

Presentation Agenda

Project I:

Measurement of actin depolymerization induced by phospho-Hsp20 peptide in keloid fibroblasts

Advisor: Dr.Padmini Komalavilas

Project II:

Differential gene expression of *Chlamydomonas reinhardtii* during State I to State II Transitions.

Advisor: Dr.Scott Bingham

Measurement of actin depolymerization induced by phospho-Hsp20 peptide in keloid fibroblasts

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Computational Biosciences

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Fibrosis

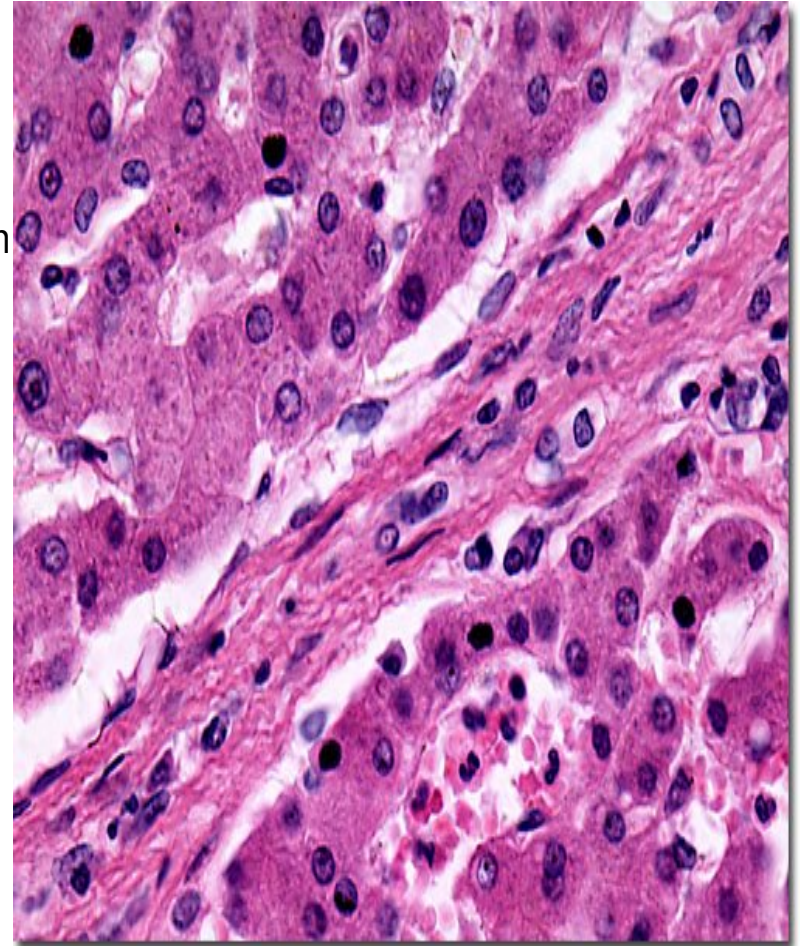
Wound healing is a natural process which results in the formation of scars.

Fibrosis: Excessive scarring occurs due to excessive deposition of collagen. Severe implications of excessive scarring are Pulmonary fibrosis, Liver cirrhosis.

Fibrosis could be mitigated by inducing actin depolymerization.

Objective

To measure Phospho-Hsp20 peptide induced actin depolymerization and amount of G actin in keloid fibroblasts.



Factors responsible for the Fibrosis

TGF- β 1 is a multifunctional protein which has a role in cell growth, differentiation and motility.

TGF- β 1 also has a role in excessive scarring and fibrosis.

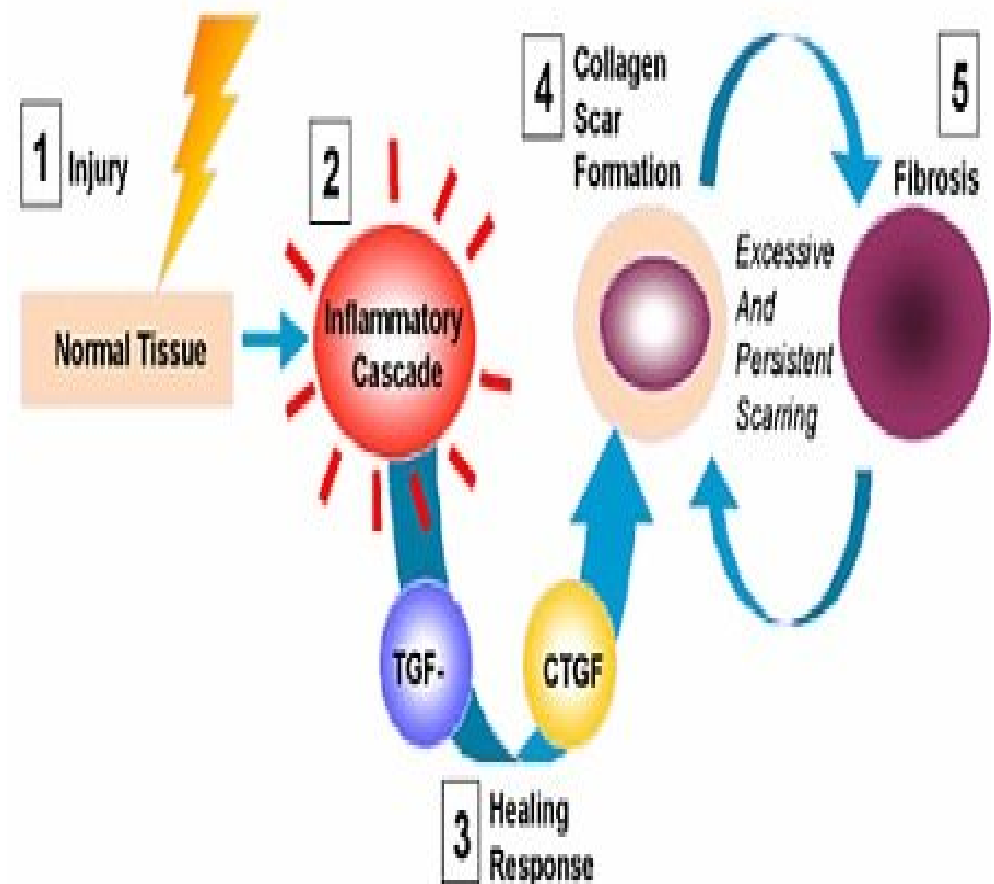
TGF- β 1 induced CTGF mediates pro-fibrotic effects and production of collagen.

TGF- β 1 and CTGF given together showed persistent fibrotic response.

Fibrotic response of TGF- β 1 can be prevented by inhibiting CTGF expression.

Note: CTGF – Connective tissue growth factor.

Pathway to Fibrosis



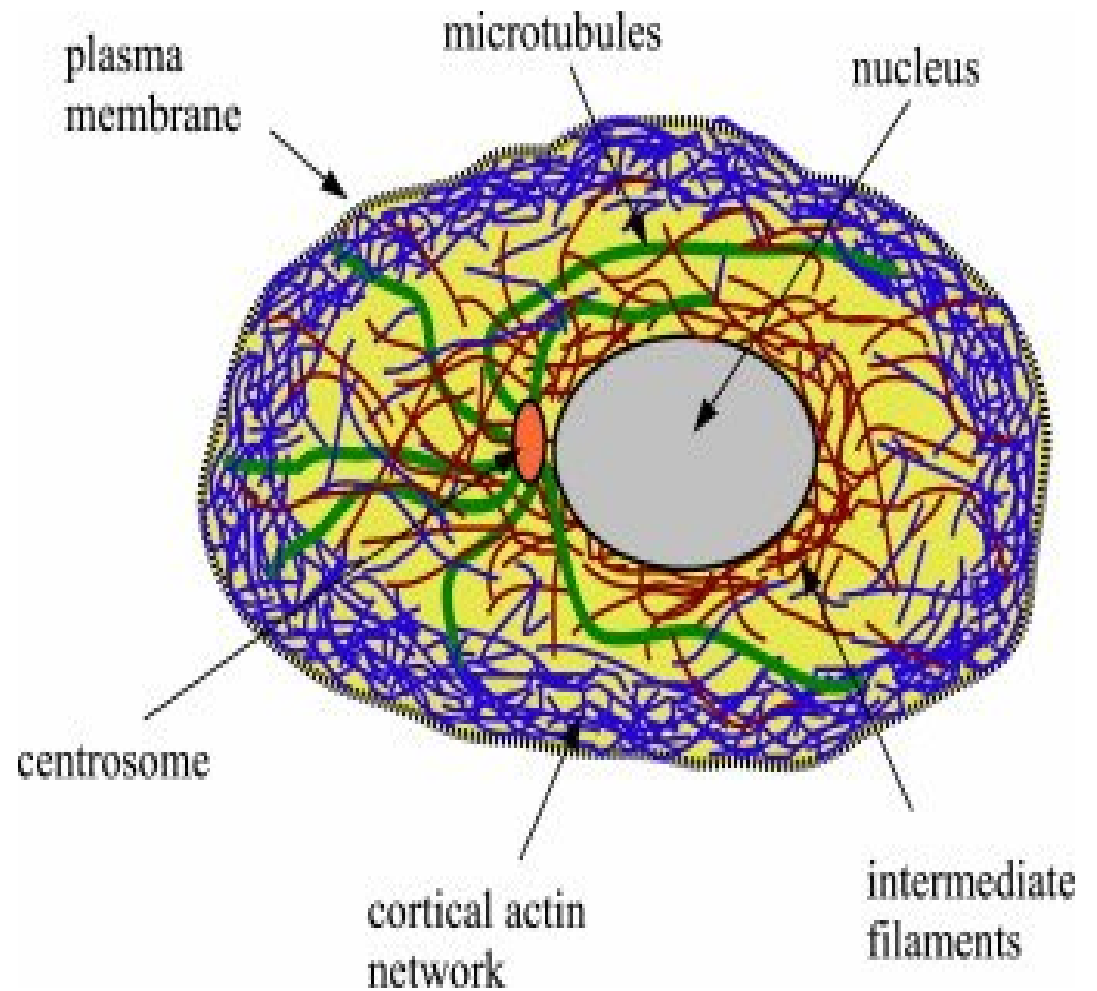
Cytoskeleton and its role in fibrosis

Cytoskeleton is a dynamic structure that is present in all cells. They maintain cell shape and protects cell.

Cytoskeleton is a network of proteins - Actin filaments, intermediary filaments and microtubules.

CTGF expression depends on cytoskeleton reorganization.

Intact cytoskeleton is required for the CTGF expression.



Effect of cAMP levels on CTGF expression

Increased cAMP levels abolish TGF- β induced cytoskeleton reorganization.

Increased levels of cAMP also abolished the TGF- β induced CTGF expression.

Phosphorylation of Hsp20 on serine 16 is one of the downstream events in cAMP signaling cascade which activates depolymerization of actin.

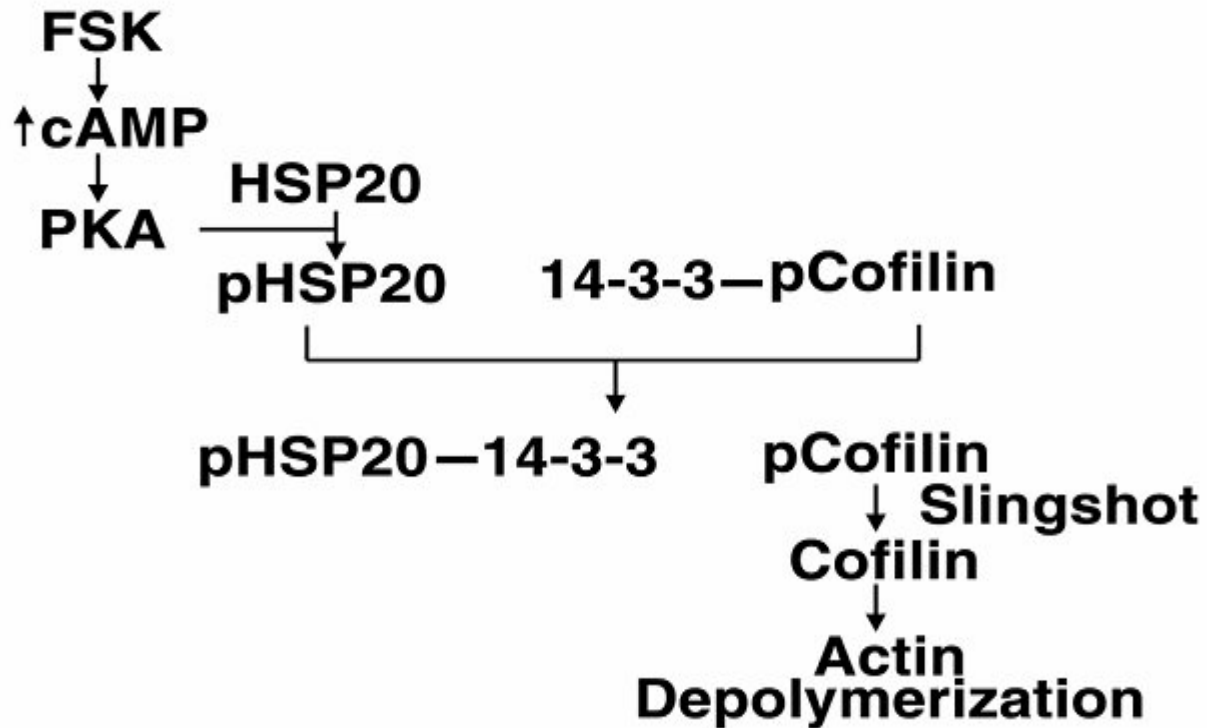


Fig: Mechanism of actin depolymerization induced by Hsp20 phosphopeptide

Effect of AZX100 on actin depolymerization

Treatment of cells with this peptide leads to changes in cellular morphology.

Loss of stress fibers and increase in monomeric G-actin.

AZX100, a phosphorylated peptide analogue of Hsp20 (WLRRAS*APLPGLK) when attached to a protein transduction domain (YARAAARQARA) enters the cell and mimics the cyclic nucleotide signaling pathway.

Activation of actin depolymerization factor cofilin.

We have attempted to measure the amount of G actin formed due to AZX100 treatment.

Methodology

Cell culture

Human Keloid Fibroblasts (HKF) were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37°C and are serum starved for 24 hours.

Cell treatment

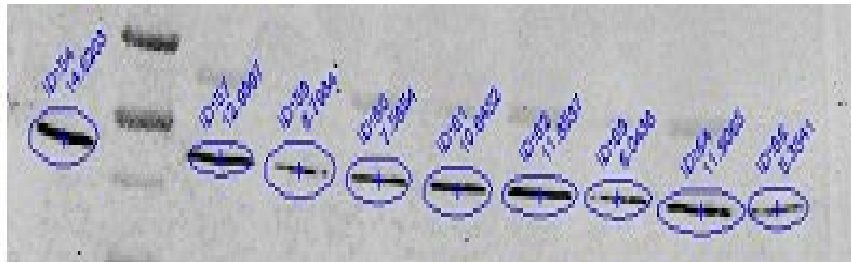
Cells are treated with TGF- β 1 (2.5 η g) , TGF- β 1 (2.5 η g) + AZX100 (100 μ M) and AZX100 alone for 30 minutes.

F/G actin assay protocol (Cytoskeleton)

Cells were homogenized and unbroken cells and nuclear debris were removed by centrifugation at 800g for 10 minutes. The supernatant was subjected to high speed ultracentrifugation at 100,000g for 1 hour results in soluble G actin and insoluble (pellet) F actin.

The pellet and supernatant were analyzed by SDS-PAGE and western blot techniques.

Results

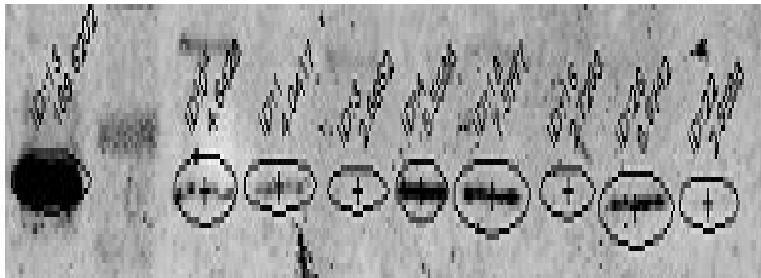


Standard G F G F G F G F
Control F-enhancer F-depolymerizer AZX100

To standardize the kit protocol

- F-actin enhancer and F-actin depolymerizer added in vitro.
- AZX100 added in vivo to keloid fibroblasts.
- As expected the F-actin is higher and G-actin is lower in cells treated with F-actin enhancer.
- F-actin lower and G-actin is higher in cells treated with F-actin depolymerizer and AZX100.
- Results suggest that kit was working adequately to measure the changes in G-actin.

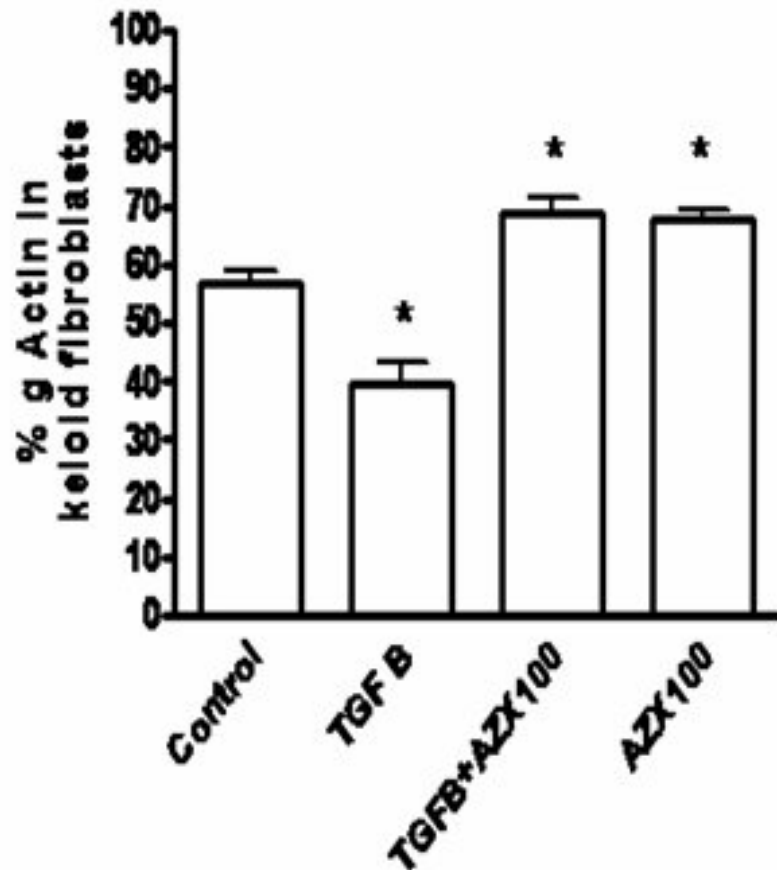
Results (contd..)



Standard	G	F	G	F	G	F	G	F
	Control	TGF beta	TGF beta+AZX100	AZX100				

- Keloid fibroblast cells were treated with TGF- β alone, TGF- β + AZX100 , and AZX100 alone to measure the amount of G actin.
- The amount of G actin is less and F actin is more in sample treated with TGF- β .
- The amount of G actin is more and F actin is less in samples treated with AZX 100 alone and TGF- β + AZX100.

Results (contd..)



- The values are means \pm SEM; n=3 experiments; *P < 0.05 is significant
- In untreated keloid fibroblasts the amount of G actin content was 56.847 \pm 1.99 % of total actin
- TGF- β - 39.483 \pm 3.686%
- TGF- β + AZX100 - 68.627 \pm 2.518 %
- AZX 100 - 67.903 \pm 1.379%
- There was a significant increase in amount of G actin when cells treated with AZX 100 + TGF- β and AZX 100.

Conclusions and Future Studies

Conclusion

- In the present study we investigated the effect of AZX100 on actin depolymerization by measuring G actin in keloid fibroblasts.
- TGF- β induced CTGF expression is required for fibrosis and excessive scarring which requires intact cytoskeleton.
- AZX 100 disrupts actin cytoskeleton by activating actin depolymerizing protein cofilin.
- The cells treated with AZX 100 has shown a significant increase in G actin.
- Activity of TGF- β is inhibited in the presence of AZX 100.
- AZX 100 is a potent F actin depolymerizer and hence is a potential drug for preventing excessive scarring and fibrotic disorders.

Future Studies

- A mathematical model describing the behavior of actin depolymerization at different time constraints and levels of AZX 100 should be done.
- A same conceptual process can be repeated in a tissue scarring condition.

References

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Differential gene expression of
Chlamydomonas reinhardtii during State I to
State II Transitions.

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Chlamydomonas reinhardtii

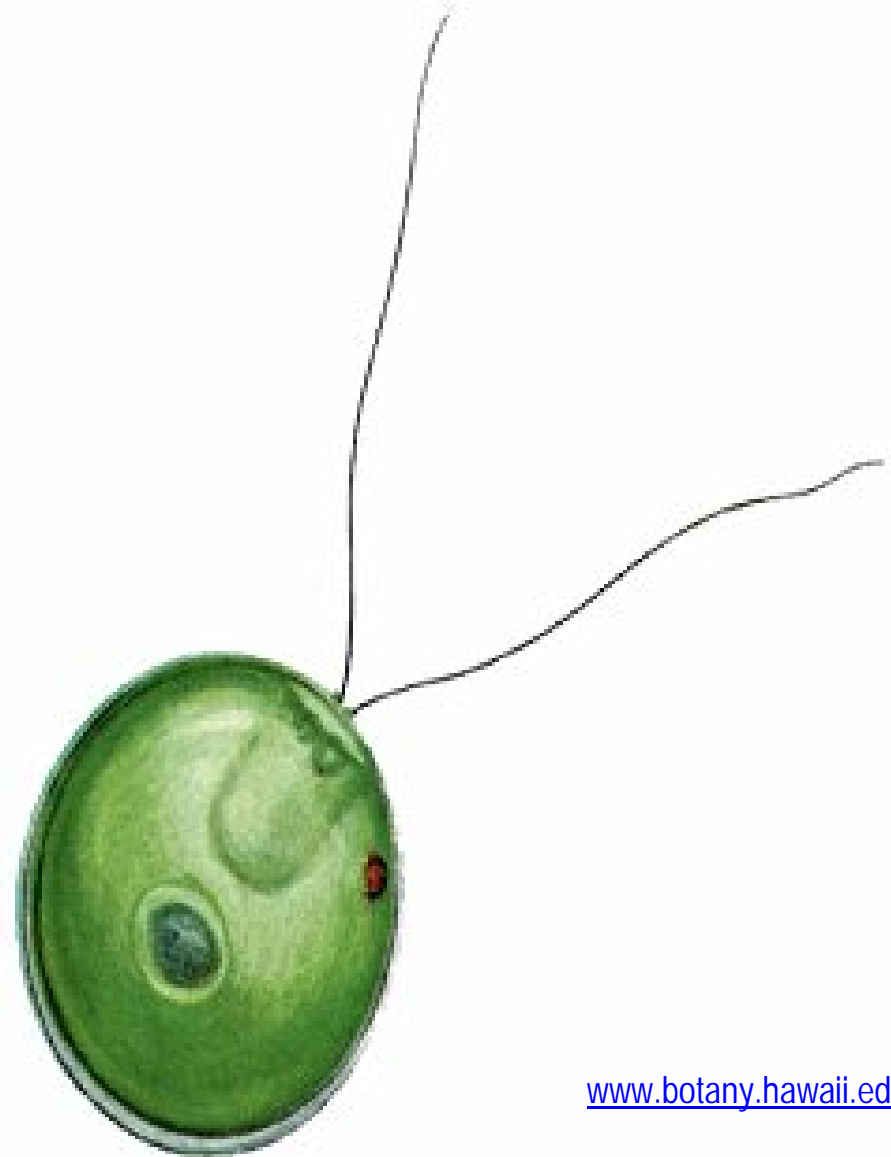
A model algae – photosynthesis and for responses to external stimuli.

Why Chlamydomonas reinhardtii?

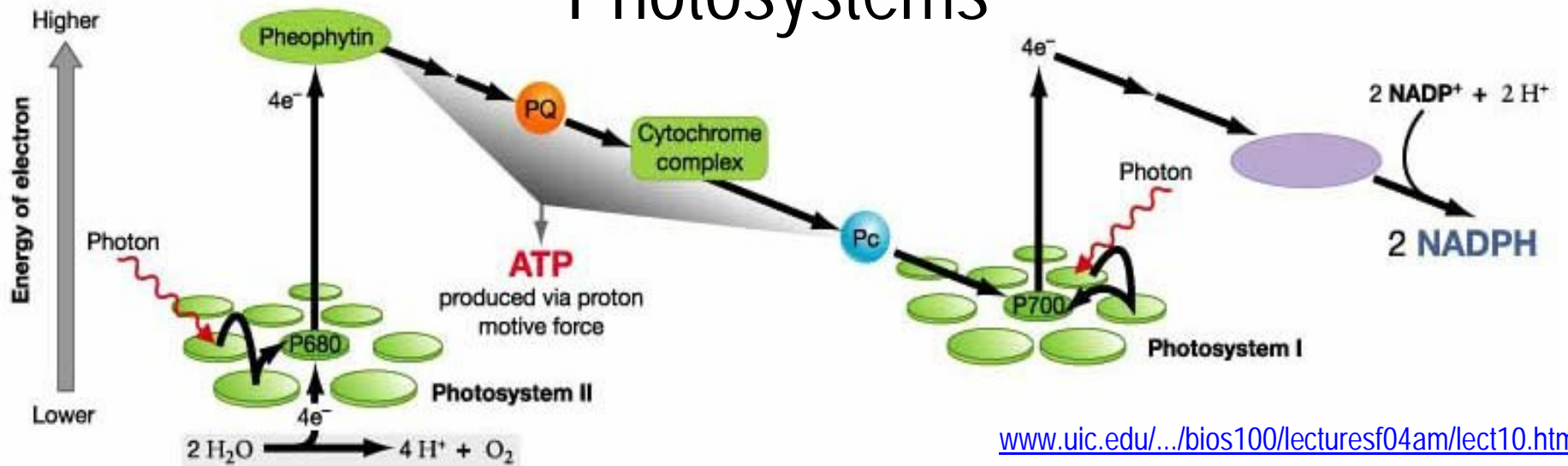
- Completely sequenced genomes
- Microarrays designed for entire genome
- Hence, expressions through out the genome are measured
- Simple and fast life cycles
- Dynamic adaptation to different light conditions

Objective

Determining patterns of gene expression in response to imbalances in the energy states of Photosystem I and Photosystem II.



Photosystems



Main Components

Chlorophyll and Antenna pigments

Accessory pigments and proteins

- Antenna pigments absorb light and transfer it to chlorophyll until light reaches reaction center
- Excited e^- are transferred from photosystem II to photosystem I
- Excited e^- reduces NADP^+ into NADPH
- Proton motive force developed is used to synthesize ATP
- ATP and NADPH fix and reduce CO_2 into carbohydrates

State I – Balanced energy condition at the two photosystems

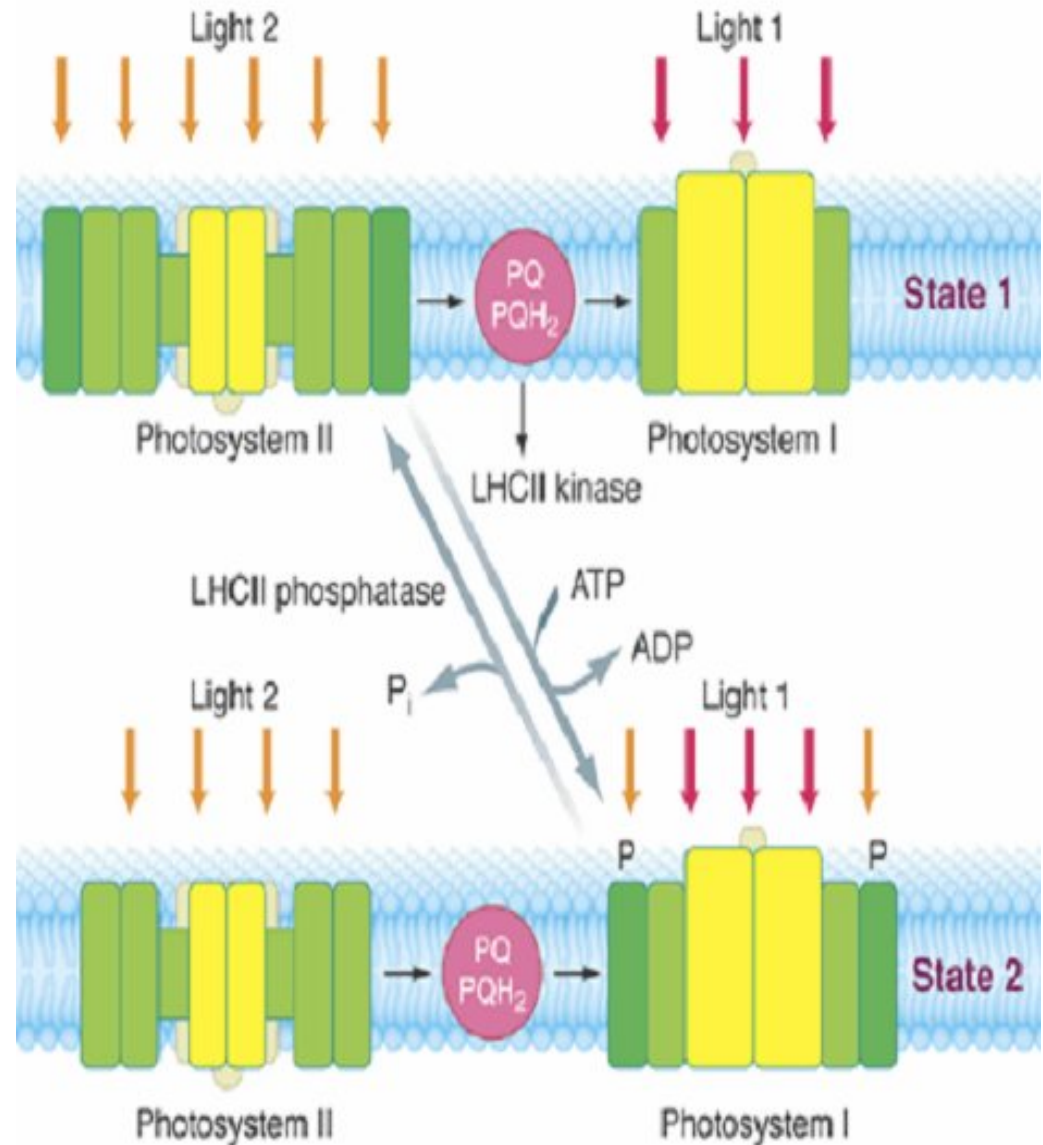
State II – Imbalanced energy condition at the two photosystems leading to movement of the light-absorbing proteins

Photosystems in detail - State transitions

Considering an imbalance at Photosystem II, a state II transition is achieved.

State II

- Excess energy is transferred from Photosystem II to Photosystem I through Light absorbing proteins
- Plastoquinone gets reduced and activates kinase
- Kinase phosphorylates LHC II which joins Photosystem I to correct energy imbalance and reach balanced energy condition



Reaching balanced energy condition, state I is achieved.

State I

- Plastoquinone gets oxidized and inactivates kinase
- Dephosphorylation of LHC II by LHC II phosphatase
- LHC-II returns to Photosystem II

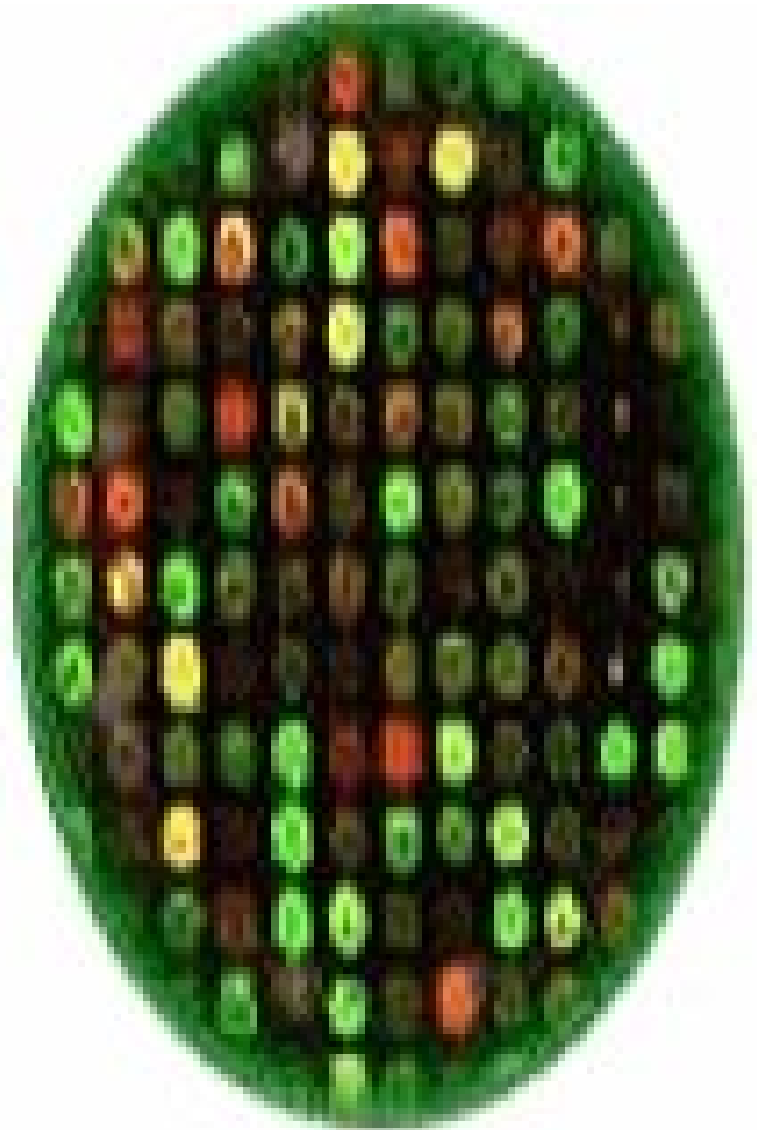
Microarray Technique

High throughput technique used to study

- protein-protein interactions
- antibody-protein interactions
- DNA-DNA interactions
- DNA-RNA interactions and etc

DNA Microarrays are

- Small solid support onto which thousands of oligonucleotides are arrayed in specific order so that each spot maps to a specific gene
- Labeled cDNA or RNA are hybridized to the spotted oligonucleotides
- Unbound RNA is removed by washing
- The color intensity of each spot corresponds to the ratio of RNA treated to control (level of expression)



Methods

For cell culturing, CC 125 strain was inoculated into Cox Chlamydomonas medium and cells were grown for 48 hours.

Anaerobic Environment to cause an Imbalance in energy conditions were created when cells were shaken in dark and bubbled with N₂ for 2 hours.

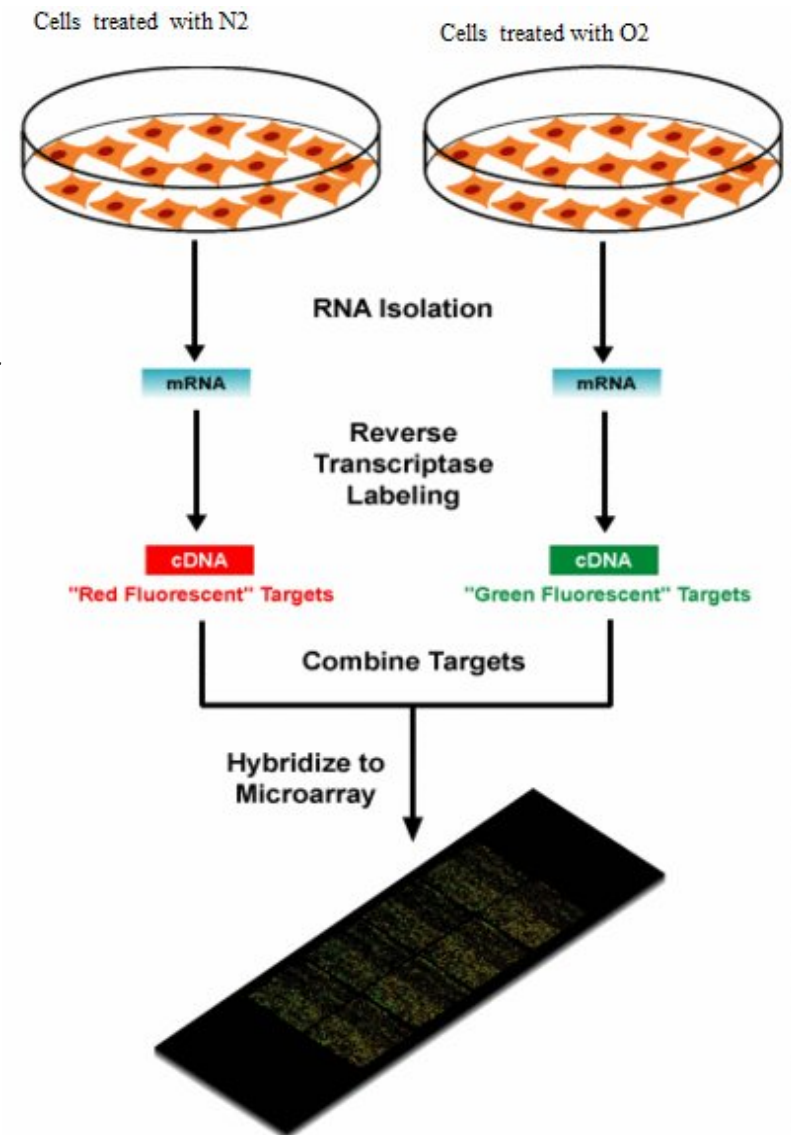
As a control, cells were allowed to grow in aerobic environment.

RNA is isolated from both cultures (control and sample).

mRNA is converted into cDNA by using Reverse Transcription.

Purified cDNA is converted into amino-allyl RNA and labeled with Cy3 and Cy5 for control and sample respectively.

Both control and sample were mixed and hybridized onto the slide and the slide was scanned using Agilent scanner.



<http://en.wikipedia.org/wiki/Image:Microarray-schema.gif>

Data Analysis

Raw images generated by Agilent scanner are stored as TIF files.

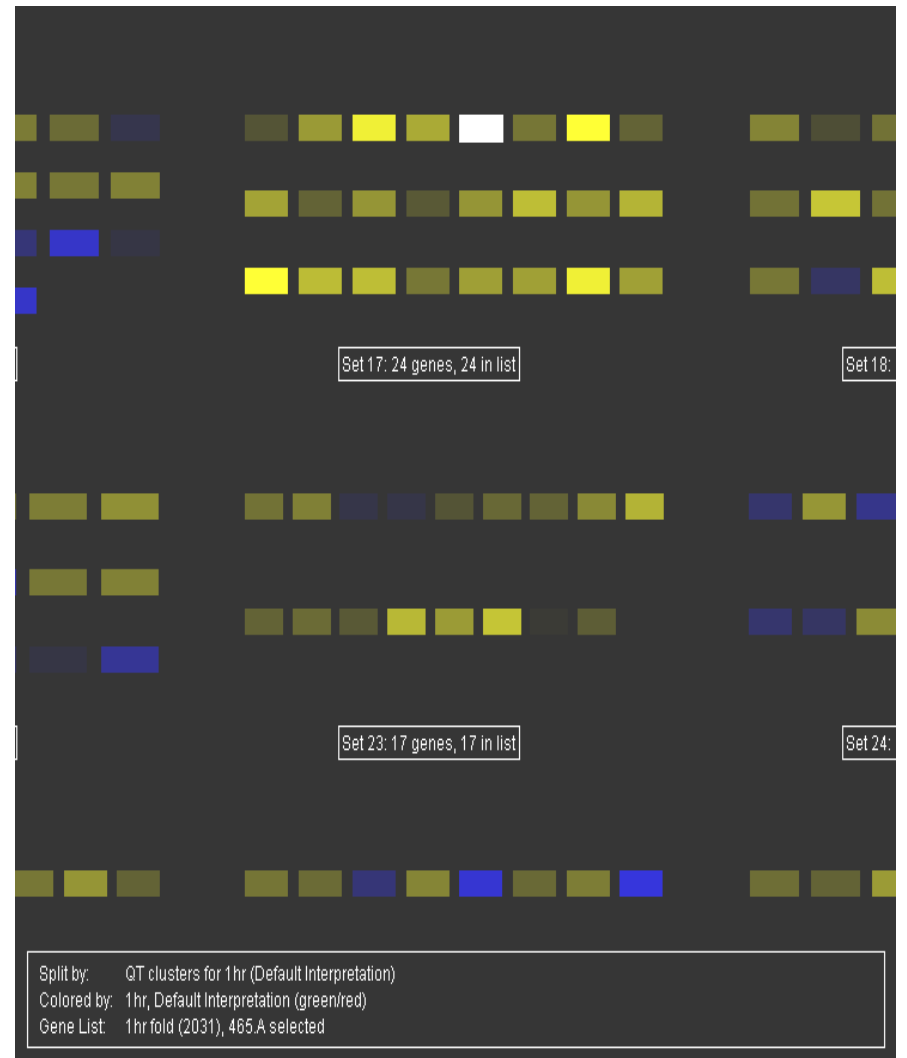
GenePix

Scanned TIF images were quantified and GPR (GenePix results) file with various attributes for each gene is generated.

Genespring

- GPR data are normalized using Loess and median normalization per chip method (by removing non biological variation).
- GPR data are filtered based on expression levels of various genes.
- The threshold for filtering is set by the user.
- QT-Clustering is performed to group genes based on their expression. This is done using Euclidean distance to partition the expression values into subsets.
- Biologically, the genes could be clustered either due to Co-expression (common transcription factor or regulatory pathway) or Common expression patterns (no biological significance).
- Genespring also integrates gene lists with Gene Ontology information to find out biological context of differentially expressed genes.
- Genespring subsets the gene lists by function or subcellular localization in order to identify interesting genes and pathways that are effected due to the anaerobic conditions.
- BLAST web server was used to determine and correlate functions to hypothetical genes.

Clustering Images



Clustering Image and its expanded view (on the right).

Results

Facets of Results

- Results are based on differential expression of genes which provides the context through which assumptions are made about functions in the cell.
- No protein levels were measured only projected levels through the proxy of transcript copy number were inferred.
- Network and pathway analysis of gene expression correlates well with observed phenotypes.
- The analysis of gene expression is a good indicator of cellular functions.

Kinase Genes

Fold change	Genbank description
6.633	(-) protein kinase
2.716	(+) (O82514) Adenylate kinase 1
4.351	(+) (P43293) Probable serine/threonine-protein kinase

Hypothetical Genes

Fold Change	Genbank Description
4.589	(+) hypothetical protein VSP-3
3.67	(-) (Q9STM2) Hypothetical
5.343	(-) (Q9LPM3) F2J10.10 protein (Hypothetical 23.3 kDa protein)

Photosynthetic Genes

Foldchange	Genbank description
3.258	(+) light-harvesting chlorophyll-a/b
2.974	(+) light harvesting complex a protein
2.794	(+) light-harvesting complex I protein
2.692	(+) Photosystem I reaction center subunit VI

Protein Metabolic Genes

Foldchange	Genbank description
2.24	(+) Carotene biosynthesis-related protein CBR, chloroplast precursor
2.7	(+) Tbc2 translation factor, chloroplast precursor [Chlamydomonas 1% id
3.48	(+) Probable menaquinone biosynthesis methyltransferase

Foldchange	Genbank description
3.116	(-) Chaperone protein dnaK (Heat shock protein 70)
	(+) CHLOROPLAST HEAT SHOCK 22 KD
2.368	PROTEIN
2.284	(+) (P51818) Heat shock protein 81-3 (HSP81-3)

Stress Genes

Results (contd..)

Kinases

Important role in state transitions by phosphorylating LHC II proteins. Here, Serine threonine kinase activity is responsible for LHC II phosphorylation and was observed by Hind and colleagues in 1986.

Stress genes

Heat shock proteins and Chaperones

Hydrogenase

Combines e^- of electron transport chain with protons to form hydrogen.

Protein metabolism genes

Proteins that control translation and post translational modifications that occur in chloroplast.

Signal transduction genes

Signal transduction pathways are induced due to external or internal signals.

Hypothetical proteins

Research has to be done to find out the functions of these hypothetical proteins.

Conclusions and Future Studies

Conclusions

- The light harvesting chlorophyll a/b complex (LHC-II) found over expressed 3.25 fold. It regulates energy distribution between the two photosystems.
- Chaperone protein dnaK (Heat shock protein 70) is over expressed 3.116 fold and has functions in refolding of misfolded proteins that are essential under stress.
- A probable Serine threonine kinase is over expressed 4.351 fold. Serine threonine kinase activity is responsible for LHC II phosphorylation.
- Tbc2 is 2.741 fold up regulated. Tbc2 is involved in the translation of the chloroplast psbC mRNA of the eukaryotic green alga *Chlamydomonas reinhardtii*. PsbC encodes polypeptides of photosystem II.
- Interestingly many hypothetical proteins were observed suggesting a need for lot more research to find out their role in state transitions.

Future Studies

- Extended Time course analysis - More than 10 time points and their comparative gene expression.
- qRT-PCR can be used to measure RNA abundance with greater precision on a small subset of interesting genes.
- Antibody array can be used to measure the abundance of proteome and post-translational modifications.

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