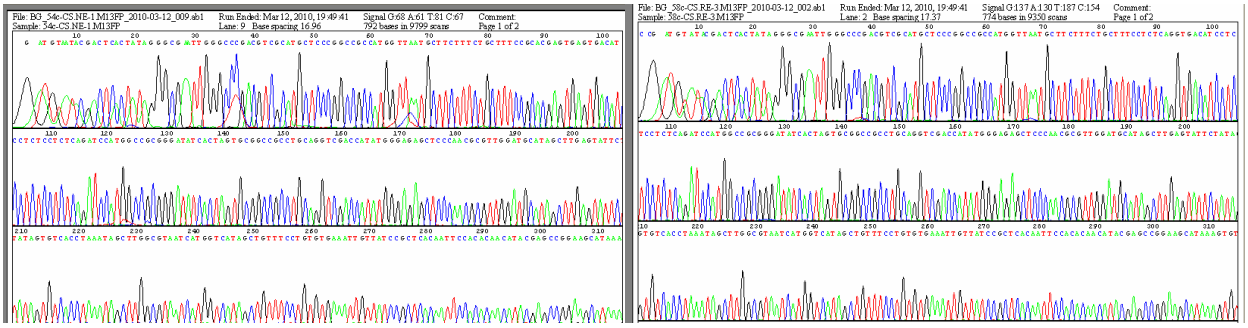
Subject sequence is Normal DNA sequence and Query sequence is regenerated lens DNA sequence. Blast technique confirms that if we usedGenespecificprimers~54bpfragmentofRXRalphadanio-reriogene. Then above mention SNP were produced. This type of SNP was come due to much reason like environment, handling and nutrition etc. But as according to our experiment and past research Vitamin A is major cause which is responsible for this type of mutation and SNP. This DNA mutation and SNP is also a cause of regeneration in Swiss albino mice.

BLAST Alignment result of Normal Lens and Regenerated Lens:

>lcl|48827 Score=66.2bits(72),
Length=50, Expect=2e-17, Identities=48/54(88%),Gaps=4/54(7%) Strand=Plus/Plus

Query 1TTAATGCTTCTTTCTGCTTTCCGCACGAGTGAGTGACATCCTCTCCTCTCAGAT 54
|||||||||||||||||||||||| |||||||||||||||||| Sbjct1TTAATGCTTCTTTCTGCTTTCCTCTC-AG---
GTGACATCCTCTCCTCTCAGAT 50
The identified SNP's are
G, A, G, T, G and A

Electropherogram: Fluorescently labeled DNA fragments were separated according to their molecular weight. All separated DNA base pair provide a peak as according to their properties these peak provide a colored graphically presentation



Normal Lens DNA **Regenerated Lens DNA**

Conclusion

In regeneration process histological study revealed that during lens regeneration after lensectomy the two layers of pigmented epithelium of the dorsal iris thickened and a cleft developed between two lamina of the dorsaliris (Figure 3 and then ucleiofiris cells changed the irshape. The nth epupillary margin of the iris becomes knob-like. The formation of this knob- like structure continued until the free margin became as wollen loop-like structure. Scattered mitotic figures were also observed. All these changes continue up today 7 after operation in vitamin A treated animals. Then the cells started to dedifferentiate: they threw out their melanosomes. These melanosomes are ingested by macrophages that entered from the wounded site. Dorsaliris cells continued to divide, forming a vesicle-like structure in the region of the removed lens. The vesicle differentiated into an ewlens. Once the new lens formed, the cells of the dorsalirisceasedmitosis. The newly formed lens was surrounded by alensepithelium whose cells were cubiodal and slightly taller. Lens fiber formation was initiated in the inner surface of the vesicular lens. At that time cells elongated and entered the lumen of the vesicle. Gradually the lumen was filled by primary lens fiber nuclei (Figure 4). Later on the secondary lens fibers differentiated and grew around the central nucleus and the regenerated lens became a better- defined structure (Figure 9 and 10).

With the help of above result we can conclude that vitamin A is major responsible factor for Trans differentiation in dorsal iris of swiss albino mice eye. In DNA base pair study with the use of DNA isolation, PCR, DNA Cloning, DNA sequencing and Blast technique its concluded that **G,A,G,T,G and A** these SNP's are present in regenerated lens DNA sequence. This is proved in SNP detection test. In SNP test Regenerated DNA gives 2 bands whereas amplification using normal DNA gives only one band. Hence it can be concluded that template #1 is normal and template # 2 is SNP type. This SNP is further concluded in BLAST comparison and Electopherogram. Now its proved that vitamin A work as a inducer for dorsal iris and its show some mutation or aberration of DNA base pair alignment in regenerated DNA. Vitamin A is responsible for all these genetic changes.

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