BIOCHEMICAL SOCIETY SUMMER VACATION STUDENTSHIP REPORT

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Identification of the biochemical pathways for histone methylation induced by nuclear envelope (NE) protein NET23

Aim of the project: To identify partner proteins of NET23 protein using cell lysates from mammalian cells transfected with C-terminal (transmembrane domains deleted) fragment of the NET23 protein. However the fragment clone did not target and express very well in the cell lines tested.

Theme of the project: The nucleus is a highly specialized organelle that serves as the information processing and administrative center of the cell. This organelle has two major functions: it stores the cell's hereditary material, or DNA, and it coordinates the cell's activities, which include growth, intermediary metabolism, protein synthesis, and reproduction (cell division). But DNA itself does not perform all the function on its own. Proteins present inside the nucleus and in the nuclear membranes interact with chromatin and regulate the expression of genes. The structure of Chromatin is regulated by histone proteins, which in turn are modified at their tails and interact with other cellular proteins. In the recent years various inherited diseases have been linked with the mutation of certain proteins in the nucleus. These mutations may render some structural changes to the proteins and structural changes in most of the cases cause loss of interaction between the protein and respective partner protein. Nuclear transmembrane proteins (NETs) have roused the interest as they have been found to affect various processes in the nucleus. Over expression of various NETs in the cell lines having H2B histone fused with reporter green fluorescent protein (GFP) has shown that NET23 has a global effect on chromatin condensation. Further characterization of this effect has indicated that histone H3 methylation accumulation results from NET23 over expression and occurs unusually before loss of histone deacetylation. But since we do not know whether NET23 recruits the histone methylase directly or act further upstream, the first step in understanding its mode of action is the determination of the partner proteins.

Description of Work: NET23 has four predicted regular hydrophobic domains indicating that it passes through the nuclear membrane many times making it difficult to purify in soluble form thus my original aim was to do the binding studies with the transmembrane domain deleted NET23 fragment however as the fragment clone did not target and express very well in the cell lines chosen we decided to use the full-length NET23 clone instead. To do the binding studies utilizing the full-length NET23 now my aim was to first transfect the cells with NET23-GFP clone DNA (due to the non availability of an antibody to the endogenous protein used) then isolate NEs from these cells. As NET23 is a membrane protein so the aim was to chemically cross-link the partner proteins, perform the co-immunoprecipitations using anti-GFP antibody, solubilise the cross-linked co-immunoprecipitated complexes and then reverse the cross-linking and analyze the NET23 bound partner proteins by Mass-spec.

I started my summer project by getting familiar with common laboratory techniques like DNA Extraction using alkaline lysis, purification of DNA by phenol chloroform extraction, learning mammalian cell culture and maintenance of cell lines, transfection of DNA into the cells etc.

I started the project with two objectives. The experiments for both the parts were done in parallel:

1. Cloning the NET23 fragment into pEGFP-N vector: My first objective was to clone NET23 fragment with additional nuclear localization signal (NLS) sequence and deletion of four hydrophobic traansmembrane regions into pEGFP-N vector. NET23 was insertion into the MCS region of the pEGFP-N2 vector using EcoRI and BamHI restriction enzyme sites as they are not present anywhere in the

NET23 gene and the insertion will be in frame fusion with GFP reporter gene. This was followed by transformation of XL10-Gold ultracompetent *E.coli* cells (Stratagene) with pEGFP-N2 vector containing NET23 fragment insertion and colonies obtained were screened for the presence of NET23 fragment by digestion with EcoRI and BamHI, followed by agarose gel analysis. The screening experiments included:

- Growing the transformed cells on agar plate containing Kanamycin as the pEGFP-N2 vector renders Kanamycin resistance to transformed XL10-Gold cell.
- The colonies obtained after 16-18 were separately grown in liquid media followed by crude preparation of plasmid DNA.
- This plasmid DNA was digested with restriction enzymes and the digested product analyzed by running it on agarose (1%) gel with DNA markers.

The cells found to be positive for vector containing NET23 fragment fused to GFP were used to make large amount of plasmid DNA by using various available methods like alkaline lysis, Qiagen Mega prep kits etc. This plasmid DNA would then be used to transfect into various mammalian cell lines.

***2. Extraction and purification of full-length NET23-GFP DNA*:** The second step of project was to prepare the large quantities of the already available plasmid containing NET23 full-length fusion with GFP for large-scale transfections into mammalian cells.

***3. Optimization of transfer of DNA into mammalian cells by transfection using full-length NET23-GFP*:** The third part was to optimize the process of transfection for over expression of the NET23 protein in different mammalian cells (Hela, 293T and Cos7) as for the preparation of NEs from mammalian cells huge amounts of NET23 over expressing cells were needed as a starting material because the yield of isolation and purification of NEs vary for various cell types. The procedures used for transfection optimization included latest techniques like Fugene lipid method, nucleofection (Amaxa) and classical approaches like Calcium Chloride and Calcium phosphate method.

***4. Isolation and purification of NEs from Mammalian cells*:** The last and the most exciting part of the project was the isolation of NEs from 293T and Hela cells (both NET23-GFP transfected and untransfected). NET23 being the transmembrane protein of nuclear membrane can be separated from rest of the cell by purifying NEs and digesting the Chromatin and extracting other nuclear material out of them. The procedure used by another member of the laboratory [2] was followed initially however as that was for developed for NEs from blood lymphocytes we had to modify and optimize it for isolation of NEs from 293-T and HeLa cells. For preparing NEs from 60-70 tissue culture dishes ($100mm^2$) every week we scraped the cells to preserve the morphology of the nucleus and extracted NEs using mild lysis and sucrose gradients.

Assessment of Results and outcomes of studentship:

1. In order to optimize the conditions for transfection various experiments were conducted modifying different variable such as number of cell, amount of DNA used and length of transfection etc. On the basis of acquired data two graphs generated are shown in Figure 1 and Figure 2.

- Comparisons between the difference mammalian cell types to deduce which gave highest transfect efficiency yielded that 293T has better transfection efficiency then Hela and Cos7 cells.
- Another set of experiments aimed at identifying the best method of transfection for large scale experiments showed that nucleofection was best for obtaining highest transfection efficiencies but $CaCl_2$ was more cost effective when using arround 100-200 million cells for transfections. 293T cells were used for transfection with NET23-GFP.
- The cloning of the NET23 fragment did not provide positive clone as the molecular weight of the cloned fragment releasded after screening restriction enzyme digestion was double the expected size , which indicated either cloning of an unrelated bacterial fragment or dimmer of the original fragment however due to the time constraints it was not be possible to repeat the whole experiment again.
- The last and the most focused experiment for the preparation of nuclear envelops from 293T and Hela (Transfected and untransfected both) yielded only 13% and 5% of the nuclei in the first two

experiments following the previous protocol. However after modification in the composition of the buffers and sucrose gradients used in later experiments the efficiency we got 70% to 95% efficiency of purity of NEs.

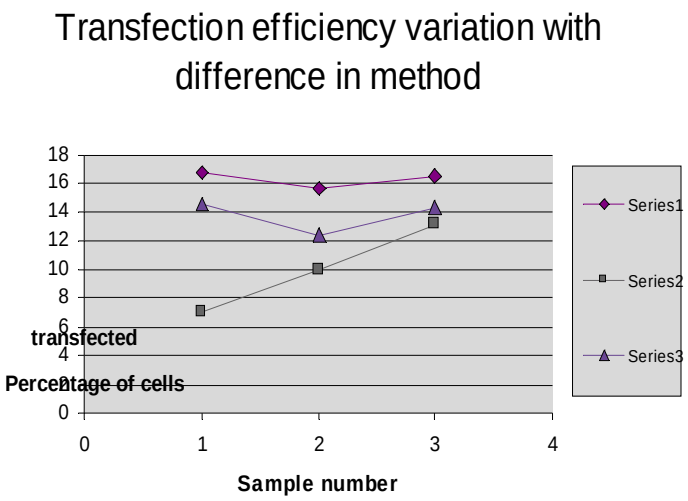

Figure 1

Series1 is nucleofection, series2 is Fugene and series3 is Calcium Chloride method.

- To analyse density of cells that is better for the transfection, 293T cells were nucleofected at different densities with NET23-GFP plasmid with same amount of DNA (5μg). The result is shown below:

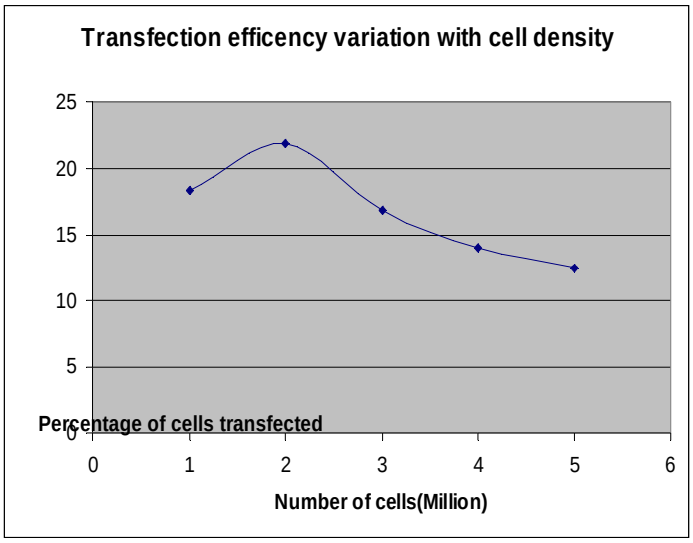

Figure 2

From the above data it can be easily concluded that:

- Though the nucleofection provided higher transfection efficiencies classical Calcium Chloride method is much more economical when transfecting huge number of cells (100-200x10^6).
- The transfection efficiency is best when 293-T cell density is 2.0 x10^6cells with 5μg DNA per transfection.

By this time I had reached the end of my laboratory time. After this my supervisor would continue with the project and perform experiments.

Future directions in which the project could be taken: Future experiments include digestion of the chromatin and other nuclear material out of the NET23 transfected and untransfected nuclei, chemical cross-linking to preserve bound complexes followed by immunoprecipitation using anti-GFP polyclonal antibodies, solubilsation of membrane protein NET23 with other partner proteins, reversing the cross-linking and analyzing the NET23 bound proteins by Mass-Spec. Initial experiments done on NET23 prove that it is quite important protein and have certain crucial role to play. More work needs to be done to understand mode of action of NET23, how it modifies the chromatin and whether it has an effect on the cell cycle.

Departures from original proposal: Original aim of the project was to identify partner proteins of NET23 protein using cell lysates from mammalian cells transfected with C-terminal (transmembrane domains deleted) fragment of the NET23 protein. However the earlier cloned fragment did not target and express very well in the cell lines chosen for NET23 chromatin analyses thus my project aim was modified and the new aim was to identify partner proteins of NET23 protein from purified NEs isolated from mammalian cells transfected with full-length (containing four transmembrane domains) NET23.

Due to the general observation and previous experiments before my arrival in the lab, we deviated slightly from the previously decided strategy and-

1. We spend lot of time on optimizing the conditions for bulk scale transfection of mammalian cells.
2. Instead of Hela cells we focused on 293T due to their high transfection efficiency and less doubling time.

Value of the studentship to the student and to the lab: The most important thing, which I learned from this studentship, is the kind of attitude and enthusiasm a student needs to pursue a career in science and research. From the point a project starts to the point the results are published, the amount of time, energy, labour and efforts put by the researchers are tremendous. Apart from the fact that I learnt very good laboratory techniques, the confidence, which I have gained to do research and take crucial decisions, is actually helping me a lot in my final year project. A vital skill I learned was to cope with failed experiments and to use their negative outcome to improve next time. For the lab I provided additional help in carrying out and optimizing large experimental set up and injected some young enthusiasm. Also working in such a multicultural and multiethnic group was a cherishable experience for me and was possible due to the funding support by Biochemical Society. All this will be extremely useful for my future career in research.

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References:

1. *"Use of sequential Chemical Extraction to purify Nuclear Membrane Proteins for Proteomics Identification"* Nadia Korfali, Elizabeth A. Fairley, Selene K. Swanson, Laurence Florens, and Eric C.Schimer.
2. *"Nuclear membrane proteins with potential disease links found by subtractive proteomics"*, Schirmer, E. C., Florens, L., Guan, T. Yates, J. R. III, and Gerace, L. (2003) *Science* **301,** 1380-1382.
3. *"The apparent absence of lamin B1 and emerin in many tissue nuclei is due to epitope masking"*,Tunnah, D., Sewry, C. A., Vaux, D., Schirmer, E. C., and Morris, G. E. (2005) *J. Mol. Histol.* **36,** 337-344.
4. *"The Nuclear Envelope Proteome: Extending the Envelope"*,Schirmer, E. C., and Gerace, L. (2005), *Trends Biochem. Sci.* **30,** 551-558.
5. *"Guilt by association: the nuclear envelope proteome and disease"*,Wilkie, G. S. and Schirmer, E. C. (2006), *Mol. Cell. Proteomics* **5,** 1865-1875.