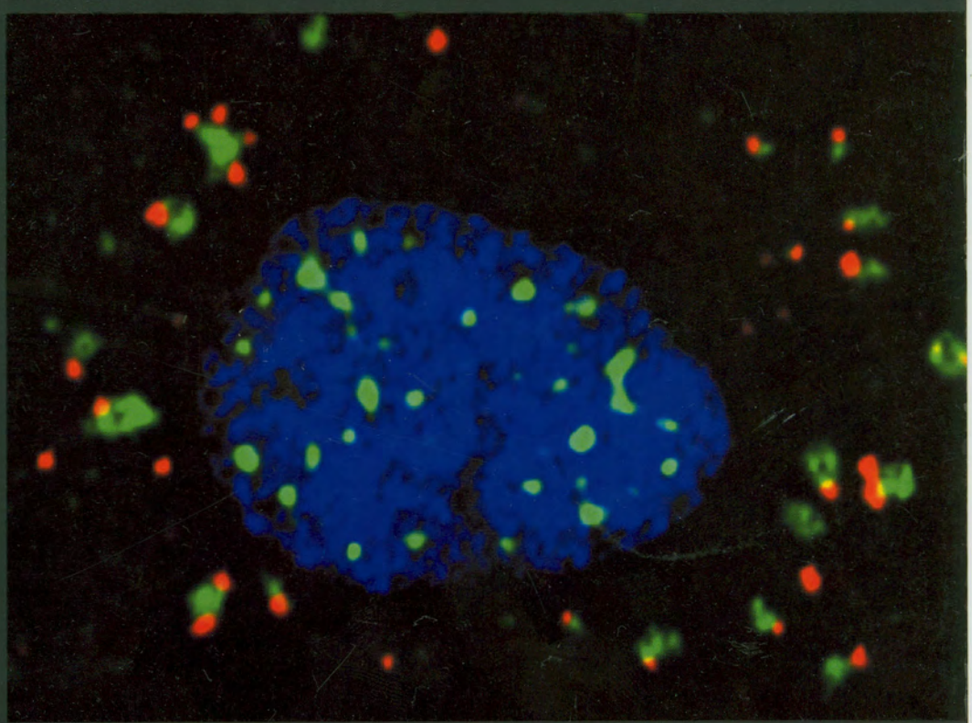
Annual Report

2010-2011

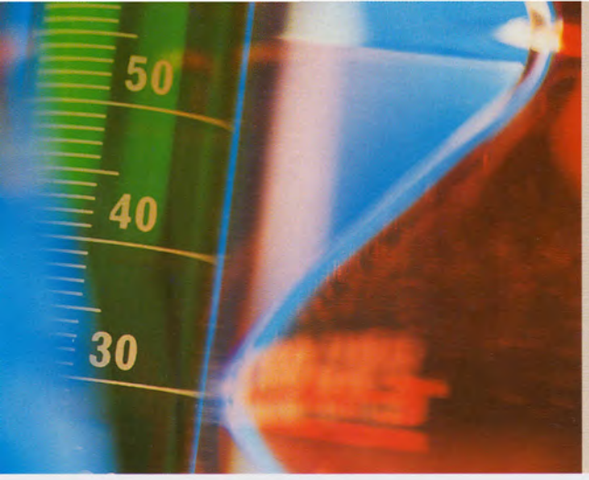


NCCS National Centre for Cell Science

National Centre for Cell Science

Annual Report 2010-2011

Content



Index of Research Reports

Mandate of NCCS

- To receive, identify, maintain, store, grow and supply:- Animal and human cell/cell cultures, cell lines of both existing (typed) and newly developed, hybrid cells including hybridomas; Tissues, organs, eggs (including fertilized) and embryos; Unicellular, obligate pathogens, parasites and vectors, plasmids, genes and genomic libraries.

- To develop, prepare quality control and supply culture media, other reagents and cell products independently and in collaboration with industry and other organizations.

- Research and development

- To establish and conduct postgraduate courses, workshops, seminars, symposia and training programs in the related fields.

- To serve as National Reference Centre for tissue culture, tissue banking and cell products and data bank etc., and to provide consultancy services to medical, veterinary and pharmaceutical institutions, public health services and industries etc. in the country.

- To provide and promote effective linkages on a continious basis between various scientific and research agencies/laboratories and other organizations including industries within the country.

- To participate in programs conducted for the betterment of society and advancement of science and technology in the country.

- To collaborate with foreign research institutions, laboratories and other international organizations in the areas relevant to the objectives of the facility.

From Director's Desk

I feel privileged to present the annual report of NCCS, Pune, which serves as a National Cell Repository, a training centre for Animal Cell & Tissue culture and a premier Research Institution. This report is an attempt to portray and reflect on the scientific activities of the NCCS in the previous year. As the National Repository, NCCS procures and maintains various cell lines and distributes them to different Universities and Research institutions in India. In the previous year, NCCS has supplied over 2300 cell lines to 120 organizations. NCCS is committed to its contribution towards Human Resource Development, by way of reaching out to individuals at all levels including students, teachers and researchers in India. Basic training as well as custom-made programmes depending on specific requirements of smaller groups of researchers were, and will continue to be, conducted at NCCS and at the user's end. In the past year, we have provided training to over 17 researchers from various organizations across the country, and 12 students have successfully completed PhD at NCCS.

Scientists at NCCS conduct research in different aspects of biology in order to generate knowledge that has potential application in the biomedical and biotechnology fields. This has been possible through the coordinated activities of scientists from various fields, motivated research students, state-of-the-art infrastructural facilities, and trained technical and administrative staff. The institute focuses on important questions relevant to human health, specifically in the area of regenerative medicine, infectious diseases and lifestyle induced health adversities. Although stem cell therapy has been used by physicians in India to treat diseases involving damage or loss of particular cell type, a major limitation is the availability/storage of viable stem cells. We have optimized the procedures for isolation, expansion, cryo-preservation and differentiation of stem cells into specific cell types. Considerable efforts are also being made to understand the cellular and molecular mechanisms defining stem cell niche in normal organs.

Human body is exposed to multitude of pathogens and the immune system functions to counteract them. The pathogens constantly evolve mechanisms to evade the host defence system, leading to infections. Identifying molecular players involved in host-pathogen interactions is of paramount importance in developing strategies to combat infections. Towards this end, scientists at NCCS study the mechanism of viral evasion of human complement system, regulation of CD40 signaling in host cells during Leishmaniasis, protein trafficking in

Leishmania, HIV biology and host-pathogen interactions during Plasmodium, Mycobacterium and Candida infections. Hyper-activation of immune system is frequently observed in many inflammatory and autoimmune disorders. We are investigating the role of the chemokines that are involved in regulating these processes.

Cancer is a complex disease caused by mis-regulation of signalling networks. In order to understand the biology of tumourigenesis, NCCS is exploring pathways involving critical players in different signalling cascades involving Osteopontin, eNOS, p53, Cyclin D1, Snail, Slug, mTOR, Wnt, etc. Efforts are also in progress to elucidate the molecular networks underlying oncogene induced senescence. The outcome from these studies is expected to provide tools for better treatment of cancer.

Epigenetic regulation of gene expression plays an important role in various cellular processes. We have shown that nuclear matrix associated proteins like SATB1 and SMAR1 regulate global gene expression by chromatin remodelling. Currently, we are exploring the functions of these two players in immuno-modulation and tumourigenesis.

Diabetes is a multi-factorial metabolic disorder and the incidence of it is increasing at an alarming rate. NCCS focuses on understanding the mechanisms involved in endocrine pancreas development and regulation of insulin biosynthesis. Scientists at NCCS have identified the molecular mechanisms regulating the insulin production and the intricate relationship between the diabetes-induced cardiomyopathy and oxidative stress. Osteoporosis is another major disease that affects a large proportion of our population, which is caused by the imbalance in bone formation and resorption. Our research has shown that immuno-modulators like IL3 play an essential role in differentiation of osteoblasts and thereby augmenting the bone regeneration.

The challenge in understanding biological processes is deciphering the underlying complex and dynamic networks. System biology approach is focused on delineating these networks and their interactions. Integrating the de novo drug design, abstract fragment based drug design and sophisticated molecular simulations is a thrust area of research at NCCS.

Microbial flora within an organism influences the metabolic processes of the host. Using advanced molecular methods such as whole genome sequencing and meta-genomic analysis, we are trying to unravel the complex microbial ecosystem in the mid-guts of humans and insects of clinical importance. For the conservation and exploitation of biodiversity in India, microbial culture collection centre (MCC) was established in NCCS, with a special mandate from DBT. The centre focuses on basic research in the areas of microbial diversity, taxonomy, genomics and proteomics, in addition to its role as a National facility for microbial culture collection and International Depositary Authority.

Identification of prognostic and diagnostic biomarkers helps in early detection of cancer and increases the chances of successful treatment. We have undertaken the efforts to identify novel biomarkers for breast cancer, using integrated proteomic, genomic and bioinformatic approaches. Also, biomolecule mining from hitherto untapped sources such as marine organisms and plants have been performed at NCCS that would aid in treatments of AIDS, diabetes, malaria and osteoporosis.

NCCS provides state of the art infrastructure facilities to its scientists. As a part of this process, we have LC-MS, MALDI-TOF, Confocal and high throughput DNA Sequencer. The challenge for biology at present is the need to deal with the incredibly complex and enormous amount of data generated. To this end, a high performance computing machine have been procured and installed which provides access to a scalable pool of computing resources.

Excellence in our research endeavours is reflected by publication of high quality papers in internationally reputed journals. Since the last report, we have published over 50 scientific papers in peer reviewed journals. Our scientific activity is facilitated by funding from various national and international agencies.

NCCS has been focusing on understanding the molecular details of biological processes that are critical to human growth, development and homeostasis. Over the years, this approach has helped in understanding the fundamental mechanisms of important cellular processes and to develop strategies for better management of diseases. We hope to contribute much more significantly in this direction in the future by coordinating our efforts using diverse research approaches.

This is my last report as the director of NCCS, and it has been a pleasure serving the centre for more than one and a half decade. I believe that the journey has been set on the right direction and the future of NCCS looks very promising. I take this opportunity to extend my heartfelt appreciation to all the scientific, technical and administrative staff and the students of the institute, past and present, who have contributed tremendously to this endeavour. I am grateful to DBT for the generous financial and administrative support, failing which it would have been impossible for me to dispense my duties effectively. I also acknowledge the support from other funding agencies. Last but not the least, I thank all the members of the institute research and management committees for their continuous support.

G.C. Mishra
Director

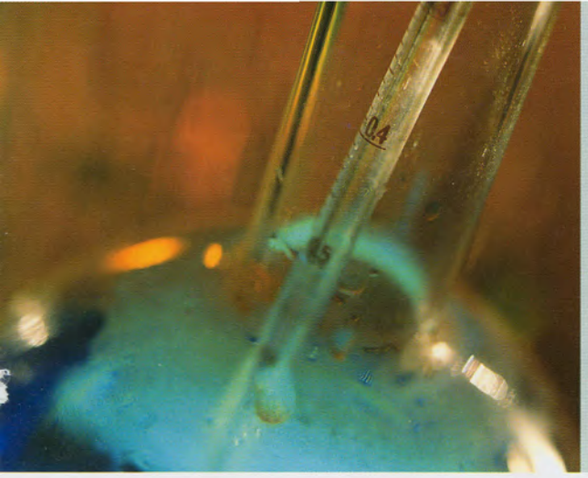
Human Resource Development

During 2010-2011, 41 students joined for pursuing Ph.D under various Scientists. This year 53 student presentations were completed and their admission is confirmed by University. The total number of Ph. D students as on 31.03.2011 was 95.

The Project Training programme is conducted twice in a year i.e. during January-June and July-December, while summer training programme is conducted during the month of May every year. The number of students attended these courses in the last year are:

Project Training - 13
Summer Training - 4

During this year 21 Research Fellows attended seminars / conferences / symposium conducted by various reputed organizations in India and 3 Research Fellows attended International seminars/ conferences/ symposium.



Repository

National Centre for Cell Science serves as a National Cell Bank for animal cell lines. The repository manages cell line procurement, expansion, cryopreservation and distribution. In this year, we have procured different cell types from different repositories. The list of cell lines, with details such as media requirement, growth conditions and its use, is available now on demand. In 2010-2011, we have supplied 2380 cell lines to 120 research institutions in the country.

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Stromal cell biology: Identification of stromal cell-mediated signals regulating hematopoietic stem cell fate.

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Background

A stem cell niche has many functions, some of which require activation of conflicting mechanisms: while the niche is expected to preserve the stem cell pool, it also has to promote a continuous formation of differentiated progenitors to achieve a steady-state hematopoiesis. How these apparently contradictory and amazingly dynamic processes are driven by the niche continues to be an intriguing issue in the stem cell research.

The importance of the niche-mediated regulation of hematopoietic stem cells (HSCs) became evident with the development of the Dexter-type long-term cultures, a landmark technological innovation in the field. Variants of these cultures comprising of irradiated/inactivated stromal cell lines-with or without genetic modifications-seeded with marrow cells, either un-fractionated mononuclear cells (MNCs) or purified HSCs, became popular tools to study the stromal function.

Though extremely useful, these cultures lacked the three-dimensional (3D) architecture of the marrow, and therefore, failed to mimic the in vivo niche. It became apparent that understanding the nitty-gritty of the complex niche-functions requires the creation of an experimental system recapitulating the specialized properties of the marrow microenvironment.

Accordingly, 3D-cultures using specialized scaffolds or extra-cellular-matrix (ECM) molecules like collagen and/or fibronectin and spheroid cultures of mesenchymal stem cells (MSCs) were developed. They have a distinct edge over traditional two-dimensional (2D)-cultures. In the last decade, remarkable progress has been made in creating three-dimensional cellular micro-environments with hydrogels-network of interacting polymer chains that are highly hydrated, with elasticity similar to natural tissues.

We have developed hydrogel-based 3D-cultures of MSCs (3D-MSCs) and shown that they recapitulate several important attributes of a functional HSC-niche and foster a superior stem cell pool on the face of a robust multi-lineage hematopoiesis, thereby

providing a physiologically relevant cellular platform to study the HSC-niche interplay in vitro.

Aims and Objectives

1. Characterization of the HSCs grown under 3D conditions in terms of stem cell-specific phenotypic and functional characters.
2. To assess the cell cycle status and division history of the HSCs growing in 3D-MSCs.
3. To examine whether 3D-MSCs mimic HSC-niche physiology.

Work Achieved

We have previously shown that the 3D cultures foster a superior growth of $CD34^+38^-Lin^-$ stem cells compared to the 2D-cultures. We carried out further phenotypic analyses of the output cells using stem cell-specific phenotypic markers to establish their hierarchical position. We also examined their cell cycle status and tracked their division history.

3D-HSCs express primitive stem cell markers: We analyzed the output cells for the expression of two important markers of primitive HSCs: CD133 and CXCR4. We found that the $CD34^+$ cells from 3D cultures contained a significantly higher percentage of cells expressing these markers (~4 and ~ 6.5 folds higher for CD133 and CXCR4 respectively, N=3, Fig.1 A-B), indicating that 3D-cultures yield significantly higher number of primitive HSCs. Although its role in HSC function *in vivo* remains controversial, N-Cadherin is an important niche-interaction molecule. When the expression of N-Cadherin was

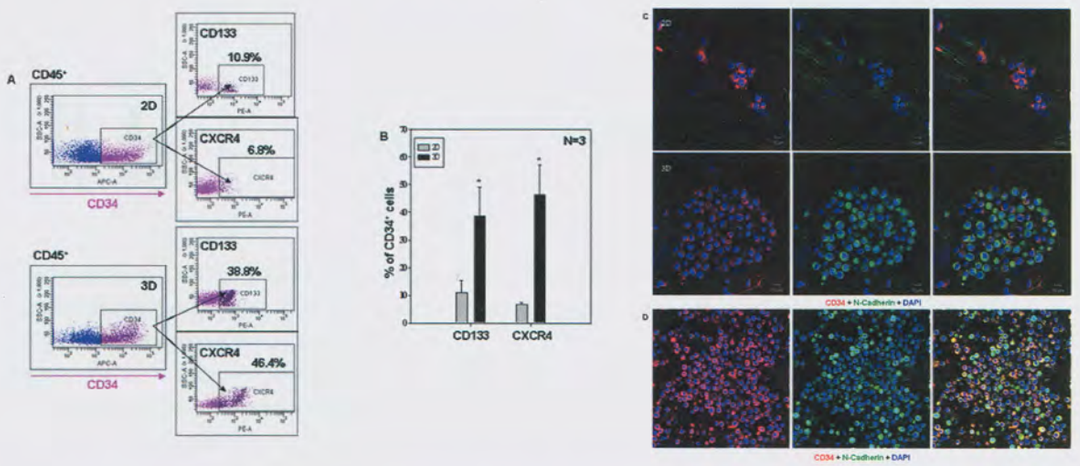


Fig. 1: 3D-MSCs foster primitive stem cell pool. **(A)** 3D-$CD34^+$ cell population contained significantly higher percentage of cells expressing primitive HSC- markers: CD133 (upper panel) and CXCR4 (lower panel) **(B)** Data from three independent experiments are depicted (N=3). **(C)** Most 3D-$CD34^+$ cells (Cy3) express N-Cadherin (FITC), albeit at variable levels. **(D)** Huge expanse of $CD34^+$N-$cadherin^+$ cells under 3D-conditions. (Tile mode, pixel size 2048 X 2048). DAPI (blue) demarcates nuclei (blue).

examined by immunofluorescence experiments, we found that most 3D-$CD34^+$ cells expressed N-Cadherin, albeit at varying levels, whereas such cells were nearly absent in the 2D-cultures (Fig. 1C-D).

3D-cultures form a functional niche: Maintaining a large pool of quiescent stem cells is another critical niche-characteristic. To examine whether the 3D cultures mimic the in vivo niche in this respect, we analyzed the cell cycle status of the output $CD45^+34^+Lin^-$ cells

from both 2D and 3D cultures. A significantly large proportion (65.56 ± 8.56 %; N=3; $p>0.01$) of the 3D-HSCs was maintained in the G0 stage of cell cycle (Fig. 2 A-B).

In order to track the division history of the 2D- and 3D-HSCs, we seeded PKH26-labelled $CD34^+$ cells in the cultures and after 7 days, tracked the division history of gated $CD45^+34^+$ Lin^- stem cell population on a flow cytometer. Results show that ~50 % of gated 3D- HSCs had retained a high PKH-fluorescence (green), and majority of them had not divided during the culture period while > 70% of their 2D-counterparts had undergone more rounds of proliferation (yellow) indicated by their low PKH26-fluorescence (Fig. 2C). Analysis of seven independent experiments (N=7) carried out with different marrow samples showed that the result was consistent and statistically highly significant (Figure 2D; 38.914± 3.705 and 16.743 ± 2.142 PKHhigh cells in 3D- vs. 2D-$CD34^+$ cells; $p<0.001$). This shows that 3D-MSCs form a functional niche and foster a significantly large pool of quiescent HSCs by actively preventing their cycling.

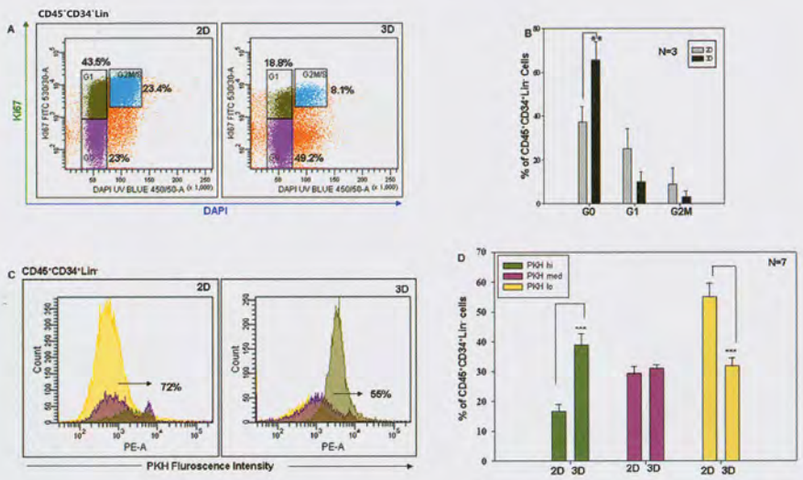


Fig. 2: HSC-quiescence is maintained in 3D-MSCs. (A) A higher % of 3D-HSCs were present in G0 state (violet color) compared to 2D-HSCs. **(B)** Data obtained in experiments performed on different marrow samples are depicted (N=3). **(C)** Division history tracking of HSCs. Data show that majority of $CD45^+34^+38$ Lin^- cells from 3D-MSCs (green histogram) did not divide during the culture period, whereas more than 70% of their 2D-counterparts (yellow histogram) had undergone proliferation. **(D)** Data from multiple experiments performed on different marrow samples shows that the observed phenomenon was consistent and statistically significant (N=7). ** $p \leq 0.01$, *** $p \leq 0.001$

Retention of HSCs via SDF1α/CXCR4 axis: The HSCs are retained in the marrow via SDF1α/CXCR4 axis. A disruption of this axis leads to an egress of the HSCs from marrow to peripheral blood circulation. The high yield of functionally and phenotypically superior HSCs from the 3D-cultures suggested that these cultures not only supported the growth of these cells, but also efficiently retained them. Earlier experiments have already shown that a higher percentage of $CD34^+$ cells expressed CXCR4. In these experiments we assessed the SDF1α expression in the MSCs by real-time PCR and immunofluorescence, and found that the 3D-MSCs expressed high level of this chemokine, at both mRNA and protein levels, as compared to 2D ones (Fig. 3 A-B). These data suggest that presence of such active chemokine axis may be responsible for a high content of HSCs in these cultures.

3D-MSCs express pro-HSC transcriptome: The BM niche is known to express some important key molecules playing critical role in the development of effective hematopoiesis: Osteopontin, Runx-2 and angiopoietin-1. It was thus imperative to

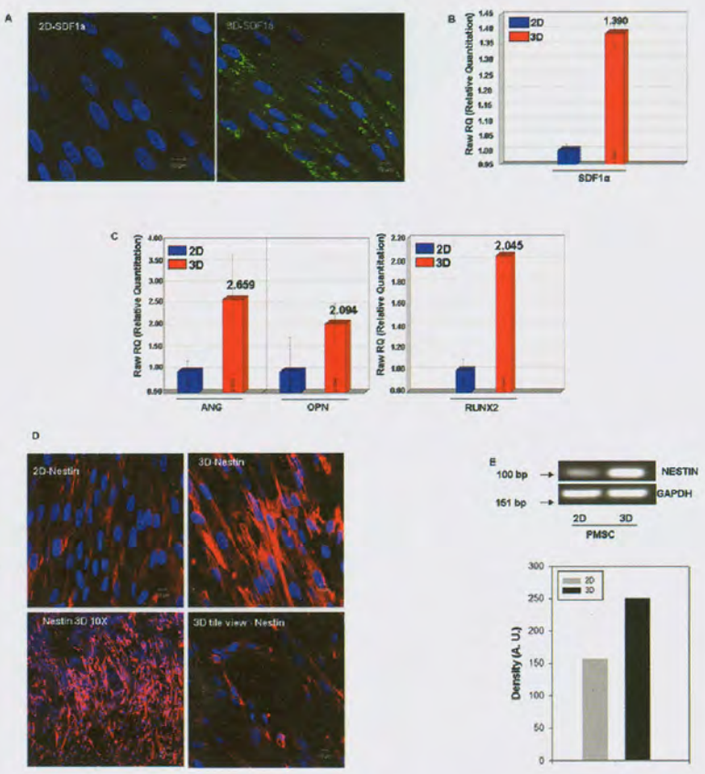


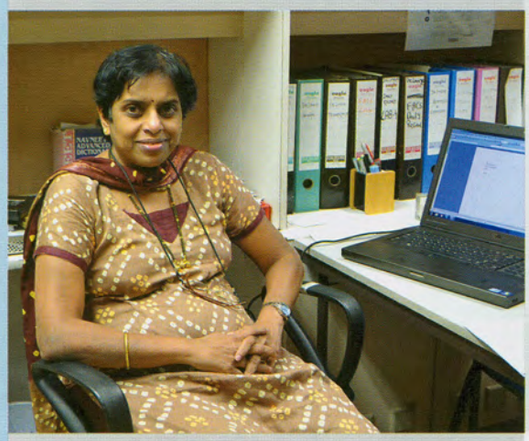
Fig. 3: 3D-MSCs retain HSCs via SDF1α-CXCR4 axis: (A) A strong intracellular fluorescence (FITC) for SDF1α is seen, indicating their potential to exert chemotactic attraction on HSCs. (B) SDF1-α is up regulated at transcriptional level in 3D-MSCs compared to 2D-MSCs. **3D-MSCs express pro-HSC transcriptome.** (C) Results of quantitative PCR experiments show 2-2.5 folds increased expression of angiopoietin-1, osteopontin and Runx-2 in 3D-MSCs. (D) 3D-MSCs are nestin-positive. 3D-MSCs exhibited a strong expression of nestin (Cy3). Both low power (10x, upper panel) and tile view (pixel size 1024X1024, lower panel) images of nestin-positive 3D-MSCs are illustrated. DAPI (blue) has been used to demarcate nuclei. (D) Semi-quantitative PCR experiments show that Nestin is transcriptionally upregulated in 3D-MSCs compared to 2D-ones.

quantify the expression of these genes in the 3D-MSCs at mRNA levels. As seen in fig. 3C, the transcripts of all these molecules were 2-2.5 folds higher in the 3D-MSCs compared to the 2D-MSCs. Nestin-positive MSCs have been recently shown to form an important HSC-niche component. We therefore examined the expression level of nestin in our cultures. As seen in the figure3 D-E, the 3D-MSCs were more strongly positive for Nestin compared to the 2D-MSCs, at both mRNA and protein levels. These results indicate that expression of pro-HSC transcriptome by 3D-MSCs contributes substantially to the maintenance of quiescent HSC-pool in these cultures.

Our data clearly show that the hydrogel-based 3D-cultures of MSCs form an in vitro counterpart of the HSC-niche and provide a physiologically relevant cellular platform to address some of the intricate issues in the niche-mediated regulation of HSCs. Besides being useful to study niche-biology, this system has several applications for translational research; the most important being its use in manipulation of stem cells in vitro for their down stream applications. Our current research is focused on creation of such "customized in vitro niches (IVNs)" to modulate stem cell functions.

Future Work

Since hydrogel-based 3D-cultures mimic the niche-physiology, we propose to harness this system to establish specialized in vitro niches (IVNs) comprising of mesenchymal cells possessing specific biochemical status - via genetic modifications or pharmacological treatments - to regulate stem cell functions for their down-stream applications.



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Studies on expansion, cryopreservation and differentiation of hematopoietic stem cells

Background

A. Generation of MSCs from cord blood and their differentiation to neural cells: Due to their self renewal and differentiation ability stem cells are being largely used in cellular replacement therapies particularly in neurodegenerative diseases. Most clinical applications of stem cells rely on development of in vitro protocols for their efficient expansion and differentiation . Although a number of investigators all over the world are involved in this type of research, the knowledge and methods of differentiating stem cells into specialized cell types is still limited. MSCs (Mesenchymal stem cells) have been derived from different sources like Bone marrow, peripheral blood, adipose tissue and umbilical cord blood. It has been described that multipotent MSCs are capable of differentiating into a variety of cell types from different germ layers.

The major advantage of UCB is the relative abundance of the source as they can be obtained easily and non invasively post delivery of new borns. Further these cells can be cryopreserved and used for transplantation even years after their harvesting. In the present proposal we are attempting to standardize culture conditions for generation of MSCs from umbilical cord blood. Once these methods are standardized then we will focus on their differentiation to neural cells. We will characterize these neural cells by using specific markers like NSE, Nestin, GFAP etc.and other functional assays.

B. Functional characterization of in vitro generated Dendritic cells: We had earlier devised a simple two step culture system for generation of DCs from UCB CD34+/MNCs. Our method uses IL-4 as one of the cytokines for DC differentiation .IL-4 has been reported to suppress PLA-1 which in turn results in reduced Arachidonic acid (AA) release from membranes. AA metabolites have important role in DC functions. Therefore we hypothesized that exogenous addition of AA in DC cultures may improve DC functions. To test our hypothesis we added AA to DC cultures at the differentiation step. The data clearly indicated that use of AA in cultures resulted in improved quality DCs.

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C. Effect of oral feeding of nutraceuticals on hematopoiesis of mice: Our earlier work had shown that inclusion of nutraceuticals belonging to the class of polyunsaturated fatty acids bring about enhancement in generation of Megakaryocytes from umbilical cord blood.We therefore hypothesized that oral feeding of mice with nutraceuticals may lead to enhanced hematopoiesis especially MK generation.

D. Ex vivo expansion of HSCs: Our earlier study revealed a novel application of two apoptotic protease inhibitors in HSPC functions and suggested that the regulation of these proteases may help in overcoming the major homing defects prevalent in the expansion cultures thereby generating a more competent graft in transplant settings.
Our findings may help in adding a further step in refining the expansion protocols to yield a graft that is more competent in stem cell transplant settings.

Aims and Objectives

1. Attempting to generate mesenchymal stem cells from Umbilical cord blood, their characterization and differentiation to neural cells.
2. Generation of DCs with or without Arachidonic acid addition at the differentiation step.
3. Effect of oral feeding of nutraceuticals on hematopoiesis of mice.
4. Use of antiapoptotic agents in expansion of HSCs in stromal based cultures.

Work Achieved

MSC generation: We attempted to generate DCs from various fractions of cord blood i.e MNCs , CD34 negative population, and cord tissues. Out of several samples cultured we could generate MSCs from 3 samples. These were characterized by typical spindle shaped fibroblastic morphology (Fig.1a). Phenotype analysis on Flow (Fig.1b) and immunofluorescence staining and imaging by pathway image analyzer (Fig.1c) show that

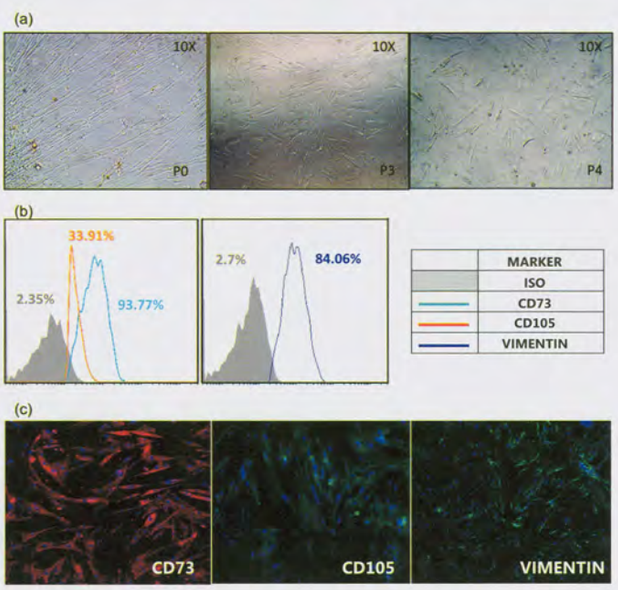


Fig. 1: MSCs generated from MNCs of human umbilical cord blood: (a) Phase contrast micrograph showing typical fibroblastic morphology in different passages; Expression profile of MSCs stained for antihuman monoclonal antibodies CD73, CD105, Vimentin.by **(b)** flow cytometry and by (c) BD Pathway image analyzer with 20x objective.

the cells expressed MSC markers like CD73,CD 105 and Vimentin. We are trying to further optimize the culture conditions and further characterize the MSCs by detecting expression levels of specific transcription factors.

DC generation: We have shown that inclusion of Arachidonic acid in DC cultures during differentiation step of our two step culture method results in improved in vitro functions of DCs. Data is depicted in Fig.2. DCs cultured in both sets showed typical veiled morphology (Fig.2a). The supernatants of arachidonic acid-DCs showed enhanced IL12 and reduced IL10 secretion as compared to control (Fig.2b). The in vitro migratory potential towards CCL19 was also improved in AA set (Fig.2C). When Mixed lymphocyte reaction of the AA -DCs was compared with the control DCs we observed significant increase in T cell proliferation at every target effector ratio tested (Fig.2d). Taken together the data show that addition of AA at the differentiation step results in superior quality of DCs especially with respect to key functions required for their use as anticancer vaccines like migration, antigen uptake, MLR and cytokine secretion.

Nutraceutical feeding: Our preliminary data shows that when mice are oral fed with DHA for ten days there is enhanced hematopoiesis in general and megakaryopoiesis in particular. The DHA fed mice demonstrated almost two fold increase in their early stem cell population as assessed by Side population (SP) analysis (Fig.3a). Similarly the platelet count in the peripheral blood was also higher as compared to PBS fed mice (Fig.3b). There was increase in number of CFU MK cells in Bone marrow of DHA fed animals as seen in Fig.3c. However there was marginal difference in CFU nos. of the two groups of animals (Fig.3d). The preliminary results suggest that oral feeding of mice with DHA promotes hematopoiesis and Megakaryopoiesis. However, we are validating the data further by carrying out long term feeding experiments as well as by using other nutraceuticals belonging to the family of omega 3 and omega 6.

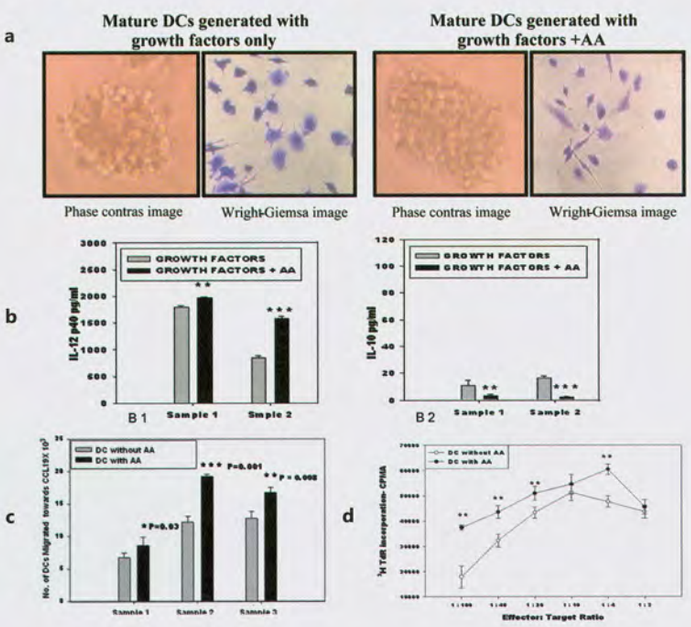
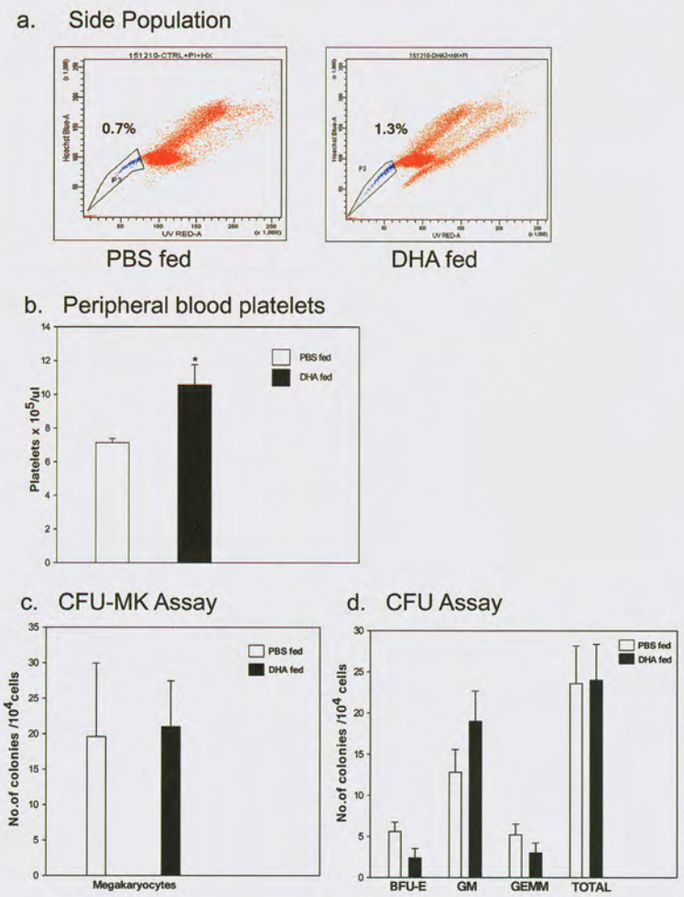


Fig. 2: Dendritic cells generated from MNC with and without Arachidonic Acid as additive at the differentiation stage: a) Phase contrast images and Wright-Giemsa stained smears show clusters of mature DCs and typical dendritic morphology in both sets. b) Detection of IL-10 and IL-12 in the culture supernatants of DCs show that AA DCs had higher levels of b1) IL12 and lower levels of b2) IL10 c) DCs with AA showed more efficient migration towards CCL-19 and d) better T-cell stimulation as seen in MLR assay.

Fig. 3: Oral feeding of mice with Docosahexaenoic acid (DHA):DHA fed mice showed enhanced generation of **(a)** side population cells **(b)** peripheral blood platelets and **(c)** CFU MK colonies in bone marrow **(d)** Progenitor content assessed by CFU assay however showed marginal difference between PBS fed and DHA fed mice.



Ex vivo expansion of HSCs: In our earlier studies we have clearly shown that inclusion of antiapoptotic agents in suspension cultures results in improved expansion of umbilical cord blood derived $CD34^+$ cells. Now we are trying to understand how the caspase and calpain inhibitors influence the stromal mediated expansion of UCB $CD34^+$ cells. These studies will be carried out using a coculture system of stromal cells and cord blood derived $CD34^+$ cells.

Future Work

1. Optimizing characterization, cryopreservation and differentiation of MSCs
2. In vitro and in vivo CTL assays using DCs
3. Feeding of animals with other polyunsaturated fatty acids and testing the effect on hematopoiesis/megakaryopoiesis.
4. Optimizing expansion protocols with stromal cultures and with use of anti-apoptotic agents.

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Assessing stemness and cell cycle properties in embryonic stem cells

Background

Stem Cells are bestowed with two of the most important characteristics such as self-renewal and differentiation. While embryonic stem cells (ESCs) display unlimited self-renewal, and they neither enter a quiescence state nor undergo senescence like the adult cells, the stem cells from adult (ASCs) have limited self-renewal potential. Strikingly though, the cancer stem cells (CSCs), which are presumed to be underlying the genesis of cancer, are basically the quiescent stem cells in adult that attain a cancerous state upon mutation, undergoing uncontrolled proliferation and growth without differentiation. Although ESCs share a couple of genes with CSCs, they differ in their cell cycle properties. While tightly regulated cell cycle machinery remains operational in case of both ESCs and ASCs, the same in cancer causing CSCs is deregulated. Furthermore, the supposedly homogenous population of ESCs, that basically undergo symmetric division unlike tissue specific progenitors, often exhibits certain levels of heterogeneity during maintenance in terms of colony morphology and size. Hence, the question arises what entails this heterogeneity in ESCs and do they accommodate different sub-populations ranging from true unspecified stem cells to early differentiating ones, despite being maintained under defined conditions to retain their undifferentiated phenotype? Further, do they also exhibit the side population (SP) phenotype similar to ASCs and CSCs and if so, what is the functional significance of the same? Needless to mention, the SP phenotype representing the stem cells/progenitors has been detected in a number of adult tissues and organs where even a single cell carries the repopulation ability within. Accordingly, we were interested in a comparative investigation of the cell cycle properties of murine ESCs during maintenance and differentiation and that of ASCs and CSCs, and deducing how it would correlate with the heterogeneity index and the stemness attributes. This may eventually provide clues to understanding the basis for ESCs induced teratoma/tumor formation.

Aims and Objectives

1. The maintenance of ESCs in undifferentiated state and assessing their stemness and pluripotent characteristics both in vitro and in vivo.

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2. Identification and purification of side population (SP) cells from ESCs and their characterizations.
3. Understanding the functional significance of the SP phenotype in ESCs and the underlying molecular basis.
4. Investigating the cell cycle properties of ESCs under maintenance and differentiation conditions and comparing the same with SP and non-SP cells from ESCs and CSCs.

Work Achieved

Identification and characterization of SP cells: Maintenance of ESCs in undifferentiated state is one of the vital requisite because of its subsequent repercussion on retention of pluripotency. Incidentally, ESCs despite being maintained under maintenance conditions display certain degree of heterogeneity in terms of colony size and morphology suggesting the possibility of asynchrony in either in their cell cycle pattern (doubling time) or development per se. Accordingly, we intended to verify whether ESCs, the supposedly homogenous cell type, include any SP phenotype and if so, how different or similar these ESCs derived SP and non-SP groups are in terms of their stemness. The ABCG2 expression, the characteristic of SP phenotype was quite low in undifferentiated ESCs as monitored by RT-PCR and immunocytochemical (Fig. 1) analysis. Strikingly, we could detect ~4-10% SP cells by FACS based on their ability to efflux Hoechst (Fig. 2A). However, ESCs exhibited higher ABCG2 expression upon over-expression of ABCG2 cDNA in the generated stable clones and the SP cells (~10-18%) were comparatively higher in those clones. Further, the SP and non-SP (MP) cells were purified separately by FACS and maintained in culture to monitor their characteristics during propagation and differentiation. The growth and morphology of sorted SP cells was comparable with parental ESCs during initial plating. Those also retained the undifferentiated ESC markers, Oct4 and Sox2 at a higher intensity compared to MP cells. Interestingly, the SP cells after a few generations when monitored for Hoechst efflux potential further gave rise to both SP

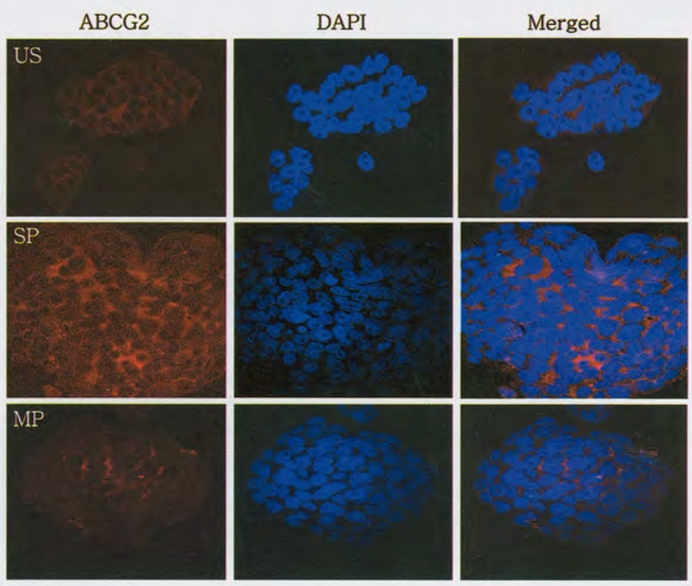


Fig. 1: Immuno-stained pattern showing expression of ABCG2 in ESCs derived SP cells that remained insignificant in MP cells. Top row represents unsorted (US) ESCs.

and MP phenotypes (Fig. 2B) indicating the preponderance of MP phenotype over the SP one. However, MP cells did not exhibit any SP phenotype following propagation (Fig. 2C) thereby suggesting the existence of subtle differences between these two groups. Detailed characterizations of the SP cells are ongoing both during passaging and differentiation in comparison with the MP group to assess the differences, if any, in their pluripotent characteristics both in vitro and in vivo and in view of deciphering the functional corollary of SP phenotype in ESCs.

Investigation of cell cycle properties: Since, ESCs have intrinsic ability of indefinite self-renewal compared to other cell types including ASCs, it is obvious that there exists a tightly regulated cell cycle machinery for them. While LIF supplementation to the medium facilitates ESCs maintenance in undifferentiated state, withdrawal of LIF leads to differentiation induction. Our earlier study has also revealed that Wnt activated conditions help in ESCs maintenance in long term culture in the medium devoid of LIF. Though the cells maintained under Wnt activated conditions retain all the undifferentiated ESCs characteristics such as expression of Oct4 and Nanog in them and retention of pluripotency both in vitro and in vivo, they exhibit a slower growth rate compared to LIF maintained ones. This prompted us to investigate their cell cycle properties. Accordingly, we analyzed the cell cycle pattern in ESCs maintained under both LIF and Wnt activated conditions and further compared the same under LIF deprived differentiation conditions (Fig. 3). Our data revealed a majority of the population remaining in S-phase with relatively small G1 and G2 on day 1 of culture, irrespective of the conditions used. This suggested that the cells were actively cycling. On day 2 too, maximum cells were seen residing in S phase of the cell cycle. Interestingly, on day 6 the G1/G2 ratio in LIF maintained cells was ≤ 1, while cells in -LIF and DMSO had G1/G2 >2 indicating the cells were differentiating in the latter case, correlating with the differentiated phenotype seen in them. However, the BIO maintained cells showed

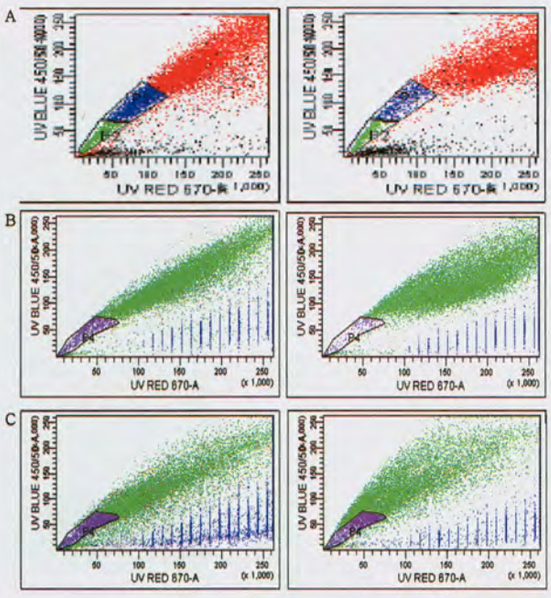
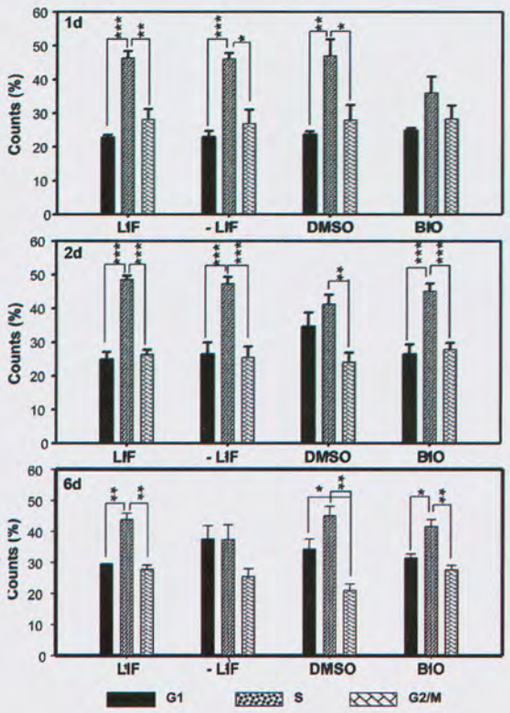


Fig. 2: (A) Detection of SP cells in murine ESCs (Left) and the same was inhibited with verapamil (Right). (B, C) Reappearance of SP phenotype in ESCs isolated SP cells following propagation in vitro (B), whereas no distinct SP phenotype was detected with isolated MP cells under the same condition (C).

Fig. 3: Cell Cycle pattern in ESCs maintained under various conditions.



intermediate G1/G2 values that remained >1. Overall, BIO and LIF maintained ESCs demonstrated a similar cell cycle pattern thereby suggesting that Wnt signaling activation could also help in ESCs maintenance even though not to the extent that can be seen with LIF. Further, we monitored the expression pattern of specific cell cycle regulators (check point genes) such as Cyclin D1, Cyclin D3, Cyclin A1, Cyclin B1, Cyclin E1, p21 and p27 in either LIF or Wnt maintained ESCs. While Cyclin D1, p21, and p27 expressions were less, higher expression of Cyclin B1 and Cyclin E1 was seen in the undifferentiated ESCs compared to the differentiated ones seen under the –LIF and vehicle control conditions. However, Cyclin A1 expression remained undetectable in them. Immunocytochemical analysis also demonstrated the expression of Cdk2, and Cdk4 in ESCs. While Cyclin D1 levels remained low in both BIO and LIF maintained conditions, an increase in its expression was seen during differentiation. The same was also true for Cyclin D3. Hence, these two cell cycle modulators among the tested ones were presumed to be the key players controlling cell cycle parameters in ESCs, the differential expressions of which tilt the balance towards either maintenance or differentiation. Attempt has also been made to assess the cell cycle properties in both SP and MP cells in order to verify any differences between these two groups and whether or not any parallel can be drawn between the cell cycle parameters and the phenotypes. However, further investigations are underway to draw a conclusive picture.

Future Work

The knowledge gained would be further utilized to investigate the significance of SP phenotype, their loss during propagation and correlation, if any, with tumorigenicity.

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Translational regulation of insulin mRNA

Background

Pancreatic β cells regulate insulin production to control blood glucose levels. These cells contain a large pool of cytoplasmic insulin mRNA (10–15% total mRNA), which is translationally quiescent at hypoglycemic (<3 mM glucose) concentrations. Recruitment to polysomes and activated translation of this mRNA occur in response to higher glucose levels, leading to about 50-fold increase in insulin biosynthesis within an hour. The level of insulin mRNA does not alter significantly during this period of glucose stimulation and transcription inhibitors do not affect this early increase in insulin biosynthesis, indicating the predominance of the posttranscriptional events in enhancing insulin biosynthesis.

Glucose induced translation of insulin in pancreatic beta cells is mediated by the 5'UTR of insulin mRNA. We have previously reported the minimal sequence/structure in the 5'UTR of rat insulin gene1 required for this regulation. We had shown that specific factors in the pancreatic islets bind to the 5'UTR of the insulin mRNA upon glucose stimulation. A minimal 29-nucleotide element in the 5'UTR was shown to be sufficient for the glucose mediated translation activation of insulin mRNA. Using RNA affinity pull down method, we isolated and identified the 5'UTR binding protein, as protein disulfide isomerise (PDI). We show that both in vitro and in vivo PDI can specifically associate with the 5'UTR of insulin mRNA. The Ins-5'UTR binding activity of PDI was confirmed further using a yeast three hybrid assay as well as RNA immunoprecipitation assay. Further, immunodepletion of PDI from the islet extract results in loss of glucose stimulated translation, indicating a critical role for PDI in insulin biosynthesis. Transient over-expression of PDI resulted in specific translation activation by glucose. We show that the RNA binding activity of PDI is mediated through PABP. PDI catalyses the reduction of PABP disulfide bond resulting in the specific binding of PABP to insulin 5'UTR. We also show that glucose stimulation of the islets results in activation of specific kinase that can phosphorylate PDI. These findings identify PDI and PABP as important players in glucose homeostasis. Further characterization of the interaction of PDI with basal translation machinery is in progress.

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Aims and Objectives

1. Isolation and characterization of the insulin mRNA UTR binding protein and its partners.
2. To understand the basic mechanism of translational regulation of insulin mRNA and the role of RNA binding proteins in this regulation.

Work Achieved

Major regulation of insulin biosynthesis occurs at the secretion and the translational level in β-islet cells. Glucose stimulates the beta cells to increase the translation of insulin mRNA but the mechanism is not completely understood. The UTR of the insulin mRNA is thought to be essential for this regulation. Rat insulin mRNA has a 57 base 5'-UTR. We synthesized radio-labeled wild type rat insulin 5' UTR and performed the RNA gel shift assay using RIN cell extracts to identify specific RNA-protein complexes. A similar specific complex was also formed with extracts from rat pancreatic islets. The complex formation was induced by high glucose treatment of the islets. We have previously identified a minimal 29 nucleotide element that is necessary and sufficient for the complex formation. Our analysis also revealed that the predicted stem loop structure is important for the complex formation. We synthesized biotinylated insulin 5'UTR and purified the binding factor(s) associated with the RNA. The insulin 5'UTR mRNP was isolated by RNA-affinity chromatography using biotinylated 5'UTR element as ligand. Protein Disulfide Isomerase (PDI) was identified as one of the proteins and was confirmed by Western analysis of the eluates. We performed RNA-EMSA in the presence of specific PDI antibodies to show that the complex formed is specific for PDI. We also showed the insulin 5'UTR specific RNA binding activity of PDI in yeast three hybrid assay and RNA immunoprecipiotation. The functional role of PDI as insulin 5'UTR specific translation activator was confirmed by the inhibition of the glucose stimulated translation activation by anti PDI antibody. We further showed that recombinant PDI was able to neutralize the anti-PDI antibody, indicating the specificity of the antibody for PDI. However bacterially expressed recombinant PDI was unable to bind to insulin 5'UTR and cause translation regulation suggesting a role for post translational modification.

Our previous experiments have shown that PDI is one of the UTR binding proteins in rat pancreatic extract. A role of PDI in translation regulation has been reported in plant system where PDI associates with the PsbA RNA and regulates the translation in response to light (22). The nuclear encoded PDI is localized to the chloroplast and associates with PABP and modulates its binding to the 5'UTR of PsbA mRNA. PDI alters the redox status of cytoplasmic PABP thus modulating its ability to associate with the 5'UTR of PsbA RNA. A similar role for PDI in regulating the translation of insulin mRNA could be envisaged, like the light regulated PsbA expression, translation of insulin mRNA is also increased very rapidly by several fold in response to glucose.

PABP binds specifically to insulin 5'UTR

We analyzed insulin 5'UTR binding activity of PABP by RNA-EMSA using bacterially expressed recombinant PABP. We expressed His tagged PABP in E.Coli and purified the

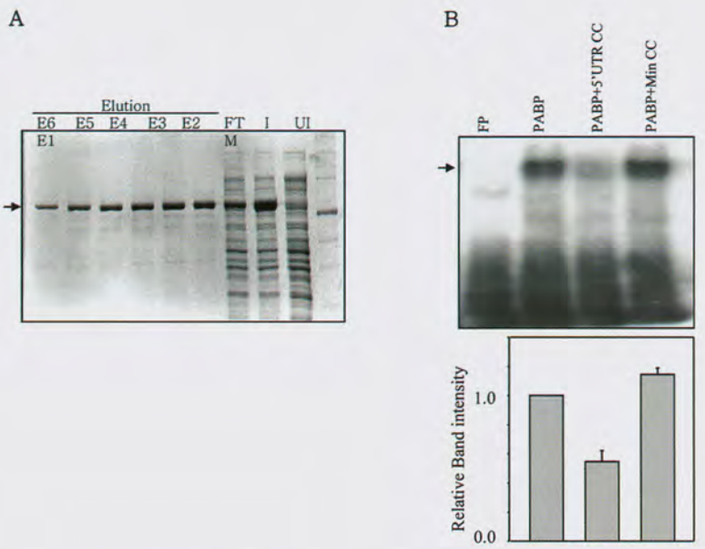


Fig. 1: Recombinant PABP binds to the insulin 5'UTR (A) PABP was cloned into pET28 vector and was expressed in E. coli as a His tag fusion protein. Protein was induced by the addition of 1 mM IPTG. The samples loaded are M; Molecular Weight Marker, UI; uninduced soluble E.coli lysate, I; induced lysate, FT; unbound Flow through from the Ni-NTA column, E1-E6 Elutions with 200 mM immidazole, The arrow indicates the recombinant PABP protein of about 70 KDa (B) Competitive RNA-EMSA was performed as mentioned in the methods. The sample in each lane is as indicated. Radio-labeled insulin 5'UTR (FP) was incubated with bacterially expressed recombinant PABP (100 ng) in presence of unlabeled insulin 5'UTR or mutant 5'UTR RNA as competitor. The gel shifted bands were quantitated densitometrically, and expressed with respect to only PABP without competitor set to 1 (Lower Panel). The graph represents the average of three independent experiments.

recombinant protein by Ni-NTA agarose beads (Fig 1A). The purified recombinant PABP was assessed for its RNA binding ability by RNA EMSA assay. The recombinant PABP was able to bind specifically to insulin 5'UTR (Fig 1B). The complex was competed out by unlabelled insulin 5'UTR but not by the mutant non binding insulin 5'UTR. These results indicate that PABP can specifically bind to 5'UTR of insulin mRNA and can potentially regulate its translation in response to altered levels of glucose.

RNA binding activity of PABP is regulated by oxido-reduction of disulfide bonds

We also assessed the regulation of the insulin 5'UTR RNA binding activity of PABP by oxidation/reduction of disulfide bonds. Recombinant PABP was oxidized with 3 mM DTNB and the binding to insulin 5'UTR was assessed (Fig 2A). Oxidation of cysteines in PABP by DTNB causes loss of binding to insulin 5'UTR, that can be restored by the addition of excess of DTT. Similar results were obtained with the islet extract (Fig 2B). The RNA binding activity of PABP can be enhanced by the addition of limiting amounts of DTT and recombinant PDI. We also assessed the modifications to PABP by DTNB by resolving the oxidized products in acid urea gels (Fig 2C), suggesting that that PABP after modification by DTNB has decreased mobility in acid urea gels and the mobility is restored upon reduction with DTT/PDI. These results suggest that like in plant chloroplast system the RNA binding activity that is responsible for translation regulation is predominantly through PABP and PDI rearranges/reduces the disulfide linkages to regulate the RNA binding activity.

Our experimental results indicate that PDI functions as an activator of translation and glucose stimulation of the cells might activate the enzymatic action of PDI by stimulating its phosphorylation. Activated PDI interact with PABP and rearranges/reduces the disulfide bonds of the PABP resulting in specific association with insulin mRNA (Fig 3). PDI-PABP may also increase the rate of ribosome recycling by promoting the interaction

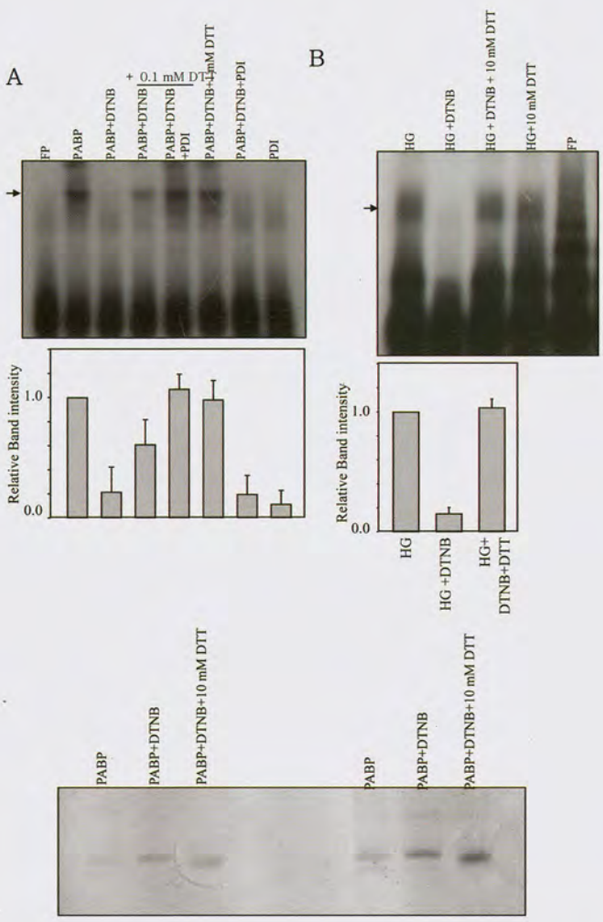


Fig 2: Oxido reduction of PABP regulates its binding to insulin 5'UTR **(A)** RNA-EMSA was performed to assess the role of free cysteines in the RNA binding activity. Radio-labeled insulin Con1 probe was incubated with PABP, PABP oxidized with DTNB, and the oxidized PABP that has been reduced with varying amounts of DTT or DTT and PDI (200 ng). The gel shifted bands were quantitated densitometrically, and expressed with respect to only PABP without competitor set to 1 (Lower Panel). The graph represents the average of three independent experiments and the error bar indicates the standard error of mean calculated by sigma plot. **(B)** Radio-labeled Con1 insulin 5'UTR was incubated with islet extracts, extracts that have been oxidized with DTNB, and the oxidized extracts that has been reduced with DTT. The gel shifted bands of HG, HG-DTNB and HG+DTNB+DTT were quantitated densitometrically, and expressed with respect to HG extract set to 1 (Lower Panel). The graph represents the average of four independent experiments and the error bar indicates the standard error of mean calculated by sigma plot. **(C)** Oxidation of PABP monitored by Acid Urea Gels. PABP was oxidized by DTNB and then later reduced with 10 mM DTT. 2µg of oxidized and/or reduced PABP were resolved on the 6% Acetic acid Urea PAGE gel to asses the oxidation status of PABP. Electrophoresis was carried out at 50 volts for 14 hrs at ambient temperature. Proteins were visualized by staining the gels with Coomassie Brilliant Blue.

between the 5' and 3' termini through its interaction with other translation factors resulting in the observed cooperativity between the 5' and 3' UTRs of insulin mRNA during glucose stimulation. PDI can also function as a chaperone for the nascent insulin peptide that emerges from the translating ribosome thereby increasing the effective insulin levels.

Conclusions and Future Work

Although the mechanism of how the binding of PABP to the insulin 5'UTR activates translation is yet to be elucidated, but it is reasonable to expect that PABP can recruit the eIF4F complex to insulin mRNA 5'-terminal resulting in increased translation. The mechanistic details of glucose-mediated insulin translation regulation have yet to be worked out. The mechanisms that underlie nutrient-induced translational regulation of insulin biosynthesis are likely to be crucial for understanding wider aspects of β-cell physiology and metabolic homeostasis, because this is the major control of insulin production in mammals under normal physiological conditions. Indeed, there is dysregulation of insulin biosynthesis in an animal model of type II diabetes, which

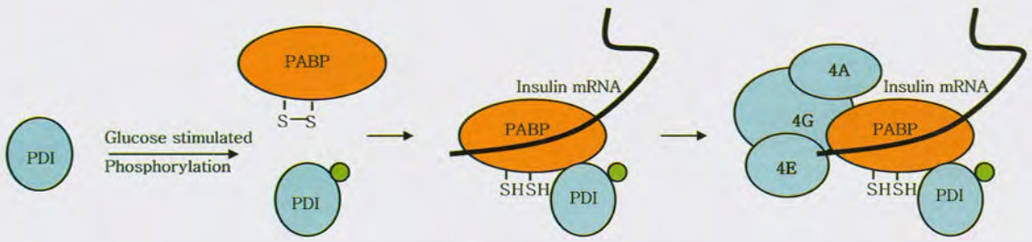
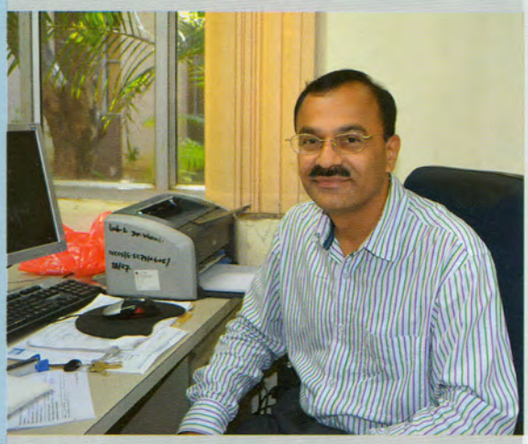


Fig. 3: Mechanism of the PABP and PDI mediated glucose induced translation of insulin

contributes to cell dysfunction and decreased availability of insulin. We have identified a protein that binds to the insulin 5'UTR and regulates its translation in response to glucose. The specific post translation modification that is responsible for the regulation of insulin translation has been described here. We plan to further characterize the 5'UTR complex and its interaction with the basic translation machinery of the cell.



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Studies on role of IL-3 on regulation of osteoblast differentiation from human mesenchymal stem cells

Background

Bone homeostasis and skeletal integrity is maintained by the co-ordinated activity of bone forming osteoblasts and bone resorbing osteoclasts. The increase in number and activity of osteoclasts, and decrease in number, activity and life span of osteoblasts results in bone loss in osteoporosis. Therefore, the regenerating potential of adult human bone is limited in osteoporosis and other bone diseases. Osteoblasts differentiate from mesenchymal stem cells (MSCs) that also give rise to adipocytes and chondrocytes. We previously reported that IL-3 is a potent inhibitor of mouse osteoclast formation and pathological bone resorption. IL-3 also prevents the development of inflammatory arthritis in mice, and protects cartilage and bone destruction in the joints. Recently we have reported that IL-3 inhibits human osteoclast formation from peripheral blood monocytes and bone marrow cells of osteoporotic individuals. These results strongly suggest that IL-3 is a potent inhibitor of osteoclast formation and bone resorption. However, the role of IL-3 on osteoblast differentiation and bone formation is not yet delineated. In this study we investigated the role of IL-3 in human osteoblast differentiation using purified population of bone marrow-derived MSCs.

Aims and Objectives

1. To investigate the role of IL-3 in regulation of osteoblast differentiation from human mesenchymal stem cells.
2. To study the effects of IL-3 on in vivo bone formation in mice.

Work Achieved

IL-3 enhances osteoblast differentiation of human MSCs: To examine the effect of IL-3 on osteoblast differentiation human MSCs were incubated for 21 days with osteogenic medium and different concentrations of IL-3. Cells were stained with Alizarin Red S for assessment of bone matrix synthesis. We found that IL-3 in a dose-dependent manner increased bone matrix mineralization (Fig. 1A). Osteoblasts express alkaline phosphatase (ALP), collagen type I (Col-1), osteocalcin (OCN), and osteopontin (OPN) at different

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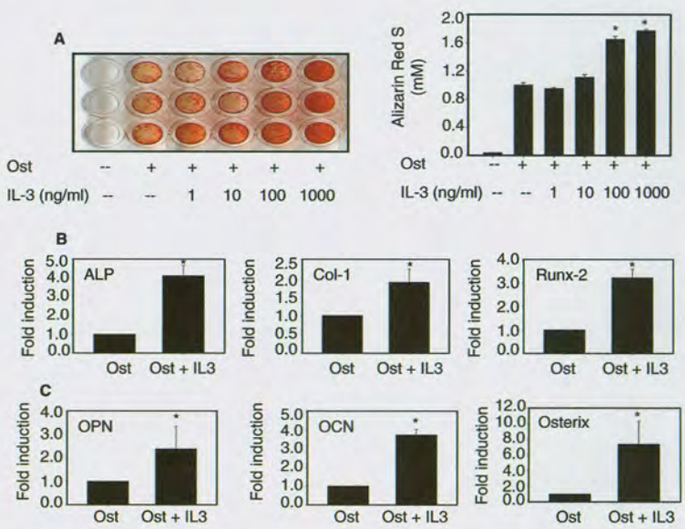


Fig. 1: IL-3 enhances osteoblast differentiation in human MSCs. (A) Human MSCs were incubated for 21 days with or without osteogenic medium and in the absence or presence of different concentrations of human IL-3. The matrix mineralization was visualized by Alizarin Red S staining. Quantitative analysis of Alizarin Red S staining was done using osteogenesis quantitation kit. Data is representative of four independent experiments. * $p \leq 0.05$ vs osteogenic control. (B and C) Human MSCs were incubated for 4 and 14 days in osteogenic medium in the presence or absence of IL-3 (100 ng/ml) and subjected to quantitative real-time PCR analysis. Fold induction of ALP, Col-1 and Runx-2 genes was analyzed after 4 days and expression of OCN, OPN and OSX genes was analyzed after 14 days. Data is representative of two independent experiments. * $p \leq 0.05$ vs osteogenic control.

stages of differentiation. The effect of IL-3 on osteoblast specific markers was analyzed by culturing human MSCs for 4 and 14 days with osteogenic medium in the absence or presence of IL-3 (100 ng/ml). Quantitative real-time PCR analysis revealed that IL-3 increases expression of ALP and Col-1 on day 4, and OPN and OCN on day 14 (Fig. 1B and C). IL-3 also increased expression of transcription factors Runx-2 on day 4 and osterix on day 14. These results suggest that IL-3 increases osteoblast differentiation and matrix mineralization in human MSCs. Next, human MSCs were incubated in osteogenic medium in the absence or presence of IL-3 (100 ng/ml) for 4, 7, 12 and 14 days and ALP activity in cell lysate was determined by colorimetric assay. IL-3 treatment significantly increased ALP activity at all the time points. Increased synthesis of OCN protein was also confirmed by quantitative analysis using ELISA.

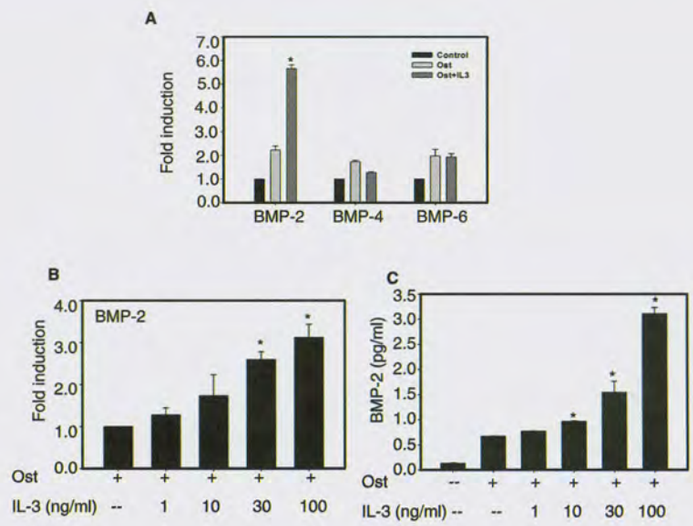


Fig. 2: IL-3 enhances osteogenesis through increase in BMP-2 expression. (A) Human MSCs were incubated for 4 days with or without osteogenic medium in the presence or absence of IL-3 (100 ng/ml). The cells were subjected to quantitative real-time PCR analysis for the expression of BMP-2, BMP-4, and BMP-6. Data is representative of two independent experiments. * $p \leq 0.05$ vs osteogenic control. (B and C) Human MSCs were incubated for 4 days in osteogenic medium with or without different concentrations of IL-3 and expression of BMP-2 was analyzed by quantitative real-time PCR. The culture supernatants were also subjected to BMP-2 ELISA. Data is representative of two independent experiments. * $p \leq 0.05$ vs osteogenic control.

IL-3 increases osteoblast differentiation through bone morphogenetic protein (BMP)-2: To further investigate the mechanism(s) of IL-3 action on osteoblast differentiation we examined the regulation of various BMPs by IL-3. BMPs including BMP-2, BMP-4 and BMP-6 are known to induce osteoblast differentiation. Therefore, the effect of IL-3 on gene expression of these BMPs was examined by quantitative real-time PCR. MSCs were incubated for 4 days in osteogenic medium in the absence or presence of IL-3 (100 ng/ml). IL-3 significantly increased the mRNA expression of BMP-2, however, showed no effect on BMP-4 and BMP-6 (Fig 2A). We found that IL-3 increased BMP-2 expression in a dose-dependent manner (Fig. 2B). Interestingly, IL-3 also increased secretion of BMP-2 protein in a dose-dependent manner (Fig. 2C). To check whether IL-3 is responsible for the increased expression of BMP-2, MSCs were incubated in osteogenic medium with or without IL-3 (100 ng/ml) in the absence or presence of different concentrations of IL-3 neutralizing antibody. We found that addition of neutralizing antibody to IL-3 abolished the inductive effect of IL-3 on BMP-2 gene expression. Our results suggest that IL-3 may be useful to induce bone formation through enhancement of BMP-2 expression.

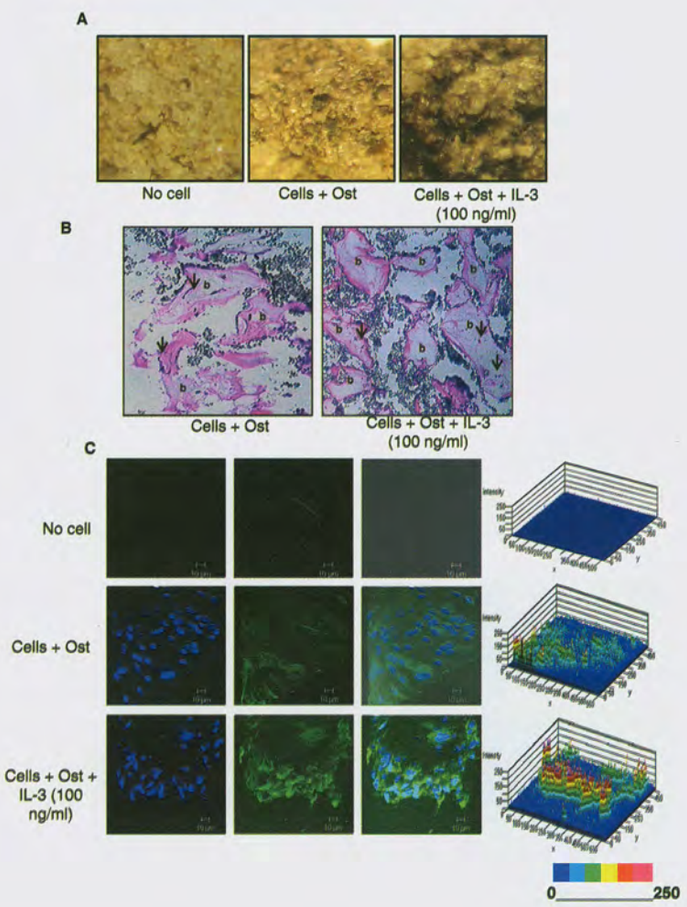
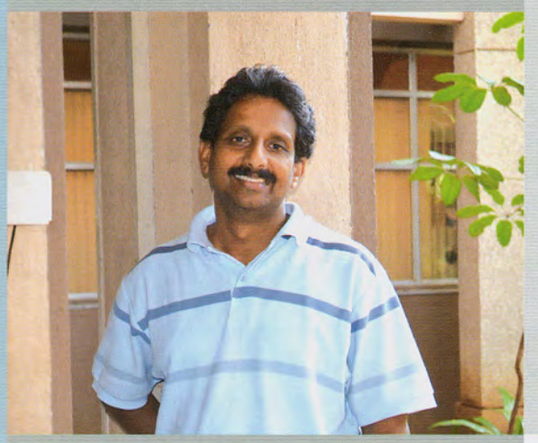


Fig 3: IL-3 augments in vivo bone regeneration capacity of human MSCs. Human MSCs seeded biografts were incubated for 10 days with osteogenic medium in presence or absence of IL-3 (100 ng/ml) and implanted subcutaneously into immunocompromised mice (n = 3 mice per group). Unseeded biografts were used as control implants. (A) After 10 weeks implants were recovered and stained for calcium deposition by von Kossa. (B) Histological sections of biografts were stained with H&E to examine in vivo bone formation. In the photographs, "b" indicates newly formed bone, black arrow indicates matrix embedded osteocytes. (C) Synthesis of OCN was detected by immunostaining of implants by antibody against human OCN. The stained implants were observed as Z-stack and all stacks were merged. Magnification, 63X. Similar results were seen in two independent experiments.

IL-3 augments in vivo bone regeneration: Since IL-3 significantly increased osteoblast differentiation and bone formation in vitro, we further examined the in vivo potential of IL-3 to induce bone formation using ectopic bone formation assay. The hydroxyapatite / tricalcium phosphate biografts were seeded with human MSCs and incubated for 10 days with osteogenic factors with or without IL-3 (100 ng/ml). These biografts were implanted subcutaneously into the immunocompromised mice. After 10 weeks the implants were harvested and examined for formation of mineralized bone matrix. von Kossa staining of biografts demonstrated significant increase in bone matrix synthesis by IL-3 (Fig. 3A). Histological examination of the explanted biografts showed that MSCs induced formation of bone in which osteocytes were embedded in mineralizing extracellular matrix, and in the presence of IL-3 significant increase in bone formation was observed (Fig. 3B). The biografts were also stained for human OCN antibody and analysed by Z stacking under the confocal microscope. The semiquantitative analysis revealed the increased human OCN deposition by IL-3 (Fig. 3C). All these results suggest that IL-3 has a potential to stimulate in vivo bone regeneration capacity of human MSCs.

Future Work

We plan to investigate the role of IL-3 on migration and wound healing abilities of MSCs.



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Dishevelled negatively regulates stress granule assembly

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Background

Stress granules (SGs) and Processing bodies (P bodies) represent mRNA-containing, non-membranous, dynamic cytoplasmic foci defined by the presence of specific and common protein components. Although P bodies are present at the stationary phase, SGs are generally assembled during diverse stress conditions, as a result of aggregation of mRNAs stalled in a translational pre-initiation complex, mediated by a set of RNA-binding proteins such as G3BP and TIA-1. The actively translating mRNAs (polysomes) are present mostly diffused in the cytoplasm. Although polysomes, P bodies and SGs represent mRNAs associated with different sets of cellular proteins present at distinct cytoplasmic locations, recent data suggests that the mRNAs and protein components may shuttle between these three pools. Thus, SGs are thought to be sites for mRNA triage, as mRNAs could be further sorted into P bodies for degradation, returned to cytoplasm for translation re-initiation or stored in the SGs. Although these studies highlight the importance of mRNA regulation for achieving cell homeostasis, the mechanism by which cells modulate the assembly/disassembly of these mRNA containing structures and determine the fate of individual mRNA in a given situation far from clear.

Wnt signaling is a conserved pathway that regulates cell fate determination, cell proliferation and cell polarization during growth and development of multi-cellular organisms. Wnt pathway is broadly categorized into canonical or non-canonical, based on their dependence on β-catenin for the signaling. In canonical Wnt signaling, β-catenin is stabilized upon binding of Wnt to the cognate receptors, essentially by inhibiting the degradation complex through a Dishevelled (Dvl)-dependent manner. The accumulated β-catenin then activates transcription of Wnt responsive genes in the nucleus. Non-canonical signaling primarily involves regulation of cytoskeleton dynamics by Dvl-dependent activation of Rac and Rho. Alternatively, Wnt could activate Ca^{2+} signaling, which also requires Dvl. Despite its demonstrated role in diverse cellular processes including Wnt signaling, the molecular function of Dvl is largely unknown. Mammalian cells express three isoforms of this protein, namely Dvl1, Dvl2 and Dvl3. An interesting feature of overexpressed Dvl is its ability to form cytoplasmic puncta, the nature of which

remains mysterious. Endogenous Dvl also localizes to cytoplasmic granules. However, overexpression and live imaging studies have shown that these cytoplasmic puncta are Dvl protein assemblages, which are in dynamic equilibrium with the cytoplasmic Dvl pool. Importantly, these structures are non-membranous and do not represent any known cytoplasmic vesicle compartments.

Owing to the structural similarities between Dvl and SGs/P bodies, we wished to test the hypothesis that the Dvl puncta represent either SGs or P bodies.

Aims and Objectives

1. Does Dvl represent stress granules or P bodies?
2. What is the connection between Dvl and mRNA metabolism?
3. Is Wnt signaling involved in mRNA metabolism?
4. What could be the mechanism and physiological relevance?

Work Achieved

Endogenous Dvl2 localizes to SGs, but not to P bodies: To investigate if the cytoplasmic Dvl puncta represented either SGs or P bodies, COS-7 cells were subjected to oxidative stress by treating with 0.5 mM sodium arsenite for 30 min. We analysed the in vivo localization of Dvl2 using affinity purified rabbit polyclonal antibodies generated against the C-terminal region of mouse Dvl2. Immunoanalysis showed that Dvl2 localized to SGs, as evidenced by co-staining with PABP1, an SG marker (Fig 1A). In untreated cells, however, both Dvl2 and PABP1 displayed uniformly diffused cytoplasmic localization, and endogenous Dvl2 was also present inside the nucleus, consistent with its reported nuclear functions. To determine, if any Dvl2 localizes to P bodies, cells were co-stained for endogenous Dvl2 and a P body marker (Dcp1a), before and after arsenite treatment. Under unstressed conditions, cytoplasmically diffused Dvl2 showed no co-localization with Dcp1a, whereas, after arsenite treatment, Dcp1a-positive P bodies often localized juxtaposed to the Dvl2 puncta, but no co-localization was observed (Fig 1B).

We also found that Dvl2 localized to SGs generated by heat stress (45oC for 45 min), ER stress (thapsigargin 1μM, 1h), or by overexpression of TIA1 or G3BP (Fig 2A, 2B), indicating that localization of Dvl2 to SGs is a general phenomenon. Taken together, these studies demonstrate that endogenous Dvl2 localizes to stress granules and not to P bodies.

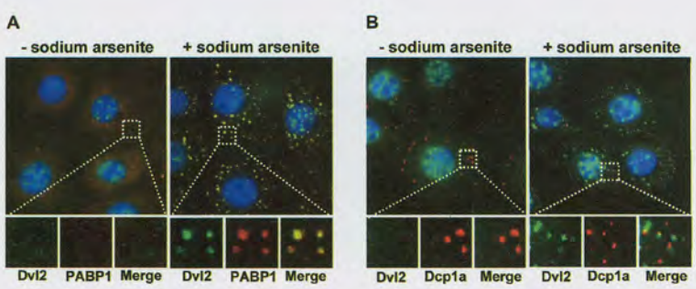


Fig. 1: Endogenous Dvl2 is targeted to SGs but not to P bodies during oxidative stress. (A and B) COS-7 cells were treated (+) or untreated (-) with 0.5 mM sodium arsenite for 30 min. Cells were fixed and stained for endogenous Dvl2 (green) and PABP1 (red; A) or Dcp1a (red; B) using specific antibodies. DNA was stained with Hoechst-33342 dye (blue). Note that Dvl2 colocalizes with PABP1 (SG marker; A) and not with Dcp1a (P body marker; B).

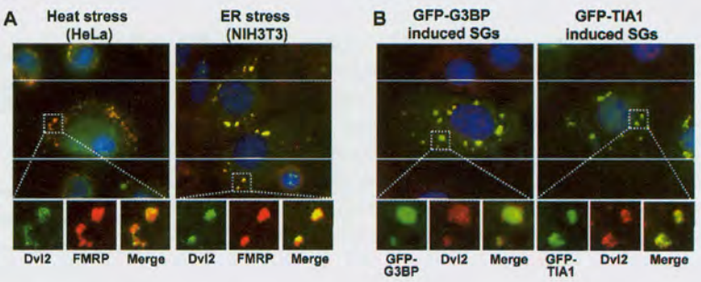


Fig. 2: Localization of Dvl2 to SGs induced by diverse stresses and by overexpression of specific SG components. (A) HeLa cells (left panel) were subjected to heat stress (45oC for 45 min) or NIH3T3 cells (right panel) to ER stress (thapsigargin 1μM, 1h). Cells were immunostained for endogenous Dvl2 (green) and FMRP (red) with specific antibodies. Note that Dvl2 localizes to both heat stress and ER stress induced SGs. (B) COS7 cells were transfected with GFP-G3BP (green; left panel) or GFP-TIA1 (green; right panel) and stained for endogenous Dvl2 using rabbit polyclonal antibodies (red). Cells were counterstained with Hoechst-33342 dye to visualize the DNA (blue).

DEP domain is sufficient for targeting Dvl2 to SGs: Dvl2 is a protein consisting of N-terminal DIX, central PDZ and C-terminal DEP domains (Fig 3A). To define the minimum region required for localization to SGs, COS-7 cells expressing different regions of Dvl2 were subjected to arsenite treatment and analyzed for their co-localization with an SG marker, FXR1. The C-terminal region, but not N-terminal or middle region, showed distinct localization to SGs (Fig 3B). Further deletion analysis demonstrated that the DEP region is sufficient for localization to SGs (Fig 3B).

Surprisingly, the full length Dvl2 fused with green fluorescent protein or other tags (HA, FLAG) failed to localize to SGs, but were often present juxtaposed to SGs. Indeed, it is known that the DIX domain of Dvl has a polymerization activity and a role in the formation of cytoplasmic puncta upon overexpression in mammalian cells. We found that Dvl2 protein devoid of the DIX domain (Dvl-ΔDIX) localized to SGs, supporting the notion that the DIX domain may sequester Dvl2 away from SGs. Removal of the PDZ domain (Dvl-ΔPDZ) also resulted in localization to SGs, albeit to a lesser extent as compared to Dvl-ΔDIX. Together, these results suggest that the regions outside DEP may have a negative effect on the localization of Dvl2 to SGs, at least when overexpressed. However, the fact that endogenous Dvl2 is still localized to SGs during environmental stress indicates that this process is tightly regulated, and may involve structural/conformational changes in Dvl2 owing to stress-induced modifications and/or interactions by other proteins to relieve the negative effect caused by DIX and PDZ domains.

Dvl destabilizes SGs in a DEP-dependent manner: We wished to study the functional relevance of the localization of Dvl2 to SGs. Upon overexpression of Dvl2 we noticed that significantly less number of transfected cells assembled SGs, suggesting a negative role for Dvl in SG assembly. We further sought to determine the regions involved in the disassembly of SGs using deletion mutants of Dvl2. Interestingly, cells expressing Dvl2 without the DEP domain failed to disassemble SGs, indicating that this region is crucial for Dvl function (Table in Fig 3). However, removal of DIX had no significant effect on SG disassembly, whereas PDZ deletion moderately affected Dvl's ability to disassemble SGs, indicating that this domain might also play a role in negatively regulating SG assembly. As DEP was found to be critical for Dvl function, we were interested to investigate the effect of K446M, which corresponds to an analogous mutation in *Drosophila* Dishevelled (*dsh*^{1}) that results in defective planar cell polarity (PCP), on SG assembly. Intriguingly, Dvl2-K446M mutant significantly affected the ability of Dvl2 to destabilize SGs, indicating that

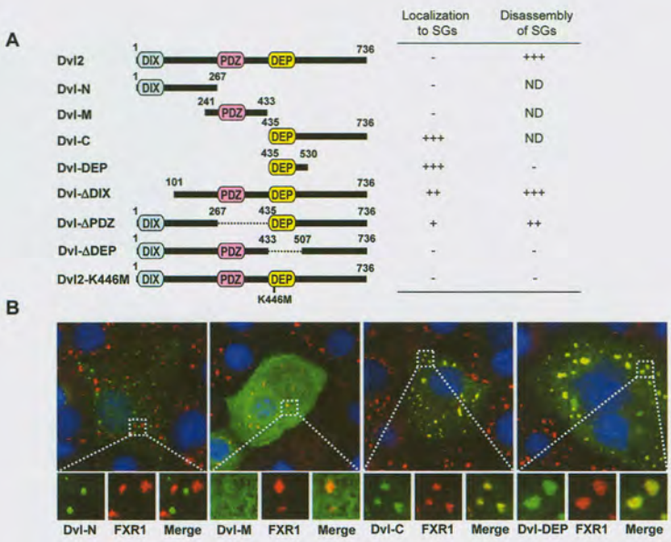


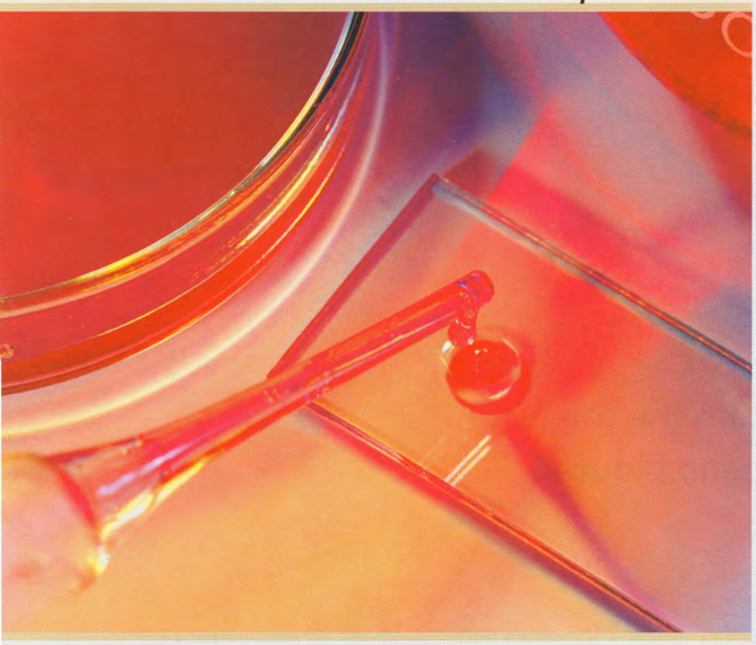
Fig. 3: DEP domain mediates targeting of Dvl2 to SGs. (A) Schematic representation of mouse Dvl2 constructs used in this study, and the ability of them to localize to and disassemble SGs, upon overexpression. Amino acid numbers are as indicated. Deleted regions are shown as doted lines. ND denotes, not determined. (B) COS-7 cells were transfected with indicated Dvl constructs (HA-tagged) and were subjected to sodium arsenite treatment. Cells were later fixed and stained for HA (green) and endogenous FXR1 (red) using specific antibodies. DNA was stained in blue. Note that the C-terminal region, particularly DEP domain, is sufficient for localization to SGs.

Dvl2 might act through the non-canonical pathway to negatively regulate SG assembly during environmental stress.

Future Work

1. Studying the molecular mechanism involved in Dvl mediated SG disassembly
2. Investigating the involvement of Wnt signaling in SG assembly
3. Understanding the functional significance of regulation of mRNA metabolism by Wnt/Dvl

Research Report



Cancer Biology

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Elucidate the role of FBXO31 in oncogene induced senescence

Background

The F-box protein FBXO31 is a candidate tumour suppressor encoded in 16q24.3, a region in which there is loss of heterozygosity in breast, ovarian, hepatocellular and prostate cancers. Using a genome-wide RNA interference (RNAi) screen, previously it has been shown that FBXO31 is required for oncogenic BRAF-induced senescence in primary human cells. However, how FBXO31 plays a role in this process is not known. Therefore, we are interested in understandanding the molecular mechanism of genotoxic stress-induced senescence and role of FBXO31 in this process.

Previously, we found that FBXO31 interacts with and mediates cyclin D1 degradation to induce G1 arrest following DNA damage. Further, following genotoxic stress, FBXO31 levels increase resulting in degradation of MDM2. The loss of MDM2 results in increased levels of p53, and hence the p53 target gene p21, leading to senescence induction. However, in cells lacking p53, FBXO31 can also induce senescence through an independent pathway involving cyclin D1 degradation. Collectively, our results indicate that FBXO31 can induce growth arrest through multiple mechanisms that differ with regard to both substrates and p53-dependence. Here we report that MDM2, the major negative regulator of the p53 tumor suppressor, is also a substrate of FBXO31.

Aims and Objectives

1. To understand how FBXO31 regulates senescence
2. To identify the cellular target(s) of FBXO31 during senescence process
3. Is it P53 dependent?

Work Achieved

Aim 1: To understand how FBXO31 regulates senescence: Previously, it has been shown that FBXO31 is involved in senescence process. In this study, we sought to understand in greater detail how FBXO31 promoted senescence. MCF7 breast cancer cells lack FBXO31 due to a heterozygous deletion and therefore provide a convenient system for studying FBXO31 function. Consistent with previous results, we found that ectopic

Collaborator(s)

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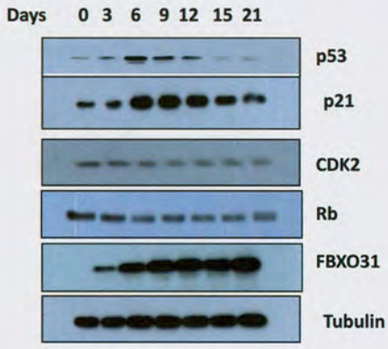
Fig.1: FBXO31 regulates p53 level during senescence process



expression of FBXO31 in MCF7 cells promoted senescence as evidenced by positive staining for senescence-associated β-galactosidase, and the induction characteristic markers of senescence (Figure 1).

One of the other genes we identified in our original RNAi screen for oncogene-induced senescence was p53, which has been shown to mediate growth arrest by multiple mechanisms including senescence induction. The finding of both FBXO31 and p53 in the screen prompted us to ask whether there is a functional relationship between these two factors. MCF7 cells contain wild type p53. Our results shows that ectopic expression of FBXO31 in MCF7 cells resulted in increased levels of p53 and the well characterized p53 target gene, p21. Thus, ectopic expression of FBXO31 results in increased p53 levels and activity.

Aim 2: To identify the cellular target(s) of FBXO31 during senescence process: The major negative regulator of p53 is MDM2, an E3 ubiquitin ligase that interacts with and directs degradation of p53. We therefore asked whether MDM2 had any role in the increased levels of p53 following ectopic expression of FBXO31. The immunoblot experiment shows that ectopic expression of FBXO31 resulted in decreased levels of MDM2. The F-box motif in F-box proteins is responsible for their ability to interact with SKP1 and subsequently induce the degradation of their target substrates. To determine

Fig. 2: FBXO31 mediated senescence is independent of p53

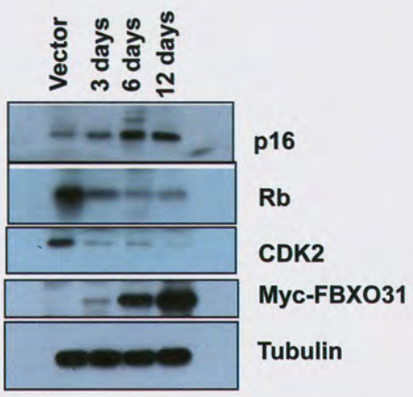
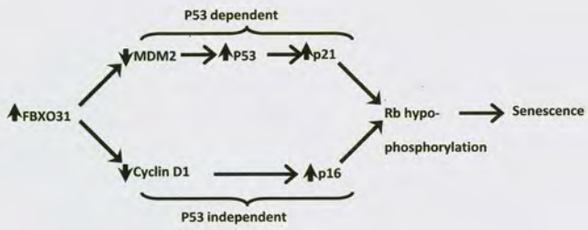


Fig. 3: Schematic model of FBXO31 induce senescence



whether the F-box motif of FBXO31 was required for MDM2 proteolysis, we used an FBXO31 derivative in which the F-box had been deleted (FBXO3ΔF). In contrast to wild type FBXO31, ectopic expression of FBXO3ΔF did not result in decreased levels of MDM2.

We next sought to determine whether the FBXO31-mediated decrease in MDM2 resulted from proteasomal degradation. We found that addition of the proteasome inhibitor lactacystin blocked the ability of ectopically expressed FBXO31 to decrease MDM2 levels. Consistent with this result, MDM2 mRNA levels were unaffected by ectopic FBXO31 expression.

Aim3: Is it P53 dependent? We previously showed that ectopic expression of FBXO31 in SK-MEL-28 cells induced G1 arrest, thereby inhibiting the growth of cultured cells and mouse xenografts. However, SK-MEL-28 cells lack wild type p53, indicating that the FBXO31 could also cause growth arrest through a p53-independent pathway(s). To determine whether in the absence of p53, FBXO31 could induce a bona fide senescence program, we ectopically expressed FBXO31 in SK-MEL-28 cells. Senescence induction was revealed by β-gal staining, decreased DNA replication as evidenced by BrDU staining, and immunoblotting for the senescence markers. These results suggest that FBXO31 mediated senescence is independent of p53 and is illustrated in the model.

Future Work

1. Does FBXO31 regulates MDM2 upon genotoxic stress?
2. Identification of cellular targets of FBXO31 under different conditions
3. Molecular Mechanism of transcriptional gene silencing
4. Genome-wide screen to identify the factors involved in DNA methyl transferase mediated epigenetic gene silencing

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Transcription factor driven epithelial-mesenchymal transition networks in ovarian cancer

Background

Epithelial-mesenchymal Transition (EMT) is a crucial phenomenon identified as a hallmark contribution to cancer. Primarily, EMT encompasses an entire repertoire of molecular changes that effect a phenotypic transition of compactly arranged epithelia to spindle shaped migratory mesenchymal-like cells. The latter is currently considered as a major contributory mechanism underlying cancer metastases. Over the last few years, an extension of this basic mechanism driven by EMT to tumor cell survival and acquisition of "stem-like" characteristics by tumor cells has been documented. A cumulative interpretation of these functionalities as "EMT Molecular Signatures" is now being correlated with aggressive tumors, further suggesting their application as predictive and/or prognostic biomarkers.

At the transcriptional level, EMT is effected by specific transcriptional repressors (TRs), the association of which were identified through a high affinity to E-boxes in the promoter region of E-cadherin (CDH1) and consequent repression of the gene. CDH1 is a component of the adherans junctions, and its down-regulation leads to dissolution of these junctions. Concurrent repression of other junctional components (Claudins, Occludins, Desmoglins, etc.) and expression of mesenchymal markers (Vimentin) are crucial mediators of the basic process that brings about a phenotypic change in the cells. TRs including Snail, Slug, Twist1, Zeb1, Zeb2, FoxC2, TCF3, TCF4, etc. have been correlated with EMT in a wide range of tumor types and grades. A crucial query that now becomes pertinent relates to the cellular context under which a specific TR is activated towards exerting its influence on the target genes.

Aims and objectives

1. Identification of the EMT-TRs specific to ovarian cancer.
2. Resolution of regulatory and co-regulated networks of these TRs towards –
   (i) Identifying the specific cellular contexts that necessitates its activation
   (ii) Assigning functions to each TR in a single tumor type

Participants

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Work Achieved

Interaction of EMT-TFs as a regulatory network in Serous ovarian carcinoma: To establish the nature of involvement of the EMT-TRs with serous ovarian carcinoma, we assessed their co-expression with the classical epithelial (CDH1, CK8, CK18) and mesenchymal (Vimentin) markers in normal (n=8) and tumor tissues (n=512) of the TCGA Serous ovarian carcinoma microarray dataset. The expression of 5 TFs *viz. TCF4, TWIST1, SNAI2, ZEB1 & ZEB2* strongly correlated with each other and with the mesenchymal marker and negatively with the epithelial markers in the tumors (Fig. 1a). These effects indicative of association with EMT were weakly evident in case of *SNAI1* and *FOXC1*. On the other hand, while *FOXC2* and *TCF4* negatively correlated with the epithelial markers, they did not correlate positively with mesenchymal VIM or the other EMT-TRs. This correlated well with the individual gene expression patterns of the makers and TRs as profiled in the TCGA tumor dataset (Fig. 1b).

Identification of individual gene targets of SNAI1, SNAI2 and TWIST1 in Serous ovarian carcinoma: Since the various EMT-TRs seemed to be capable of trans-repressing and trans-activating a common set of epithelial and mesenchymal markers, we extended these comparisons by determining the effects of these TRs on global targets within cells. Towards this, we derived the individual gene targets of SNAI1, SNAI2 and TWIST1 by carrying out ChIP-on-chip in "A4" cells established earlier in the lab from a serous ovarian adenocarcinoma ascites sample as a single cell clone (Bapat et al. 2005). This led to cataloguing of all probes on the Agilent 244k human promoter arrays that bound to the 3 EMT-TRs. Probe intensity normalization to identify significantly enriched probes ($p<0.05$) was carried out using BRB tools. The gene target lists thus generated were further compared with the gene expression profile of these cells to identify genes that were either totally not expressed or expressed at low levels. The entire exercise resulted in the identification of 99 genes that were common targets of the 3 TRs - SNAI1, SNAI2 and TWIST1 or combinations thereof (Fig. 2a). Additional, exclusive targets of each TR were also identified.

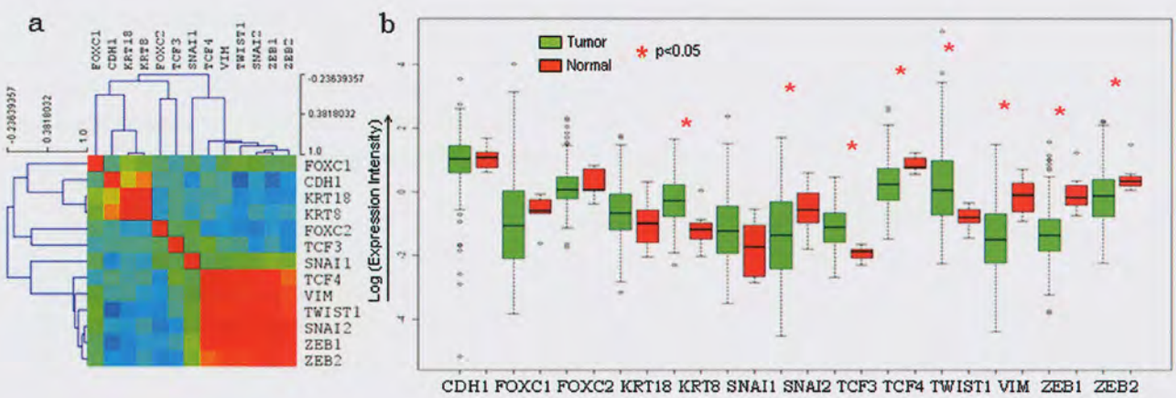
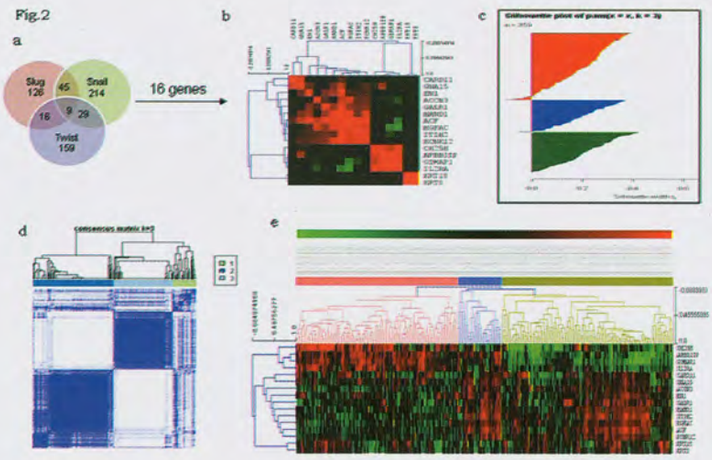


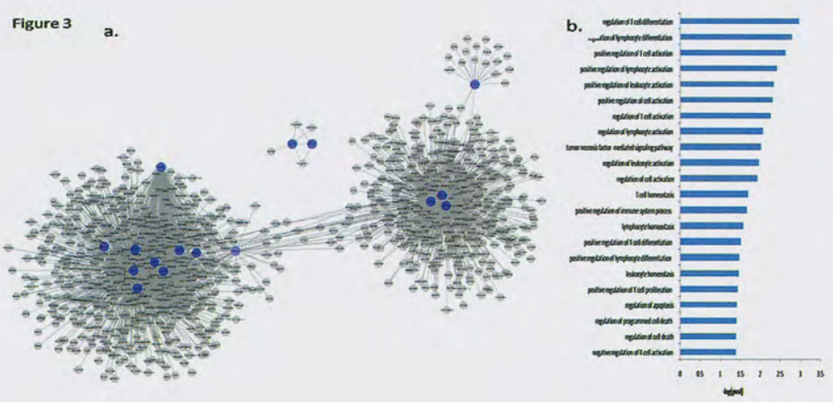
Fig.1: **a.** Correlation of EMT-TFs, epithelial and mesenchymal markers in TCGA serous ovarian adenocarcinoma datasets; **b.** Comparative Expression of EMT-TFs and markers between tumor and normal tissues; significance determined using Welch Two sample t-test at 0.99% confidence interval

Fig.2: **a.** Identification of 99 genes that were common targets of the 3 transcrptional repressors viz. SNAI1, SNAI2 and TWIST1. **2b**. Pearson Correlation analyses to extract a core set of 16 target genes with maximum correlation. **2c**. Identification of patient classes using silhouette clustering. **2d.** Identification of patient classes using consensus clustering. **2e.** Identification of patient classes using hierarchial clustering.



Resolution of regulatory and co-regulated networks defined by the common targets of these TRs and application of the same towards patient classification: Using the same TCGA serous adenocarcinoma dataset, we probed the differential expression of these 99 genes towards patient groups to identify 55 differentially expressed genes in the 359 patient cohort. Further stringency of analyses was introduced through Pearson Correlation analyses to extract a core set of 16 target genes that maximally correlate with each other (Fig. 2c). The patient dataset was then examined by alternative computational data clustering techniques with this 16 gene-set. The various methods included consensus clustering, hierarchical clustering and silhouette clustering that were effectively used to group the cohort. Concordance between these techniques led to the assignment of three different core classes within the 359 patients while 75 (20.8%) patients were of indeterminate or mixed class. This serves to confirm that key clinical phenotypes of ovarian cancer can be identified within the highly heterogeneous ovarian cancer phenotypes, based on the activation of a few EMT- associated transcription factors.

Fig. 3: ARACNe generated gene interaction networks for node-interacting gene interactions. Blue circles represent the 16 least redundant nodes **(a).** DAVID pathway analyses of the enriched networks **(b)**



Network and Pathway analyses of the common targets of these TRs: Network analysis was carried out through application of ARACNe (Algorithm for the reconstruction of Cellular Networks) as described in the previous year. A compact network consisting of one isolated node and a bi-centered network was thus generated (Fig. 3a). We further performed a functionality correlation based on the gene hubs around each node using DAVID. Surprisingly, apart from the expected cell junction components and apoptosis pathways which have been earlier documented as being down regulated by these repressor molecules, several pathways involved in immune function were identified (Fig. 3b). This reveals a novel involvement of the EMT–associated transcription factors in disease progression and will be investigated further.

Future Work

1. Correlation between the two classes of patients and their clinical characteristics.
2. Functional correlation that would assigning significance to each TR in a single tumor type
3. Identifying the specific cellular contexts that necessitates the activation of a specific TR

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Chemotherapeutic drugs mediated cell killing and functional activation of p53 in cancer cells

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Background

There is a medical need for advances in cancer treatment, since surgery, radiotherapy and conventional cytotoxic chemotherapy have made only a modest overall impact on mortality. Thus, the discovery of new targets and identification of novel pathways and strategies for therapeutic intervention in cancer are extremely important. Therefore, understanding the molecular events that contribute to cancer development as well as those which enhance drug-induced cell death will not only help in explaining the relationship between cancer genetics and chemotherapeutic drugs but also will improve the sensitivity and specificity of the treatment. In addition, the activation of molecules responsible for cell growth check, which otherwise are ineffective in certain cancers, may also significantly reduce the growth of cancer cells.

One of such molecule is p53. It is a major tumor suppressor or guardian of the genome that is inactivated in various cancers. Almost all human papillomavirus (HPV) infected cancer cells contain wild-type p53, but is non-functional as HPVE6 protein abrogates its function by ubiquitin-dependent and -independent degradation, by inhibition of acetylation or by repressing p53-dependent downstream molecular pathways.

Ectopic expression of p53 in cancer cells lacking p53 or harboring mutant and/or abrogated wild-type p53, have contrasting effects on cell-fate. In p53 null cancer cells, p53 overexpression causes cell cycle arrest and apoptosis. However, in virus infected cells harboring wild-type p53, overexpression of p53 does not induce cell cycle arrest and apoptosis. Thus, the role of p53 overexpression in HPV-positive cells remains obscure. p53 executes its tumor suppressor activity by triggering cell cycle arrest and apoptosis. The factors that facilitate selection between cell cycle arrest and/or apoptosis are not well-understood. It has been reported that p21 is most important transcriptional targets of p53 that causes cell cycle arrest, and p53 executes apoptosis through Bax transcription. To study the role of p53 in E6-positive cells, we developed a novel isogenic HeLa cells with Tet-On-regulated p53 expression. Tet-On system exhibits tight-on/off regulation and is

devoid of pleiotropic effects. Moreover, high induction levels are achieved rapidly in this system, and the inducer, doxycycline (Dox), is a well-characterized chemical.

p53 overexpression does not promote cell cycle arrest and apoptosis in HeLa cells. We demonstrate that protein phosphatase 2A (PP2A) controls p53 functions and its inhibition activates p53, causing cell cycle arrest/apoptosis *in vitro* and tumor growth inhibition *in vivo*. Interestingly, cyclin dependent kinase 5 (Cdk5) regulates p53 phosphorylation essential for its activation. Taken together, we propose that non-genotoxically overexpressed p53 can be activated by inhibiting its dephosphorylation in HPV-positive cervical cancer cells. This strategy may be of therapeutic importance in case of p53 associated gene therapy.

Aims and objectives

1. To investigate the mechanism of activation of overexpressed p53 in HPV E6 positive human cervical cells.
2. To investigate In-vivo implications and molecular events involved.

Work achieved

I. Development and screening of unique HeLaTet-On p53 inducible cell-system: HPV positive human cervical cells (HeLa cells) were transfected with pTet-On and selected on G418 for 21 days. Media was changed every 4th day with fresh media containing same concentration of G418. HeLaTet-On cells were then verified by transfecting pBIEGFP and observing GFP fluorescence after 48 h induction with 2000 ng/ml of Dox. Clones expressing high GFP and low leaky expression were selected for the development of double stable cell-line. HeLaTet-On cells were cotransfected with pTREp53 or pBIEGFP

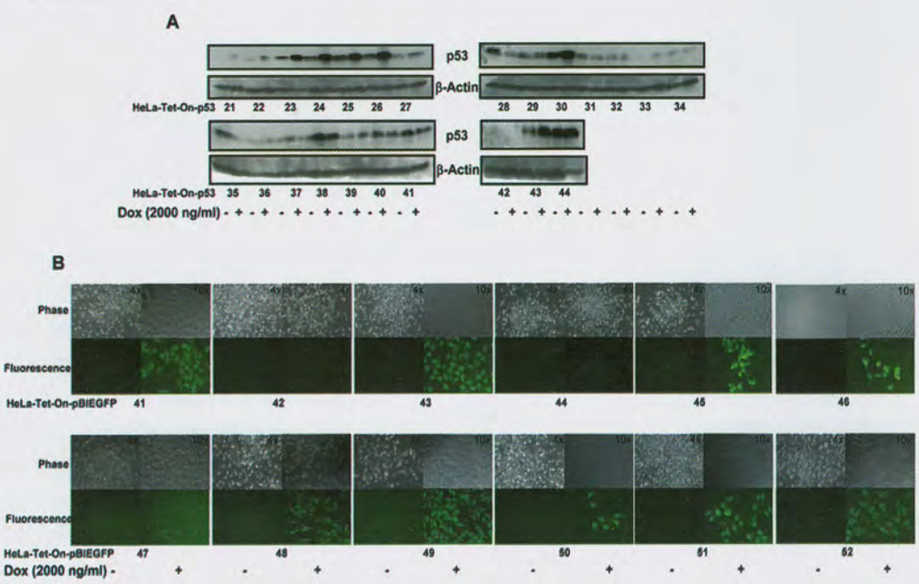

Fig. 1: Verification and screening for Tet-On responsive p53/GFP clones. (A) For screening of Tet-On as well as pTREp53 transfected cells, clones (HeLa-Tet-On-p53 numbered 21 to 44) were treated with 2000 ng/ml Dox for 48 h and processed for western blotting with p53 specific antibody. β-Actin served as a loading control. Two clones with low leaky and high inducible expression, HeLa-Tet-On-p53 23 and 26, were selected for further studies. (B) Screening for Tet-On regulated GFP clones (HeLa-Tet-On-pBIGFP number 41 to 52) was performed by incubating cells with 2000 ng/ml Dox for 48 h followed by observation under fluorescent microscope. One clone HeLa-Tet-On-pBIEGFP (number 43) having low leaky and high inducible expression was selected for further studies.

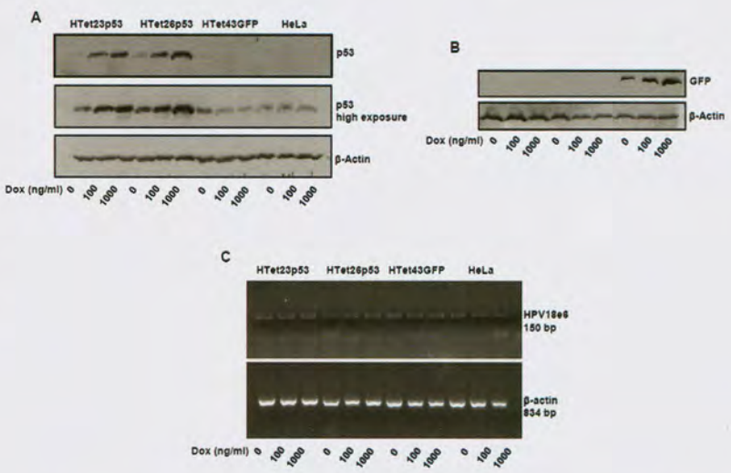

Fig. 2: p53/GFP are tightly regulated by Dox and it does not alter HPV18E6, a viral cell cycle deregulator (A) Selected clones (HTet23p53, HTet26p53 and HTet43GFP from Figure 1) were treated with 100 and 1000 ng/ml of Dox and after 48 h processed for western blotting for p53. β-Actin was used as a loading control. (B) Cells were treated as mentioned in (A) and processed for western blotting for GFP. β-Actin was used as a loading control. (C) HPV18 E6 mRNA levels were determined by semi-quantitative PCR. Cells treated with Dox were processed for RT-PCR. β-actin was used as a loading control. HTet43GFP and HeLa cells served as experimental controls.

and pTKHyg. Cells were selected for 21 days on 200 µg/ml of hygromycin B. Total 24 colonies were picked up and screened for p53 expression after addition of 2000 ng/ml of Dox for 48 h. For GFP 12 colonies were picked up and after 2000 ng/ml Dox addition cells were observed under fluorescent microscope.

Seven out of 24 p53 transfected clones (HeLaTet-On-p53 21 to 44) and nine out of 12 GFP transfected clones (HeLaTet-On-pBIEGFP 41 to 52) exhibited induction in the presence of Dox (Fig. 1A and B). Two clones HeLaTet-On-p53-23S and HeLaTet-On-p53-26S (represented as HTet23p53 and HTet26p53) along with HeLaTet-On-BIEGFP-43 (represented as HTet43GFP) with low-leaky and high regulatory expression were selected for further studies. Growth properties of clones for 6 days were similar to parental HeLa cells and also protein concentration did not alter between the clones and parental cells. Dox upto 2000 µg/ml was non-toxic (data not shown).

Tight-regulation of p53 expression was confirmed by addition of 100 and 1000 ng/ml of Dox. p53 expression was induced in response to Dox in a dose-dependent manner (Fig. 2A). Also, GFP protein expression was tightly-regulated (Fig. 2B). As E6 downregulation induces cell-death, E6 mRNA levels in p53 and GFP expressing clones as well as in parental HeLa cells was detected by RT-PCR. No alteration in e6 expression following treatment with Dox was observed (Fig. 2C). p53 localization and nuclear retention is essential for execution of its transcriptional and tumor suppressor activities. Level of p53 was increased in response to Dox in a dose dependent manner, which is predominantly localized in the nucleus (green represents p53 staining and blue represents DAPI for DNA (Fig. 3A and 3B). No alteration in p53 protein expression was detected in Dox treated HTet43GFP (red) and parental HeLa cells (green) (Fig. 3C and 3D). In HTet43GFP cells, GFP protein expression (green) is tightly-regulated by Dox (Fig. 3C).

II **p53 overexpression does not cause cell cycle arrest or growth inhibition although it possesses DNA binding activity:** Propidium iodide staining for the cell cycle analysis

indicated no alteration in cell cycle in p53 over expressing cells as compared to HTet43GFP or HeLa cells. Also long-term consequence of p53 overexpression was investigated by clonogenic-survival assay. Almost equal numbered and sized colonies were formed by p53 over expressing HTet23p53 and control HTet26p53 cells. As Dox-induced p53 was localized in the nucleus, there could be an increased binding of p53 to its consensus sequence in HTet23p53 and HTet26p53, but not in HTet43GFP and HeLa cells. Also, an increase in CAT reporter activity was detected in only in p53 over expressing HTet23p53 and HTet26p53 cells

Unlike stress conditions, wherein p53 induction promotes cell cycle arrest or apoptosis, our results demonstrates that p53 overexpression in HPV-positive cells does not induce cell cycle arrest or apoptosis. One reason could be the inhibition of cellular machineries that carry out critical posttranslational modifications, which are required for sequence specific selection of promoters of those genes responsible for induction of cell cycle arrest or apoptosis by HPV. To inhibit the phosphatase, okadaic acid (OA), a potent and specific inhibitor of PP1A and PP2A, was used. Okadaic acid (5 nM) specifically inhibits almost 100% of phosphatase (PP2A) activity in the cells. Dose-dependent growth inhibition was observed in p53 over expressing HTet23p53 and HTet26p53 (2.5 nM OA caused 25%, whereas 5 nM caused 60%) as compared to HTet43GFP and HeLa cells (Fig. 4A). OA alone did not significantly affect cell-survival (Fig. 4A). Decrease in colony number and colony size in p53 over expressing OA treated cells (HTet23p53 and HTet26p53) was also observed (Fig. 4B). To confirm that indeed p53 specifically inhibits cell-growth in the presence of OA, p53 siRNA was used which decreased the levels of overexpressed p53 as compared to Ctrl siRNA (Fig. 4C inset). Silencing of p53 reduces cell-death by 30-35% in p53 over expressing cells as compared to HTet43GFP cells or Ctrl siRNA transfected cells (Fig. 4C).

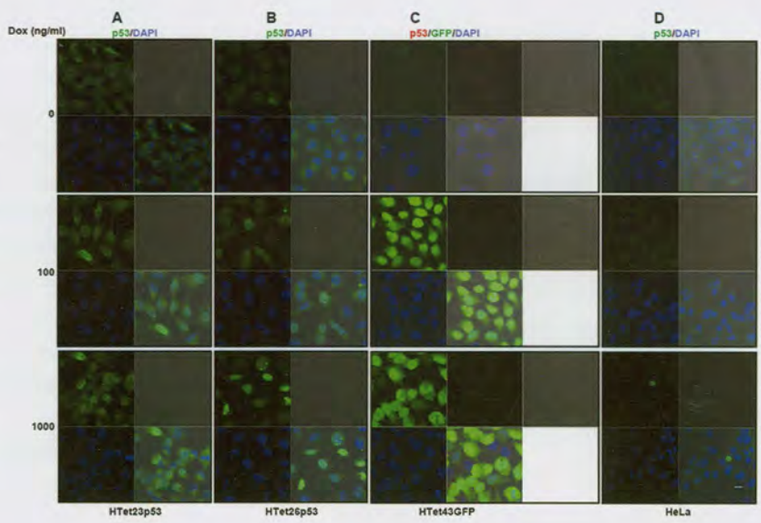

Fig. 3: Overexpressed p53 exhibits nuclear localization. (A) HTet23p53, (B) HTet26p53 and (D) HeLa cells were treated with 100 and 1000 ng/ml of Dox for 48 h and processed for immunofluorescence study with p53 specific antibody. HTet23p53, HTet26p53 and HeLa cells were probed with FITC conjugated secondary antibody (green). Upper left section of image represents p53 staining (green); lower left represents DAPI (blue), upper right phase contrast and lower right (overlay of them). (C) HTet43GFP cells were probed with rhodamine conjugated (red) secondary antibody. Green staining in HTet43GFP cells depicts GFP staining. DAPI (blue) represents nuclear staining. Upper left section of image represents GFP (green), upper middle section represents p53 staining (red), lower left represents DAPI (blue), upper right phase contrast and lower middle represents (overlay of them). Bar: 10 µm.

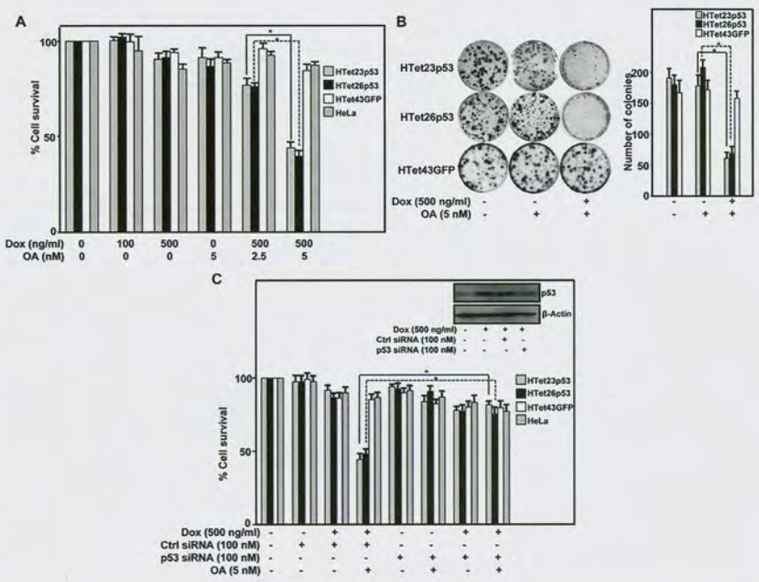

Fig. 4: Inhibition of PP2A causes cell-growth inhibition in a p53 dependent manner. (A) HTet23p53, HTet26p53, HTet43GFP and HeLa cells were treated with indicated concentrations of OA with or without Dox for 48 h. Thereafter MTT assay was performed. Bar represents variation within the wells of an experiment (±S.E.). *Represents P<0.01. (B) Five hundred cells plated were treated as mentioned in (A). After 48 h cells were washed and incubated for 21 days. Cells were stained with crystal-violet and colonies were counted. Bar graph represents average of colony number per plate (±S.E.) from three experiments. *Represents P<0.01. (C) HTet23p53, HTet26p53, HTet43GFP and HeLa cells were transfected with control or p53 siRNA in 96 well-plate, 18 h post-transfection cells were treated with Dox and/or OA and MTT assay was performed after 48 h. Bar graph represents variation within the wells of an experiment (±S.E.). *Represents P<0.05. HTet26p53 cells were transfected with control or p53 siRNA. Eighteen hour post-transfection, cells were treated with Dox and further incubated for 48 h. MTT for cell survival evaluation or western blot analysis was performed for p53.

p53 executes its apoptotic function through intrinsic or extrinsic pathways. We investigated the status of Bax, an important transcriptional target of p53 that mediates intrinsic mitochondrial apoptosis. Bax translocates to mitochondrial outer membrane causing MOMP and releases cytochrome-C into cytosol. Cells lacking Bax or over expressing Bcl-2 are profoundly resistant to a broad range of apoptotic stimuli, including chemotherapeutic drugs treatment and serum starvation. In HPV-positive cancers, Bcl-2 overexpression and Bax degradation by E6 facilitates cancer progression. Our results demonstrate that upregulated Bax translocates to mitochondria upon PP2A-inhibition in p53 over expressing cells. Therefore, only phosphorylated p53 triggers Bax transcription, which causes apoptosis. In addition, the cell cycle arrest caused by inhibition of PP2A in p53 over expressing cells may be dependent on transcriptional upregulation of p21 gene. These data provide evidence for the reactivation of E6 disrupted p21 and Bax pathways.

III **Activated and not overexpressed p53 inhibits tumor growth:** To validate that these *in vitro* findings have *in vivo* implications, HTet23p53 or HTet43GFP cells were administered in NOD/SCID mice and monitored weekly for tumor growth. Upto three weeks after implanting cells, tumors grew to almost similar extent in mice supplemented with or without Dox. Thereafter, tumor growth was rapid in mice injected with untreated HTet23p53 cells and treated with OA without being supplemented with Dox. Similarly, in mice injected with HTet43GFP cells, tumors grew rapidly in those treated with OA and supplemented with or without Dox. Interestingly, in mice injected with HTet23p53 cells and treated with OA, in addition to being supplemented with Dox, tumor growth was significantly retarded (Fig. 5A). Reduced tumor-growth is reflected in differences in size and weight of the excised tumors (Fig. 5B). Tumor samples were analyzed to ascertain the involvement of stabilized p53 and also for the activation of its downstream growth inhibitory factors. In tumor samples from OA treated mice bearing HTet23p53 cells, p53

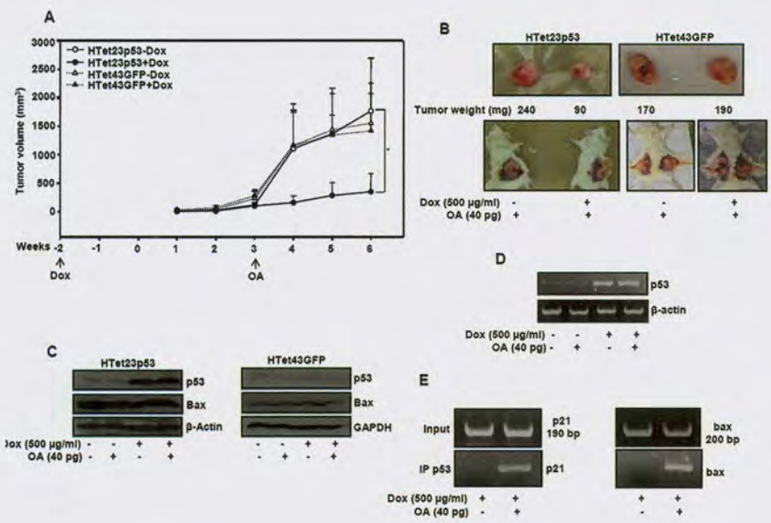

Fig. 5: Activated p53 inhibits tumor-growth by transcriptional activation of its downstream pathways. (A) HTet23p53 (n=12 mice) or HTet43GFP (n=4 mice growing 2 tumors each) were divided in two groups and one was fed on Dox. After tumor size reached to 5-10 mm diameter, OA was administered to all mice. Tumor-growth were measured weekly and average tumor volume was plotted (+S.E.). *Represents $P<0.05$. (B) Tumor image and weight after mice were sacrificed. (C) Western blotting of p53 and bax in HTet23p53 and HTet43GFP tumors with or without Dox in the presence and absence of OA. (D) R-TPCR for p53 was performed from tumors samples with or without Dox and treated with or without OA. β-actin is loading control. (E) Tumors from mice fed on Dox with or without OA treatment were processed for ChIP assay. Input and eluted DNA was used for RT-PCR with p21 or bax promoter primers.

and bax protein levels were higher (Fig. 5C) and the levels did not increase in tumors of HTet43GFP cells. The p53 transcript and protein levels were higher in HTet23p53 cells-derived tumors from mice supplemented with Dox and, levels were not enhanced further by OA treatment (Fig. 5D). These results clearly indicate that the stabilization of p53 protein also occurs in *in vivo* tumors. Conclusively, the ChIP assays performed on lysates of tumors excised from mice provided with Dox in water, with or without OA treatment revealed enhanced promoter occupancy of activated p53 on p21 and bax promoters in vivo (Fig. 5E).

Future work plan

1. The alterations in the expression profile of genes will be looked into in non-activated and activated p53 in human cervical cells.
2. The effects of chemotherapeutic drugs on cell lines derived from various human solid tumors will be investigated.
3. Involvement of metabolic malfunctioning on the cancer cell growth and the efficacy as well as outcome of chemotherapeutic drugs will be investigated.

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Role of mTOR signaling pathway in survival, proliferation and invasion of human gliomas

Background

Glioblastoma multiforme (GBM) is a highly aggressive and most common primary brain tumor in adults with a dismal survival of one year post diagnosis. Malignant gliomas display extensive infiltration into the surrounding brain tissue making them resistant to the existing therapeutic strategies. Monocytes are recruited to the primary tumor site where they differentiate into tumor associated macrophages (TAMs) and secrete inflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α), Interleukin-1β (IL-1β), VEGF and other chemokines that positively regulate invasion and angiogenesis aided by the secretion of high levels of proteolytic enzymes such as Matrix Metalloproteinases (MMPs). TNF-α is an activator of the major survival pathways–NF-κB and PI3K/Akt. The mammalian Target of Rapamycin (mTOR), functions downstream of PI3K/Akt pathway by forming two distinct complexes, mTORC1 and mTORC2, to regulate cell growth, proliferation and survival. The specific role of mTORC2 and its component proteins in cancer progression has gained attention only in the recent years. The mTORC2 component, Rictor, is important for phosphorylation of Akt (S473) that is constitutively activated in gliomas and is involved in tumor progression.

Aims and Objectives

1. Identification of the downstream targets of Akt and NF-κB pathways in TNF-α-mediated responses in gliomas.
2. To understand the mechanism(s) that contributes to resistance in these tumors with focus on activation and consequence of Akt /mTOR in gliomas.

Work achieved

We investigated the role of Rictor, a core component of mTORC2, in proliferation, survival and invasion of gliomas. Our earlier studies showed that siRNA mediated knock down of Rictor resulted in enhanced MMP-9 activity and invasion in glioma cells. Further studies were done to unravel the molecular mechanism underlying Rictor-regulated MMP-9 expression and activity. Analysis of whole cell lysates of human glioma cell lines

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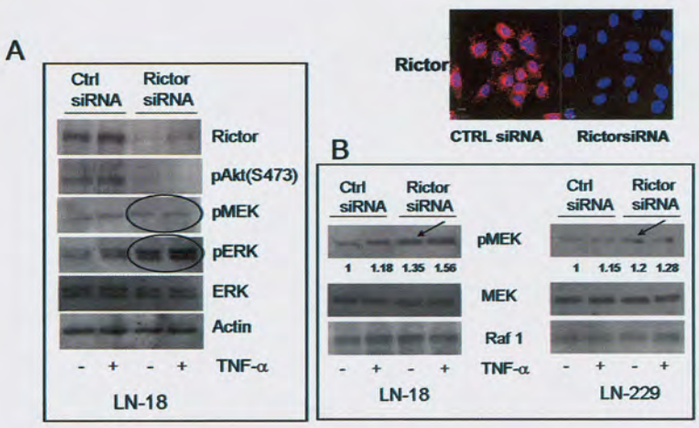

Fig. 1: (A) LN18 cells transfected with control (ctrl) and Rictor siRNA were treated with TNF-α (10ng/ml). Whole cell lysates were analyzed by western blotting for Rictor, phosphorylated forms of Akt (S473), MEK and ERK and total ERK. (B) LN18 and LN229 cells transfected with control (ctrl) and Rictor siRNA were treated with TNF-α (10ng/ml) and lysates were analyzed for Raf-1 kinase activity The fold changes of pMEK are with respect to levels in control untreated cells. The confocal image shows expression of rictor in ctrl and rictor siRNA transfected cells.

transfected with Rictor siRNA displayed a significant increase in phosphorylated MEK and ERK as compared to control siRNA transfected cells (Fig 1A). TNF-α-mediated activation of MEK and ERK was comparable between Rictor-silenced and control transfected cells. The activation of ERK and MEK is regulated by Raf-1 kinase in many cell types. The phosphorylation of Raf-1 at a highly conserved S259 residue by Akt inhibits the activation of Raf-1/MEK/ERK pathway. Rictor ablation resulted in significant increase in the Raf-1 kinase activity as seen by increase in phosphorylated, active MEK1 compared to control transfected cells. The upregulation was greater in Rictor silenced cells compared to control cells exposed to TNF-α (Fig 1B). Treatment of Rictor-silenced cells with GW5074 resulted in reduction of phosphorylated ERK in LN-18 and LN-229 cell lines, confirming that activation of ERK was a downstream event of Raf-1-mediated signaling (Fig. 2). To elucidate the significance of the activation of ERK in upregulation of MMP-9, the effect of inhibitors to Raf-1 (GW5074) and ERK (PD98059) on MMP-9 activity was examined by zymography analyses. The inhibitors effectively reduced gelatinase activity induced on silencing Rictor in the cell lines (Fig. 3A). Invasion assay performed with LN18 cells transfected with control or Rictor siRNA and treated with TNF-α in the presence of

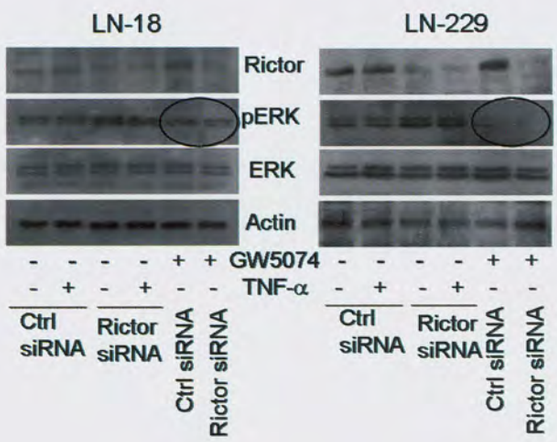

Fig. 2: LN18 and LN229 cells transfected with control (ctrl) and Rictor siRNA were treated with Raf-1 kinase inhibitor, GW5074 (10mM) or with TNF-a (10ng/ml) for 12h. Whole cell lysates were analyzed for the expression of phosphorylated ERK, Rictor and total ERK. Actin was used as the loading control.

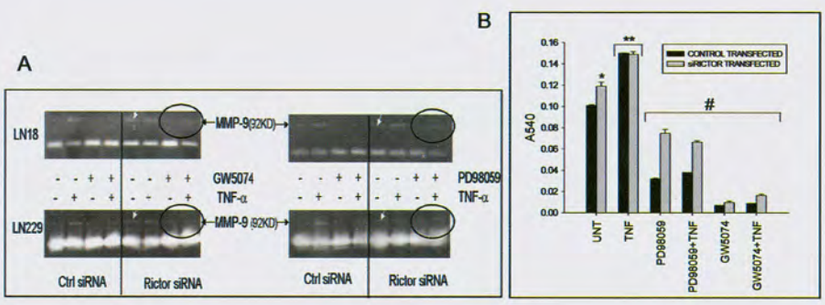

Fig. 3: (A) LN18 and LN229 cells transfected with control (ctrl) or Rictor siRNA were pretreated with GW5074 (10mM) or ERK inhibitor- PD98059 (40mM) for 2h, followed by treatment with TNF-a (10ng/ml) for 12h. Conditioned medium of the cells subjected to gelatin zymography analysis for MMP-9 levels.. (B) LN18 cells transfected with control (ctrl) or Rictor siRNA were incubated with TNF-a and PD98059 (40mM) or GW5074 (10mM) and invasion assay was performed. Data is represented as mean absorbance values +/- SEM of the two similar experiments performed in duplicates. * $p<0.001$ control vs Rictor siRNA transfected, ** $p<0.001$ untreated vs TNF-a treated cells in corresponding control and Rictor siRNA transfected cells, # $p<0.001$ in absence vs presence of inhibitor.

GW5074 and PD98059 revealed that the inhibitors effectively reduced the invasion in control cells and that induced by silencing Rictor. GW5074 was more effective in inhibiting invasion than PD98059. Together, these results suggested that loss of function of Rictor resulted in enhanced MMP-9-mediated invasion of cells via activation of Raf-1/MEK/ERK pathway (Fig. 3B).

Conclusions: The findings suggest an undescribed role for Rictor in regulating invasion by enhancing MMP-9 activty via Raf-1/MEK/ERK pathway in gliomas.

Future work

Various signaling pathways play crucial role in invasion in tumors, further studies will explore the interactions of mTOR signaling with such pathways. The studies will also include factors in microenvironment such as inflammatory cytokines that influence tumor progression and invasive potential of gliomas.

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Understanding mechanisms of transformation elicited by 600bp non-coding RNA gene – Ginir

Background

The genetic programmes of complex organisms including humans are now shown to be transacted by non-coding RNAs that rival that of proteins. Rather, the extent of control exerted by non-protein-coding RNAs increases with increasing complexity of the organisms, perhaps exceeding that of the proteins in well developed organisms, reaching an estimated value of ~ 98.8% in humans. A majority of these ncRNAs are dynamically transcribed, with many showing specific expression patterns and subcellular localizations, but their exact functions in cells remain to be defined. The emerging evidence indicates, however, that several of these RNAs control the epigenetic states that govern development, and that many of them are dys-regulated in cancer and other complex diseases. Moreover, it appears that plasticity in these ncRNA functions might exist, especially in the brain. Thus, unravelling the role of non-coding RNAs in specific tissue functions is likely to be crucial for the understanding of growth, differentiation, development and overall function of the organs in higher animals.

Aims and Objectives

1 To determine the expression patterns of Ginir and Giniras (anti-sense transcripts Ginir) during mouse embryonic development

2 To identify the targets for Ginir and Giniras that may unravel the molecular signalling mechanisms regulated by them during cellular growth

Work Achieved

A vast majority of non-coding RNAs are transcribed in a developmentally regulated manner. In our laboratory, we have focused on a pair of non-coding, sense (Ginir) and anti-sense (Giniras) RNAs to understand their function during development and cellular growth. We have determined that a temporal variation in the expression pattern occurs in the pair of Ginir and Giniras RNAs in developing mouse embryos with highest expression of Giniras RNA (>400 fold) found in 15.5 dpc of embryonic development (Fig.1). Localization of the Ginir and Giniras RNAs using their specific LNA-probes in RNA-fluorescence in situ hybridization (RNA-FISH) experiments in developing mouse embryos of 7.5-10 days age, demonstrated a predominant Ginir and Giniras expression in the brain

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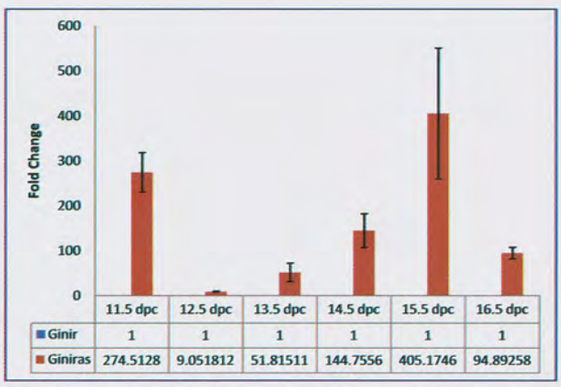
Fig. 1: qRT-PCR analyses: Expression of Ginir and Giniras in various days of mouse embryo development using orientation specific cDNA's for Ginir and Giniras and gene specific primers.Fold values for Giniras expression are calculated by normalization of Ginir levels to one and GAPDH as internal control

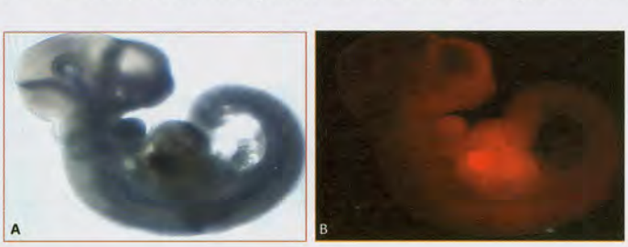


	11.5 dpc	12.5 dpc	13.5 dpc	14.5 dpc	15.5 dpc	16.5 dpc
Ginir	1	1	1	1	1	1
Giniras	274.5128	9.051812	51.81511	144.7556	405.1746	94.89258

and heart regions indicating that they may have significant role in the development of these tissues or organs (Fig. 2).

Further, our studies show that homeostasis of a pair of naturally expressed, non-coding and overlapping sense and anti-sense transcripts (SAST) is critical to normal cell cycle progression. Our studies provide evidence for the role of sense transcript (Ginir) as a genomic instability-inducing RNA promoting oncogenic transformation, while its anti-sense counterpart (Giniras) acts as its negative regulator. A detailed analyses of the signalling pathways through which genomic instability may be induced by Ginir led us to discover defects in the ubiquitin- mediated degradation pathways that led to accumulation of Cyclin D1 and Cyclin E promoting cell cycle progression in NIH-Ginir cells. This also became evident in the increased levels of cyclin-dependent kinases CDKs – 4 and 6 being present in these cells, which are partners of Cyclin D1 function, required for inducing transactivation of S-phase genes in cells. Significantly, the enhanced expression of Cyclin-D1 in Ginir cells was accompanied by high levels of phosphorylation of it's down-stream target protein pRb, which was not found to happen in NIH-EV cells or NIH-Giniras cells. Protein ubiquitination is known to be regulated by a cascade of reactions mediated by three enzyme complexes- E1, E2 and the E3-ubiquitin ligases. Cullin family of ubiquitin ligases (E3) target numerous cellular proteins for proteasomal degradation. Cyclin D1 is a known substrate of poly-ubiquitination complex SKP1/CUL1/F-box (SCF) and is targeted for degradation by the SCF ubiquitin ligase during cell cycle progression and in response to DNA damage. The proteasomal inhibitor MG132 stabilized the endogenous cyclin D1 and Cyclin E protein levels in NIH 3T3 cells by inhibiting their degradation. However, in NIH-Ginir cells, the presence MG-132 did not show additional accumulation of Cyclin D1 and Cyclin-E, suggesting that Ginir interferes with the formation of the proteasomal degradation complex of SCF, leading to the maintenance of

Fig. 2: Expression of Ginir in 9.5dpc mouse embryo A). Phase contrast Micrograph of mouse embryo and B). In situ hybridization with Ginir specific LNA probe.



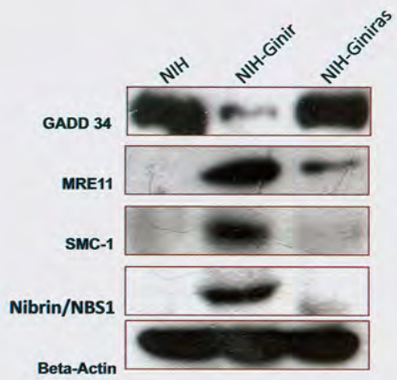


Fig. 3: Activation of DNA damage molecules in Ginir Cells. Western blot analyses showing over-expression of molecules involved in DNADamage in Ginir cells. Gadd34 is specifically down-regulated in NIH-Ginir cells

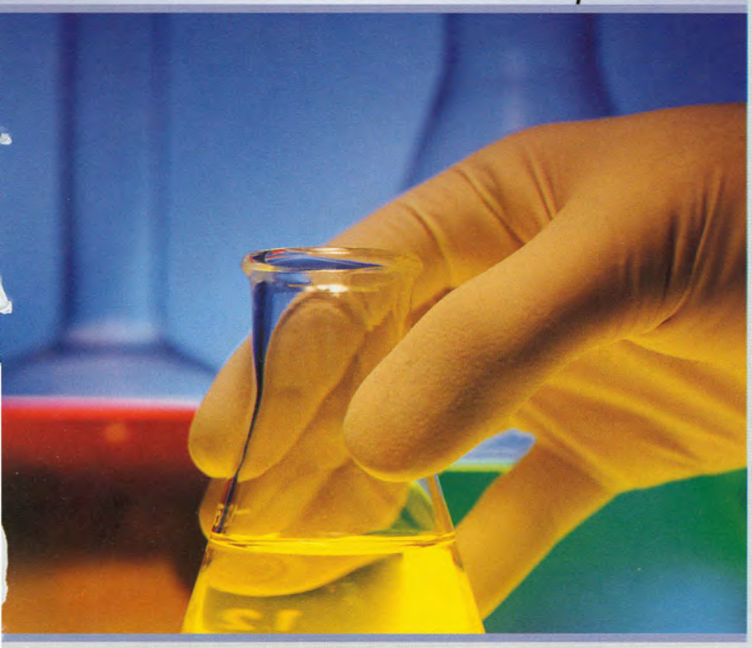
a high level of Cyclin D1 in these cells. Also, these cells show low levels of tumor suppressor genes Cullin1 and Cullin3 compared to NIH3T3 cells and our detailed studies provided evidence suggesting that Cullin1 and Cullin3 are the likely direct targets of Ginir.

Interestingly, we found low levels of *Gadd34* gene (also known as MyD116) expression in NIH-Ginir cells compared to NIH-EV and NIH-Giniras cells. The *Gadd34* gene expression is known to be regulated by genotoxic stress, nutrient deprivation and differentiation. Considering that increased levels of *Gadd34* RNA correlates with apoptosis induced by various signals and that it's over expression can elicit apoptosis, the reduced levels of *Gadd34* RNA in the NIH-Ginir cells correlate well with their resistance to apoptosis. The other genes related to DNA damage like Mre11 and Nibrin were over-activated in Ginir cells as compared to the other Ginir derivatives (Fig.3). Ginir expression also induced increases in the activation levels of check-point proteins that are closely involved in monitoring fidelity of DNA replication and chromosome segregation, such as, Ataxia telangiectasia mutated (ATM), ATM-Rad52-related protein (ATR), check point kinase1 (Chk-1) and check point kinase 2 (Chk-2). The NIH-Ginir cells, but not the NIH-EV cells, consistently demonstrated a greater density of stalled replication forks, demonstrating that Ginir overexpression resulted in an inappropriate G1-S transition and/or progression through the S-phase. In conclusion, our studies indicate that the high level of genomic instability induced by Ginir predisposes the target cells to tumorigenicity, which is an outcome of an over-activated DNA damage response pathway and faulty proteasomal degradation machinery, leading to inhibition of G_0 growth arrest, erroneous and faster cell cycle progressions, inhibition of differentiation, and loss of genomic integrity. We propose that Ginir and Giniras represent a pair of novel ncRNA regulators impacting the normal cell cycle progression, embryonic development and tumour suppression and their deregulation may lead to cancer. Our results provide support for the hypothesis that mechanisms like SAST may be effective in orchestrating gene regulation, chromatin remodelling, animal development, nuclear reprogramming and stem cell differentiation.

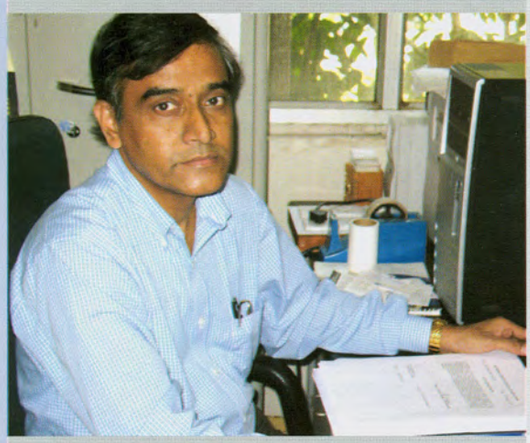
Future Work

1) Study the epigenetic changes induced during cellular reprogramming by Ginir in various cell systems including human adult dermal fibroblasts
2) Investigate the role of Ginir during development and differentiation in mouse and human systems

Research Report



Signal Transduction



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Studies on Role of Stroma- and Tumor-derived Osteopontin in Tumor Microenvironment

Background

Recent advancement in cancer research is focused on understanding the paradigm that tumor progression involves an intricate crosstalk between tumor and host/stromal environment. Osteopontin (OPN), a member of SIBLING family of chemokine like metastasis associated protein plays key role in regulation of various physiological and pathophysiological processes. OPN through αvβ3 integrin or CD44 regulates a series of signaling cascades leading to expressions of matrix metalloproteinase (MMP-2 and MMP-9), cyclooxygenase-2, vascular endothelial growth factor, ICAM-1 and cyclin D1 that controls cell migration, apoptosis, angiogenesis and tumor progression. Earlier data have revealed that increased expression of OPN correlates with enhanced melanoma progression. However, the mechanism by which stromal OPN regulates melanoma growth is not well defined. In this study, using melanoma model in wild type (OPN WT) and OPN knockout (OPN KO) mice, we have demonstrated that deficiency of host OPN effectively curbs melanoma growth, angiogenesis and metastasis. Melanoma cells isolated from wild type mice (B16 WT) exhibit elevated tumorigenic potential as compared to the parental cell line (B16F10) or cells isolated from the tumors of knockout mice (B16 KO). This study provides evidence that host derived OPN plays important role in melanoma progression and angiogenesis.

Aims and Objectives

1. To generate the melanoma tumor using B16F10 cells in OPN WT and OPN KO mice.
2. To establish the primary cultures from OPN WT (B16 WT) and OPN KO (B16 KO) mice.
3. To examine whether stromal OPN (B16 WT) regulates vasculogenic mimicry, melanoma cell migration, angiogenesis and tumor-endothelial cell interaction leading to melanoma growth and metastasis and to delineate the mechanism underlying this process.

Work Achieved:

In this study, we have injected B16F10 cells to the OPN WT and OPN KO mice and observed the reduced tumor growth in OPN-knockout mice. We have further established

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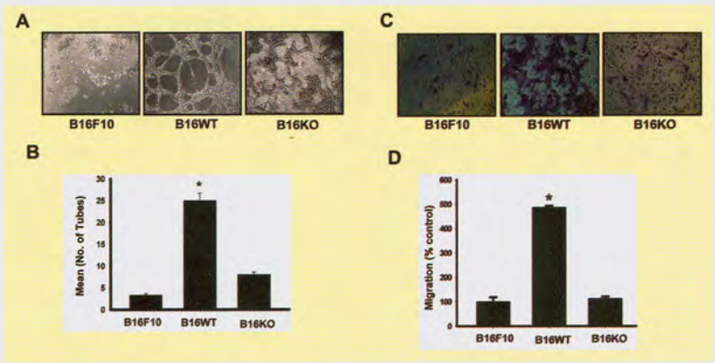
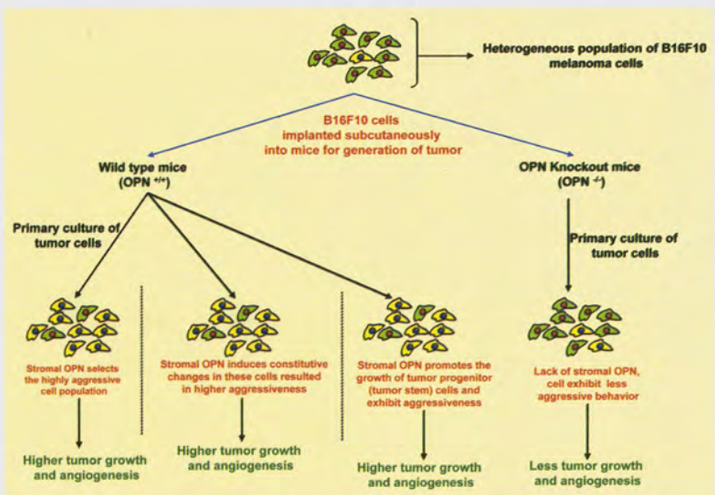
Fig. 1: B16 WT cells showed enhanced cell migration and angiogenesis as compared to B16 KO or parental B16F10 cells



two independent primary cultures, one from the tumor of OPN WT (B16 WT) and the other from OPN KO mice (B16 KO). Our data revealed that B16 WT cells showed enhanced vasculogenic mimicry, cell migration, angiogenesis and tumor-endothelial interaction as compared to the parental B16F10 cells or B16 KO cells (Fig. 1). We have reinoculated B16 WT, B16 KO and parental B16F10 cells to OPN KO mice and further confirmed that stromal OPN plays critical role in regulation of tumor growth and angiogenesis. Based on these findings, we have proposed that stromal OPN might control some constitutive changes in melanoma cells resulted in enhanced activation of kinases and transcription factors and elevated expression of oncogenic molecules which in turn regulates the belligerent behavior of B16 WT cells. Moreover, stromal OPN might select the aggressive subpopulation from the heterogeneous parental B16F10 cells and produce a highly tumorigenic population of melanoma cells which exhibit enhanced belligerent behavior. Our further experiments are underway to study that stromal OPN might promote the growth of stem cell population of B16F10 cells and exhibit the aggressive behavior in a selective manner. However, due to lack of stromal/host OPN in OPN KO mice, B16 KO retain the characteristics of parental B16F10 cells and showed less aggressive behavior. Taken together, our study demonstrated, at least in part, the crucial

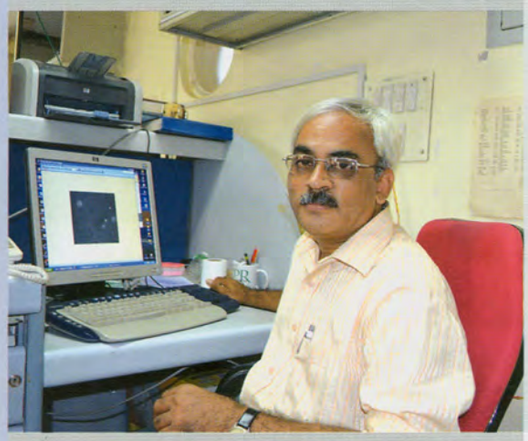
Fig. 2: Schematic representation by which stromal OPN regulates melanoma growth and angiogenesis



role of host/stromal OPN in regulation of tumor growth, metastasis and angiogenesis in melanoma model (Fig. 2).

Future Work

Several lines of evidences indicated that epithelial-mesenchymal transition plays crucial role in control of tumor growth and metastasis. The role of OPN in regulation of epithelial-mesenchymal transition and its correlation with activation of transcription factor and expression of epithelial and mesenchymal specific genes leading to cancer progression and angiogenesis are in progress. Moreover, the functional role of intracellular and secretory tumor derived as well as stromal OPN in control of tumor angiogenesis is the primary focus of the laboratory. The therapeutic and diagnostic significances of OPN in regulation of tumor angiogenesis in various cancer models will be further studied.



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Molecular characterization of tlyA gene product, Rv1694 of Mycobacterium tuberculosis: A non-conventional hemolysin and a ribosomal RNA methyl transferase

Participants

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Background

The complete genome sequence of the *Mycobacterium tuberculosis (M. tb)*, H37Rv, has been determined and its bioinformatic analysis improved our understanding of the life-cycle of this facultative, intracellular, human pathogen. Although re-annotation of the genome sequence of M. tb strain, H37Rv, changed some assignments, the significant portion of the annotation remained unchanged. Bioinformatic analysis has allowed us to predict the function for 2058 proteins out of 3995 (52%) and more than 150 of these have been experimentally identified in mycobacterial function. Among the 1051 conserved hypothetical proteins, 376 putative proteins showed no similarity to any known protein and it is possible that some of them may be specific to *M. tb* only. In H37Rv, among many virulence factors, it was predicted that *tlyA* gene is a part of an operon containing at least three other genes viz. the first being *tlyA* (Rv1694), second is *ppnk* (Rv1695) and the third is RecN (Rv1696), homologous to *E. coli* RecN. Since H37Rv is a well characterized pathogen an obvious question surfaces about the role of Rv1694 in pathogenicity and virulence. During the initial annotation, Rv1694 was predicted to be a 268 amino acid long, hemolytic protein, which may function as a virulence determinant as it showed homology with the *tlyA* gene products of swine pathogen *Serpulina hyodysenteriae*, a causative agent of swine dysentery and almost identical to *tlyA* of *Mycobacterium leprae*. This annotation was consistent with experimental observations that described the presence of hemolytic molecules in *M. tb*. For example, King and co-workers detected contact dependent hemolytic activity in the virulent strain H37Rv which was reduced by several fold in attenuated strain H37Ra, and not present in vaccine strain BCG. Moreover, two more proteins of *M. tb*, which showed homology with Phospholipase C of *Pseudomonas aeruginosa* and 30% homology to hemolysin A, precursor of Vibreo cholerae, which have been reported to possess hemolytic activity. In addition, Wren et al. showed the presence of *tlyA* homologues in *M. tb*, *Mycobacterium leprae*, *Mycobacterium avium* and *Mycobacterium bovis* BCG, but appeared to be absent in non-pathogenic strain *Mycobacterium smegmatis*. Interestingly, introduction of the *tlyA* gene into *Mycobacterium smegmatis* using a mycobacterial shuttle expression plasmid increased the contact dependent hemolysis orchestrated by it. However, no further information is available on Rv1694 until recent studies showed that Rv1694 functions as a ribosomal

RNA methyltransferase. The methylase activity resulted in methylation of ribosomal RNA which can alter the susceptibility to capreomycin, a macrocyclic peptide antibiotic. It has been observed that mutations in the *tlyA* gene may make the bacteria resistant towards capreomycin. Hence, the question arises as to whether the tlyA is a hemolysin or a ribosomal methyltransferase or it is a dually active molecule.

Multi-functionality for a protein can arise due to the presence of distinct/separate domains to carry out an individual function or upon post-translational modification such as proteolytic cleavage which may result in acquisition of a new function. In the present example Rv1694 has been suspected to possess two entirely diverse activities i.e. haemolytic and RNA methylation activities, which is rather unusual. Since mycobacterium can have a complicated external environment after entry into macrophages, the role of an individual protein like Rv1694 needs a thorough characterization in order to decipher its location specific function during the initial phase of infection. Such studies are important to prove or disprove its diverse/distinct functions to correlate its probable role in pathogenicity of *M. tb.* The answers to the above questions can be unambiguously obtained only with the help of detailed characterization of Rv1694 as purified Rv1694, per se, must exhibit the said activities under in vitro, conditions.

Aims and objectives

In view of the importance, the aim of the present study is to answering the following questions viz., (i) Can this protein be present in two distinct locations and perform two diverse functions? (ii) If yes, any other molecule(s) assist this protein to perform these diverse functions? (iii) How the hemolytic function is orchestrated by it?

Work Achieved

Homology of Rv1694 of *M. tb*: Rv1694, *tlyA* gene product of H37Rv, is an alkaline protein that has >40% similarity with other cytotoxins/hemolysins. Initial annotation has referred the *tlyA* gene product as a cytotoxin/hemolysin homologue and it was classified as a hypothetical 'pore forming hemolysin'. The Rv1694 also contains a motifs that align well with rRNA methyltransferase and RNA binding proteins as it displays considerable homology with FtsJ/RrmJ (~48% homology, 23S rRNA methylase) of *E. coli.* Since Rv1694 also has well conserved K^{69}-D^{154}-K^{182}-E^{238} tetrad, it has been re-annotated as rRNA methyltransferase. Hence, the *tlyA* gene product exhibits homology with two diverse classes of proteins viz. hemolysin/cytolysin as well as ribosomal RNA methyltransferases. The full-length gene of Rv1694 (828 bp) was cloned in pT7Nc (without 6-his tag) and pET28a(+) (with 6-histidine tag at the carboxy terminus) vectors were expressed in *E. coli* BL21(DE3) CodonPlus-RIPL. The CD data was also consistent with a homology model shown in Figure 1, which was built with Swiss-Model server using 3hp7A.pdb as template which represents the putative hemolysin from *Streptococcus thermophilus*.

Rv1694 has hemolytic activity: *E. coli* has no natural homolog of Rv1694, thus, it was not expected to exhibit any contact dependent hemolysis of red blood cells. In order to confirm the hemolytic activity of Rv1694 expressing *E. coli*, we have analyzed for contact dependent haemolytic property. Our data unambiguously detects the contact

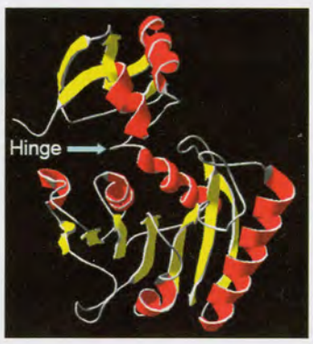

Fig. 1: Model of Rv1694: Model of Rv1694 was built using the putative hemolysin from *Streptococcus thermophilus*, using 3HP7.pdb as template with the help of Swiss Model Server . The Rv1694 has a typical fold of a ribosomal RNA polymerase consisting of 7 β-sheets surrounded by 5 α-helices. Please note that the amino acids 246-268(QTD...EGP) are not present in the depicted model. The white arrow points the region containing basic amino acids between the domains susceptible to proteases like trypsin/Proteinase K.

dependent hemolysis of rabbit RBCs (rRBC) in comparison to uninduced controls in ~30 hrs of incubation time. In order to rule out any pre-existent, loose, oligomeric aggregates responsible for the haemolysis of rRBCs in our experiments, we have generated the Rv1694 by *in vitro* transcription and translation (IVTT) and examined for hemolytic property. The haemolytic activity of Rv1694 generated by IVTT, which is unambiguous and consistent with the slow hemolysis data just described.

Visualization of Rv1694 on *E. coli* surface: The confocal microscopic pictures of *E. coli* stained for the Rv1694 show that the Rv1694 was present on the outer cell wall of *E. coli* as seen in Figure 2 with the help of specific staining of the polyclonal antibody raised by us. It is important to note that neither mock transformed *E. coli* (stained with Rv1694 specific immune serum) nor secondary antibody used in the study showed any staining. The hemolysis of Rv1694 expressing bacteria was reduced by about 50% in comparison to the hemolysis in the absence of immune serum. These observations suggest that the Rv1694 was expressed on the cell wall of *E. coli* and the anti-Rv1694 antibody neutralizes the contacts needed for the assembly and lysis.

Binding and oligomerization of Rv1694 on RBC membranes: We further examined whether or not Rv1694 can form ordered oligomers which must be present and responsible for the slow but consistent hemolysis observed by us. Hydropathy plot analysis of Rv1694 primary sequence has revealed that there are two probable transmembrane regions viz. amino acids 67-87 and 150-170. The Rv1694 showed strong binding to rRBC membranes and formed stable oligomers and showed concentration dependency, as shown in Figure 3, and the kinetics of oligomerization on rRBC

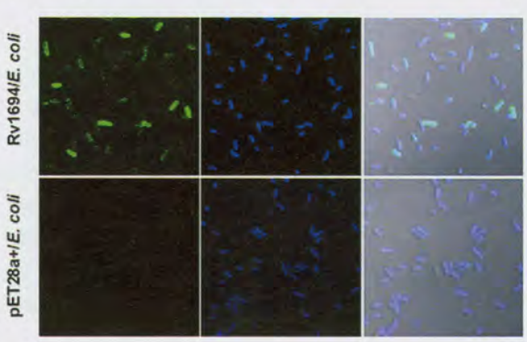

Fig. 2: (A) Immuno-fluorescence localization of Rv1694 on *E. coli*: *E. coli* transformed with Rv1694 or pET28a+ was immunostained with immune rabbit serum followed by detection with Cy2 conjugated goat anti-rabbit IgG (green fluorescent). Left side of the panels show the Rv1694 localization (Green), middle panels show the DAPI stained bacteria (Blue) and right panels show the merged images.

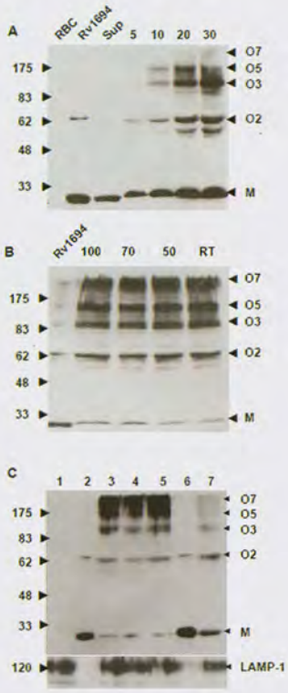

Fig. 3: **(A) Binding and oligomerization of Rv1694 on rRBC membrane:** The concentration dependant binding and oligomerization of Rv1694 was examined by incubating the Rv1694 with 2% rRBC for 30 min. Samples were electrophoresed under reducing condition on 8% SDS-PAGE and immuno probed with anti-6-histidine-antibody. Lanes indicated with RBC (rRBC membrane only), Rv1694 (Rv1694 purified protein only), Sup (Supernatant of rRBC incubated with Rv1694) and lanes indicated 5, 10, 20 and 30 stands for rRBC incubated with 5.0 µg, 10.0 µg, 20.0 µg and 30.0 µg Rv1694 respectively. **(B) Oligomerization of Rv1694 on RBC membrane in the absence of reducing agents:** Rv1694 (10 g) mixed with rRBC (2%) were mixed in the absence of reducing agent for 30 min at room temperature and samples were washed and dissolved in 4% SDS and 5x laemmli sample buffer without β-mercaptoethanol. Samples were electrophoresed on 8% SDS-PAGE and probed with anti-6-histidine-antibody after transfer to nitrocellulose membrane. Lane 1. Rv1694 purified protein only; Lanes 2-5 show the oligomers of Rv1694 on the RBC membrane at different temperatures, i.e., Lane 2: heated at 100oC; Lane 3: heated at 70oC; Lane 4: heated at 50oC; Lane 5: at room temperature. **(C) Binding and oligomerization of Rv1694 protein on the Phagosomal membrane:** Oligomerization of Rv1694 was observed by incubation of purified Rv1694 and phagosomal preparations of RAW 264.7 cells. An equal amount of protein was taken out at indicated time points. Samples were electrophoresed under non-reducing conditions on 8% SDS-PAGE and immuno probed with anti-6-histidine-antibody. Lane 1: Phagosomal preparation alone; Lane 2: Unboiled Rv1694 protein; Lanes 3 to 5: Unboiled samples of phagosomal preparations and Rv1694 incubated for 10.0, 30.0, and 60.0 min respectively. Lane 6: Boiled Rv1694 protein and Lane 7: Boiled sample of 60 min incubated phagosomal preparation and Rv1694. Labels viz. M, O3, O5, O6 and O7 indicate monomers, trimers, pentamers, hexamers and heptamers respectively. Unboiled samples prove the absence of any pre-existing oligomer of Rv1694 added to phagosomal preparation. The blot was stripped and re-probed with anti-LAMP-1 antibody to ascertain the phagosomal preparation. LAMP-1 is 120-kDa late-phagosomal glycoprotein marker. The data shown is one of the three independent experiments.

membranes was found to be slow in comparison to well studied, conventional hemolysins.

Rv1694 binds to and oligomerizes on macrophage phagosomal membranes: To further understand the role for Rv1694 in this process, we have examined the binding and oligomerization of Rv1694 on phagosomes isolated from RAW264.7 cells for different time periods i.e. 10, 30, 60 min. The Rv1694 showed strong binding to the phagosomal membranes and formed stable oligomers, as shown in Fig.3, which are very similar to the ones seen on rRBCs including their stability in 4% SDS and susceptibility to near boiling temperatures under non-reducing conditions. Moreover, unboiled samples prove the absence of any pre-existing oligomer of Rv1694 added to phagosomal preparation. In summary, all these observations suggest that the Rv1694 has the ability to bind to target membranes such as phagosomal membranes and can form multimeric structures for destabilization. In this process it appears that a disulfide bond seems to play a key role.

Rv1694 is a ribosomal RNA methylase: In order to evaluate the ribosomal RNA methyltransferase activity of Rv1694, we attempted several approaches: In our first approach, we monitored the growth rates of *E. coli* expressing the Rv1694 and mock vector (pET28a+) transformed *E. coli* without or with capreomycin (100 µg/ml) as shown in Figure 4. In the presence of capreomycin, the mock vector transformed *E. coli* growth was retarded by only ~10% while the *E. coli* expressing the Rv1694 construct did not grow efficiently (>40% loss). We also ensured that these cultures expressed the Rv1694 by examining for hemolysis for each time point. This experiment confirms the Rv1694

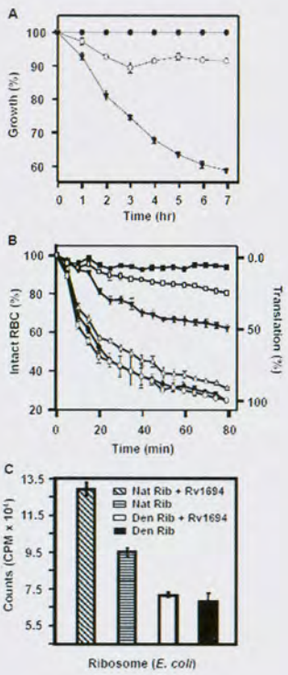

Fig. 4: (A) Growth of *E. coli* expressing Rv1694 in presence of capreomycin: Growth *E. coli* expressing Rv1694 in the presence and absence of capreomycin (100 g/ml), was monitored vis-a-vis the mock vector (pET28a+) transformed *E. coli*. Symbols represent (●) growth of Rv1694 transformed *E. coli* in the absence of capreomycin. (▼) growth Rv1694 transformed *E. coli* culture in presence of capreomycin. (○) growth curve of mock vector transformed *E. coli* in the presence of capreomycin. Error bars represent standard errors from two independent experiments. **(B) Inhibition of coupled *in vitro* transcription-translation by capreomycin:** S30 extracts of Rv1694 (without carboxy terminal 6-histidine tag) transformed and of mock vector (pT7Nc) transformed BL21(DE3) *E. coli* were prepared and set for the *in vitro* transcription and translation reactions. Translation reactions contain two concentrations of capreomycin (16 and 80 ng/ml) and all other constituents such as S30 premix, S30 extract, DNA concentration, rifampicin, RNasin H (20 units) are identical for all reactions. We have used supercoiled plasmid DNA of staphylococcal α-hemolysin as a reporter gene whose hemolytic activity was assayed by monitoring the hemolysis at OD_{595}. All points for all curves were calculated with respect to 100% lysis of rRBCs and average of three independent experiments is shown in the graph. The symbols are defined as follows: (●) S30 extract of Rv1694 without capreomycin; (○) S30 extract of mock vector without capreomycin; (▼) S30 extract of Rv1694 with 16 ng/ml capreomycin; (Δ) S30 extract of mock vector with 16 ng/ml capreomycin; (■) S30 extract of Rv1694 80 ng/ml capreomycin; (□) S30 extract of mock vector with 80 ng/ml capreomycin. **(C) *In vitro* methylation activity of purified Rv1694 protein on isolated Ribosome:** Methylation of ribosomes (prepared from *E. coli* strain) in presence or absence of purified Rv1694 and [^{3}H]S-adenosylmethionine. From left, first bar (Crossed pattern) represents the reaction mix with native ribosome and Rv1694, second bar (horizontal pattern) represents reaction mix with native ribosome only, third bar (open) represents reaction mix with denatured ribosome (65oC) and Rv1694 and fourth bar (filled) represents reaction mix with denatured ribosome only. Abbreviations: 'Nat Rib' for native ribosome, 'Den Rib' for heat denatured ribosome, 'Prot' for Rv1694 purified protein. Error bars represent standard errors from triplicate experiments.

mediated methylation of ribosomal RNA of *E. coli* under in vivo conditions, as the mock vector transformed control has practically showed no dramatic retardation of growth in the presence of capreomycin. In the second approach, we also analyzed the *in vivo* methylation activity of Rv1694 by coupled *in vitro* transcription-translation with capreomycin. In this experiment, we prepared the S30 extracts of *E. coli* expressing the Rv1694 along with controls. We have carried out the in vitro transcription and translation experiments at two different concentrations of capreomycin (16 ng/ml and 80 ng/ml), in order to closely match the observations reported earlier. In Figure 4, in the absence of capreomycin, the translational efficiency of S30 extracts of Rv1694 expressing *E. coli* and mock vector *E. coli* showed nearly identical translational efficiency of the staphylococcal α-hemolysin reporter (filled circles vs. open circles) and this value was taken as 100% translation efficiency. At 16 ng/ml capreomycin, the S30 extract made from Rv1694 expressing *E. coli* showed reduced translation (~50% loss) i.e. considerable loss of hemolysis (solid inverted triangles) where as the mock vector transformed *E. coli* showed no loss of translation (open triangles). At 80 ng/ml capreomycin concentration, the S30 extract made from Rv1694 expressing *E. coli* showed no translation of reporter (solid squares), whereas the vector transformed *E. coli* showed about 26% lysis (solid squares vs. open squares) compared to the translation without capreomycin. It should be noted that the *E. coli* is partially sensitive to capreomycin at higher concentrations i.e. >80 ng/ml. This experiment, once again, confirms the ribosomal RNA methylation activity of Rv1694.

Future Work

We will be investigating the mechanism of action of TlyA of M.tb and other homologues of other pathogenic bacteria in detail.

Research Report

Diabetes

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Investigation of mitochondrial dysfunction, oxidative damage and apoptotic cell death stress mechanism in diabetic cardiomyopathy

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Background

High glucose-induced cardiac apoptosis plays an important role in diabetic complications. However, the molecular mechanisms of cardiac damage by glucose are incompletely understood. Perhaps, the cytotoxic actions of glucose are mediated, in part, through oxidative stress and intracellular Ca^{2+} overload. These two insults are linked to each other, viz., the oxidative stress induces an increase in $[Ca^{2+}]i$ while the increase in $[Ca^{2+}]i$ increases oxidative stress. Furthermore, mitochondria being a storehouse for intracellular calcium, a source of reactive oxygen species (ROS), and a sensor of oxidative stress, play a key role in regulation of apoptosis under a variety of pathological conditions including diabetes. Hyperglycemia-induced mitochondrial superoxide overproduction through activation of three pathways—activation of protein kinase C isoforms, increased formation of glucose-derived advanced glycation endproducts and increased glucose flux through the aldose reductase pathway, have been implicated in the pathogenesis of diabetic complications. In the mitochondria, superoxide ($O_2\bullet-$) is the primary ROS made, which is converted to H_2O_2 by the enzyme manganese-superoxide dismutase (Mn-SOD) that catalyze the dismutation of $O_2\bullet-$ to hydrogen peroxide and molecular oxygen {Reactions (1) and (2)}.

$$M^{n+} + O_2^{\bullet -} \longrightarrow M^{(n-1)} + O_2 \qquad (1)$$

$$M^{(n-1)} + O_2^{\bullet -} + 2H+ \longrightarrow M^{n+} + H_2O_2 \qquad (2)$$

Therefore, modulating mitochondrial activity could possibly control ROS production. Hence, novel approaches to block superoxide generation in diabetic mitochondria may offer a therapeutic benefit by preventing the development of diabetic cardiomyopathy.

Our collaborators have recently synthesized a low molecular weight SOD mimic, ML3 for the treatment of various oxidative stress-mediated pathogenesis. It is a five co-ordinate manganese complex of 2-formylpyridine thiosemicarbazone, 2-acetylpyridine thiosemicarbazone and 2-quinolinecarboxaldehyde thiosemicarbazone ligand (Fig 1A). We evaluated the protective effect of ML3 on isolated adult rat ventricular myocytes (ARVMs) and a rat cardiac myoblast H9c2 against Hyper glycemia (HG)-induced

cardiotoxicity. Our results indicate that the novel SOD mimic ML3 display significant inhibition of HG-induced cardiac contractile dysfunction and cytoprotective activity in H9c2 cells, which might be of therapeutic use in the treatment of diabetic cardiomyopathy.

Aims and Objectives

To study the protective role of novel Mn-SOD mimics against high glucose induced H9c2 cell death and to explore their protection against oxidative stress

Work achieved

We have previously studied the precise mechanism(s) of high glucose induced calcium mobilization, mitochondrial calcium overload and subsequently mitochondria dependent and independent cell death cascade in cardiac myoblast H9c2 cells. The role of Ca2+ in calpain activation and caspase deployment in high glucose mediated cell death was studied and results from our work provided valuable insight about ca2+ overload induced calpain pathway in high glucose induced cell death.

In the present study we have examined the protective effect of Mn-SOD mimic ML3 on high glucose induced H9c2 Cell death. Cells were treated with 5.5 mM and 33 mM of glucose for a time course study of up to 24h, 48h, 72h and 96h and the cell viability was assessed by MTT assay. It was previously observed in our laboratory that 33mM glucose concentration resulted in significant reduction of cell viability of H9c2 cells in 72 and 96h. Therefore only these two time points 72h and 96h are considered in this study. Mannitol, used for osmotic control, exhibited that the loss of cell viability after 35 mM concentration is a result of osmotic distress and contribution of glucose concentration alone will be difficult to assess. Therefore, glucose concentration of 33 mM was used for all further experiments.

To evaluate the protective effect of Mn-SOD mimics ML-3 on the in vitro system, H9c2 cells were treated with normal glucose 5.5mM glucose (NG or control) and 33mM (high glucose, HG) along with different concentrations (10, 50, 100nM) of ML3 for 72 and 96h. Superoxide generator mix, 1mM xanthine and 0.05U xanthine oxidase (X+XO), was used as positive control either alone or along with above mentioned concentration of ML-3 to see the superoxide scavenging activity of drug and its protective effect against X+XO induced H9c2 cell death. (X/XO treatment was given after pretreatment of cells in normal glucose media with different concentration of drugs and before two hours of completion of time point). Mn SOD (100U) was used as specific scavenger for superoxide. Mn-SOD mimic ML-3 was first standardized for the concentrations which have maximum protection against high glucose induced H9c2 cell death and also against X+XO (superoxide generator used as positive control) induced H9c2 cell death. ML-3 showed maximum protection at concentrations 10nM, 50nM and 100nM. Above this concentration it exhibited toxicity. Therefore for further experiments only these three concentrations were used to test the protective effect against high glucose induced cell death. Out of the three concentrations used (10, 50 and 100nM) it was found that both

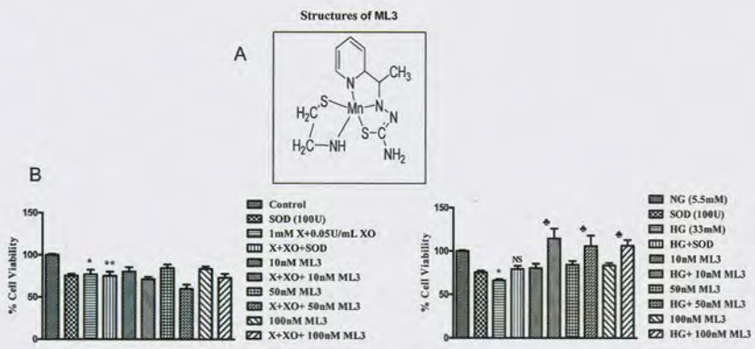


Fig. 1: A. Structure of Mn-SOD mimic ML-3, B. Effect of high gluose on % cell viability by MTT assey. H9c2 cells were treated with different concentration of ML3 (10, 50, and 100 nM), 100U SOD and 1mM Xantthine+0.05U/ml Xanthine Oxidase (X+XO) for 96 h. The columns represent the % viability of H9c2 cells. Results were expressed as mean ± SEM of $n = 3$.

ML-3 showed maximum protection at 10 and 50nM concentrations against high glucose and X+XO induced H9c2 cell death (Fig 1).

ML-3 decreased high glucose-induced intracellular peroxynitrite (ONOO-) levels and retained MMP (mitochondrial membrane potential): Exposure of H9c2 cells to HG for 48h led to an increase in intensity of fluorescent positive cells from normal glucose condition to high glucose condition. Treatment with of ML-3 reduced the generation of ONOO- under high glucose condition as can be seen in Figure 2, by the decrease in intensity of fluorescent positive cells in high glucose condition, to treatment of Mn-SOD mimics under high glucose condition and retains MMP. This result indicates that Mn-SOD mimics have the activity to reduce the production of highly reactive intracellular oxidant peroxinitrite under high glucose condition.

These studies provide further evidences that Mn-SOD mimics ML-3 is protective against the high glucose induced H9C2 cell death, and these mimics also have antioxidant property as they are showing a marked decrease in levels of mitochondrial superoxide and peroxynitrite in high glucose condition.

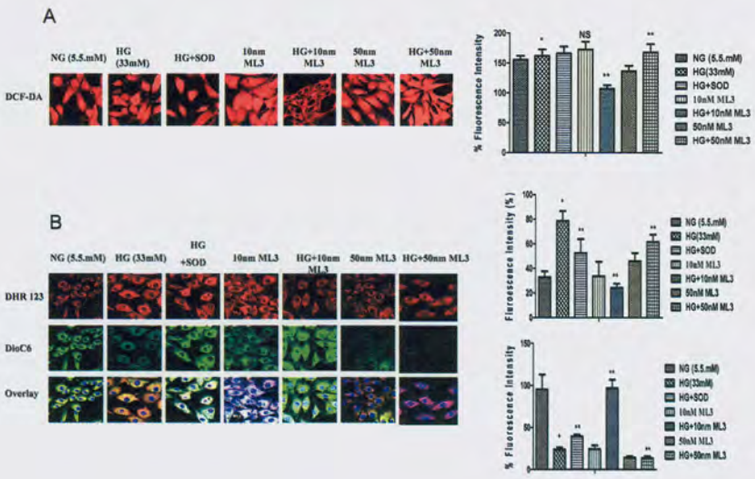
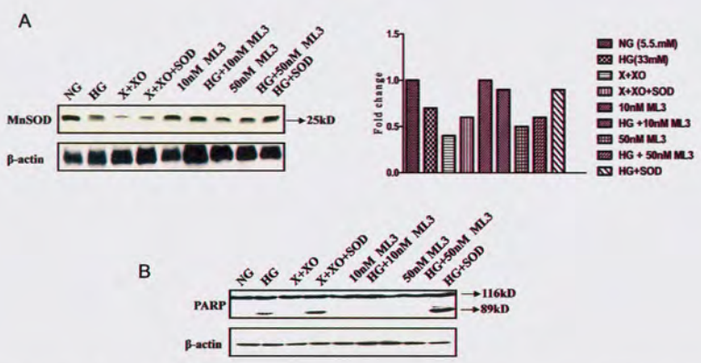


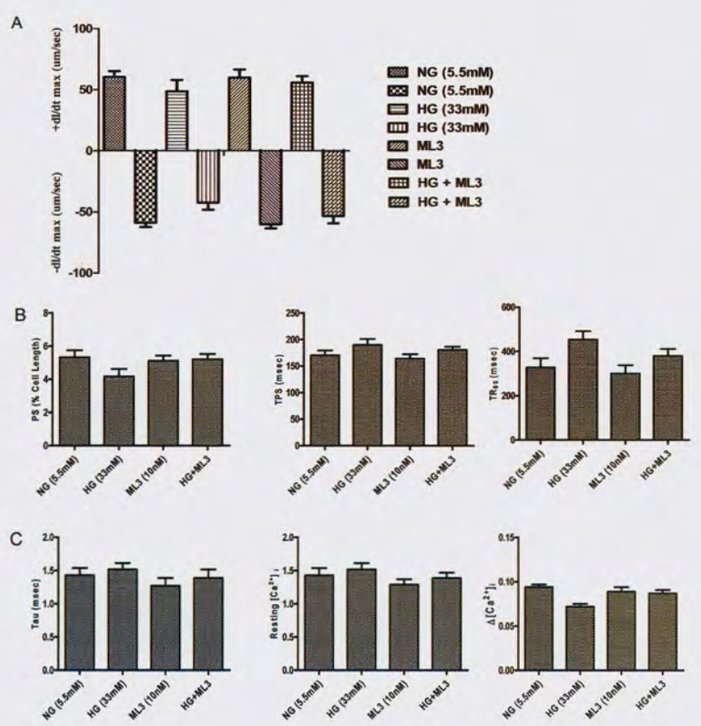
Fig. 2: Detection of A. superoxide, B. peoxynitrite and MMP in H9c2 cells by confocal microscopy. Cells were treated with Mn SOD mimetic ML-33 in high glucose condition for 48h, .stained with fluorescent probe DHR-123 (5μM) and DiOC6 (100nM) and images are acquired using confocal microscopy. Xanthine (1 mM) + xanthine oxidase (0.05U) served as positive control where as Mn SOD (100U) served as scavenger for $O2^{\bullet-}$.

Fig. 3: Effect of Ml-3 on A. Mn-SOD expression and B. PARP cleavagein H9C2 cells under high glucose condition. H9c2 cells were treated with 5.5mM glucose (control C, lane 1), 33mM high glucose HG (lane 2), positive control X+XO (lane 3), X+XO with superoxide scavenger 100U Mn SOD (lane 4), 10nM and 50nM of drugs ML-3 (lane 5 and lane 8) and 10nM and 50nM ML-3 along with 33mM high glucose HG (lane 6 and 10). 6b represents the densitometry result showing the fold change in the Mn SOD expression. Actin served as a loading control.



To elucidate the mechanisms of how these synthetic low molecular mass Mn-SOD mimics have the ability to augment the intracellular antioxidant defence mechanism, Mn-SOD expression was studied by western blot. Exposure of H9c2 cells to HG as well as X/XO showed a decrease in intracellular Mn-SOD expression that was restored after ML3 treatment thus indicating that ML3 also has the property to augment the intracellular antioxidant defense (Fig 3). Since earlier we have observed that increased generation of intracellular ROS and RNS in response to sustained high glucose condition triggers DNA single strand breakage, which induces a rapid activation of poly(ADP-ribose)polymerase (PARP), we further studied the expression of PARP and show that PARP cleavage induced by the high glucose and X/XO treatment, was reduced after ML3 treatment (Fig 3).

Fig. 4: Effect of ML-3 on contractile properties of adult rat ventricular cardiomyocytes. (A) Mechanical indices are maximal velocities of shortening/relengthening (±dL/dt), (B) peak shortening (PS), (C) time to peak shortening (TPS), and (D) time to 90% relengthening (TR 90). Means±SEM, n=15–20 cells/group.

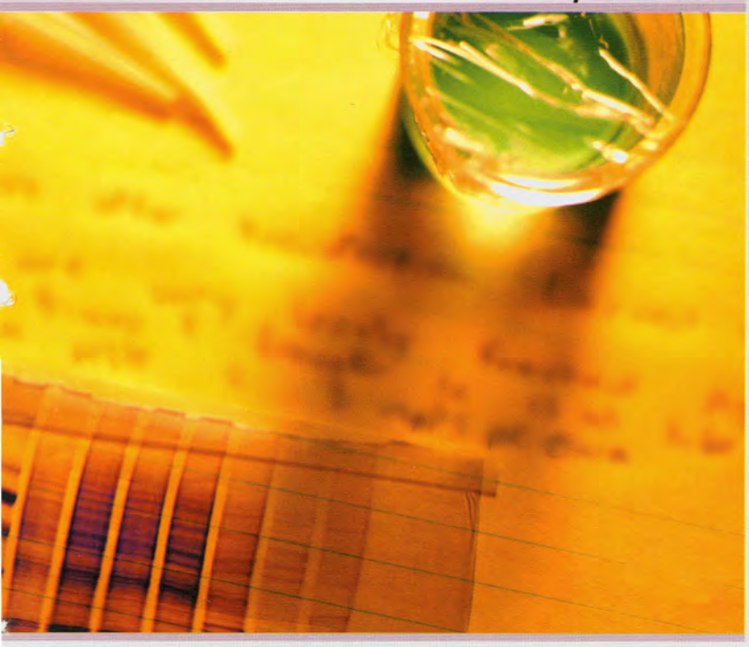


Further studies on primary cardiomyocytes indicated that ML-3 prevents high glucose induced mechanical abnormalities. To ascertain the role of ML-3 on contractile indices the myocytes were treated with or without ML-3 under high glucose condition. Myocytes treated with high glucose showed reduced peak shortening amplitude associated with a decrease in ±dL/dt, prolonged TPS, and prolonged relengthening (TR90) which was restored by ML-3 (Fig 4A, 4B). Mechanical dysfunction in cardiomyocytes is caused by abnormal calcium accumulation. To investigate the probable mechanism of action involved in high gluose induced mechanical dysfunction, the effect of ML-3 on intracellular calcium levels were observed in adult cardiomyocytes. Resting calcium levels were observed to be increase in high glucose. Cytosolic free calcium decrease rate (Tau) was evaluated in Fura-2AM-loaded cardiomyocytes after treatment with high glucose. Treatment of adult cardiomyocytes with high glucose significantly reduced the cytosolic free calcium decrease rate compared to normal cardiomyocytes ($p<0.05$) (Fig 4C). This was consistent with reduced peak shortening and time to 90% re lengthening. However, all these high glucose induced defects were restored by ML-3

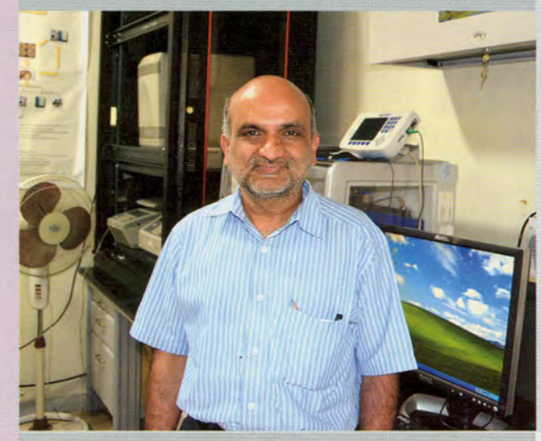
Future work

We will study the mechanical indices of the protective effects of ML3 in vivo in detail.

Research Report



Biodiversity



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Microbial diversity and genomics

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Background

Microbial Genomics: Microorganisms represent about half of the global biomass as they are ubiquitous and form predominant form of life on earth. They not only outdo the eukaryotic cells in number but their metabolic, physiologic and genetic diversity is far greater than any other life form. However, very little information is available about their diversity as compared to higher life forms due to lack of appropriate methods to study them. Initially, 16S rRNA sequence studies enabled researchers to have a glimpse of bacterial diversity. In the recent years high-throughput sequencing technologies enable us to have a better understanding of uncultivable majority of microbes. Our laboratory uses these methodologies to understand microbial community structure and function of selected unique ecological niches.

Aims and Objectives:

Understand the structure-function relationship of microbes in unique ecosystems like insect gut, human gut and hyper-saline hyper-alkaline lake.

Work Achieved

1. INSECT GUT MICROBIOLOGY

All insect species are known to harbor a rich and complex community of microorganisms in their guts and other body regions. These microorganisms participate in many types of interactions ranging from pathogenesis to obligate mutualism. As different groups of insects have different feeding habits, vast microbial diversity found associated different gut structures and functions and promotes the establishment of different bacterial phylotypes.

A. Study of Midgut bacteria of *Aedes* mosquitoes and their role in the infectivity of dengue virus: Bacteria present in the midgut of *Aedes* mosquito might have important roles as determinants of virus survival and their development in mosquito. Their role as contributor to the modulation of vector competence is unknown as of today. We are investigating the role of midgut bacteria of *Aedes* mosquitoes in the process of infectivity of dengue virus.

In this study, midgut bacteria of three *Aedes* species (*Ae. aegypti, Ae. albopictus and Ae. vittatus*) from dengue endemic and non endemic areas were studied using culture dependant and independent method and quantified using Real-Time PCR. We identified *Staphylococcus* sp., *Exiguobacterium* sp., *Bacillus* sp., *Enterococcus* sp., *Aeromonas* sp., *Pseudomonas* sp., *Serratia* sp., *Shigella* sp. which were earlier reported from midgut of different mosquitoes while *Rahnella* sp., *Burkholderia* sp., *Ralstonia* sp., *Moraxella* sp., *Sphingomonas* sp., and *Roseomonas* sp. were previously reported from other insects' gut.

Culture dependant identification studies revealed significant differences between the midgut bacteria of Ae. *Aegypti* from two sampling areas. We observed considerable bacterial diversity in the midgut of three Aedes species. At the same time number and type of midgut bacteria from dengue endemic and non-endemic areas in 16S rRNA gene found variable.

The most significant feature of the study was the dominance of *Serratia* spp. in all the three culture independent studies from dengue non-endemic areas. *Serratia* spp. was totally absent in clone libraries from dengue endemic areas. The other notable feature was the dominance of *Aeromonas* sp. in Ae. *aegypti* from dengue endemic areas.

The study highlights the link between the disease prevalence in some areas and the bacterial diversity and richness in midguts of wild mosquitoes. It is now established that viruses transmitted by mosquitoes co-exist with associated midgut bacteria that can affect their vectorial ability to carry virus. Characterizing the bacterial composition and diversity of *Aedes* mosquitoes in their natural environment is a step forward in understanding the ecology and the multipartite interactions occurring in these vectors of arbovirus.

B. Phylogenetic profiling and molecular characterization of gut-associated bacteria in larvae and adults of the flesh fly, *Sarcophaga* sp. (Diptera: Sarcophagidae): Flesh flies (Diptera: Sarcophagidae) are necrophagous insects, well known causal agents of myiasis and considered as carrion flies which provide important clues for the estimation of the postmortem interval (PMI) which is of forensic importance. Gut-associated bacteria in larvae and adults of flesh fly, *Sarcophaga* sp., were studied using both culture-dependent and culture-independent methods.

Members of the Gammaproteobacteria and Firmicutes were found in the larvae of the flesh fly gut. Cultured based study showed the presence of genus *Acinetobacter, Ignatzschineria, Citrobacter, Budvicia, Proteus, Pasteurella, Lysinibacillus, Bacillus, Staphylococcus* and *Vagococcus* in the midgut of flesh fly. Communities in adult flesh fly guts were found consisted of members of the Actinobacteria, Gamma-proteobacteria, firmicutes and Bacteroidetes. Cultured isolates showed the affiliation to the genus *Providencia, Ignatzschineria, Bacillus, Myroides* and *Dermacoccus*. Clone sequences from adult guts were grouped into 10 operational taxonomic units (OTUs) at 3% difference among sequences.

numerous genera, species and strains of bacteria. Most studies of intestinal microbiota of infants have been based on the fecal samples using the conventional culture techniques which have the several limitations evident with the varied results of the different studies.

In this study, the development of the gut flora in the normal delivered breast fed infants starting from the day 0 up to the 2nd year of their life was monitored using culture-independent 16S rDNA cloning and sequencing approach. It was observed that colonization is gradual process; it starts immediately after the birth. Initially with the normal environmental microbes and microbes from the mother, and it goes through rapid changes till a stable flora established. Weaning (introduction of solid food) is found to be an important event in the shaping up of the infant gut microflora, rapid changes occur after that as it is evident from our data (shift from Proteobacteria dominance to Firmicutes). It is also observed that no significant changes occur after first year of the infant life at the gut microbial community structure level in infants born with vaginal delivery and their community profile resemble that of normal adult gut flora mainly dominated by Bacteroids and Firmicutes.

B. Effect of mode of delivery on the gut microflora of infants: The neonatal period is crucial for intestinal colonization. Infants gut at the time of birth use to be sterile and soon after delivery it undergoes colonization with different type of bacteria. There are several factors like gestational age, type of delivery, feeding mode which affect the initial colonization.

In this study the effect of mode of delivery was studied. It is found that fecal microbiota of vaginally born (VB) infants was found to be distinctly different from their counterpart cesarean section born (CB) infants. The most abundant bacterial species present in VB infants were *Acinetobacter* sp., *Bifidobacterium* sp. and *Staphylococcus* sp. while CB infants' fecal microbiota was dominated with *Citrobacter* sp., *Escherichia coli* and *Clostridium difficile*. An interesting finding of our study was recovery of large number of *Acinetobacter* sp.; a noso-comial pathogen, in the feces of the VB infants. Although none of the infants had shown any clinical symptoms of disease, this observation emphasizes the potential risk of *Acinetobacter* related epidemic outbreak in infants from this region. In addition variation in the microbial community structure due to genetic and environmental factors is being investigated.

C. Assessment of micro-eukaryotic Diversity in the human gastrointestinal tract: Human gut is a complex ecosystem. Millions of bacteria along with other micro-eukaryotic organism i.e. protozoan, fungi are known to live in dynamic equilibrium. Majority of the studies carried over to understand the exact community structure of organism in this ecosystem has been focused on the bacterial diversity. No such thorough studies were done to elucidate the diversity of micro-eukaryotic organisms present in this habitat. These micro-eukaryotic organism are believed to have role in the maintaining the balance of this niche through predation and production of anti-bacterial secondary metabolites.

We studied the micro-eukaryotic diversity in infants up to 1st year of their life and in their respective mothers were examined for such diversity. A distinct pattern of micro eukaryotic organism which includes fungi and small protozoan was observed in the mother's stool samples. However, we found that human infants devoid of any micro-eukaryotic organism at least till one year of their life. As against the earlier reports; Blastocystis sp. which has been implicated into several gastrointestinal disorders; none of our subject has shown clinically significant symptoms of any related disease.

D. Comparative study of gut and oral microbiota of diabetic and non-diabetic individuals: Diabetes is one of the most prevalent genetic diseases. We aimed to study if there is any correlation between the microbial community structure and development of diabetes using culture-independent methods. Focus is also put on if there is any correlation of microbial diversity among the individuals from same family (Father, mother and kids).

The dominant bacterial groups observed in the current study from the gut and oral samples are being further investigated. We have designed the experiments to study compounds originating from the human gut microbial metabolism, which have ability to affect the host's physiological processes. The influence of these metabolites on differences in disease risk among individuals will be monitored by correlating gut microbial community composition and subjects' response.

3. MICROBIAL DIVERSITY OF EXTREME ENVIRONMENTS

Microbial populations are the major contributors to the transformation of organic carbon, sulfur, nitrogenous compounds and metals, and play an important role in ecosystem food webs and nutrient cycling. However, majority of the microbes can not be cultivated in the laboratory and hence at present we have little or no knowledge of the metabolic potential and ecological roles of most microbial species. Isotope probing methods offer great potential to identify the microorganisms that metabolize and assimilate specific substrates in environmental samples. The Indian team has earlier carried out 16S rRNA based studies on the diversity of the Lonar lake ecosystem and the proposed project is aimed at using functional genes and Stable Isotope Probing to understand metabolic potentials and activities of the indigenous microbial communities.

Lonar Lake:

Lonar Crater Lake is situated in the Buldhana district (Maharashtra, India) in the formerly volcanic, Deccan-Trap geological region. Lonar Lake is unique aquatic habitat as harbours substantial amount of salts, metals and carbonates. Our endeavour to study on molecular ecology of Lonar Lake started with phylogenetic analysis of the microbial diversity in the Lake. Strikingly the results had shown that Lonar Lake entertains huge uncultured microbial diversity despite its extreme conditions.

We extended the work to test the genetic and metabolic capacity of this microbial diversity in such alkaline and saline environment using new and advanced molecular ecology techniques such as Stable isotope probing (SIP), Single Strand Conformation

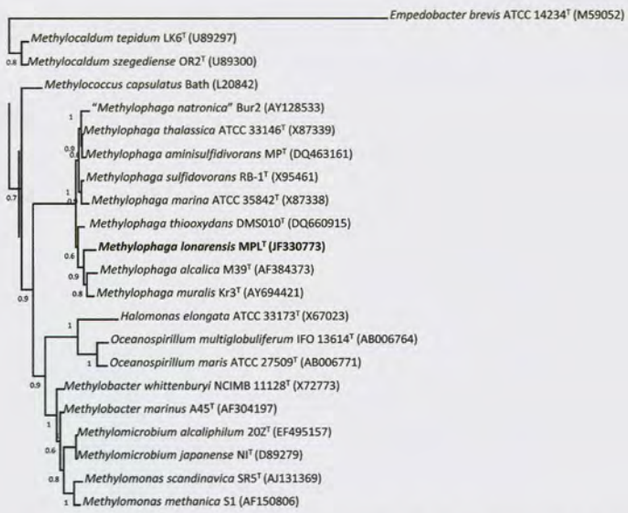
Polymorphism (SSCP), Differential Gradient Gel Electrophoresis (DGGE), Real time- PCR. We undertook culture-dependent strategies to isolate novel methylotrophs, ribosomal RNA based stable isotope probing (rRNA-SIP) using methanol as a substrate and study of seasonal variation in microbial diversity of acetate utilizing microrganisms by means of DNA-SIP experiments.

Transmission electron micrograph image of the novel isolate. Magnification (25000X)

The use of culture based strategies resulted in the isolation of Methylophaga lonarensis, sp. nov, (strain MPLT), a novel obligately methylotrophic bacterium from the sediments of the saline and alkaline Lonar Lake. The isolate, which utilized methanol, was an aerobic, Gram-negative, asporogenous, motile short rod multiplying by binary fission. It required sodium bi-carbonate or sodium chloride for growth in alkaline medium and although not auxotrophic for vitamin B12, the addition of vitamin B12 enhanced its growth. Optimal growth occurred with 0.5-2% (w/v) NaCl at 28-30 °C and pH 9.0-10.0. The cellular fatty acid profile of the strain consisted primarily of straight-chain saturated C16:0 and unsaturated C16:1 and C18:1 acids. The major ubiquinone was Q-8. The dominant phospholipids were phosphatidylethanolamine and phosphatidylglycerol. Cells accumulated the cyclicimino acid ectoine as the main compatible solute. The DNA G+C content was 50.0 mol%. Based on 16S rRNA gene sequence similarity (94.0-95.4 %) with all the type strains of methylotrophs belonging to the genus Methylophaga and DNA-DNA relatedness (31%) with the nearest soda lake strain M39T, it can be concluded that isolate MPLT represents a novel species. Strain MPL exhibited 99.8 % 16S rRNA and functional gene similarity to the sequences retrieved from our earlier methanol stable isotope probing (SIP) experiment and thereby, suggesting an important ecological role for this novel organism in the extreme lake environment.

The Lonar Lake strain stands out from other Methlophaga spp. isolated so far in its ability to utilize only methanol as sole carbon and energy source and not any other substrates such as methylamine, dimethylamine, trimethylamine, DMS, DMSO, Dimethylformamide or fructose. The successful isolation of this unique methylotrophic bacterium has profound implications towards conservation of the Lonar Lake biodiversity and its potential use in industry as a source of valuable metabolic products such as ectoine.

DNA-SIP experiments for exploring seasonal variation with Sodium acetate as a substrate showed the effect of climatic changes on the microbial diversity of Lonar Lake. Three



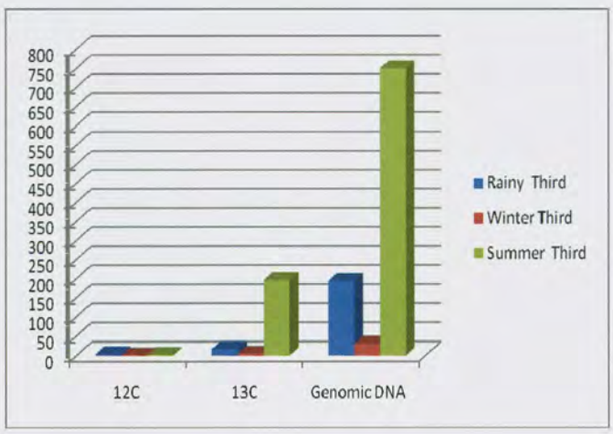
Phylogenetic affiliation of novel Lonar Lake strain MPL with other methylotrophs in Gammaproteobacteria

libraries constructed from the heavy DNA fractions of 13C DNA-SIP experiments showed that the number of Operational taxonomic units (OTU's) increases significantly with changes in season. Similar results were also confirmed by Real Time PCR analysis as well.

Future Work

- Exploration of biotechnological potential of microbes present in insect gut using various approaches.
- Understanding the role of mid gut bacteria in the capacity of mosquito to transmit disease.
- Understanding of role of human gut microbes in health and diseases.
- Metagenomic analysis of Lonar Lake and marine sediment/water for exploration of biotechnological potential in order to dissect the ecological structure.

Bar chart showing Real time analysis for Seasonal and Spatial data for 16 rRNA gene libraries.



Research Report

Bioinformatics & Proteomics

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Molecular simulation to biochemical network perturbation in infectious disease

Background

Computational systems biology aims to develop a class of integrated mathematical, computational and experimental techniques with the goal of linking the knowledge of different molecular parts of a living cell in comprehending the structure, dynamics, control and design of biological systems. Systems based biological concordance offers predictive reproducibility in infectious diseases. Looking at diseases as 'perturbation of networks' can provide such a framework which may provide insights from systems biology into the practicalities of personalized, preventive, predictive and participative medicine. In number of diseases lipid metabolism plays an important role. Moreover, lipids play an active role in a variety of dynamic processes involving the membranes that compartmentalize the cell. To achieve this, cell must regulate the mechanical properties of the membrane and it can do so partly by controlling its lipid composition. The underlying biophysical question is to understand the variability and chemical diversity of membrane lipid composition, the mechanical properties of the membrane and the associated protein functions. Also, some drugs bind to the lipid and modulate the structure of membranes. Understanding the effect of drug permeation through lipid membranes may minimize the investment in drug discovery and development. It may be possible to move beyond the characterization of membranes to explore the thermodynamic and kinetic features governing the membrane binding and transport. This may be directly applied to the design and optimization of liposomal delivery systems. Though there are extensive bodies of published experimental efforts, there have been few attempts to consider theoretically the interdependence of these two phenomena by modeling the lipid biosynthetic network as an integrated system in which the biochemical and biophysical descriptions of the metabolic network are fundamentally linked. In the current project we focused on tropical disease Leishmaniasis.

The global burden of leishmaniasis has taken a new dimension in the recent years due to emergence of drug resistant varieties of *Leishmania major*, posing a major threat to leishmaniasis eradication. Understanding the roles of lipid metabolism in Leishmania

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parasites could have important implications in guiding the development of novel phospholipid analogues that possess anti-protozoan activity. The cell envelope is enclosed with a number of unique lipids that play an important role in Leishmania major virulence. Most cellular lipids are embedded within membranes; membrane composition adds another level of complexity in the studied biological network. Proteins related to GPI and GIPL synthesis, ubiquinone biosynthetic process, LPG biosynthesis, fatty acid ß oxidation and fatty acid elongation were sorted out according to their relative abundance as they may be considered as putative vaccine antigen candidates and may contribute towards immunogenicity. The focus is laid on studying leishmanial proteome upon infection into their host cells. The answer is being seeked for possible global evolutionary strategies employed by leishmanial proteome to rewire the host cellular networks and by doing so forcing the host to reorganize its cellular activities. We chose LPG biosynthetic processes as LPGs are essential for adhesion of the parasite to the midgut of the insect and therefore are important for transmission of the parasite to the human host. The basic LPG structure in all Leishmania species consists of a 1-O-alkyl-2-lyso-phosphatidyl inositol lipid anchor, a heptasaccharide glycan core, a long phosphoglycan polymer composed of (Galß1-4manα1-PO_4)n repeat units (n=10-40), and a small oligosaccharide cap. In different *Leishmania* species the phosphoglycan repeat units contain additional substitutions that mediate key roles in stage-specific adhesion. Deletion of LPG in *L.major* indicates that the glycosylated structures are involved in resistance to oxidative stress and the human immune system. Henceforth, it is evident that lipids are essential cell constituents and therefore must be constantly synthesized to allow multiplication of the parasite. This suggests that the pathways leading to their synthesis are essential for parasite proliferation and pathogenesis and thus offers a reasonable target for rational design of novel antileishmanial drugs.

It is pertinent to keep in perspective that for building comprehensive, system-wide computational models of complex disease pathophysiologies, different modeling techniques from top-down physiological models to bottom-up atomic and molecular interaction techniques have to be integrated in a common platform. In this light, stochastic modeling and simulation of biological processes are problems of high interest today. The multitude of research opportunities related to the development of effective and reliable simulation tools for these stochastic models as well as for formulating the theoretical foundation to support them, makes this area particularly attractive for numerical analysts.

In nutshell, multiscale modeling and simulation techniques may permit us to study the spatial and temporal properties of large systems to be simulated using atomic-detail structures. On the other hand, the estimation of kinetic parameters for the mathematical modeling of biochemical pathways may provide a basis for iterative manipulation of biochemical pathways.

Aims and Objectives

Specific Aim 1: Cellular model of Lipid Membranes and its interaction with

Miltefosine

1. To understand the drug partitioning in lipid bilayer and the underlying interactions between lipid and drug.
2. To study the drug conformational changes, the perturbation in membrane structure, and changes of membrane properties as permeability barrier.

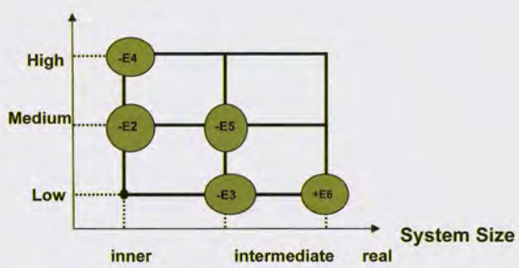
Specific Aim 2: Stochastic simulation for Lipophosphoglycan biosynthetic network in *L.major*

1. To build the gene regulatory network for L.major
2. To transform the regulatory network into a mathematical model depending upon the Flux Based Analysis.
3. To perform numerical simulations for the built network model. Kinetics modeling is useful in this for constraining the range of possible dynamic behaviors.
4. To lay focus onto reviewing the mathematical foundations for analyzing chemical reactions and describing how these systems of coupled chemical reactions can provide insight into the behavior of regulatory mechanisms.

Work Achieved

Cellular model of Lipid Membranes and Drug Partitioning: The model of the DPPC bilayer was used for simulation of the phospholipid environment around the drug. The lipid parameters were taken from the literature. The partial atomic charges were calculated at DFT/B3LYP/6- 31G** level using Gaussian 09 package. Also, the partitioning of large molecular systems was done by following ONIOM (Our-own-N-layered integrated molecular orbital and molecular mechanics) method, whereby the whole system is partitioned into onion-skin-like layers, using QM/MM. The total energy can be obtained from following:

E= E[Low, real] + E[Med, int] + E[High, inner]- E[Low,int]-E[Med, inner]
= E6+E5+E4-E3-E2



where real denotes the entire system, which is treated at low level, while int denotes the part of the system partitioned to be the intermediate layers for which the energy is calculated at both medium and high levels. Here inner denotes the inner layer of the system partitioning, whose energy is calculated at both high and medium levels.

Chemical potential profile, μ(z), of solute across a lipid bilayer and adjacent water phase is obtained by inserting the solute numerous times into randomly selected positions in the system obtained by MD simulation and calculating the interaction energy, E(z), between

the inserted solute and all the molecules in the system. From <exp (−E(z)/RT)>t, where <...>t denotes the thermal average over insertions of solute with randomly chosen orientations into configurations of the system at depth z unperturbed by the solute, the excess chemical potential, μ(z), and thereby the free energy of transfer, ΔG(z), from bulk water to the bilayer interior at the depth z are obtained: $\Delta G(z) = \mu(z) - \mu(\text{water}) = -RT\ln\langle e^{-E(z)/RT}\rangle / \langle e^{-E(\text{water})/RT}\rangle$

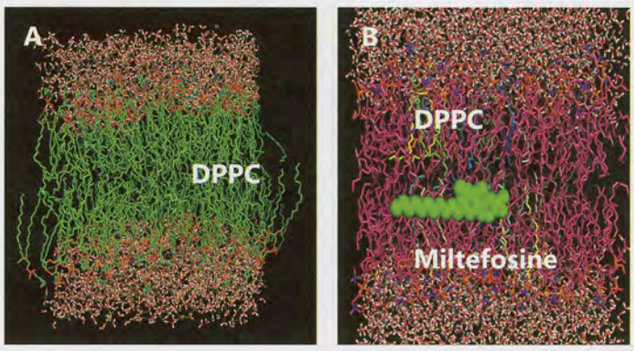


Fig. 1A : Movement executed by phospholipids in a bilayer system. Total energy and force on the bilayer were extracted during the entire simulation process

Fig.1B: Drug bound to the lipid bilayer system

This simulation may enable us to understand the thermodynamic interactions between the lipid and drug molecules, the role of electrostatics in such adducts, and the permeability of drug (Fig. 1A & 1B). Degree of penetration into the membrane interior obtained from computer simulations may also be useful in estimating the extent to which a drug or prodrug that is unstable in a given solution or biological fluid might be protected when bound to a lipid bilayer membrane. Multi-scale modeling and simulation revealed that the interactions between the drug and membrane are both electrostatic and hydrophobic. Increasing the chain length and lipophilicity may strengthen the binding interactions and overcome the problem of drug resistance.

Stochastic simulation for Lipophosphoglycan biosynthetic network in *L.major*: Interrelated set of genes can be represented effectively through Cytoscape as a network that describes lipid metabolic pathway. From the Gene Regulatory Network of the Lipid Metabolism Pathway (Fig.2A), it was found that the maximum number of the genes and the resultant gene products (enzymes), distinctive to the *L.major*, were specifically clustered in the LPG and GIPL Biosynthesis Pathway, followed by the fatty acid Biosynthesis (Fig.2B).

Network-centric modeling presented in Fig. 2A and simulation approach systematically allowed us to study the stochastic dynamics of cellular processes at molecular level. We all know cellular processes are typically viewed as systems of chemical reactions. Often such processes involve some species with low population numbers, for which a traditional deterministic model of classical chemical kinetics fails to accurately capture the dynamics of the system. In this case, stochastic models are needed to account for the random fluctuations observed at the level of a single cell. We surveyed the stochastic models of LPG biosynthetic network which is a well-stirred biochemical system and then discuss important recent advances in the development of numerical methods for simulating them. Finally, we identified some key topics for future research.

Fig. 2A: Gene Regulatory Network built through Cytoscape. Emphasis is laid on degree distribution of few nodes with high probability of interaction. Edges of high connectivity between the sub-networks are also noted.

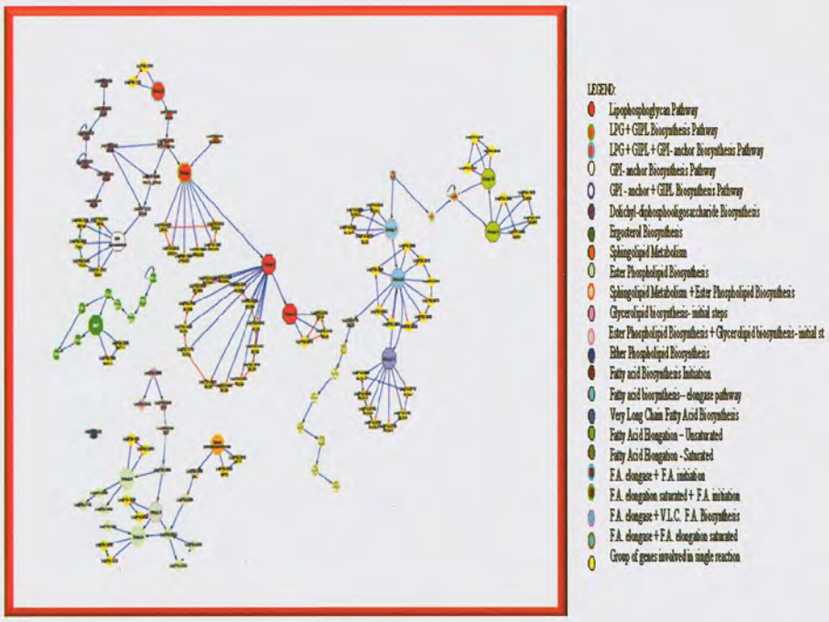


Fig.2C marks the transition of the qualitative knowledge of the biological system into a computational format captured in the LPG pathway network structure. Interactome modeling techniques gives computational advantages for molecular level study of biologic pathways and the impact of stochasticity in them by mapping available knowledge into parameterized model of events. Also, with different order of time-scales for biological events typically, between gene regulatory and metabolic reaction events, the stochastic simulation approach provides the flexibility of capturing their interactions in time. This is accomplished using the simulation mechanisms based on the certain defined kinetic laws such as Convenience Kinetics, Generalized Mass Action Kinetics and the Hill-Hinze equation. These techniques are carried out upon treating a biological process as a system of equations, represented by their rate constants and other parameters and simulating their interactions through numerical techniques. The numerical simulations capture the effects of genes and their expression level through the time-course of evolution at molecular concentration.

Fig.2B: Abundance of Enzymes in Lipid Metabolism of *L.major*

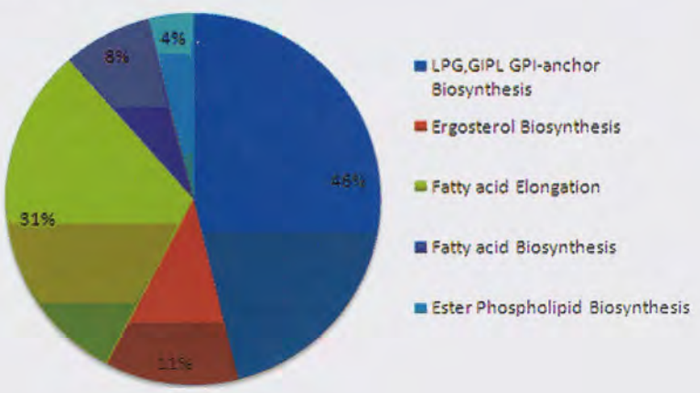
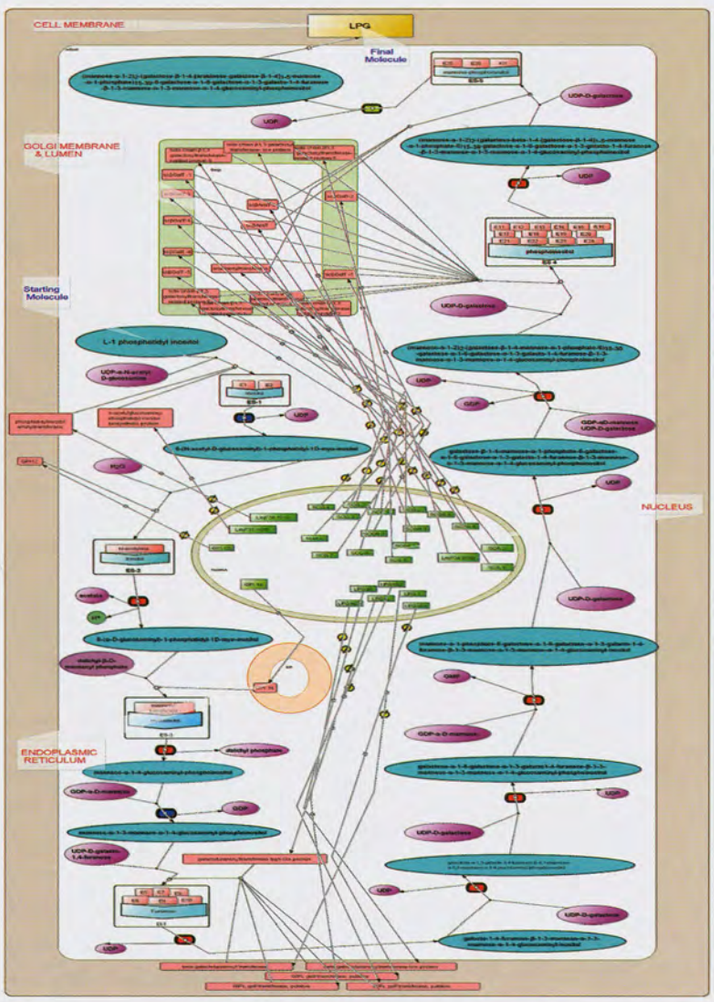
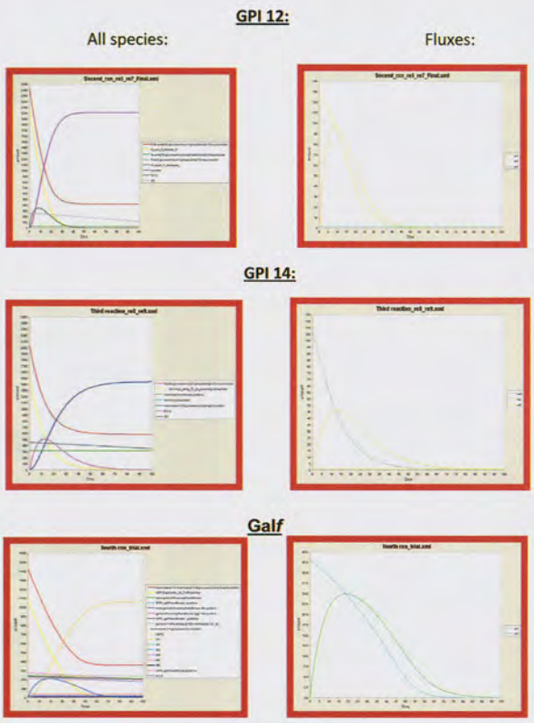


Fig. 2C: The LPG biochemical pathway representing a structured (Extensible Markup Language (XML)) format data which can be easily rendered into the discrete event simulation framework. The red indicates the chokepoint reactions and yellow represents the flux through each reaction.



Two of the enzymes controlling LPG flux viz., GlcNAc-PI-de-N-acetylase and D-mannosyltransferase are positively regulated by GPI 12 and GPI 14 (Fig.3). Thus, knockout of GPI12 and GPI14 may cause these proteins and their corresponding enzymes to operate at basal levels only instead of higher levels under GPI12 and GPI14 non-mutant conditions causing the flux to decrease. In Leishmania major, the core of the abundant surface lipophosphoglycan (LPG) is structurally related to that of the smaller glycosylinositolphospholipids (GIPLs) in containing galactosylfuranose (Galf) residues. However, deletion of the putative Galf -transferase (Galf T) *LPG1* affected Galf incorporation in LPG but not GIPLs. Thus, LPG and *LPG1* satisfy the requirements for virulence factors genes as set forth by the modern "Molecular Koch's postulates", establishing LPG itself as a Leishmania virulence factor. Since Galf is not present in humans, the Galf biosynthetic pathway is an attractive target for the development of novel anti-parasitic drugs. In this pathway, UDP-galactopyranose mutase (UGM) catalyzes the conversion of UDP-galactopyranose to form UPD-Galf, which serves as a

Fig. 3: Fluxes across GPI12, GPI 14 and Galf predicted through discrete stochastic simulation event.



precursor for all the Galf found on the cell surface. Deletion of UGM gene may lead to the identification of specific inhibitors of this enzyme which is being pursued in this project.

The success of simulation methodologies depends on validation of results with wet lab experiments. The present study provides a framework for verification of LPG biosynthetic processes and their subsequent hypothesis testing of biological functions prior to more advanced and costly in vitro and in vivo experiments. This study provides further interrogation of human pathogens and a platform for the integration of high throughput data. Focusing on metabolic causes of pathogenicity and virulence will lead to discovery of unique therapeutic targets dissimilar from human metabolism.

Future Work

1. To examine precisely how do molecules diffuse across lipid membranes and what factors govern their rate of transport. Also, membrane drug interaction induces fusion under physiological conditions which emphasizes the notion of local surface dehydration and perturbation of lipid packing. This might be possible through penetration of apolar amino acid segments into the hydrophobic membrane interior. We further want to investigate the outcome of such a study.
2. To study the computer simulation of lipid bilayer systems with peptide nanotube channel.
3. To propose experimental measurements and extensions of the stochastic modeling framework.

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Discovering novel biomarkers for breast cancer using gel-based (2-D DIGE) and gel-free (2-D LC-MS/MS) proteomic approaches

Background

In women, breast cancer is the most common malignancy and the second most common cause of cancer-related mortality. Three subtypes of breast cancer are identified ER and PR positive, HER2 positive and triple negative. As yet very few drugs are available for breast cancer, which remains a major cause of morbidity and mortality in women. Early diagnosis of breast cancer improves the likelihood of successful treatment and can save many lives. But, it requires successful strategies for early detection and screening of the disease. However, current techniques like mammography to detect breast tumor has intrinsic limitations. Thus early diagnostic biomarkers are critically important for detection, diagnosis, and monitoring disease progression in breast cancer. There is an urgent need to discover novel biomarkers of breast cancer for early detection and diagnosis. Quantitative proteomic profiling of body fluids, tissues, or other biological samples to identify differentially expressed proteins represents a very promising approach for improving the outcome of this disease. Proteins associated with breast cancer identified through proteomic profiling technologies could be useful as biomarkers for the early diagnosis, assessment of prognosis, prediction of therapeutic effect and treatment monitoring.

Mass spectrometry (MS) based proteomic technologies have experienced major improvements in recent years for simultaneous analysis of thousands of proteins on the basis of differences in their expression levels and characterization of post-translational modifications involved in cancer progression. The most commonly used proteomic approach is a combination of either two-dimensional electrophoresis (2-DE) or liquid chromatography (LC) to separate proteins/peptides and MS to identify and characterize them. Such rapidly developing technology enables us to discover novel cancer biomarkers to a greater extent. Such biomarkers may have broad applications, such as early indication of disease, monitoring of disease progression, and monitoring of drug targets. In general, MS based proteomic approaches begin with the collection of biological specimens representing two different physiological conditions. One of the samples is obtained from cancer patients and the other reference subjects, such as normal individuals, or patients with benign conditions. Proteins or peptides from plasma,

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tissue or cell line samples are extracted and separated to reduce sample complexity. Protein profiles are obtained after separation and then compared against each other using statistical algorithms in order to detect differentially expressed proteins.

Already, several putative breast cancer biomarkers with potential clinical applications have been reported using proteomic technology. Estrogen receptor, progesterone receptor, Her-2/neu, CA125, CEA, CA15.3 are among reported biomarkers and have been widely accepted for routine clinical use. These biomarkers have been serving as prognostic and predictive factors for targeted therapy. However, their detection sensitivity, and specificity to predict metastasis potential is limited. With reported breast cancer biomarkers, much is left to be desired in terms of clinical applicability. We need novel breast cancer biomarkers that will further enhance our ability to diagnose, prognoses, and predict therapeutic response. Because biomarkers can be analyzed relatively noninvasive methods and are economical as compared with other expensive techniques, hence it is worth to investigate novel biomarkers. In this work, we plan to discover novel biomarkers for breast cancer of Indian patients using high throughput mass spectrometry based proteomics. In addition, quantitative measurement of biomarkers in these patients will be helpful to detect the grade of breast cancer, which is important in diagnosis and prognosis of the disease. Further characterization of post-translational modifications of biomarkers will be useful to understand the process of development and metastasis in Indian scenario.

Aims and Objectives

1. Discovering novel biomarkers for breast cancer using high-throughput proteomic technology.
2. Quantitative measurement of biomarkers in breast cancer using 2-D DIGE gel based method and iTRAQ labeling LC-MS based method.
3. Characterization of post-translational modification (PTM) changes in biomarkers involving in breast cancer using LC-MRM-MS method.

Work Achieved

We have started the optimization of the protocols for gel based and gel free proteomic analysis from serum and tumor tissue.

I) **Gel-based proteomic approach:**

(A) **2-D Electrophoresis (2-DE):** We have optimized the protocols for proteins extraction from serum. We were run 2-DE using 18 cm IPG gel and 24 cm IPG gel in three different ways i) direct crude serum, ii) TCA-acetone precipitation and iii) TCA-acetone precipitation followed by high abundance proteins depletion (Figure 1A). More proteins were obtained by high abundance proteins depletion followed by TCA-acetone precipitation. The protein pattern obtained for the serum protein extract indicates that 24 cm IPG gel offers good separation and resolution. When comparing with the 18 cm IPG gel, approximately 25 % more spots were detected with the 24 cm IPG gel (Figure 1B). To identify the proteins using mass spectrometry requires intense protein spots in the gel. So that, 24 cm gel method found to be a good method of choice to identify proteins from serum. We also stained the gels with coomassie blue and silver stain. Coomassie blue

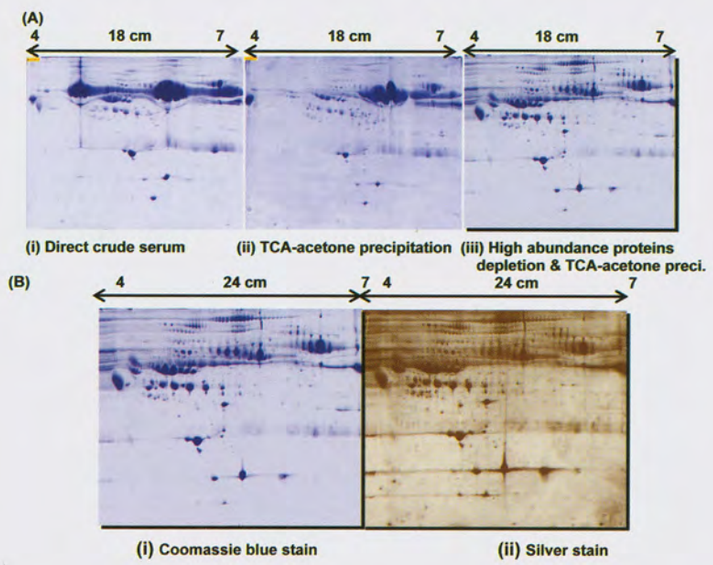

Fig. 1: (A) 2-DE separation of 18 cm IPG gel, (B) 2-DE seperation of 24 cm IPG gel.

stain was found to be a good stain for mass spectrometry identification. AB-Sciex 4800 MALDI-TOF/TOF and 4000 Q-Trap LC-MS/MS mass spectrometers were used to identify the proteins.

(B) 2-D Difference gel electrophoresis (2-D DIGE): Albumin/IgG-depleted samples were labelled with the cyanine dyes Cy2, Cy3, and Cy5 from GE Healthcare. Fifty micrograms of total protein taken from patient and control samples was minimally labelled with 400 pmol of Cy3 or Cy5, respectively. For one set of experiments, samples were labelled in reverse order of CyDyes (patient samples with Cy5 and control samples with Cy3). Cy2 was used to label the same amount of internal standard, resulting from the pooling of aliquots of all patients and controls. Triplicate experiments were performed to minimize the error. We identified the up regulated and down regulated proteins of Breast cancer serum samples using 2-D DIGE software.

ii) LCMS-based proteomic approach:

(A) LC-MS/MS: All LC-MS/MS experiments were performed on a 4000 Q-Trap system (Applied Biosystems, MDS Sciex) coupled to a Tempo nano MDLC system equipped with

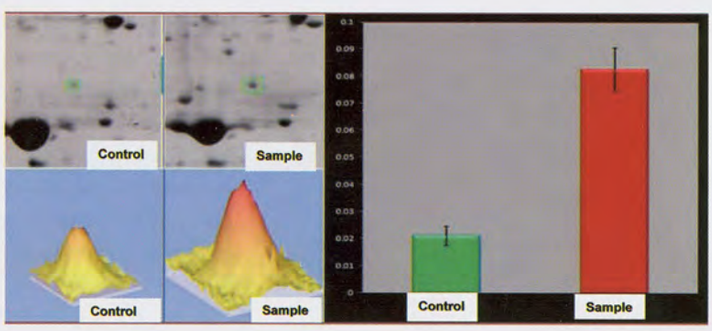

Fig. 2: Example showing up regulation of protein identified using 2-D DIGE method.

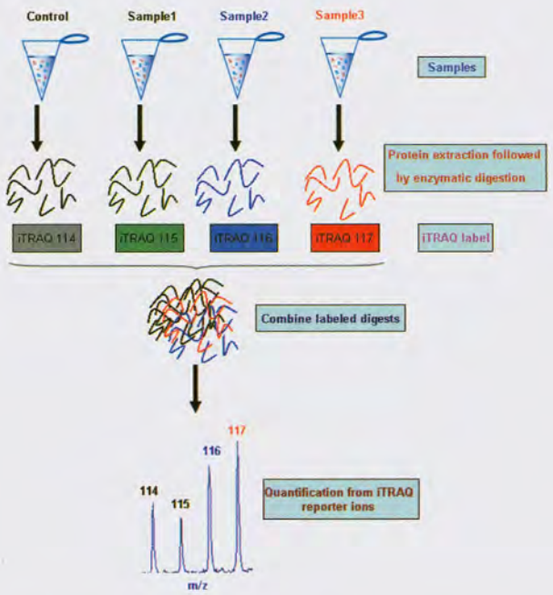

Fig. 3: General scheme of a multiplex reaction of four different samples (control, grades II, III and IV of breast cancer, designated as Control, Sample1, Sample2 and Sample3, respectively) using iTRAQ technology.

an autosampler (Eksigent Technologies). Peptides were separated using a nano peptide 200A magic C18 column (Michrom Bioresources). MS analysis were performed in positive ion mode with a mass range of 300–1600 m/z with the three most abundant peptides selected for fragmentation. MS data were searched using Mascot search v1.6b13 (Matrix science, London, UK) and ProteinPilot2.0.1 database searching engine (Applied Biosystems).

(B) iTRAQ based quantitative proteomic method: We are optimizing iTRAQ quantitative method for serum and tissue samples as shown in Fig 3. Protein aliquot containing 100 µg of total protein in 20 µL of 0.5M triethylammonium bicarbonate/0.1% SDS were reduced by the addition of 2 µL of 50 mM Tris-(2-carboxyethyl) phosphine followed by incubation at 60◦C for 1 h. The reduced cysteine residues were blocked by the addition of 1 µL of 200 mM methylmethanethiosulfonate in isopropanol followed by incubation at room temperature for 10 min. The total protein was then digested by the addition of 10 µL of trypsin at 0.5 µg/µL and incubated at 37◦C for 12 h. One unit of each iTRAQ reagent label (defined as the amount needed to label 100 µg of protein) was thawed and reconstituted in 70 µL of ethanol, with vortex mixing for 1 min. The contents of each reagent solution were added to the digest and incubated at room temperature for 1 h. The resulting labeled peptide samples were analyzed using 4800 MALDI-TOF/TOF followed by proteinpilot data base.

Future work

In future studies we plan to discover novel biomarkers for breast cancer using above mentioned optimized protocols of 2-DE, 2-D DIGE and LC-MS/MS. Further, we are also going to quantify the novel biomarkers using iTRAQ LC-MS/MS based technology.

Research Report

Infection & Immunity

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Understanding the role of chemokine and chemokine receptors to control the inflammation and autoimmunity

Background

Inflammation is the complex set of reactions involves a set of cytokines, chemokines, adhesion molecules and various cell types. Inflammation can happen in any organs or tissues in response to traumatic reaction, infection, early cancer, post-ischemic, toxic or autoimmune injury. The functional relationship between inflammation, innate immunity and autoimmunity are well recognized. However, the cellular and molecular mechanism that regulates inflammation and autoimmune diseases are still not well defined.

There are several secreted chemokines, cytokines and its specific ligands expressed into the inflamed microenvironment, and a joint venture of pro- and anti-inflammatory functions are initiated together by infiltrating leukocytes, tissue fibroblasts, epithelial and endothelial cells. Chemokine receptors and cell adhesion molecules presents on the cell surface are known to be involved in the migration of immune cells into the inflamed tissue but also prevents the reverse migration to secondary lymphoid tissues. These chemokines and cell adhesion molecules are well studied in the migration of cells. However, do intrinsic signaling from these receptors perturb the cell differentiation and function is not well defined.

Most of the chemokine receptors are G protein coupled receptors (GPCRs). G-proteins are heterotrimer molecular consist of α-, β-, and γ-subunits and transduce signals from surface receptors to intracellular effectors. Upon receptor activation, G-protein complex dissociate into α-and βγ-subunits which in turn recruit various signaling components at the inner surface of the plasma membrane followed by production array of intracellular second messengers such as IP3, DAG, Ca^{2}+, cAMP and IP3. G-protein signaling regulates number of cellular events such as induction of cell polarity, actin polymerization, reversible cell adhesion, myosin dynamics, transmigration, cell activation, differentiation and functions. There are number of targets remain to be elucidated and discovered. It is important to note that signaling experiments are often performed on isolated cells, chemokine–engineered cells, only carried out with one chemokine ligand, mostly using one concentration and time points. Furthermore most of the experiments are performed

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in the absence of other important factors such as intigrins, growth factors, and pro-inflammatory cytokines- thus requiring careful data analysis and interpretation in order to draw a conclusions compared to the cells in natural three-dimensional microenvironment. How do intrinsic signaling from these chemokine receptors, cytokine receptors and cell adhesion molecules help in controlling the inflammation and autoimmune diseases need to be investigated?

Aims and Objectives

Specific aims

1. How does inflammatory chemokine receptors such as CCR6 activation along with other co-stimulatory or inflammatory signals affect Th1, Th2, Th17 and Treg differentiation and function both in vitro and in vivo?
2. What epigenetic modifications are induced by CCR6 activation at different regulatory regions of genes associated with different subsets of T cell differentiation, and their function under inflammatory and tolerogenic conditions?

Work Achieved

We have established an experimental animal model for studying autoimmune colitis. In this model, C57BL/6 mice were given 2% Dextran Sodium Sulphate (DSS) in drinking water. These mice developed colitis within 5-8 days as seen by body-weight loss, loose stools, bloody diarrhea, and rectal prolepses. Immunohistochemical analysis of colon showed the infiltration of mononuclear cells in the colon. Further, we showed that DSS treatment increased the $CCD6^{+}CD44^{+}CD4^{+}$ cells in spleen and mesenteric lymph nodes.

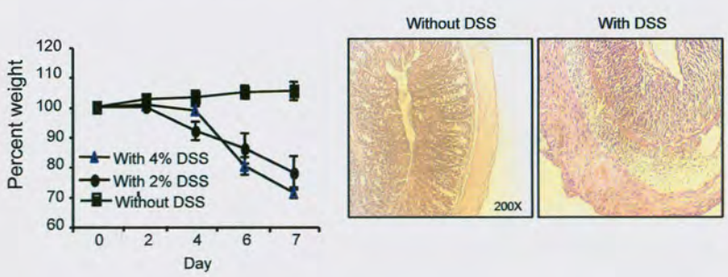

Fig.1: Dextran sodium sulfate treatment induced autoimmune colitis. C57BL/6 mice were given different concentration of dextran sodium sulfate (DSS) in autoclaved drinking water (w/v) for indicated time points. Weight of each mouse was recorded each days. Mean percentage of weight of mice shown (left). (n=6 mice/group). Mice were sacrificed on day 7, colon were collected and stained with hematoxylin and eosin (right).

Future Work

Since CCR6 is G protein-coupled receptor, we hypothesize that downstream signaling together with other signals not only responsible for activation of cell migration, but also might be regulating gene expression associated with other cellular functions. How CCR6 activation affects Th1, Th2, Th17 and Treg function, how CCR6 signaling regulates epigenetic modification of regulatory regions of different subset of $CD4^{+}$ T cell specific genes and how CCR6 activation affects the antigen presenting function of B cells and DC under inflammatory and tolerogenic conditions will be addressed by this research project. Understanding the cellular and molecular mechanisms of CCL20 and CCR6 regulation and function will help to develop novel therapeutics for the control of inflammation and autoimmune diseases.

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Monocytes/macrophages interaction with Plasmodium falciparum malarial parasite components

Background

In continuation with previous studies, we are studying interaction of Plasmodium falciparum parasite components with MO/MQ. Synthetic Plasmodium like heomozoin and parasite derived malaria pigment induced activation of innate immune responses has been reported recently and the use of malarial metabolite–hemozoin as vaccine adjuvant has also been suggested. Polarization of macrophages phenotype plays a role in the parasitic and inflammatory diseases. M1 macrophages have high IL-12 and low IL-10 phenotype and produce high amount of inflammatory cytokines. Activation of M2 macrophages are induced by TH2 cytokines IL-4 and IL-13. These macrophages have low IL-12 and high IL-10 phenotypes. These MQ display progressive functional changes resulting from change in the microenvironment. The significance of macrophage polarization in the context of pathophysiology in parasitic infectious diseases and role of parasite components & the malaria pigment hemozoin in the induction of regulatory macrophage phenotype are being investigated. A clear knowledge of the parasite components and host receptors involved in the immune responses is crucial for gaining a better insight into malaria pathogenesis and protective immunity. We have investigated effect of synthetic β-hematin (a structural analogue of malaria pigment hemozoin) and parasite derived hemozoin on comparative cytokine secretion in human MO/MQ and human derived cell line-THP-1 monocytes & macrophages in *vitro*. We are also investigating curcumin, an anti-inflammatory moiety, which has been reported to reduce mice malaria parasite density considerably and enhanced survival rate in the cytokine alteration in MO/MQ & parasite interaction.

Aims and Objectives:

1. Interaction of Plasmodium falciparum malaria parasites & its components with human MO/MQ & THP-1cells and its effect on the expression pattern of different molecules, Co-stimulatory molecules etc, DC maturation, Cytokine Profiles etc.
2. Alteration in the intracellular-signaling molecules due to phagocytosis of the parasites, free hemozoin & synthetic beta-heamozoin crystals. Manipulations of macrophage functions by parasites and its pigment.

Participants

Ms. Priyanka Fernandes

Mr. Mangesh S.Deval

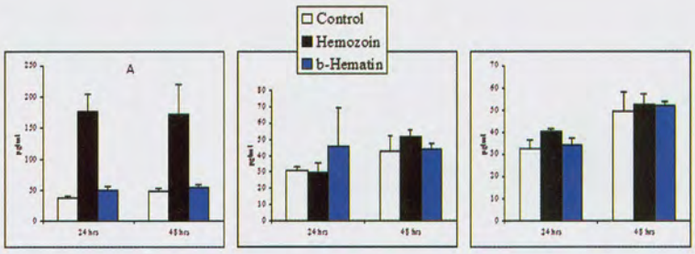


Fig 1: IL-10 Cytokine Production in Culture Supernatants of (A) PBL (MQ) (B) THP-1 Monocytes and (C) THP-1 MQ-C, phagocytosed with *Plasmodium falciparum* hemozoin and synthetic β-hematin.

Worked achieved

Though they are structurally similar, β-hematin and hemazoin induce different cytokine in vitro when phagocytosed by MO/MQ. When the hemozoin extract was used the human PBL-MQ cells secrete very high levels of IL-10 in the culture supernatant while the synthetic β-hematin pigment failed to induce the cytokine production in spite of its phagocytosis (Fig 1). Both the pigments, malaria parasite hemozoin and β-hematin do not induces IL-12 (p40) production in THP-1 cells. The LPS stimulated THP-1 macrophages secretes very high levels of IL-10 and low levels of IL-12, but when the cells were fed with hemazoin, the secretion of these cytokines were completely reversed i.e. IL-10 cytokine goes down and IL-12 levels increases (Fig 2). β-hematin extract do not show any effect on the either of cytokines secretion. THP-1 cell produces very high levels of 1L-12 when treated with Curcumin but in the hemozoin fed cells, the levels of this cytokines production decreases considerably and induces IL-10 secretion. However, when the hemozoin fed cells were treated with curcumin and chloroquine, the IL-10 secretion fell to half without affecting the levels of IL-12 (90).

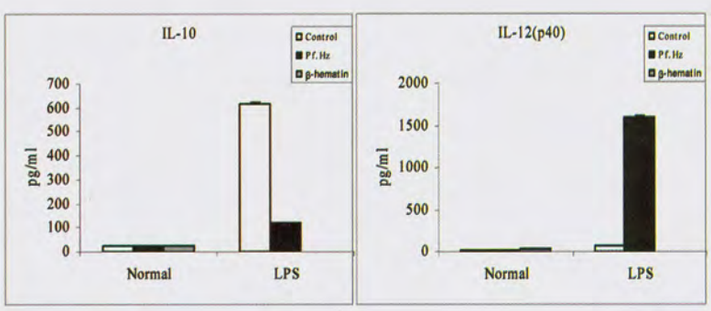
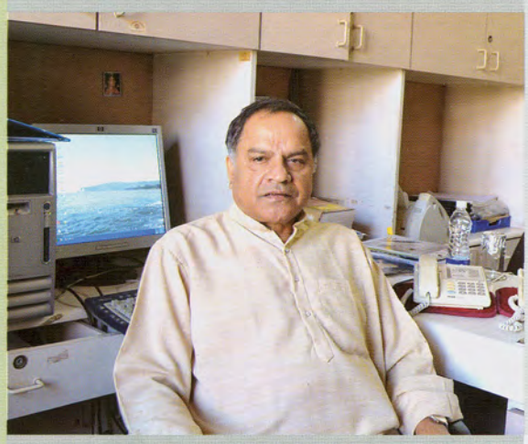


Fig. 2: *P. Falciparum* - Hz induced IL-10 & IL-12(p40) secretion in Normal & LPS stimulated THP-1 MQ.

Future Work

We are studying IL-10 cytokine secretion in both THP-1 and human MO/MQ at the transcriptional signaling path way as well as identification of hemozoin associated parasite component responsible for the cytokine production.



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Studies on role of interleukin-3 in development of T_{reg} and Th17 cells

Background

Regulatory T (T_{reg}) cells prevent the development of chronic inflammatory and autoimmune diseases by suppressing autoreactive T cells. In rheumatoid arthritis (RA) and collagen-induced arthritis (CIA) T_{reg} cells are defective and there is increased osteoclastogenesis. Recent studies suggest that T_{reg} cells inhibit osteoclast differentiation and bone resorption, and prevent development of CIA, whereas Th17 cells have pathogenic role in inflammatory arthritis. Interleukin-17 (IL-17) secreted by Th17 cells exacerbates joint destruction in CIA. Interleukin-3 (IL-3), a cytokine secreted by T helper cells, stimulates the proliferation, differentiation and survival of pluripotent hematopoietic stem cells. IL-3 is a potent inhibitor of osteoclastogenesis and bone resorption and has an anti-inflammatory activity. These observations led us to hypothesize that the anti-inflammatory activity of IL-3 may be through regulation of Treg and Th17 cells development. In previous studies we have reported that IL-3 promotes in vitro expansion of both induced and natural Treg cells, and potently suppresses the proliferation of effector T cells. IL-3 enhances expression of Foxp3 in Treg cells via IL-2. In further studies we investigated the role of IL-3 in development of T reg cells in vivo in CIA mice.

Aims and Objectives

1. To investigate the in vivo role of IL-3 on natural and peripheral Treg cells.
2. To investigate the role of IL-3 in development of Th17 cells.

Work Achieved

In vivo role of IL-3 in regulation of Treg cells in CIA mice: IL-3 has an anti-inflammatory activity and it also enhances the development of T_{reg} cells in vitro. Therefore, to investigate the in vivo role of IL-3 in development of Treg cells, we induced CIA in DBA/1J mice and IL-3 treatment was given for 15 days. CIA mice developed severe inflammation as evidenced by marked swelling and erythema of the hind paws. We found that in mice treated with IL-3 there was a significant reduction in paw thickness (Fig. 1A) and mean arthritic score (Fig. 1B). Photographs of hind paws in Fig. 1C show significant reduction of inflammation in

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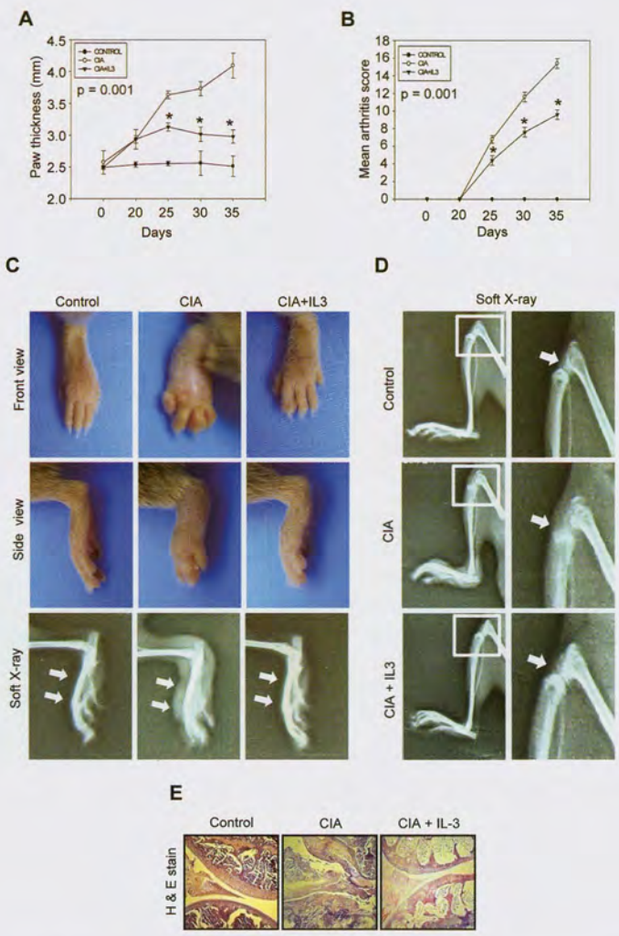

Fig. 1: IL-3 suppresses inflammation and prevents joint damage in CIA mice. CIA was induced in DBA/1J mice by collagen type II emulsion and at the time of booster dose of collagen type II on day 21 mice were treated with IL-3 for next 15 days. Mice were assessed every fifth day after booster injection for the severity of arthritis by measuring hind paw thickness (A) and clinical arthritic score (B). Data is represented as mean ± SEM, n = 5 mice per group. (C) Front and side views of hind paws showing inflammatory swelling, and X-rays showing inflammation of surrounding soft tissues. (D) X-rays of knee joints showing bone and cartilage structure. (E) Histological sections of knee joints showing inflammation and cartilage damage. Magnification, X10. Similar results were obtained in two independent experiments.

mice treated with IL-3. Also, thickness of the inflammatory soft tissues was decreased by IL-3 (Fig. 1D). RA is a chronic inflammatory disorder that ultimately leads to the destruction of joint architecture. By radiological examination we observed that IL-3 treatment prevented damage to articular cartilage (Fig. 1D, enlarged regions). We also noticed that CIA mice had markedly enlarged spleen and inguinal lymph nodes which were normal in size in IL-3-treated mice. By histological examinations of knee joints on day 36, we observed massive infiltration of polymorphonuclear and other inflammatory cells in knee joints of CIA mice, and there was multiple superficial cartilage erosion. In contrast, knee joints of mice treated with IL-3 showed infiltration of few inflammatory cells, and no erosion of articular cartilage (Fig. 1E). Using microcomputed tomography (μ-CT) we further assessed the structure of tibiae. We observed significant loss of trabecular and cortical bones in CIA mice, which was prevented by IL-3 (Fig. 2A). Also, a significant increase in trabecular thickness (Tb. Th.), trabecular number (Tb. N.), bone volume fraction (bone volume/tissue volume; BV/TV), connectivity density (conn. Dn.), and cortical thickness (Ct. Th.) was observed in IL-3–treated mice as compared to CIA mice (Fig. 2B). Bone architecture denoted by the structure model index was not altered.

These results suggest that IL-3 treatment reduces arthritic score and inflammation and prevents damage to bone and cartilage tissues in knee joints of CIA mice.

To further investigate the in vivo mechanism of IL-3 action in prevention of CIA, we analyzed the effect of IL-3 on Foxp3$^+$ T_{reg} cell development. Total lymphocyte populations derived from lymph nodes, spleen, and thymus tissues were analyzed by FACS for percentage of CD4$^+$Foxp3$^+$ T_{reg} cells. The percentage of T_{reg} cells was drastically decreased in thymus, lymph nodes, and spleen of CIA mice. There was a 50% decrease in percentage of T_{reg} cells in lymph nodes and around 70% decrease in spleen and thymus. Interestingly, in IL-3 treated mice the percentage of T_{reg} cells in all the three tissues was restored to near normal levels (Fig. 3A). Fig. 3B represents the average percentage of T_{reg} cells in lymph nodes, spleen, and thymus of mice treated with or without IL-3. These results suggest that IL-3 has a potential to augment the percentage of Foxp3$^+$ T_{reg} cells in vivo.

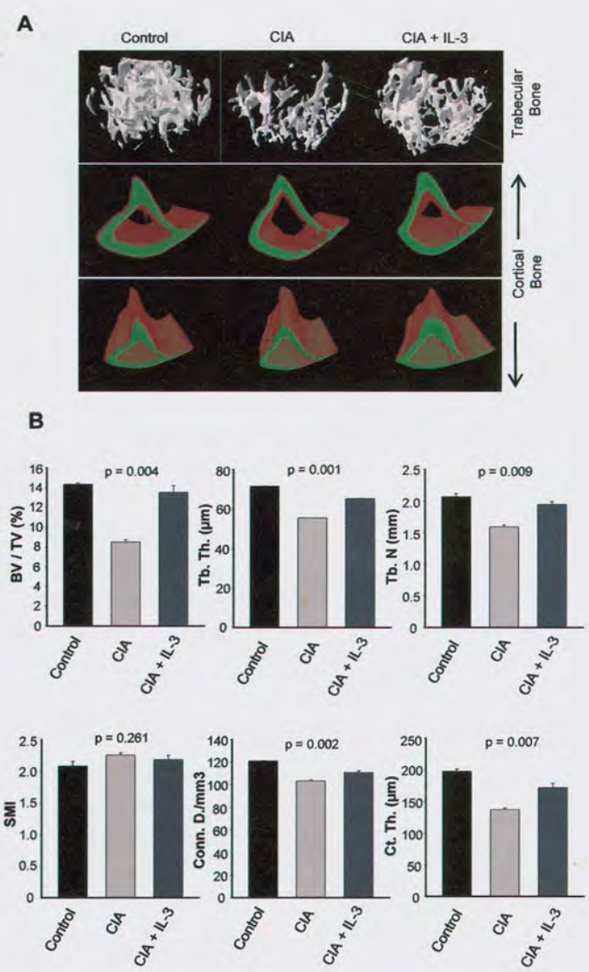

Fig. 2: IL-3 prevents trabecular and cortical bone loss in CIA mice. Mice were sacrificed on day 36 and tibiae were analyzed for bone parameters. (A) µ-CT reconstructions showing trabecular and cortical bone structures of representative tibiae in control, CIA and IL-3 treated CIA mice. (B) Morphological measurements of trabecular and cortical bone indices such as BV/TV, Tb. N, Tb. Th, SMI, Conn. Dn. and Ct. Th. computed from µ-CT reconstructions of tibias of control, CIA and IL-3 treated CIA mice. Similar results were obtained in two independent experiments.

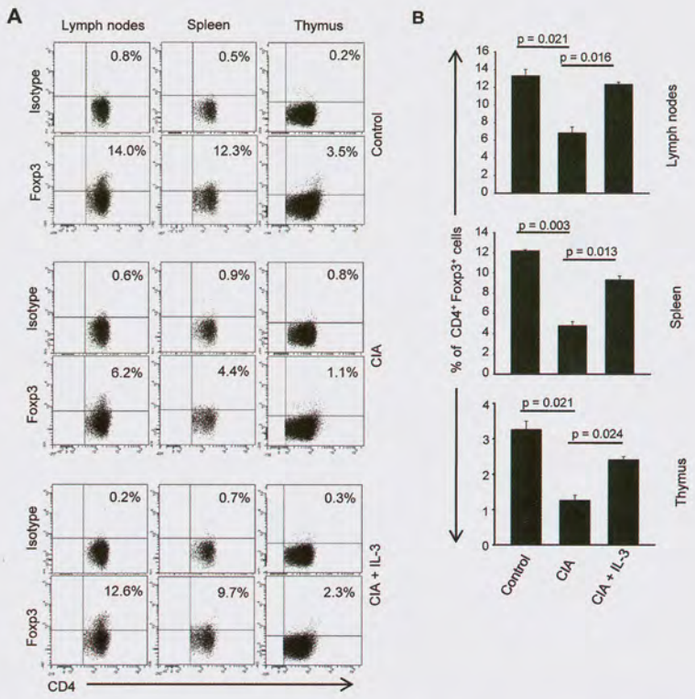

Fig. 3: IL-3 enhances the percentage of Foxp3+ Treg cells in vivo. (A) Total cells derived from lymph nodes, spleen and thymus tissues were analyzed for percentage of $CD4^+Foxp3^+$ Treg cells by FACS. (B) Average percentage of $CD4^+Foxp3^+$ cells from tissues of 3 mice. Mean ± SEM. $p < 0.05$ in all the groups.

IL-3 inhibits synthesis of proinflammatory cytokines in CIA mice: Although the pivotal role of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 is documented in the induction and maintenance of RA, the pathogenic events that lead to the development of human RA are not fully understood. These cytokines promote the deleterious imbalance in bone metabolism and contribute to enhanced bone destruction. T_{reg} cells in active RA are defective in controlling production of proinflammatory cytokines. T_{reg} cells suppress immune responses through numerous mechanisms, including the production of anti-inflammatory cytokines, direct cell to cell contact, and by modulating the activation state and function of APCs. Therefore, we further examined the in vivo effect of IL-3 on the levels of various proinflammatory and anti-inflammatory cytokines. In CIA mice there was a significant increase in the production of IL-6, IL-17A, TNF-α, IL-1, and IFN-γ and decrease in secretion of IL-5 and IL-10. IL-2 production was also decreased in CIA mice. Interestingly, we observed that IL-3 significantly increased the production of IL-10, IL-2, IL-5, and IFN-γ and decreased the production of IL-6, IL-17A, TNF-α, and IL-1. These results suggest that IL-3 has a potential to regulate the synthesis of proinflammatory and anti-inflammatory cytokines in CIA.

We also found that Th17 cells express IL-3Rα and IL-3 decreases the number of Rorγt+ Th17 cells in a dose-dependent manner. We are further investigating in detail the regulation of development of Th17 cells by IL-3

Future Work

To investigate the role of IL-3 in development of human T reg and Th17 cells.

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Host cell factors in HIV pathogenesis and identification of new anti-viral lead molecules

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Background

Human immunodeficiency virus is the causative agent of acquired immunodeficiency syndrome (AIDS). The hallmark of the disease is gradual depletion in the number of $CD4^+$ T cells, leading to the onset of opportunistic infections. The incidence of HIV infection has reached pandemic levels worldwide including in India. The therapeutic regimen being used at present can reduce the viral load remarkably but are not the ultimate answer to AIDS patients. Our group has been working on different aspects of HIV pathogenesis, related to host-virus interactions, immune response and drug discovery. The primary objective is to gain better understanding of the virus and its interaction with the host cell, which may lead to new antiviral strategies.

Aims and Objectives

1. Role of viral regulatory proteins Tat and Nef in HIV pathogenesis, and differential gene expression studies in HIV-1 infected cells.
2. CD40–CD40L signaling in HIV infection.
3. Identification of novel molecules with anti-HIV activity from plant source and their potential for use as microbicides.

Work Achieved

Role of viral regulatory proteins Tat and Nef in HIV pathogenesis, and differential gene expression studies in HIV-1 infected cells: The Human Immunodeficiency Virus Type 1 encodes a 27 KDa protein, Nef, which has come a long way from being termed as a negative factor to being one of the most important proteins of HIV-1. However, its role in HIV-1 replication and gene expression remains to be clearly understood. We have shown earlier that Nef not only interacts with the heat shock protein 40 (Hsp40) but it also induces the expression of Hsp40 in HIV-1 infected cells. This interaction seems to be necessary for Nef mediated up regulation of viral gene expression. Although involvement of heat shock proteins in viral pathogenesis has been reported earlier, a clear understanding of their role remains to be elucidated. We have now shown that Hsp40

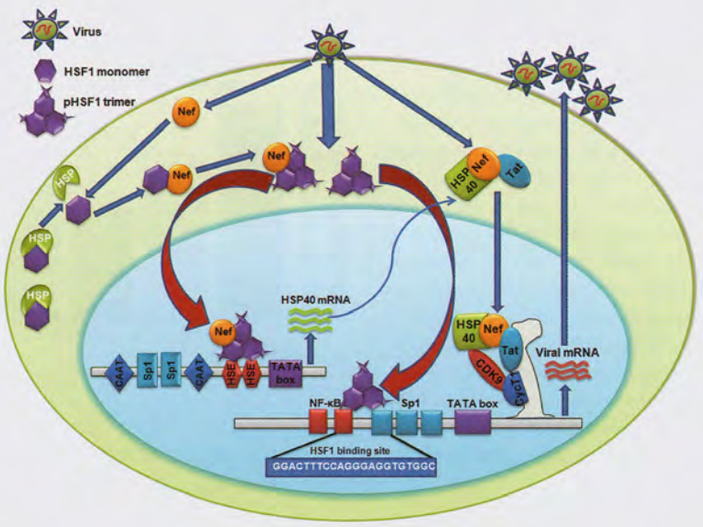

Fig.1: Proposed model showing HSF1 mediated regulation of HIV-1 transcription and replication by two distinct pathways. First pathway involves Nef dependent activation of HSP40 promoter activity leading to increased viral gene expression whereas the second pathway involves Nef independent direct activation of HIV-1 LTR promoter activity to increase viral gene expression and replication.

and Hsp70 reciprocally regulate HIV-1 gene expression and replication. We have also initiated a comprehensive study of all the HSP protein family members during HIV infection. Our results indicate that HSPs are differentially regulated during infection. As Heat shock factor-1 (HSF-1) is the major transcription factor regulating HSP gene expression, we have also analyzed the role of HSF-1 in viral replication and pathogenesis. Our results now clearly indicate that HSF-1 positively regulates HIV-1 gene expression and replication by two distinct mechanisms. Firstly, along with Nef, it activates HSP40 promoter and the increased HSP40 then promotes viral gene expression and replication as reported earlier. Secondly, HSF1 directly interacts with a novel HSF-1 binding sequence in the HIV-1 long terminal repeat (LTR) promoter and induces viral gene expression and replication (Fig. 1). Our results thus provide convincing evidence for the first time that HSF-1 is involved in regulation of viral gene expression and replication.

We have recently identified Cyclin K as a novel Nef-interacting protein and have shown that CycK inhibits HIV-1 gene expression and replication in Nef-dependent manner. The positive elongation factor b complex, comprising of CDK9 and Cyclin T1, is a host factor required for viral gene expression and replication. Enhanced expression of CycK in the presence of Nef induces CycK-CDK9 binding, which prevents CDK9-Cyclin T1 complex formation and nuclear translocation of CDK9, resulting in inhibition of HIV-1 LTR driven gene expression. To confirm the above-mentioned finding, we directly examined the localization of CDK9 in wild type and Nef deleted NL4-3 virus transfected cells in the presence of CycK by immunofluorescence. The result presented in Fig. 2 clearly shows that CDK9 was localized more in the nucleus of pNL4-3ΔNef transfected cells as compared to pNL4-3 transfected cells. Furthermore, this inhibitory effect of CycK was not observed with Nef deleted virus, indicating the requirement of Nef in this phenomenon. Finally, silencing of CycK in HIV-1 infected cells resulted in increased translocation of CDK9 in to the nucleus leading to increased viral gene expression and replication. This data also suggests that endogenous CycK might act as an inhibitory factor for HIV-1 gene

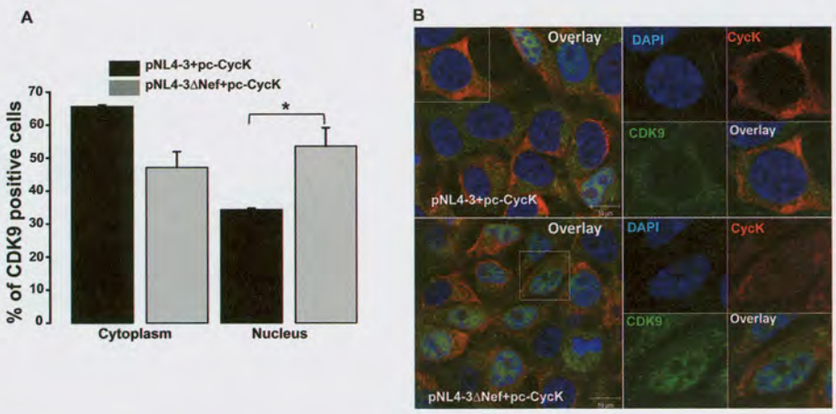

Fig. 2: Cyclin K restricts CDK9 nuclear translocation in the presence of Nef. (A) CDK9 translocation in to the nucleus of HEK-293T cells is increased in the cells transfected with Nef deleted molecular clone. HEK 293T cells were transfected with pNL4-3 or pNL4-3ΔNef molecular clone along with pc-CycK. Immunofluorescence staining was performed with CDK9 and CycK antibodies. Cells stained for CDK9 in nucleus or cytoplasm were counted and plotted as a bar diagram with data from three independent experiments. Black and grey bars indicate CDK9-stained cells transfected with pNL4-3 and pNL4-3ΔNef respectively. (B) Representative immunofluorescence images of CDK9 localization in the experiment described in (A).

expression and replication. Thus our results clearly demonstrate that CycK utilizes HIV-1 Nef protein to displace CycT1 from P-TEFb complex resulting in inhibition of HIV-1 gene expression and replication. At the end, identification of CycK as a Nef interacting protein and the consequences of this interaction provide another example of a viral protein being utilized by the host in combating the pathogen.

CD40–CD40L signaling in HIV infection: Impaired antigen–presenting function is thought to be a critical component of HIV–associated immunodeficiency. However, the mechanisms underlying these defects have not been clearly understood. Among the various ligand–receptor pairs important for $CD4^+$ T cell–antigen presenting cell (APC) communication, CD40–CD154 interaction is very important. CD40 is a member of the tumor necrosis factor (TNF)-receptor super family, which is constitutively expressed on the surface of APCs, whereas CD40 ligand or CD154, a member of the TNF superfamily, displays tightly regulated expression on the surface of $CD4^+$ T cell as a result of signaling via T-cell receptor. CD40-CD154 interactions are critical for the induction and regulation of cell-mediated immunity. Defective activation of APC by T cells that do not express CD40L could thus represent a primary event in the establishment of immunosuppression. Thus, studies of changes in CD40 and CD40L expression in presence or absence of HIV-1 may provide useful insights into the immunological abnormalities in HIV infected individuals. Hence, we have studied the expression of CD40 and CD40L on different subsets of immune cells such as monocytes, B cells, NK cells, CD4 and CD8 T cells of HIV infected individuals. We have observed that CD40 expression on B and NK cells gets down-regulated during HIV infection but without any significant change in CD40 expression on monocytes. The expression of CD40L on $CD4^+$ T cells was down-regulated, while on CD8+ T cells it showed up-regulation during HIV infection. Thus, the overall expression levels of CD40 and CD154 showed inverse regulation during HIV infection.

Identification of novel molecules with anti-HIV activity from plant source: The current therapeutic strategy involving the use of reverse transcriptase and protease inhibitors in combination (HAART) has proven to be useful in controlling the virus, but is not sufficient to eradicate the virus from the patients. Extensive work is being done throughout the

globe to identify new ant-HIV therapeutic strategies. One of the strategies has been to identify anti-HIV compounds from natural resources. We have been working along with NIPER for screening of compounds isolated from medicinal plant extracts and new synthetic compounds for identification of anti-HIV molecules along with their potential to be used as a microbicide. Our studies have resulted in identification of a number of novel derivatives of quinoline 2, 4-diol, β-carboline and dimeric phloroglucinol molecules showing potent anti-HIV activity. Several of these molecules have shown high safety or therapeutic index in cell based assays, which are now being further studied for potential development of a microbicide formulation. In addition to these, efforts have been made to develop combination of two or more molecules which target multiple stages of viral life cycle, which are called as Portmanteau inhibitors. Derivatives of Caffeic acid phenylethyl ester (CAPE) and benzanilides have been shown to inhibit HIV by two distinct mechanisms, the first targeting the Integrase enzyme, and the second acting as a CCR5 antagonist. We have recently reported synthesis and anti-viral activity of several novel caffeoyl anilide compounds, which show significant inhibition of virus by both inhibiting entry through CCR5 co-receptor and by inhibiting the Integrase function. We have also recently initiated structure based development of new Integrase inhibitors in collaboration with Birla Institute of Technology, Mesra.

Future Work

Our results till date indicate that heat shock proteins 40 and 70 play an important role during HIV-1 infection. We are now trying to elucidate the role of other heat shock proteins like Hsp27, Hsp60, Hsp90 and Hsp105 in HIV replication and pathogenesis, which will provide us a comprehensive knowledge about the role of HSPs during HIV infection. We are also continuing characterization of several new Nef interacting host cell proteins identified previously by yeast two hybrid screening for their functional relevance in HIV lifecycle. Furthermore, we are studying the recruitment of Tat protein on the chromatin during HIV infection that might lead to the elucidation of the mechanism of Tat mediated regulation of both viral and cellular gene expression. Identification of differentially expressed genes and their relevance to HIV induced cell death is being continued with a focus on mitochondrial oxidative phosphorylation system in infected cells. Our studies on the role of CD40 and CD40L in HIV infection will be taken further by initiating studies on human peripheral blood mononuclear cells and monocyte derived macrophages. Finally, studies to identify novel anti-HIV molecules will be continued with the objective to identify novel lead molecules with potential for use as anti-HIV microbicides.

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Study of *Idli* Fermentations

Background

Staple foods worldwide are cereals and pulses that are consumed after cooking. But large amounts of cereals are fermented to improve its biological value prior to consumption. *Idli* is one such fermented cereal preparation and the quality of steamed product is a subject of interest being a very popular breakfast food.

Idli batter fermentation has been the subject of few research investigations involving many aspects. These studies include optimization of ingredients, microbiological aspects of batter fermentation, physico-chemical changes during batter incubation and nutritional improvement after fermentation. The only fundamental work describing the fermentation of Idli batter was published in 1965 [Mukherjee et al, 1965]*. The paper ascribed role of acid and gas production to L. mesenteroides, S. faecalis and P. pentosaceus during fermentation. In the light of this situation, the aim of this study was to isolate microorganisms from samples of *Idli* batter from vendors where *Idli* fermentations are routinely carried out on large scale.

*1. Mukherjee SK, et al. Role of leuconostoc mesenteroides in leavening the batter of idli, a fermented food of india. Appl Microbiol. 1965;13:227-31.

Aims and Objectives

1. Identification of bacterial isolates using routine biochemical tests as well by 16S ribosomal RNA sequence analysis. This will impart the idea regarding the diverse microbial flora present during fermentation.
2. Most isolates from cereal fermentations play important role as probiotic microorganisms, for that reason isolates from *Idli* batter will be assessed for different probiotic criteria.
3. In this study, another intention is to correlate microbial activity with the changes in rheology.

Participants

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4. Many conventional fermented foods from Asia have been upgraded to high technology production methods. This could happen because of the strong research traditions in fermented food technology. This research component is minimal in *Idli* manufacturing. Therefore, attempt has been made to throw some light on type of microorganisms and their role in making of *Idli*.

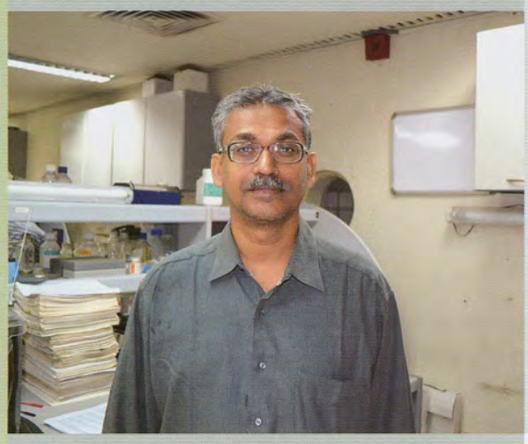
Work Achieved

Isolation of Bacteria from *Idli* batter: Naturally fermented *Idli* batter from three different samples was used for analysis of bacterial species responsible for fermentation. Total of 354 well isolated colonies were picked up based on colony characters for further studies. All 354 isolates from different *Idli* batter samples were sequenced for partial 16S rDNA gene and were identified up to species level. In Bangalore samples, that showed maximum diversity based on cultural characters, total of 36 different bacterial species could be detected [Fig. 1] while in Pune sample only 19 diverse bacterial species were found. In Laboratory-fermented sample 22 bacterial species could be identified. There exists wide bacterial diversity in three ready to steam *Idli* batter as compared to that reported earlier. Most of the isolates from *Idli* batter belong to *Lactobacillales* [Firmicutes]

Fig.1: : Bacteria from Idli batter – Bangalore sample

and Enterobacteriaceae [Gamma-proteobacteria] family. Bacteria belonging to both these families are known to be amylolytic that degrade the amylopectin and amylose starch present in endosperm of rice, producing mono- and di-saccharides. It is reported that glucose and maltose increases during initial part of soaking of cereals as well during batter incubation due to cereal amylases also. These sugars are further metabolized to give acid that will lower the pH of the batter and produce sour taste. Some members of these families are hetero-fermentative in nature and these produce carbon dioxide that is responsible for leavening of the batter. When isolates from *Idli* batter were tested for maltose fermentation, some cultures like Citrobacter and Klebsiella did not produce acid and gas. Most cultures belonging to genus Enterococcus, Enterobacter and Pediococcus could produce acid. While, most cultures belonging to genus Weissella produced both acid and gas during maltose fermentation. The heterofermentative bacteria and chief leavening agent *L. mesenteroides* described in Mukherjee et al's (1965) report was not found in any sample tested in this study. The leavening action is therefore mainly carried out by different species of genus *Weissella* isolated from *Idli* batter sampled used in this study.

It is interesting to note that three samples collected from three geographical regions had same bacterial flora in common. Bacteria [at genus level] common to all three samples were *Pantoea, Staphylococcus, Citrobacter, Enterobacteria, Klebsiella, Pediococcus, Lactobacillus* and *Weissella*. The search for most of the bacterial isolates from Idli batter was done to find out whether they are present in other traditional cereal fermentations and it showed that most of the isolates were also detected in other fermented foods. Detection of bacteria by non-culturable technique also showed presence of similar flora in Bangalore sample. Two of the samples namely Pune and Laboratory-fermented were found to be lacking yeast flora even after screening multiple samples. But the sample from Bangalore showed consistently presence of single type of yeast. The same yeast was isolated from different batches of samples. The yeast was identified by sequencing of partial gene sequence of 18S rDNA gene by PCR as *Candida tropicalis*. In addition to soaking of raw material, microbial action was found to be essential for leavening and souring action as well in the rheological changes.



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CD40 signaling in the regulation of immune response

Background

Co-stimulation through CD40-CD40-L system plays important roles in Immune responses to auto-antigens, allo-antigens, pathogens and tumor antigens. The interaction between T-cell-expressed CD40-ligand (CD40-L) and antigen presenting cell-expressed CD40 plays a crucial role in T-cell activation. We have shown during the past eight years that CD40-CD40-L interaction significantly modulates the functions of macrophages and dendritic cells. The effects were mediated by the reciprocally signaling kinases, inducing counteractive effector functions. Last year, we studied the role of Protein Kinase C isoforms and dual-specific phosphatases in CD40-induced effector functions.

Work Achieved

The macrophage-expressed CD40 reciprocally signals through p38MAPK and ERK1/2 and modulates anti-leishmanial immune responses by altering productions of IL-10 and IL-12, the cytokines that play crucial roles in the promotion or protection from L. major infection, respectively. The p38 MAPK and ERK-1/2 are dephosphorylated by dual-specificity MAPK phosphatases (MKPs), we examined the role of CD40 in the regulation of MKPs in *L. major* infection. MKP-1 expression and activity increased whereas MKP-3 expression and activity decreased in virulent *L. major*-infected macrophages. CD40 differentially regulated the expression and activity of MKP-1 and MKP-3, which, in turn, reciprocally regulated CD40-induced p38MAPK and ERK-1/2 phosphorylation and effector functions in macrophages. Triptolide and lentivirally expressed MKP-1 short hairpin RNA that inhibits MKP-1 expression enhanced CD40-induced anti-leishmanial functions and significantly protected susceptible BALB/c mice from *L. major* infection. Similarly, lentivirally over-expressed MKP-3 significantly reduced disease progression and parasite burden in susceptible BALB/c mice. Thus, CD40 reciprocally regulates MKP-1 and MKP-3 expression and activity while the MKPs contribute to the reciprocal CD40 signaling-regulated anti-leishmanial functions. The data reveal a novel parasite-devised immune evasion strategy and identify a target to redirect CD40-regulated immune responses.

Participants

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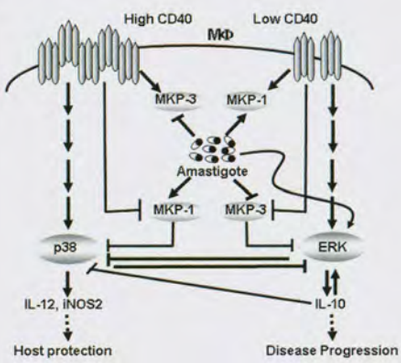
On the role of PKCs in CD40-induced anti-leishmanial functions, we examined whether a single receptor uses PKC isoforms differentially to regulate cellular responsiveness, for example, the differential usage of ten PKC isoforms, which are integral to receptor-triggered cellular responses, by CD40 in macrophages. We report that in mouse macrophages, higher doses of CD40 stimulation induces optimum phosphorylation and translocation of PKCα, βI, βII and ε whereas a lower dose of anti-CD40 activates PKCδ, ζ, λ. Infection of macrophages with the protozoan parasite Leishmania major impairs PKCα, βI, βII isoforms but enhances PKCδ, ζ, λ isoforms. PKC α, βI, βII, ε isoforms induce p38MAPK phosphorylation, IL-12 expression and Leishmania killing. PKCδ, ζ, λ promote ERK-1/2 phosphorylation, IL10 production and parasite growth, identifying these as pro-leishmanial isoforms. Treatment of BALB/c mice with lentivirally expressed PKCδ- or ζ-specific shRNA significantly reduces the infection and reinstated host-protective IFN-γ-dominated T cell response, defining the differential roles for PKC isoforms in immune homeostasis.



The above figure shows the MKP-1 and MKP-3- regulated reciprocal CD40 signaling in L. major infection. Here, we show that differential activation of two phosphatases, MKP-1 and MKP-3, can also lead to the reciprocity between the MAPKs. A higher dose of anti-CD40 Ab stimulation inhibits MKP-1 expression, but enhances MKP-3 expression, but lower doses of anti-CD40 Ab stimulation does the reverse. As MKP-1 preferentially dephosphorylates p38 MAPK, thus MKP-1 inhibition results in host protection through enhanced IL-12 and NO production. In contrast, because MKP-3 preferentially dephosphorylates ERK1/2, over-expression of MKP-3 results in host protection.

The diseases caused by Leishmania, a protozoan parasite that resides and replicates obligatorily within macrophages, have significant socio-economic impact through gross disfiguration, morbidity and mortality worldwide. Despite these problems, an effective anti-leishmanial vaccine remains elusive. Herein, we have analyzed the immunogenicity and protective efficacy of L. major MAP kinase 10 (LmjMAPK10) against the challenge infection with the parasite. We observe significant protection against the infection by LmjMAPK10 priming of BALB/c mouse strain, a susceptible host. The resistance to the infection is generally associated with mixed Th1/Th2 responses to the infection following immunization with LmjMAPK10 DNA or protein or a combination of both DNA and protein. Therefore, LmjMAPK10 is a probable vaccine candidate against the infection.

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Development of complement inhibitors to block host cell damage

Background

The complement system is one of the most powerful effector mechanisms involved in the body's defense. It is a key component of the innate immunity, playing a central role in the host defense against pathogens. Although the complement system is designed to target pathogens, inadvertent complement activation is known to result in host tissue injury. Studies from various laboratories clearly show that complement is a pathological factor in a large number of diseases including, but not limited to, experimental allergic neuritis, type II collagen induced arthritis, myasthenia gravis, hemolytic anemia, glomerulonephritis, ARDS, stroke, heart attack and burn injuries. In addition, deficiencies, mutations and polymorphism in complement regulatory proteins and/or its components have also been linked to diseases such as paroxysmal nocturnal hemoglobinuria (PNH), hereditary angioedema (HAE), age-related macular degeneration (AMD), atypical hemolytic uremic syndrome (aHUS) and membranoproliferative glomerulonephritis type II (MPGN type II). Thus, it is apparent that there is a critical need of complement inhibitors. Our laboratory primarily focuses on development of complement inhibitors against human complement proteins factor B (fB) and C2, which are the key proteases of the complement system involved in initiation and activation of alternative and classical/lectin pathways, respectively.

Aims and Objectives

1. Identification and characterization of factor B/C2-specific complement inhibitors
2. Investigation of the mechanism of complement inhibition by factor B/C2-specific complement inhibitors
3. Evaluation of therapeutic potential of factor B/C2-specific complement inhibitors

Work Achieved

Identification of Complin, a peptide inhibitor of complement proteins factor B and C2: In an effort to identify small molecular weight complement inhibitors against fB, we screened random peptide phage-displayed libraries against this protein. The screening

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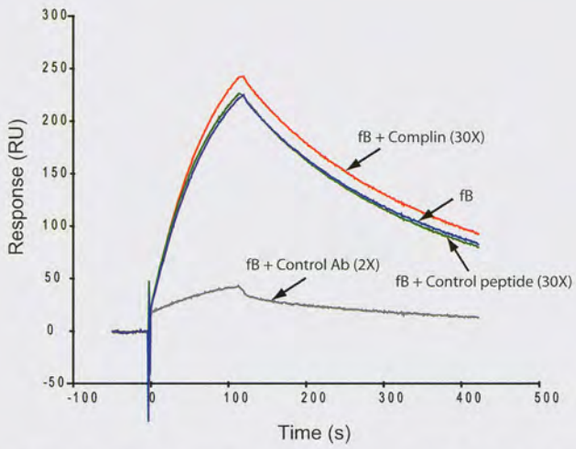
of about 1500 clones from the cysteine-constrained as well as the linear library yielded about 75 positive clones and sequencing of these clones confirmed the presence of five unique clones. We then synthesized the peptides corresponding to phage-displayed sequences. Of the five synthetic peptides examined, only one peptide displayed inhibitory activity. The IC_{50} value of this linear peptide for inhibition of fB activation was 56 μM i.e., the 50% inhibition of fB activation required about 52-fold molar excess of this peptide. Next, in an effort to design a peptide with enhanced inhibitory activity, we examined the phage-displayed peptide sequences for the presence of a motif. Alignment of five phage-displayed peptide sequences showed the presence of eight conserved residues with a highly conserved four-residue core. Based on the motif, we then synthesized fifteen different disulfide constrained peptides. Although all the phage-displayed peptides except one were linear, we designed motif-based peptides as cyclic peptides, as they are likely to be structured and have increased half life in serum as compared to the linear peptides. To optimize the structure of the peptide we primarily varied the ring size and residues outside the four-residue core. Functional characterization of these peptides for inhibition of fB activation demonstrated that only three of the fifteen peptides showed increased inhibitory activity compared to the parent inhibitory peptide. The most active peptide showed an IC_{50} of 9.5 μM, indicating that about 9-fold molar excess concentration of this peptide was sufficient to inhibit 50% of fB activation. As expected, this peptide also inhibited alternative pathway-mediated lysis of rabbit erythrocytes (IC_{50} = 33 μM). The complement components fB and C2 are structural homologs. We therefore also looked whether the peptide inhibits C2 activation. Interestingly, the peptide did show inhibition of C2 activation, and also the classical and lectin pathways. Based on its activities, we have named this peptide as Complin (complement inhibitor).

During designing the motif-based constrained peptides, we made an assumption that cyclization would result in stabilization of the peptide structure. Thus, to validate our premise, we synthesized a linear analog of Complin wherein cysteines were replaced with Ala. This analog did not show any inhibitory activity even at 500 μM concentration, suggesting that indeed disulfide bond plays an important role in the maintenance of a preferred structure of the peptide required for its inhibitory activity. In addition, we also determined the importance of amino acid residues of Complin between Cys 2 and Cys 10 in its inhibitory activity by performing an Ala scan analysis. Substitutions of each of the residues between Cys 2 and Cys 10 with Ala showed that substitutions at all the positions except at positions 4 and 9 drastically hampered its activity. These data therefore suggested that three of the four core residues play critical role in maintaining the functional activity of the peptide.

Development of inhibitory monoclonal antibodies against factor B: Apart from developing a small molecular inhibitor against fB, we have also developed neutralizing monoclonal antibodies (mAbs) against human fB. Our fusion experiment resulted in generation of 95 hybridoma clones against fB. Based on reactivity to native and denatured fB in a dot blot assay we subcloned eight antibody producing clones.

Fig. 1: Effect of Complin on binding of factor B to C3b.



Characterization of these clones for isotypes showed that of the eight, four were IgGκ producing clones, two were IgAκ producing clones, and two were IgMκ producing clones. Our data on localization of mAb binding region on fB showed that the IgM and IgA antibodies bound to the Bb region, while the IgG antibodies bound to the Ba region of fB. Functional characterization of all the mAbs revealed that only three IgG mAbs (74, 59 and 34) possessed fB neutralizing activities. The IC_{50} values for inhibition of fB activation were 2.3μM, 2.5μM and 5.5μM, respectively. These mAbs also inhibited the activation of the alternative pathway with IC_{50} values 740 nM, 780 nM and 1100 nM, respectively.

Investigation of the mechanism of complement inhibition of Complin and factor B-specific monoclonal antibodies: Surface plasmon resonance binding data for Complin to fB showed that the peptide binds to fB in a bivalent manner ($\chi 2 = 1.58$); the apparent equilibrium rate constants (K_{D1} and K_{D2}) were 3.05 μM and 4.16 μM. We therefore analyzed binding of Complin to both the fragments of fB i.e., Ba as well as Bb. As expected, it bound to both Ba as well as Bb fragments and data fitted well the 1:1 Langmuir binding model.

Activation of fB occurs in two steps. The first step involves binding of fB to C3b and the second step involves cleavage of C3b bound fB by factor D. Thus to investigate how Complin inhibits fB activation we designed a Biacore assay wherein we measured binding of fB or fB pre-incubated with Complin to C3b immobilized on a sensor chip. If Complin inhibits C3b-fB interaction then there should be a decrease in binding response when fB is pre-incubated with Complin as compared to binding response of fB alone. We did not observe any inhibition in binding response in fB pre-incubated with Complin (Fig. 1). It is therefore apparent that Complin inhibits the second step i.e., cleavage of fB by factor D. Based on these data we hypothesize that by binding to Ba and Bb fragments, Complin locks the conformation of fB in its "proenzyme state" where the scissile bond (Arg^{234}-Lys^{235}) remains occluded, and thereby inhibits its cleavage. Because Complin showed binding to Bb, we also examined whether it has the ability to inhibit the activities of pre-formed convertases C3b,Bb and CVF,Bb. Our data showed that it has only partial effect if any at high concentration on these enzymes. Thus the peptide primarily inhibits the alternative pathway by inhibiting activation of fB.

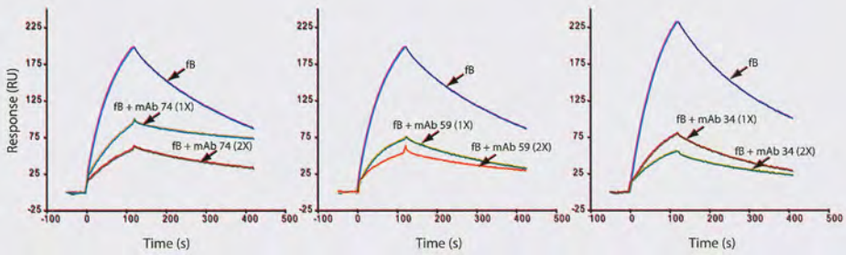


Fig. 2: Inhibition of binding of factor B to C3b by monoclonal antibodies.

The IgG mAbs 74, 59 and 34 showed inhibition of activation of fB as well as the alternative pathway of complement activation. We thus performed a series of experiments to explore the mechanism by which they inhibit activation of fB and the alternative pathway. First we examined if they bind to Ba or Bb fragment: SPR analysis performed by immobilizing Ba and Bb and flowing mAbs indicated that all the antibodies bound only to the Ba fragment. Detailed analysis showed that IgG's, 74, 59 and 34, bound to the Ba fragment with a K_D of $3.51X10^{-10}$ M, $8.1X10^{-10}$ M, $2.97X10^{-10}$ M, respectively. Next we examined if they have any effect on pre-formed C3- and C5-convertases. This was an unlikely possibility since mAbs showed binding to Ba fragments only. Nevertheless, to rule out this possibility we looked at their effect on the pre-formed enzymes. Addition of graded concentration of mAbs to the preformed C3- and C5- convertases showed no effect on the enzyme activity i.e., cleavages of their substrates C3 or C5, suggesting that these mAbs inhibit the alternative pathway primarily by inhibiting fB activation. As discussed above, inhibition of fB activation could be either due to inhibition of binding of fB to C3b or due to cleavage of fB by factor D. Inhibition of fB binding to C3b was studied by ELISA as well as SPR assays. All the three IgG mAbs showed dose dependent inhibition of fB binding to C3b in an ELISA assay. These results were further corroborated by SPR assays (Fig. 2), suggesting that unlike Complin, these mAbs inhibit the first step of fB activation.

Evaluation of therapeutic potential of factor B-specific monoclonal antibodies: When blood is exposed in extra-corporeal circuits during hemodialysis, hemofiltration, plasmapheresis, apheresis, and cardiopulmonary bypass (CPB) surgery, complement activation occur that can lead to post-therapy complications like systemic inflammatory reactions, stroke and end organ failure. The complement-mediated injury that occurs is directly mediated by the membrane attack complex (C5b-9) and indirectly by C3a and

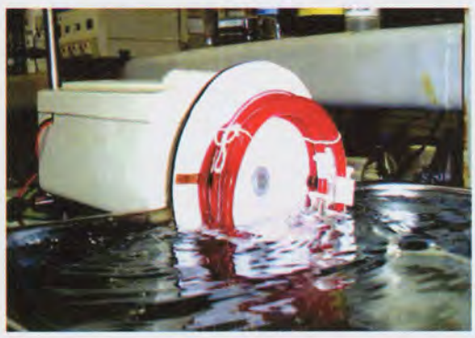


Fig. 3: Extra-corporeal circuit setup used for evaluation of effect of monoclonal antibodies on complement activation.

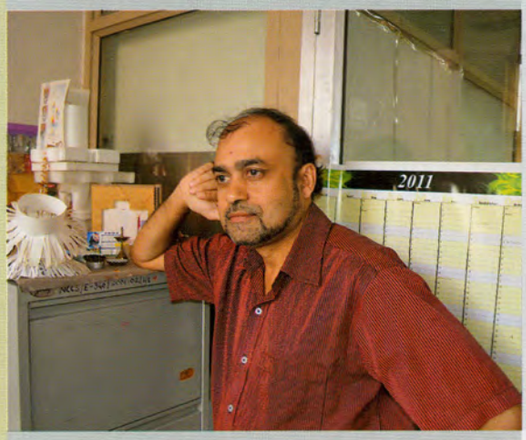
C5a through activation of mast cells, monocytes and PMNs. We thus utilized the extra-corporeal circuit model to evaluate the therapeutic potential of neutralizing mAbs (Fig. 3). All the three mAbs showed dose dependent inhibition of C3a, C5a, C5b-9 and PMN elastase in this model, but the most effective was mAb 74. It inhibited 50% generation of above complement activation products and PMN elastase at a concentration which is 4- to 8-fold less than the concentration of fB in the plasma.

Future Work

1. Evaluation of therapeutic potential of Complin.
2. Determination of solution structure of Complin by 2D NMR.
3. Determination of crystal structure of Complin in complex with fB.
4. Development of potent analogs of Complin based on its structure.

Research Report

Chromatin Architecture & Gene Regulation



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Global gene regulation by tumor suppressor SMAR1

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Background

Changes in composition of nuclear matrix associated proteins contribute to alterations in nuclear structure, one of the major phenotype of malignant cancer cells. The malignancy-induced changes in this structure lead to alterations in chromatin folding, the fidelity of genome replication and gene expression programs. The nuclear matrix forms a scaffold upon which the chromatin is organized into periodic loop domains called Matrix Attachment Regions (MAR) by binding to various MAR binding proteins (MARBPs). Aberrant expression of MARBPs modulates the chromatin organization and alters transcriptional network that leads to oncogenesis. Dysregulation of nuclear matrix associated MARBPs have been reported in different types of cancers. Some of these proteins have tumor specific expression and are therefore considered as promising diagnostic or prognostic markers in few cancers. SMAR1 (Scaffold/ Matrix attachment region binding protein 1), is one such nuclear matrix associated candidate tumor suppressor whose expression is drastically reduced in higher grades of breast cancer. *SMAR1* gene is located on human chromosome 16q24.3 locus, the loss of heterozygosity (LOH) of which has been reported in several types of cancers.

SMAR1 was identified as novel DNA binding protein from murine thymocyte expression library screen. The human homolog of SMAR1 named BANP was isolated as a BTG3 associated nuclear protein using yeast two hybrid approach. The BTG3 (B- cell translocation gene) is the member of anti-proliferative gene family which includes BTG1 and BTG2. The human SMAR1 is present on chromosome 16q24.3 (Fig. 1A), while mouse SMAR1 maps to distal portion of chromosome 8 at a distance of 111.8 cM. The sequence comparison revealed that human and mouse SMAR1 exhibit more than 95% homology. Both human and mouse SMAR1 contains 14 exons, but in mouse there is 23kb insertion between exon 1 and 2 (Fig. 1B). Open reading frame of human *SMAR1* encodes two alternatively spliced forms, one of which has 117 bp deletions at N-terminus. *SMAR1* gene encodes a 2.1 kb long mRNA that corresponds to 60 kDa full length protein (548 amino acids). Northern blot analysis showed that *SMAR1* transcript is differentially

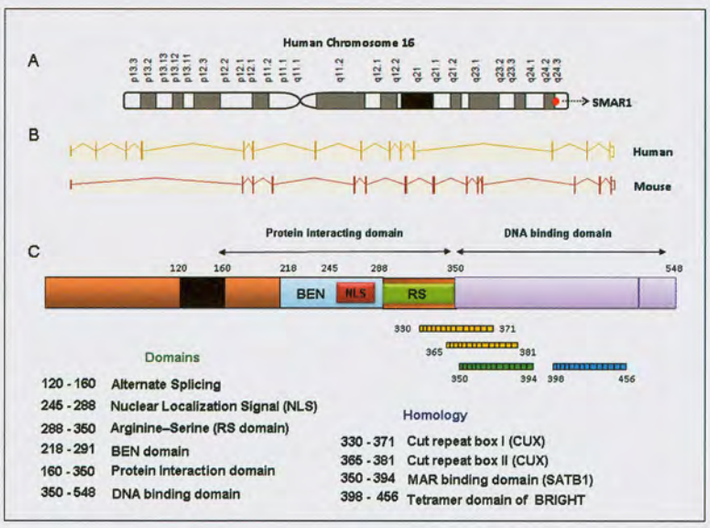

Fig.1: (A) Genomic localization of SMAR1 on chromosome 16 (B) Organization of introns and exons in human and mouse SMAR1 gene loci (C) Different functional domains of SMAR1 (Alternate splicing, nuclear localization signal, RS motif and BEN domain). The homology domains of Cux, SATB1 and Bright are depicted. Yellow bar represent the Cut repeat box 1 and 2, green bar represent the homology domain of SATB1 and blue bar represent the tetramer domain of Bright.

expressed in all organs like heart, lung, thymus, spleen, liver, brain and kidneys. However, the expression is high in thymus compared to other organs. Protein sequence analysis revealed that SMAR1 shares homology with other MARBPs such as Cux/CDP, Bright and SATB1 in the MAR binding regions. The MAR binding domain of SMAR1 resides within 352-394 amino acids and is identical to that of SATB1. An extended stretch of same region (328-420 aa) share significant homology with Cut repeat box I (330-371 aa) and box II (365-381 aa) of Cux/CDP. In addition to this, 398- 456 aa region of SMAR1 is homologous to the tetramer domain of Bright. The small domain of SMAR1 within 160-350 aa contains nuclear localization signal (NLS). 160-350 aa region of SMAR1 also contains arginine-serine rich motifs and serves as a protein interaction domain, while the C-terminal region 350-548 aa is characterized as a DNA binding domain (Fig.1C). Additionally, SMAR1 also contains the BEN domain residing between 218-291 amino acid residues (Fig.1C). The BEN domain is predicted to mediate protein-DNA and protein-protein interactions during chromatin organization. The 160-350 aa region which contains BEN domain interacts with MAR-binding protein Cux/CDP, and the HDAC/SIN3complexes. The C-terminal region of the BEN domain overlaps with the MAR-binding region in SMAR1. The BEN domain is highly conserved and is predicted to be present in proteins that function as transcription factors. It also functions as an adaptor for the higher-order chromatin structure and recruitment of chromatin modifying factors in transcriptional regulation.

Recently we have elaborated on the current understanding of molecular mechanisms involved in tumor suppressor functions of SMAR1 and highlight the regulation of its various cellular target genes involved in cell growth, apoptosis and tumorigenesis (Figure 2). Various molecular and biochemical studies convincingly demonstrate that SMAR1 can modulate the functional activity of two major cellular homeostatic regulators, p53 and NF-κB. While p53 is mutated in over more than 50% of human cancers of various histological origin and favors cellular transformation, the aberrant NF-κB activity

promotes tumor cell survival, metastasis and angiogenesis. To this effect, we demonstrated that restoration of SMAR1 function in breast cancer derived cells can activate p53 and block aberrant NF-κB activity. Combination therapy is emerging as a key to overcome problems of drug-related toxicity and resistance, and simultaneously targeting the p53 and NF-κB pathways has been an attractive strategy to combat cancer, with several lead compounds identified in recent years. Identification of small molecules which can activate or stabilize SMAR1 could have great potential in combination therapy for cancer, because activation of SMAR1 can modulate both these pathways and hence can be an effective player in tumor suppression.

Aims and Objectives

1. Regulation of CD44 gene splicing and its Implication in cancer
2. DNA damage repair by SMAR1 through Ku70 deacetylation
3. Control of cytokine genes for T_H1-T_H2- T_H17 and T-reg differentiation

Work Achieved

Regulation of CD44 gene splicing and its Implication in cancer: Nuclear matrix is the site for pre-mRNA splicing and the nuclear matrix proteins are reported to be involved in RNA packaging, processing and nuclear export. Chromatin remodeling machineries in consort with the histone modifications, etch the splicing code and acts as scaffold for the recruitment of spliceosomal machinery co-transcriptionally. Incorporation of the variable exons is sometimes dependent on the external stimuli. The signal transduction activators of RNA (STAR) family proteins, like Sam68, ASD-2 are reported to regulate the incorporation of the variable exons by directly binding to the RNA elements. The promoter structure modulated by the transcription factors plays an important role in the regulation of alternative splicing. But it is highly unclear how chromatin modulators and especially nuclear matrix proteins regulate transcription coupled constitutive and alternative pre-mRNA splicing.

The spatial and temporal generation of splice variants of pre-mRNA demands that alternative splicing is subjected to stringent regulation. It has also been proposed that chromatin could play a role in the chaperoning of the neosynthesized pre-mRNA with consequences on splicing. We first identified that nuclear matrix protein SMAR1 interacts with splicing co-activator SRm160 which is known to regulate Ras dependent CD44 alternative splicing and also enhances constitutive splicing. Inclusion of variable exons in CD44 mRNA is dependent on MAP kinase signaling pathway. CD44 has 10 constant exons and 10 variable exons residing between constant exon 5 and 6. Higher levels of CD44 variants confer strong metastatic potential to tumors. In the context of SRm160, our studies deals with the regulation of CD44 alternative splicing by nuclear matrix protein SMAR1 in an ERK dependent manner. Knock down of SMAR1 enhances the inclusion of CD44 variable exons. We found SMAR1 interacts with Sam68 endogenously, another protein of Signal Transducer and Activator of RNA splicing (STAR) family, and MAP kinase mediated activation causes post translational modification of SMAR1 by ERK and

Other equipments in the facility :

FACS Vantage equipment was given to BD under buy back scheme. Aria III SORP has been purchased from BD under buy back scheme and has been installed in March 2011. New Canto II machine was supplied FOC by BD under buy back scheme in March 2011.BDPathway 855 installation was completed by end of April 2010. The first training for operators was conducted by BD in November 2010. It will be made functional soon.

Confocal Microscopy

The Confocal Facility has two microscopes:

Zeiss LSM510 META

Advanced Spectral Confocal Microscope, Zeiss LSM510 META, with programmable CO2 incubator and temperature-humidity control. This system comprising offully motorized and computer controlled Inverted Fluorescence microscope, is being used for regular confocal as well as FRET, FRAP, Live Imaging etc.The Lasers available are Blue Diode laser (405nm), Argon laser(458/477/488/514 nm), 543nm He-Ne and 633nm He-Ne. The spectral detector permits separation of upto eight emission signals, even if the fluorescence spectra are strongly overlapping.

Leica SP5 II

A new Leica SP5 II system has been installed. It is a high-end Broadband Confocal Laser Scanning Microscope with 4 cooled spectral PMTs and unique AOBS,technology equipped with CO2 incubator,Fully motorized, automated and computer controlled Microscope Leica DMI 6000.The Lasers are Blue Diode Laser 405nm, Ar Laser 458, 488, 476, 496, 514, DPSS 561nm, HeNe 594nm and HeNe 633nm with Scanning Stage and incubation chamber for live cell experiments. Software for Confocal imaging 3D imaging and reconstruction, Dye Finder, Time lapse, colocalization , FRET (SE & AB), FRAP is also available.

The numbers of samples imaged during 2010-11 were approximately 3873 in-house and 209 came from various other institutes. Number of samples for live-imaging was 13.

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mediates the translocation of protein from the nucleus to cytoplasm. We are now investigating, whether a dual control of CD44 expression exists; one via upregulation of p53 by certain anticancer drugs, wherein p53 transcriptionally inactivates the expression of CD44 and secondly whether this p53 can bind to the SMAR1 promoter, increasing its expression and thereby preventing the abnormal alternative splicing of CD44, in higher grades of cancer.

DNA damage repair by SMAR1 through Ku70 deacetylation: The DNA damage response pathway is a signal transduction pathway consisting of sensors, transducers and effectors. Two related and conserved proteins, ATM and ATR, are central components of the DNA damage response (DDR). Scaffold/matrix attachment region binding protein (SMAR1) is one such tumor suppressor stress responsive protein which is known to globally modify the topology of chromatin. DNA replication and efficient DNA repair depends on chromatin architecture because anchoring of the damaged DNA to the nuclear matrix is prerequisite for efficient repair.

Panoptic studies on SMAR1 has revealed that SMAR1 is a stress responsive protein as Doxorubicin, Camptothecin and UV mediated damage induces the expression levels of SMAR1. This has rationalized our intrigue to understand the regulation of DNA double strand breaks by SMAR1. In response to γ-irradiation SMAR1 is rapidly induced, and phosphorylated at serine 370 by ATM which causes its nuclear translocation and forms irradiation induced foci (IRIF) which colocalizes with γH2AX and. Upon nuclear translocation, SMAR1 facilitates in the DSB repair but still the molecular mechanism is unknown. SMAR1 directly interacts with Ku70 protein and favors DNA repair by enhancing deacetylation of Ku70 via recruiting histone deacetylase HDAC1. The Ku protein plays a key role in multiple nuclear processes, e.g., DNA repair, chromosome maintenance, transcription regulation, and V(D)J recombination. Consistent with this, chromosomal structural analysis upon SMAR1 knockdown and overexpression showed that cells lacking SMAR1 had increased sensitivity to ionizing radiation, and contained significantly higher levels of chromosome aberrations after exposure to ionizing radiation while cells over expressing SMAR1 were having less chromosome aberrations and showed high survival. DNA damage quantification also suggests crucial role of SMAR1 in DNA damage repair (Fig. 2). SMAR1 promotes phosphorylation of an indispensible checkpoint kinase ChK2 at Thr68 and thus decreased phosphorylation of histone H3 Ser10, further acting as an important regulator of G2/M cell-cycle transition. Functionally SMAR1 facilitates DSB repair by assisting in anchorage of DNA repair factors like Ku70 at the site of repair and causing cell-cycle arrest hence providing ample time for repair. These observations provide new insights into how the nuclear matrix binding protein SMAR1 critically modulates DNA repair activity and contributes for the maintenance of genomic integrity. DNA repair mechanism is a promising target of many novel cancer therapies as inefficient repair in the cell is the prime reason for mutations in the genome and development of cancer and involves mutation in the DNA repair genes and tumor suppressor genes. Drugs that target DNA damage checkpoint proteins are already in

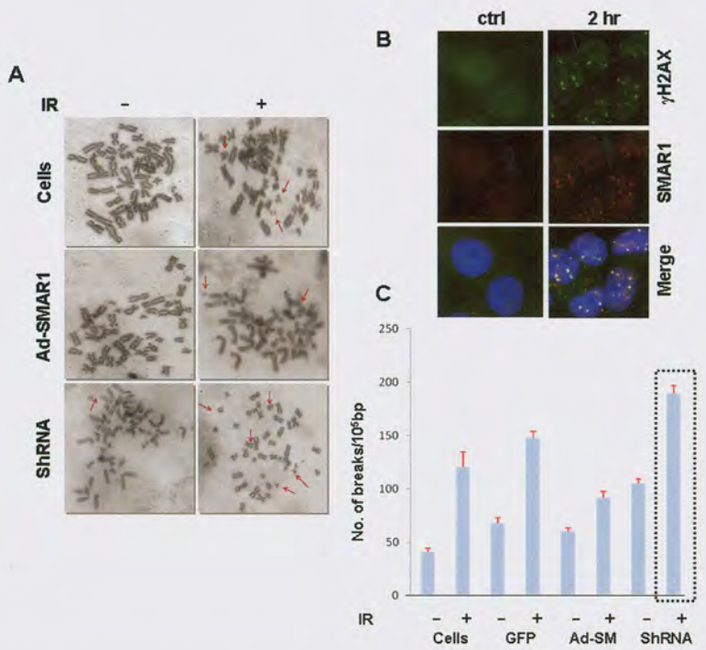

Fig. 2:. A. SMAR1 knockdown causes higher chromosomal aberrations upon IR. B. Colocalizationt experiment using confocal microscope showing direct interaction of SMAR1 with γH2AX. C. Assay for DNA damage repair shows that maximum DNA breaks remain unrepaired in the absence of SMAR1

clinical trials as anticancer drugs and studies on different cellular factors that can regulate DNA damage checkpoints in cancer stem cells and radio-resistance will provide insights into novel cellular targets which can lead to improvement in treatment modalities.

Control of cytokine genes for T_H1-T_H2- T_H17 and T-reg differentiation: We are working on a matrix attachment region binding protein (MARBP) SMAR1 that globally regulates gene transcription through recruitment of HDAC1-Sin3 complex at various promoters. Previous results from our lab suggested a critical role of SMAR1 in the differentiation of T helper cells to Th1 and Th2 subtypes by the regulation of T-bet promoter. Further extension of SMAR1 mediated regulation of T cell lineage elucidated on yet another function of SMAR1 in regulating Th17 differentiation. The expression level of SMAR1 is downregulated in naïve T cells polarized *in-vitro* towards Th17. Induced expression of SMAR1 inhibits Th17 polarization probably by binding to the MAR regions on the IL17 locus. Research on SMAR1 further assumes it to be a global regulator of gene transcription having multifarious functions in the regulation of other cytokine genes that drives specific T cell lineages. Th17 cells are the most important candidate for the immune response against inflammatory conditions. Hence, regulation of Th17 by a cell intrinsic factor can be a potent regulator of inflammatory responses. Understanding the regulation of the inflammatory responses by SMAR1 will be assessed using over-expressed and T cell specific conditional knock-down mice. In this regard, chemically induced colitis and rheumatoid arthritis models are under study to better understand the function of nuclear matrix proteins in T cell differentiation and thus in immunity through T cell polarization. Mice experimental models of chemically induced colitis showed a protective role of SMAR1 through the disease progression. Conserved MAR sequences

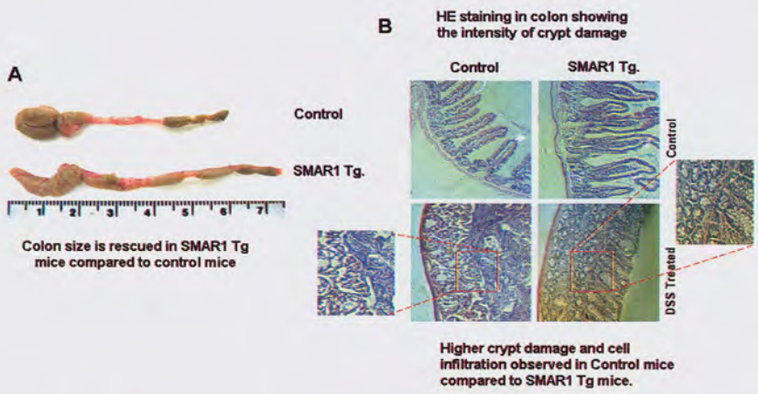

Fig. 3: Comparison of the colon architecture in SMAR1 transgenic and control mice of DSS induced colitis. (A) Colon size of control and SMAR1 Tg. mice after 15 days of DSS induced colitis are shown at the same scale. (B) Histological analysis done using hematoxylin / eosin-staining method of colon from untreated and DSS treated control and SMAR1 Tg. mice are displayed by photographs of colon cross-sections 15 days after colitis induction.

were observed in IFNγ and IL17 locus which are the signature cytokines for Th1 and Th17 cell lineages respectively suggesting the regulation of these genes by SMAR1 (Fig. 3). Many reports described the chromatin modulation associated with the polarization of naïve T cells to different lineages. Inter and intra chromosomal interactions and the histone modifications culminate in the activation of genes specific for one particular lineage and the repression of others. The molecular mechanisms associated with the chromatin changes are still to be understood clearly.

Future Work

Regulation of CD44 variants will be studied in detail. Since SMAR1 is downregulated in advanced cancer, we shall look into possible mechanism of inverse correlation in more detail. Regarding the DNA damage repair function, we shall study more detail about the protein-protein interactions and colocalization of repair protein at the site of damage. The work on the T cell differentiation is in good progress. We shall include asthma as one of the disease model to better understand the Th1-Th2 differentiation as possible role of SMAR1 in transgenic and knock-out mice.

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Mechanisms of global gene regulation by SATB1

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Debashis Mitra, NCCS

Background

Eukaryotic chromatin is compartmentalized or distributed into functional components. Higher-order organization of chromatin is mediated by tethering of high affinity sequences or MARs onto nuclear scaffold, which then participate in dynamic arrangement of chromatin into topologically independent loop domains. Special AT-rich Binding protein 1 (SATB1) imparts a 'cage-like' structure within the nucleus circumscribing the heterochromatin and euchromatin regions by tethering the MARs onto the intra-nuclear framework and act as docking site for several chromatin modifiers SATB1 exerts its regulation on target genes by distinct mechanisms. SATB1 also binds to promoters and upstream regulatory regions, thereby directly influencing their transcriptional status. SATB1 recruits different chromatin remodelers such as HDAC1 and HATs via its PDZ-like domain and thereby regulates its target genes. Interaction of SATB1 with CtBP1 and β-catenin underscores its role in regulation of Wnt target genes. Furthermore, post-translational modifications of SATB1 act as molecular switches in regulation of SATB1 target genes. The microarray based gene expression profiling revealed multiple targets of SATB1. Locus-wide occupancy study indicated that SATB1 is clustered at structural and regulatory elements across the MHC locus - at MARs forming the bases of chromatin loops and at upstream regulatory regions of genes within the chromatin loops respectively. Thus, SATB1 exhibits two distinct modes of regulation by directly regulating individual target genes and by regulating distant genes in a coordinated manner. SATB1 interacts with PML and regulates the MHC class I cluster in response to physiological stimuli. SATB1 is responsible for maintenance of dynamic chromatin 'loopscape' and organization of transcriptionally poised chromatin. An additional mechanism responsible for SATB1 mediated regulation of genes is alternative splicing of SATB1 itself. Upon TCR mediated activation Satb1 is alternatively spliced to generate its longer isoform $SATB1^{L}$. $SATB1^{L}$ exhibits differential affinities to various SBSs and modifies the transcriptional activity at the target genes. Thus $SATB1^{L}$ expression induces further complexity in regulating SATB1 target genes. Furthermore, SATB1L exhibits a different nuclear localization along with the differential regulation exerted on target genes.

Within the interphase nuclei, chromosomes occupy distinct territories referred to as chromosome territories (CTs). These chromosome territories are open structures

comprising of condensed chromatin domains and considerable amount of interchromatin space. The interchromatin space has chromatin loops emanating from compact chromatin domains. SATB1 circumscribes different chromatin territories, while inter-chromatin space is occupied by loops of gene dense regions and transcriptionally poised genes. This interchromatin void is in turn filled by SATB1 binding at the bases of the loops and upstream regulatory region. Thus SATB1 presumably plays a major role in creating transcriptionally conducive environment in the interchromatin space. As cellular differentiation proceeds, changes in transcriptional activity are often coupled with changes in sub-nuclear localization of chromosomes. Transcriptional regulatory elements act by repositioning specific genetic loci to regions with active or silent transcription. Although initially the CTs were thought to be arranged in radial fashion with the gene-rich and active or transcriptionally poised chromatin in the nuclear interior to the gene-poor and silent chromatin localized to the nuclear periphery, recent evidences suggest that nuclear positioning and gene activation may be reconciled by the potential presence of the microdomains at the nuclear periphery. We found that thymocyte chromatin is actively redistributed upon TCR mediated activation and these events are linked with repositioning of multiple gene loci. Further, SATB1L in association of lamin-associated domains (LADs) is responsible for dynamic gene positioning within the nucleus.

Aims and Objectives

1. Functional characterization of alternatively spliced form $SATB1^{L}$.
2. To delineate the differential regulation imparted by SATB1 and $SATB1^{L}$.
3. To study the role of $SATB1^{L}$ in T cell development.

Work Achieved

1. **T cell activation leads to reorganization of chromatin territories and domains in thymocyte nuclei:** The thymocyte nuclei highlight distinct genomic organization marked by heterogenous DNA content. Nuclear dye DAPI that stains DNA exhibits differential staining of the nucleus into DAPI intense and DAPI light areas indicative of open and highly condensed DNA respectively (Fig. 1A). The three-dimensional network SATB1 protein network circumscribes the heterochromatic regions in thymocyte nuclei. However, upon TCR activation, the chromatin organization seems to undergo gross alterations. Firstly, DAPI stained nuclei exhibited almost homogenous DNA content such that obvious differential staining of hetero- and eu-chromatin was not observed. TCR mediated activation of thymocytes induced $SATB1^{L}$ expression, accompanied with loss of cage-like structure of SATB1. $SATB1^{L}$ was shown to localize at the peri-nuclear region along with Lamin B1, and differentially regulate multiple SATB1 target genes (NCCS annual report 2009-10). To reconcile the change in genomic organization depicted by DAPI staining and co-localization of $SATB1^{L}$ with Lamin B1, naïve, activated and activated + Rottlerin treated thymocytes were stained for H3K9 acetylation to mark the micro domains of the thymocyte nuclei for active chromatin (ENCODE Project Consortium). The co-immunostaining of H3K9 acetylation (green) and SATB1 (red) imparted a strikingly similar pattern in naïve thymocytes (Fig. 1B). Upon activation though the intricate staining of both SATB1 and H3K9 acetylation was not detected, but they were still found to co-localize more at the periphery than in the interior of the nucleus. Indeed it can be noted

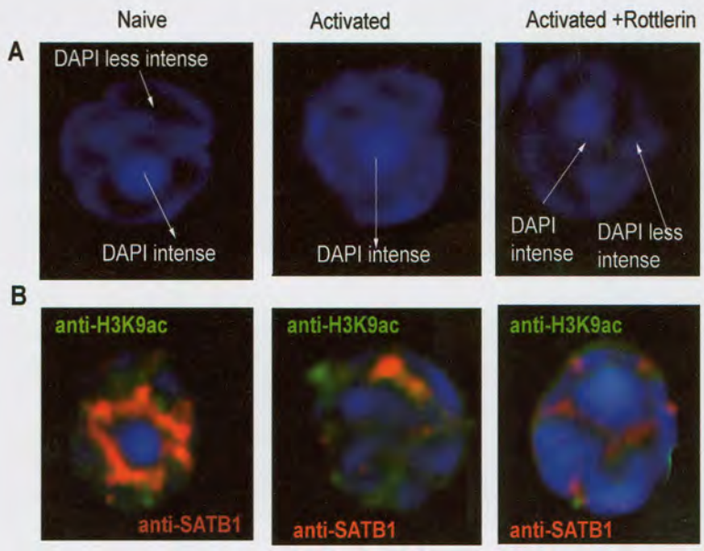


Fig.1: TCR activation leads to reorganization of the chromatin territories in thymocyte nuclei. Immunofluorescence images depicting gross level chromatin domain organization within thymocyte nuclei. A. Thymocyte nuclei are stained with DAPI to reveal the DAPI intense and less intense areas in naïve thymocytes, activated thymocytes and thymocytes activated in presence of Rottlerin. Shown here are 0.2 µM optical sections of the images captured using 'Apotome' module of Axiolmager Z1 upright fluorescence microscope (Carl Zeiss). The images were further processed using inverse theorem in Deconvolution software. B. Naïve thymocytes, activated thymocytes and thymocytes activated in presence of Rottlerin are triple stained with SATB1 (Alexafluor 594 nm), H3K9 acetylation (Alexafluor 488 nm) and counterstained with DAPI. The images were captured using 'Apotome' module of Axiolmager Z1 upright fluorescence microscope (Carl Zeiss).

that, acetylated histone H3 at lysine 9 (H3K9ac) colocalizes with SATB1^{L}, as TCR activation induces expression of SATB1L which is detectable by anti-SATB1 immunostaining. This was further corroborated using Rottlerin treatment that inhibits SATB1^{L} production, which failed to highlight any such organizational changes at the nuclear periphery (Fig. 1B). This observation suggested that although SATB1^{L} localizes to peri-nuclear region, which is known to have active and silent micro-domains sub-nuclear areas marked with SATB1^{L} were also associated with an activation marker. This implicated that SATB1^{L} does not localize with heterochromatic sub-domains of Lamin B1.

2. TCR activation results in repositioning of SATB1 target genes in murine thymocytes: SATB1L is specifically generated by alternative splicing of SATB1 transcript and its role in TCR mediated regulation for maturation of developing thymocytes has been documented before. SATB1^{L} exerts its regulation via differential binding affinity to different SBS and thus potentially regulates multiple genes. SATB1 organizes chromatin of various loci and brings about co-ordinated regulation of genes. SATB1 tethers different SBSs lying at the base of chromatin loops onto the nuclear matrix. Differentiation of cells is known to introduce 'looping-out' of different genes from their chromosome territories into inter-chromosome voids. This differential partitioning into matrix and loop was also detected upon TCR activation as a result of presence of SATB1^{L}. To detect if this matrix-loop partitioning is also accompanied with physical re-localization within the nuclei we performed FISH using two SBSs. We detected a SATB1^{L}-dependent change in re-localization of the SBS-700 probe from interior to periphery using Florescence In Situ Hybridization (FISH) against SBS-700, whereas upon Rottlerin treatment the probe was found to be localized back in the interior region (Fig. 2A). However, FISH analysis also revealed that SBS-336 is not relocated under similar conditions (Fig. 2B). It can therefore be postulated that SATB1L expression results in relocation of only those genomic targets that are bound by SATB1^{L}. Furthermore, to find out if SATB1^{L} targets these loci to nuclear periphery, immuno-FISH for SBS-700 and -336 was performed. The in situ hybridized

cells were then immunostained with anti-LaminB1 in naive and activated thymocytes in presence and absence of Rottlerin. Interestingly, both SBS-700 and SBS-336 were found to be targeted to LaminB1 at the nuclear periphery (Fig. 2C and 2D). Although these SBSs co-localized irrespective of expression of SATB1L, but their position in the interior or at the nuclear periphery is dependent upon SATB1^{L} expression. Targeting of genes to nuclear periphery leads to both transcriptional activation and repression depending upon their context. Nuclear periphery is known to be site of active transcription and silencing. To delineate whether these SBS associate with active sites of transcription or hetero-chromatinization upon activation FISH was followed with immunostaining using anti-Pol

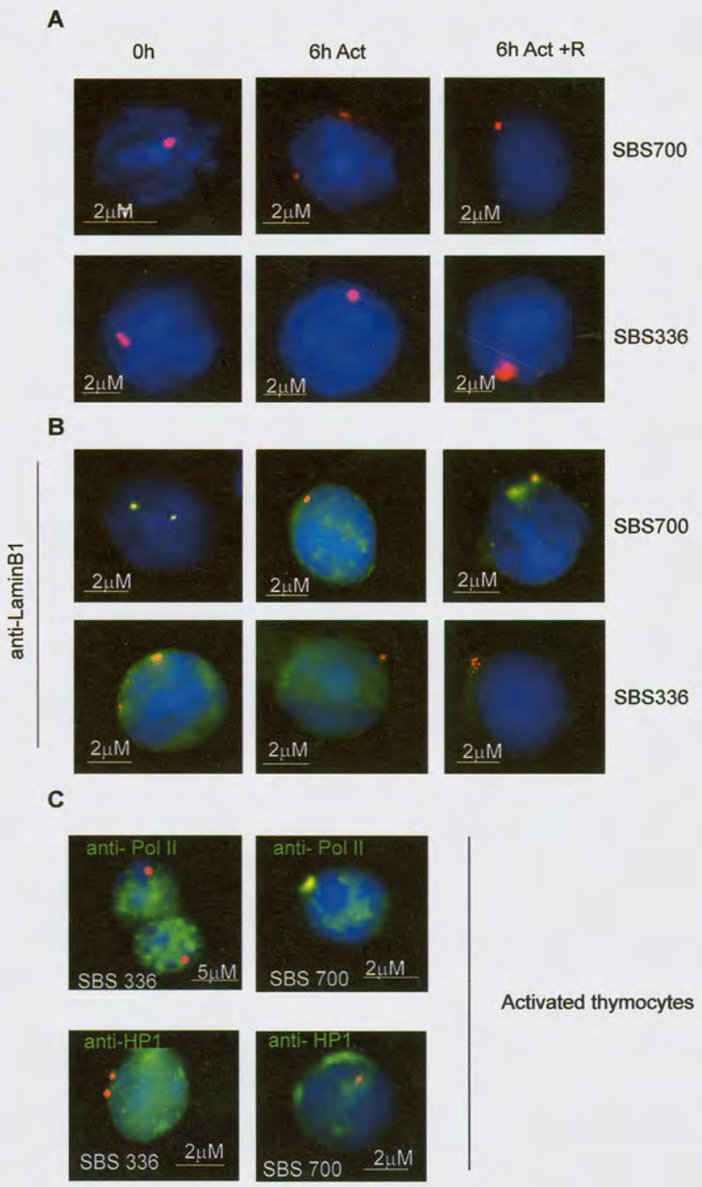


Fig. 2: TCR activation results in repositioning of SATB1 target genes. Immuno-FISH was performed with two SATB1 targets SBS-700 and SBS-336. **A.** Fluorescence in situ hybridization (FISH) was performed for SBS 700 and SBS 336 using naïve, activated and activated and rottlerin treated thymocytes. The thymocyte nuclei were counterstained with DAPI. FISH signals were captured using 'Apotome' module of AxioImager Z1 upright fluorescence microscope (Carl Zeiss). Images shown are of 0.2 μM optical sections with FISH signals. **B.** FISH followed by immunostaining was performed for detecting co-localization of SBS-700 (upper panel) and SBS-336 (lower panel) with LaminB1. Images were acquired on 'Apotome' module of AxioImager Z1 upright fluorescence microscope (Carl Zeiss). To confirm co-localization images were taken at different depths of the nuclei. The merged images of anti-Lamin B1 (green) and FISH signals (red) shown here are acquired at 0.2 μM resolution. **C.** Immuno-FISH was performed on activated thymocytes for SBS-700 and SBS-336 as probes to detect co-localization of SBS-700 and -336 with pol II and HP1. Immuno-FISH merged images for the two probes with anti-pol II (Alexafluor 488 nm) and anti-HP1 (Alexafluor 488nm) independently, are shown in upper and lower panels. The images show cells counter stained with DAPI, captured at 0.2 μM resolution on Apotome' module of AxioImager Z1 upright fluorescence microscope (Carl Zeiss).

II and HP-1 as markers of poised and silenced chromatin. SBS-336 did not co-localize with Pol II whereas SBS-700 was found to co-localize with Pol II at the nuclear periphery. This suggested that SBS-336 was not associated with the transcriptional hub marked by Pol II, whereas SBS-700 although located at the nuclear periphery was associated with the transcriptional hub. Further to delineate whether lack of association of SBS-336 with pol II implicated association with HP1 resulting in silencing, we performed immuno-FISH with anti-HP-1. Indeed, SBS-336 co-localized with HP1 indicating its association with the hetereochromatic regions at the nuclear periphery upon TCR activation. SBS-700 was used as a negative control, to demonstrate lack of association with HP-1 (Fig. 2C and 2D). Taken together, these results suggest that SATB1^{L} is not only responsible for targeting genes to nuclear territory, but also allocates them to different micro-domains at the nuclear lamina eventually deciding the activity status of the gene.

3. **SATB1 targets genes to Lamin associated domains (LADs):** Lamins are known to play a key role in chromatin organization and in affecting the transcriptional state of the genes targeted to nuclear periphery. SBS-700 and SBS-336 which are differentially bound by SATB1 and SATB1^{L} co-localize with Lamin B1 although only SBS-700 which is bound by SATB1^{L} actually exhibits re-localization. To monitor the differential binding of SATB1 and SATB1^{L}, EMSA was performed for SBS-700 and SBS-336 using GST:SATB1 and GST:SATB1L DNA-binding regions. We observed that both SATB1 and SATB1^{L} were bound to SBS-700. However, SBS-336 was not bound by both these proteins suggesting that SBS-336 is not a direct target of SATB1 and SATB1^{L} (Fig. 3A). SATB1 bound SBS-336 at higher concentration whereas SATB1L was unable to do so. Thus the affinities of SATB1 and SATB1^{L} seem to be different for different SBSs. The differential behavior is also evident from re-localization of gene within the nucleus (Fig. 2). To ascertain the functional significance of re-localization of genes depending upon SATB1^{L} expression status, we monitored occupancy of SATB1, SATB1^{L}, Lamin B1, H3K9 acetylation and H3K9 tri-methylation. Chromatin was immunoprecipitated from naïve, activated and activated+Rottlerin treated thymocytes. The eluted chromatin was amplified by real-time PCR using primers flanking SBS-700 and SBS-336. We detected that upon activation SATB1 loses its occupancy at the SBS-700 equivalent to almost 8 folds, accompanied with an equivalent enrichment of SATB1^{L} (compare bars 1 and 2 of SATB1 and SATB1^{L} graphs). Accompanied with SATB1^{L} enrichment at the SBS-700 we also observed enrichment of Lamin B1 from 2- 5 fold (bars 1 and 2). However this occupancy of Lamin B1 on the SBS-700 was not dependent on SATB1^{L} expression, because despite inhibiting SATB1^{L} with Rottlerin, no change was detected in Lamin B1 occupancy on SBS-700 (bars 3 of SATB1^{L} and Lamin B1). This suggested loss of SATB1 from the SBS-700 increases Lamin B1 occupancy at SBS-700. Surprisingly, the histone modifications suggested otherwise. We noticed SATB1^{L}-dependent enrichment of 20 -old of H3K9 acetylation with modest or no change in H3K9 trimethylation levels. This is indicative of 'open' chromatin at SBS-700 (graphs for H3K9ac and H3K9(me)3. SBS-336 which is not regulated directly by SATB1^{L} showed a different profile. We noticed activation-dependent loss of SATB1 at the locus whereas there was very little enrichment of 2-folds over naïve of SATB1^{L}. Although association of LaminB1 at SBS336 did not enrich upon SATB1^{L} induction post activation, SATB1^{L} inhibition resulted in loss of LaminB1 occupancy at SBS-336. We have noticed that SATB1 and SATB1^{L} occupancy at different target genes brings about histone

modifications at those loci. Indeed we noticed increase in H3K9 tri-methylation which was found to be SATB1^{L}-dependent. Noteworthy was an observation that H3K9ac was enriched at SBS-336 in absence of SATB1^{L} upon T cell activation. This implicated that SATB1 represses SBS-336 in naïve thymocytes. Upon activation although SATB1 loses its occupancy, the marginal gain of SATB1L at SBS-336 presumably brings about tri-methylation of H3K9. However, this histone modification was found to be replaced with acetylation mark in absence of SATB1 post activation implying activation of the locus in absence of both SATB1 and SATB1^{L}.

Discussion

Transcriptional status of genes is influenced by nuclear organization. The nuclear organization reveals that interphase chromosomes are confined to chromosome territories. Thus chromosomes are folded into three-dimensional flexible networks allowing the possibility that positioning of the genes and chromosome domains may exert regulatory effect on gene expression. Correlation of gene positioning to gene activation and repression in relation to nuclear periphery occurs during differentiation. During development and differentiation the T cells undergo dramatic chromatin organization accompanied with gene re-localization and repositioning. Large-scale genomic reorganization within the thymocyte nuclei as an effect of SATB1^{L} expression is evident from the change in counter-staining pattern exhibited by DAPI. Thymocyte nuclei, which in resting phase exhibit distinct DAPI intense and less intense areas, appear to be homogenously stained upon TCR activation. Accompanied with this was loss of SATB1 staining that circumscribes the CTs while filling the inter-chromosomal voids. Developing thymocytes exhibit number of histone modifications coupled with reorganization of chromatin architecture. Upon activation a similar effect induced by SATB1^{L} in reorganizing

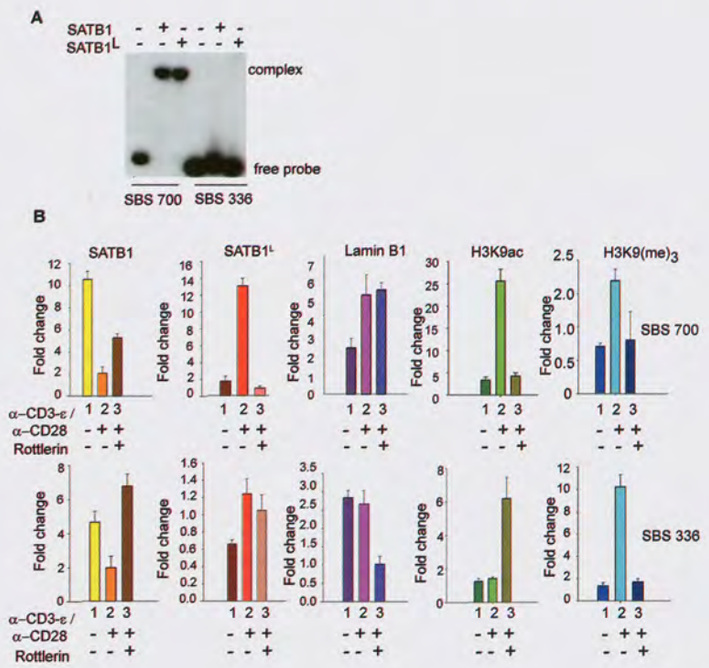


Fig. 3: SATB1^{L} target genes to Lamin-associated domains. SATB1^{L} binds differentially to different SBSs. **A.** SATB1^{L} and SATB1 GST-tagged proteins were used for in vitro binding assays. EMSA was performed using 32P-labeled SBS 700 and SBS 336 regions that showed in vivo SATB1 occupancy. Autoradiogram shows differential binding of SATB1 and SATB1^{L} proteins with SBS 700 and SBS 336. **B.** Occupancy of SATB1, SATB1^{L}, LaminB1, H3K9ac and H3K9(me)3 was monitored over SBS 700 and SBS 336 using ChIP. Chromatin was isolated from naïve thymocytes, thymocytes activated with CD3ε and CD28 and thymocytes activated in presence of Rottlerin for 6h. The DNA purified from immunoprecipited chromatin was amplified by real-time PCR. Relative occupancy was calculated by normalizing the Ct values of Input and IgG controls for treatments over the naïve samples. Each error bar represents standard deviation from triplicates. The names of the SBSs are indicated on the right side of graphical panels and the antibodies used are marked above each graph.

the acetylation mark across the genome was detected. Histone acetylation is a mark of active and open chromatin (ENCODE consortium). Acetylation of histones at K9 position mimicked the cage-like pattern generated by anti-SATB1 in naïve thymocytes. This staining pattern was found to change with TCR activation. This suggested that there were gross genomic reorganization occurring within the thymocyte nuclei as an effect of TCR activation. H3K9ac marks were also found to occur in close vicinity of the perinuclear region upon TCR activation. This indicated that post activation there would be some micro-domains at the nuclear periphery that are transcriptionally active or poised. This could probably indicate $SATB1^{L}$-dependent repositioning of 'active chromatin hubs' within the nuclear territory upon TCR activation.

The nuclear periphery can be associated with both transcriptionally active and inactive genes. Spatial positioning of gene is not always associated with its function however is dependent upon the sub-nuclear position of the gene relative to nuclear landmarks that influence gene activity. Dynamic repositioning of genes occurs in lymphocyte undergoing proliferation and or differentiation induced gene silencing. Thus, large-scale spatial reorganization of the genome might potentiate a role of locus relocation in maintaining heritable gene activation or repression. Upon TCR activation $SATB1^{L}$ dependent re-localization of a target gene was observed. Further analyses with Rottlerin treatment confirmed this. SBS-700, which is bound both by SATB1 and $SATB1^{L}$ is relocalized from the interior to the nuclear periphery. However, SBS-336 that is not bound directly by $SATB1^{L}$ remains constrained at the nuclear periphery. Association of genes to nuclear periphery or nuclear lamina does not always implicate gene repression. The potential presence of different micro-domains at the nuclear periphery decides the transcriptional state of the gene. Thus it can be concluded that $SATB1^{L}$ not only introduces differential affinities with which it binds multiple SBSs but it is also responsible for repositioning of target genes to different micro-domains at the Lamins (LADs) that either induce transcriptional activation or repression of the target genes.

To summarize, TCR-mediated activation that results in $SATB1^{L}$ expression introduces differential regulation of SATB1 target genes at different levels. Firstly, $SATB1^{L}$ introduces differential affinities with which it binds to different SBS. Thus introduces stringency in the regulation of target gene pool. Secondly, it alters the transcriptional activity of certain key genes required in the T cell development and differentiation. Thirdly, it potentiates re-localization of different $SATB1^{L}$ regulated genes while the mobility of those that are not regulated by $SATB1^{L}$ is constrained. Most importantly, those SATB1 targets that are not regulated by $SATB1^{L}$ are repressed by their association with LADs. Taken together, $SATB1^{L}$ imparts a multilayer functional regulation on target genes required during development and differentiation of T cells.

Future Work

1. To study the role of SATB1 in partitioning chromatin into active and inactive compartments.
2. To study the effect of repositioning of SATB1 targets during T cell development and differentiation.

Bioinformatics and High performance Computing Facility

Shailza Singh

The bioinformatics facility at NCCS provides access to high-performance compute resources and programming expertise. The compute infrastructure serves scientists at NCCS to master the informatics needs of their research in a proficient and cost-effective manner.

Hardware Infrastructure:

SGI Altix XE 1300 Cluster
Head Node:
SGI Altix XE 270 Serve.
Dual Quad Core XEON 5620 @ 2.4GHz / 12MB cache,12GB Memory,5 x 2TB SATA Disk @ 7.2K RPM RAID 5

Compute Nodes:
SGI Altix 340 Servers
2 x HEXA Core XEON 5670 @ 2.93GHz / 12MB cache, 24GB Memory, 250GB SATA Disk @ 7.2K RPM, Dual Gigabit Ethernet Card

SGI Cluster Software Stack:
SLES Ver 11
SGI ProPack 7
SGI Foundation Software Ver 2.0

Interconnect:
24-Ports Gigabit Ethernet Switch

Sunfire X 4150 (Intel Xeon 2.33 GHz Quad Core Dual Processor) Standard Libraries and compilers.

Specialized Workstations: HP workstations of Intel Core 2 Duo @3.00GHz with 8 GB of DDR2 memory, 320 GB of SATA storage and 19" LCD wide Display with Linux operating system

Desktop Computers: Desktop computers with Intel core 2 duo processor @1.8Ghz to 2.8GHz with 2 GB to 4 GB of DRR2 memory, 160GB to 320GB of SATA storage with 17" wide and normal LCD display and loaded with Windows XP.

Printer: HP Laserjet M1136MFP, Canon Network Printer

UPS: 10KVA (APC make) to run clusters

Software infrastructure: Bioinformatics Facility at NCCS has procured several software for scientific research with commercial and/or academic licenses. These are:

Sequence analysis: BLAST, CLUSTAL-W, MEGA, Eisen

Molecular Modeling: Modeler (DISCOVERY STUDIO 3.0), Protein Families (DISCOVERY STUDIO 3.0), Protein Health (DISCOVERY STUDIO 3.0), Protein Refine (DISCOVERY STUDIO 3.0), Profiles-3D (DISCOVERY STUDIO 3.0)

Molecular Docking: Flexible Docking (DISCOVERY STUDIO 3.0), LibDock (DISCOVERY STUDIO 3.0), Ludi (DISCOVERY STUDIO 3.0), LigPrep (DISCOVERY STUDIO 3.0), LigandFIT (DISCOVERY STUDIO 3.0),LigandScore (DISCOVERY STUDIO 3.0), AUTODOCK, Database of 1.5million Compound Library (DISCOVERY STUDIO 3.0)

Pharmacophore Modeling: Auto Pharmacophore generation, Receptor-ligand pharmacophore egeneration,3D QSAR pharmacophore generation, Steric Refinements with excluded volumes. (DISCOVERYSTUDIO3.0)

Toxicity Prediction: ADMET (DISCOVERY STUDIO 3.0), TOPKAT (DISCOVERY STUDIO 3.0),

QSAR: Create Bayesian Model, Recusrive Partioning Model, Multiple Linear Regression Model, partial least squares model, genetic function approximation model, 3D QSAR model (Discovery Studio 3.0). Intelligent QSAR using molecular fragments of interest and their features, evaluation of descriptors from template scaffold to form relationship with the activity.

Molecular Dynamics: CHARMM, GROMACS, NAMD, MOIL

Molecular Visualisation: Rasmol, MolMol, WinCoot, Swiss PDB viewer, MolScript, VMD

Ab intio modeling: GAUSSIAN

Systems Biology Tools: Virtual Cell, M-cell, Cell Designer, GEPASI, Cytoscape, Osprey, E-Cell, SimBiology

Artificial Intelligence: SVMlight and SNNS

Material Modeling and Simulation: Material Studio 5.5

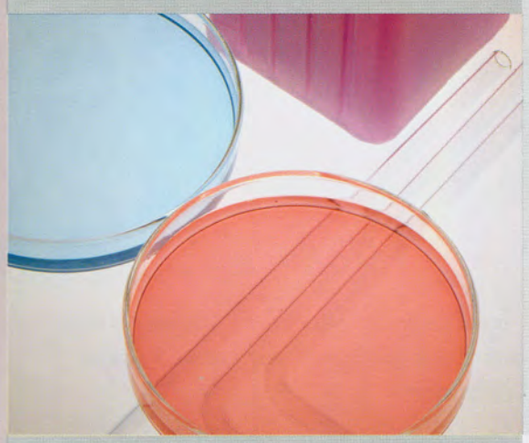
Graphs and Graphics: Sigma Plot, GNU Plot, Corel Draw and Adobe PhotoShop

Statistical packages: MATLAB and R

The total number of mice strains, inbred, mutant and hybrids, being maintained at the Experimental Animal Facility stands at 42. The foundation/nuclear colonies of mice are housed in Individually Ventilated Caging systems.

Complete scientific support and advice has been extended regularly to Scientists and their group members for the conduct of experiments under IAEC approved projects. The following services have been provided as per user requirements: collection of blood and other samples, immunizations, surgical procedures, injection of tumor cells in SCID/nude mice etc., assistance in the writing of Animal Study Protocols, education and assistance regarding interpretations of animal use regulations and the procurement of animals.

The breeding of laboratory animals has been planned to meet the needs of Scientists / Research Scholars for various animal experiments. A total of 9,088 laboratory animals were supplied on demand for the ongoing research projects during the period.



Proteomics Facility

Srikanth Rapole

The facility has three instruments:

1. 4800 MALDI TOF/TOF system is a tandem time-of-flight MS/MS system that is used for high-throughput proteomics research. The system can identify proteins by determining accurate masses of peptides formed by enzymatic digestion. Additionally, the system can more definitely identify and characterize proteins by isolating and fragmenting a molecular ion of interest and measuring the fragment ion masses. The number of samples analyzed is approximately 432 samples including 286 external samples from July-2010 to May-2011.

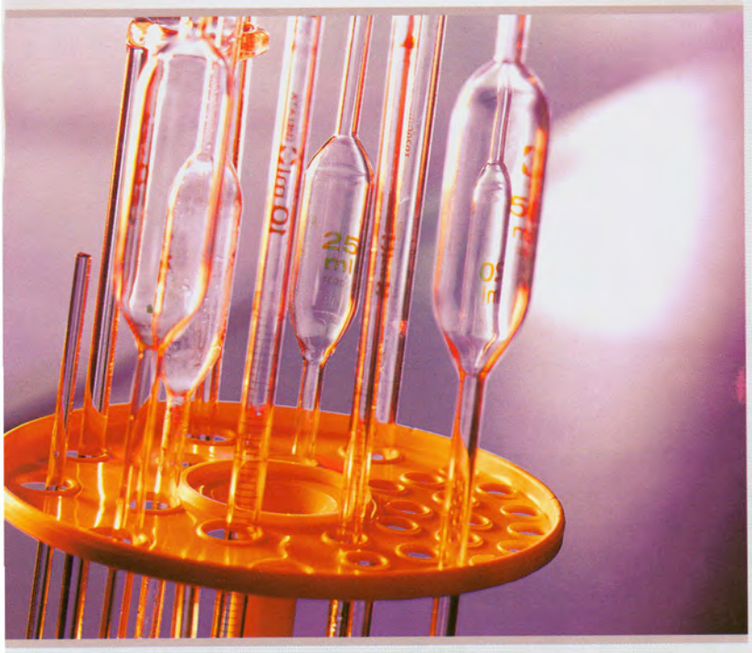
2. 4000 Q-Trap MS/MS system is hybrid triple quadrupole/linear ion trap mass spectrometer. The system is ideal for proteomic applications including post-translationally modified proteins discovery, protein identification, and biomarker validation. The number of samples (small molecules) using infusion syringe pump analyzed is approximately 15 samples including 9 external samples from July-2010 to May-2011.

3. Tempo Nano MDLC system is high performance, reliable, nano-scale liquid chromatography for proteomic applications. This Nano-LC system delivers precise and reproducible micro-scale gradients at constant flow-rates, creating ideal conditions for optimized mass analysis and a stable ionization spray.

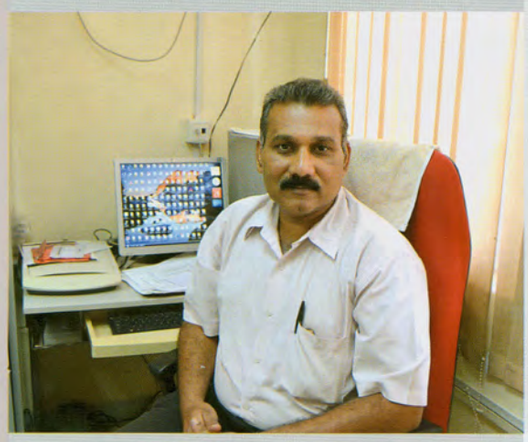
Participants

Dr. Vijay Sathe, *Technician*

Snigdha Dhali, *Technician*



Support Units



Ramanamurthy Bopanna

Rahul Bankar

Experimental Animal Facility

The Experimental Animal Facility is a core scientific department of the Institute with an objective to breed, maintain and supply quality laboratory animals for research and development. The section also extends complete research support to facilitate animal experimentation in the Institute. It is a barrier-maintained facility for the breeding, maintenance and supply of high quality and standardized laboratory animals viz. inbred and mutant mice, rats, rabbits etc. for the ongoing research projects of the Institute. The following is the list of various laboratory animals maintained at the facility:

MICE:

- BALB/cJ
- C57BL/6J
- DBA/2J
- DBA/1(2 lines)
- 129/SvJ
- FVB/NJ
- NOD/LtJ
- SWISS#
- BALB/c*
- NMRI $^{nu/nu}$
- NZB
- AKR#

Genetically engineered mutant mice (knock-out, transgenic and mutant mice -**29 lines**)

RATS:

- WISTAR

RABBITS:

- NEWZEALAND WHITE

Defined barrier practices are followed in the maintenance of the laboratory animals.

The breeding program for the propagation of the inbred mice is based on the established principles of genetics and breeding. The breeding program involving mutant mice is structured as per the genetic requirement of the specific strain concerned.

The Team:

Mr. Md. Shaikh
Mr. A. Inamdar
Mr. P.T. Shelke
Ms. Vaishali Bajare
Mr. Rahul B. Kavitake
Mr. Ganesh B. Yadav
Mr. Sanjay Gade
Mr. Harshal G. Gaonkar
Mr. Dilip B. Thorat

Library

The NCCS library has collection in the frontier areas of biotechnology having relevance to NCCS research activities. The library holds approximately ten thousand six hundred twenty two bound journals, two thousands five hundred twenty seven books and one hundred fifty seven NCCS Ph.D. thesis. Currently it subscribes to sixty nine scientific journals and twenty eight other periodicals in print form and six scientific online journals.

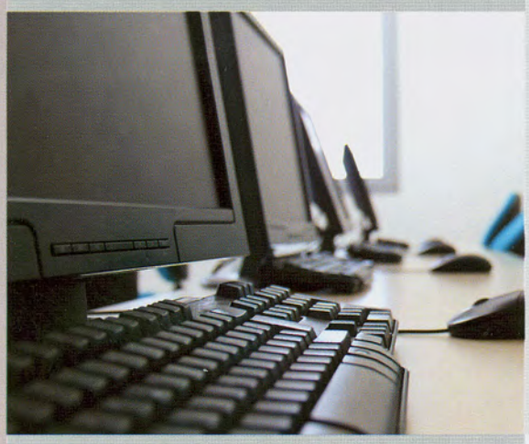
In the development of its collection, the library's priority is to support NCCS research activities. The library collection is expanded in consultation with NCCS scientists. The library's print collections are growing by approximately 1000 volumes per year. The library is equipped with Linux based SLIM21 with RFID Interfaced library software for the library house keeping operation and Web-OPAC for online searching of the library documents. The library has also installed barcode technology for circulation (Issue & Return) of library documents. It maintains library information (in Hindi & English) on its webpage which includes free Online Medical database link, NCCS research publication list, library forms, NCCS in News, Ph.D. thesis collection and list of NCCS Alumni and other Scientific Grants\Funds and fellowship related links. During the period under review, the library has created Digital Archive of NCCS Ph.D. thesis.

Additional documentation facilities include local area network for library activities, PubMed database access, a number of CD-ROMs for books, journals & Ph.D. thesis. The library is listed in the Union Catalogue of Biomedical Serials in India created by National Informatics Centre, New Delhi and continues to be a member of the Medical Library Association of India.

The Library is a member of DBT Online Journal Consortia "DeLCON" which provides access to 918 Online Journals (including one Online Book Methods in Enzymolgoy series) from various publishers and "SCOPUS" citation database.

Team

Mr. Krupasindhu Behera
Mr. Rameshwar Nema
Mrs. Aparna V. Panse
Mr. M.V. Randive



Computer Section

Video-conferencing Facility (VCF) was installed at NCCS and is working satisfactorily.

The VCF was initiated by DBT to connect all its institutes. It has been installed, configured and tested satisfactorily with NII, New Delhi, CDFD Hyderabad and RCB, Thiruvananthapuram.

NKN Internet Connectivity from NIC was installed.

The National Knowledge Network connectivity commissioned by National Informatics Centre has been installed and is functional in Computer Department.

Virtualization of servers on HP Storage MSA 2000

An HP Storage Box with 10TB capacity and RAID configuration has been installed and all the critical servers have been virtualized using VMWARE4.0 to optimize CPU and hard disk space utilization.

TATA (formerly VSNL) Internet Leased Line Up-gradation

The ILL from TATA Communications has been upgraded to 2Mbps (1:1) for general purpose use in the institute.

New website development is in progress

The NCCS new website development is almost complete and will be hosted soon.

Buy-back of old ACER Computer

The buy-back proposal of old ACER Computer (30NOs.) have been completed with new Dual Core ACER computers with Win.7 OS installed and configuration of all necessary software and LAN connectivity.

AMC of servers and computers

The 3 yrs. AMC contract of HP servers and Desktop PCs has been awarded for smooth functioning of systems.

Team

Mr. Rajesh Solanki
Mr. Shivaji Jadhav
Ms. Rajashri Patwardhan
Ms. Kirti Jadhav

NCCS Facilities

DNA Sequencer

A total of 10, 000 samples were run on the machine during this period.

FACS core facility

There were four FACS equipments in the FACS core facility of the Institute under my supervision during the period under consideration. These were operated on rotation basis by five dedicated operators.

Names of technicians in the facility:

Hemangini Shikhare
Pratibha Khot
Amit Salunkhe (Joined in July 2010)
Rupali Jadhav (Joined in Feb.2011)
Ashwini Kore (Joined in March 2011)

The usage of the four equipments for the period under consideration is summarized in the tables below.

IMMUNOPHENOTYPING & CELL CYCLE analysis:

Equipment	Surface /Intracellular staining	DNA Cell cycle	CBA flex	CBA	Total Samples Acquired
FACS Calibur	2354	1271	-	-	3625
FACS Canto II	5296	-	50	-	5346

SORTING:

EQUIPMENT	SORTING	ACQUISITION	TOTAL
*FACSAria SORP	230	221	451
# FACSAria II	63	98	161

*The FACSAria SORP was functional till February 2011 and now the equipment is in the process of Upgradation to Aria III standard.

Aria II was installed in April2010 .However till Dec. 2010, due to shortage of manpower, the equipment was operated only for daily Startup & Shutdown procedures .It was made fully functional from Dec. 2010.

Samples from outsiders:

We have acquired following samples for outsiders like IRSHA,NCL:
Surface/ Intracellular staining -- 83 and DNA cell cycle -- 95 on FACS Calibur.

Other equipments in the facility :

FACS Vantage equipment was given to BD under buy back scheme. Aria III SORP has been purchased from BD under buy back scheme and has been installed in March 2011. New Canto II machine was supplied FOC by BD under buy back scheme in March 2011.BDPathway 855 installation was completed by end of April 2010. The first training for operators was conducted by BD in November 2010. It will be made functional soon.

Confocal Microscopy

The Confocal Facility has two microscopes:

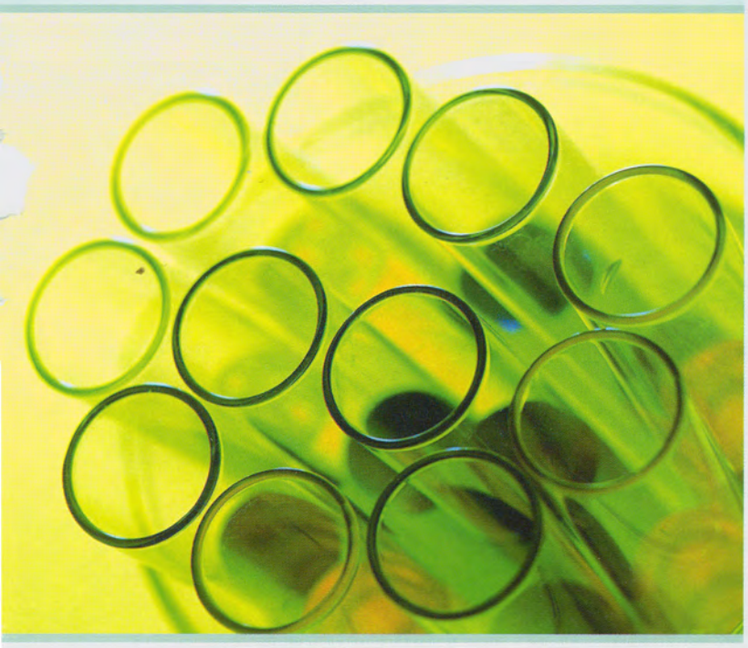
Zeiss LSM510 META

Advanced Spectral Confocal Microscope, Zeiss LSM510 META, with programmable CO2 incubator and temperature-humidity control. This system comprising offully motorized and computer controlled Inverted Fluorescence microscope, is being used for regular confocal as well as FRET, FRAP, Live Imaging etc.The Lasers available are Blue Diode laser (405nm), Argon laser(458/477/488/514 nm), 543nm He-Ne and 633nm He-Ne. The spectral detector permits separation of upto eight emission signals, even if the fluorescence spectra are strongly overlapping.

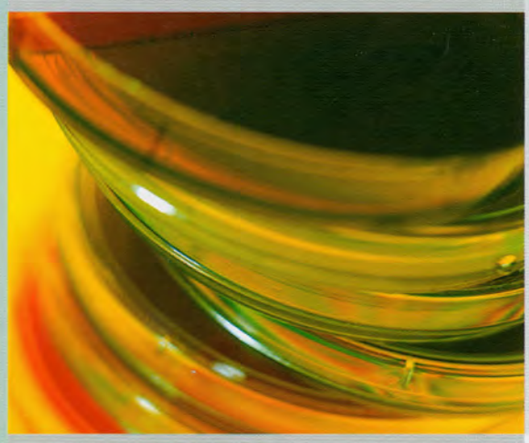
Leica SP5 II

A new Leica SP5 II system has been installed. It is a high-end Broadband Confocal Laser Scanning Microscope with 4 cooled spectral PMTs and unique AOBS,technology equipped with CO2 incubator,Fully motorized, automated and computer controlled Microscope Leica DMI 6000.The Lasers are Blue Diode Laser 405nm, Ar Laser 458, 488, 476, 496, 514, DPSS 561nm, HeNe 594nm and HeNe 633nm with Scanning Stage and incubation chamber for live cell experiments. Software for Confocal imaging 3D imaging and reconstruction, Dye Finder, Time lapse, colocalization , FRET (SE & AB), FRAP is also available.

The numbers of samples imaged during 2010-11 were approximately 3873 in-house and 209 came from various other institutes. Number of samples for live-imaging was 13.



Publications & Awards



Publications & Patents

1. Bajaj M, Hinge A, Limaye LS, Gupta RK, Surolia A, Kale VP (2010). Mannose-binding dietary lectins induce adipogenic differentiation of the marrow-derived mesenchymal cells via an active insulin-like signaling mechanism. *Glycobiology*, 21(4): 521-529

2. Hinge AS, Limaye LS, Surolia A, Kale VP (2010). In vitro protection of umbilical cord blood-derived primitive hematopoietic stem progenitor cell pool by mannose-specific lectins via anti-oxidant mechanisms. *Transfusion* 50(8): 1815-1826.

3. Hinge A, Bajaj M, Limaye L, Surolia A, Kale VP (2010). Oral administration of insulin receptor-interacting lectins leads to an enhancement in the hematopoietic stem and progenitor cell pool of mice. *Stem Cells and Development* 19(2) 163-174.

4. Sangeetha VM, Kale VP, Limaye LS (2010). Expansion of Cord Blood CD34+ Cells in Presence of zVADfmk and zLLYfmk Improved Their In Vitro Functionality and In Vivo Engraftment in NOD/SCID Mouse *PLoS ONE* Vol 5: 8 e12221

5. Kulkarni SD, Muralidharan B, Panda AC, Bakthavachalu B, Vindu A, Seshadri V (2011). Glucose-stimulated Translation Regulation of Insulin by the 5' UTR-binding Proteins. *J. Biol. Chem.* 286: 14146-14156.

6. Raju R, Balakrishnan L, Nanjappa V, Bhattacharjee M, Getnet D, Muthusamy B, Kurian Thomas J, Sharma J, Rahiman BA, Harsha HC, Shankar S, Prasad TS, Mohan SS, Bader GD, Wani MR, Pandey A (2011). A comprehensive manually curated reaction map of RANKL/RANK signaling pathway. *Database (Oxford)* (In press).

7. Khapli SM, Tomar GB, Barhanpurkar AP, Gupta N, Yogesha SD, Pote ST, Wani MR (2010). Irreversible inhibition of RANK expression as a possible mechanism for IL-3 inhibition of RANKL-induced osteoclastogenesis. *Biochem. Biophys. Res. Commun.* 399: 688-693.

8. Pandey V, Vijayakumar MV, Ajay AK, Malvi P, Bhat MK (2011). Diet induced obesity increases melanoma progression: involvement of Cav-1 and FASN. *Int. J. Cancer* (In press).

9. Vijayakumar MV, Ajay AK, Bhat MK (2010). Demonstration of a visual cell-based assay for screening glucose transporter 4 translocation modulators in real time. *J. Biosci.* 35(4): 525-531.

10. Ajay AK, Upadhyay AK, Singh S, Vijayakumar MV, Kumari R, Pandey V, Boppana R, Bhat MK (2010). Cdk5 Phosphorylates Non-genotoxically Overexpressed p53 following Inhibition of PP2A to Induce Cell Cycle Arrest/apoptosis and Inhibits Tumor Progression. *Mol. Cancer* 9: 204.

11. Das G, Shiras A, Shanmuganandam K, Shastry P (2011). Rictor regulates MMP-9 activity and invasion through Raf-1-MEK-ERK signaling pathway in glioma cells. *Mol. Carcinog.* (6): 412-23.

12. Pujari R, Nagre NN, Chachadi VB, Inamdar SR, Swamy BM, Shastry P (2010). Rhizoctonia bataticola lectin (RBL) induces mitogenesis and cytokine production in human PBMC via p38 MAPK and STAT-5 signaling pathways. *Biochim. Biophys.* Acta 1800(12): 1268-75.

13. Kale A, Kale S, Yadav P, Gholap H, Pasricha R, Jog JP, Lefez B, Hannoyer B, Shastry P, Ogale S (2011). Magnetite/CdTe magnetic-fluorescent composite nanosystem for magnetic separation and bio-imaging. *Nanotechnology.* 22(22): 225101.

14. Rajendran G, Shanmuganandam K, Bendre A, Mujumdar D, Goel A, Shiras A (2011). Epigenetic regulation of DNA methyltransferases: DNMT1 and DNMT3B in gliomas. *Neurooncol.* 104(2): 483-94.

15. Reddy EM, Chettiar ST, Kaur N, Ganeshkumar R, Shepal V, Shanbhag NC, Shiras A (2011). Dlxin-1, a member of MAGE family, inhibits cell proliferation, invasion and tumorigenicity of glioma stem cells. *Cancer Gene Ther.* 18(3): 206-18.

16. Polisetty RV, Gupta MK, Nair SC, Ramamoorthy K, Tiwary S, Shiras A, Chandak GR, Sirdeshmukh R (2011). Glioblastoma cell secretome: Analysis of three glioblastoma cell lines reveal 148 non-redundant proteins *J. Proteomics.* 74(10): 1918-25.

17. Mruthyunjaya S, Rumma M, Ravibhushan G, Anjali S, Padma S (2011). c-Jun/AP-1 transcription factor regulates laminin-1-induced neurite outgrowth in human bone marrow mesenchymal stem cells: Role of multiple signaling pathways. *FEBS Lett.* 585(12): 1915-22.

18. Joshi-Navare K, Shiras A, Prabhune A (2011). Differentiation-inducing ability of sophorolipids of oleic and linoleic acids using a glioma cell line. *Biotechnol. J.* 6(5): 509-12.

19. Venkatpurwar V, Shiras A, Pokharkar V (2011). Porphyran capped gold nanoparticles as a novel carrier for delivery of anticancer drug: In vitro cytotoxicity study. *Int. J. Pharm.* 409(1-2): 314-20.

20. Dhar S, Reddy EM, Prabhune A, Pokharkar V, Shiras A, Prasad BL (2011). Cytotoxicity of sophorolipid-gellan gum-gold nanoparticle conjugates and their doxorubicin loaded derivatives towards human glioma and human glioma stem cell lines. *Nanoscale* 3(2): 575-80.

21. Pradhan S, Mishra R, Sharma P, Kundu GC (2010). Quercetin and sulforaphane in combination suppress the melanoma progression through down regulation of matrix metalloproteinase-9. Expt. Ther. Med, 1: 915-920.

22. Kumar V, Behera R, Lohite K, Karnik S, Kundu GC (2010). p38 Kinase is Crucial for Osteopontin-induced Furin Expression that Supports Cervical Cancer Progression. *Cancer Res.* 70 (24): 10381-10391.

23. Bulbule A, Saraswati S and Kundu GC (2011). Status of Research on Matrix Metalloproteinases (MMPs) in India. *Expert Opin. Ther. Targets* 15(6): 671-5.

24. Prakash M, Kale S, Ghosh I, Kundu GC, Datta K (2011). Hyaluronan-binding protein 1 (HABP1/p32/gC1qR) induces melanoma cell migration and tumor growth by NF-kappa B dependent MMP-2 activation through integrin α(v)β(3) interaction. *Cell Signal* 23(10): 1563-77.

25. Rahman A, Srivastava SS, Sneh A, Ahmed N, Krishnasastry MV. (2010) Molecular characterization of tlyA gene product, Rv1694 of Mycobacterium tuberculosis: A non-conventional hemolysin and a ribosomal RNA methyl transferase. *BMC Biochem.* 11: 35.

26. Kain V, Kumar S, Puranik AS, Sitasawad SL (2010) Azelnidipine protects myocardium in hyperglycemia-induced cardiac damage. *Cardiovasc. Diabetol.* 9: 82.

27. Gower DJ, Mauro DS, Giri V, Bhatta G, Govindappa V, Kotharambath R, Oommen OV, Fatih FA, Mackenzie-Dodds JA, Nussbaum RA, Biju SD, Shouche YS, Wilkinson M (2011). Molecular systematics of caeciliid caecilians (Amphibia: Gymnophiona) of the Western Ghats, India. *Mol. Phylogenet. Evol.* 59(3): 698-707.

28. Kantak JB, Bagade AV, Mahajan SA, Pawar SP, Shouche YS, Prabhune AA (2011). Isolation, Identification and Optimization of a New Extracellular Lipase Producing Strain of Rhizopus sp. *Appl. Biochem. Biotechnol.* 164(7): 969-78.

29. Prakash G, Varma AJ, Prabhune A, Shouche Y, Rao M (2011). Microbial production of xylitol from D-xylose and sugarcane bagasse hemicellulose using newly isolated thermotolerant yeast Debaryomyces hansenii. *Bioresour. Technol.* 102(3): 3304-8.

30. Singh P, Raghukumar C, Verma P, Shouche Y (2011). Fungal community analysis in the deep-sea sediments of the Central Indian Basin by culture-independent approach. *Microb. Ecol.* 61(3): 507-17.

31. Sharakhova MV, Xia A, Tu Z, Shouche YS, Unger MF, Sharakhov IV (2010). A physical map for an Asian malaria mosquito, *Anopheles stephensi*. *Am. J. Trop. Med. Hyg.* 83(5): 1023-7.

32. Pednekar SN, Dohe VB, Deshpande SM, Kamble SS, Bharadwaj RS, Shouche YS (2010). An outbreak of Pseudomonas aeruginosa in a burn unit. *Burns* 36(7): e130-1.

33. Verma P, Pandey PK, Gupta AK, Kim HJ, Baik KS, Seong CN, Patole MS, Shouche YS (2010). Shewanella indica sp. nov., isolated from the sediment of Arabian Sea. *Int. J. Syst. Evol. Microbiol.* 61(9): 2058-64.

34. Salunke BK, Salunkhe RC, Patole MS, Shouche YS (2010). Wolbachia and termite association: present status and future implications. *J. Biosci.* 35(2): 171-5

35. Shailza Singh (2011). Membrane permeability in Biological Systems: A Systems Biology Perspective. Journal of *Computer Sci. Syst. Biol.* 4: 027-032.

36. Xu J, Yang Y, Qiu G, Lal G, Yin N, Wu Z, Bromberg JS and Ding Y (2011). Stat4 Is Critical for the Balance between Th17 Cells and Regulatory T Cells in Colitis. *J. Immunol.* 186(11): 6597-606.

37. Lal G*, Yin N, Xu J, Lin M, Schroppel B, Marie I, Levy DE, Ding Y and Bromberg JS* (2011). Distinct inflammatory signals have physiologically divergent effects on epigenetic regulation of Foxp3 expression and Treg function. *Am. J. Transplant.* 11(2): 203-14. (*co-corresponding author).

38. Srivastava RK, Tomar GB, Barhanpurkar AP, Gupta N, Pote ST, Mishra GC*, Wani MR* (2011). IL-3 attenuates collagen-induced arthritis by modulating the development of Foxp3+ regulatory T cells. *J.Immunol.* 186: 2262-2272 *(*co-corresponding authors).*

39. Tripathy MK, Ahmed Z, Ladha JS, Mitra D (2010). The cell death regulator GRIM-19 is involved in HIV-1 induced T-cell apoptosis. *Apoptosis* 15: 1453- 1460.

40. Bodiwala HS, Sabde S, Gupta P, Mukherjee R, Kumar R, Garg P, Bhutani KK, Mitra D, Singh IP (2011). Design and synthesis of caffeoyl-anilides as portmanteau inhibitors of HIV-1 integrase and CCR5. *Bioorg. Med Chem.* 19: 1256-1263.

41. Bodiwala HS, Sabde S, Mitra D, Bhutani KK, Singh IP (2011). Synthesis of 9-substituted derivatives of berberine as anti-HIV agents. *Eur. J. Med. Chem.* 46:1045-1049.

42. Sabde S, Bodiwala HS, Karmase A, Deshpande PJ, Kaur A, Ahmed N, Chauthe SK, Brahmbhatt KG, Phadke RU, Mitra D, Bhutani KK, Singh IP (2011). Anti-HIV activity of Indian medicinal plants. *J. Nat. Med.* 65(3-4): 662-9.

43. Rawat P, Mitra D (2011). Cellular heat shock factor 1 positively regulates human immunodeficiency virus-1 gene expression and replication by two distinct pathways. *Nucleic Acids Res.* 39(14): 5879-92.

44. Khan SZ, Mitra D (2011). Cyclin K inhibits HIV-1 gene expression and replication by interfering with CDK9-Cyclin T1 interaction in Nef dependent manner. *J. Biol. Chem.* 286(26): 22943-54.

45. Kumar M, Rawat P, Khan SZ, Dhamija N, Chaudhary P, Ravi DS, Mitra D (2011). Reciprocal Regulation of Human Immunodeficiency Virus-1 Gene Expression and Replication by Heat Shock Proteins 40 and 70. *J. Mol. Biol.* 410(5):944-58.

46. Padamwar MN, Patole MS, Pokharkar VB (2011). Chitosan-reduced gold nanoparticles: a novel carrier for the preparation of spray-dried liposomes for topical delivery. *J. Liposome Res.* (In press).

47. Oturkar CC, Nemade HN, Mulik PM, Patole MS, Hawaldar RR, Gawai KR (2011). Mechanistic investigation of decolorization and degradation of reactive red 120 by Bacillus lentus BI377. *Bioresour. Technol.* 102(2): 758-64.

48. Srivastava N, Sudan R, Saha B (2011). CD40-Modulated Dual-Specificity Phosphatases MAPK Phosphatase (MKP)-1 and MKP-3 Reciprocally Regulate Leishmania major Infection. *J. Immunol.* 186: 5863-72.

49. Kumari S, Singh S, Saha B, Paliwal PK (2011). Leishmania major MAP kinase 10 is protective against experimental L. major infection. *Vaccine* (In press).

50. Ahmad M, Raut S, Pyaram K, Kamble A, Mullick J, Sahu A (2010). Domain swapping reveals complement control protein modules critical for imparting cofactor and decay-accelerating activities in vaccinia virus complement control protein. *J. Immunol.* 185(10): 6128–6137.

51. Pyaram K, Yadav VN, Reza MJ, Sahu A (2010). Virus-complement interactions: an assiduous struggle for dominance. *Future Virol.* 5(6): 709-730.

52. Yamayoshi A, Yasuhara M, Galande S, Kobori A, Murakami A (2011). Decoy-DNA Against Special AT-Rich Sequence Binding Protein 1 Inhibits the Growth and Invasive Ability of Human Breast Cancer. *Oligonucleotides* 21: 115-21.

53. Hamada M, Haeger A, Jeganathan KB, van Ree JH, Malureanu L, Wälde S, Joseph J, Kehlenbach RH, and van Deursen JM. Ran-dependent docking of importin β to RanBP2/Nup358 filaments of nuclear pores is essential for adequate cNLS-mediated protein import and cell viability. *J. Cell Biol.* 194(4): 597-612.

Book Chapters / Invited Reviews:

- Pavithra L, Chavali S, Chattopadhyay, S (2011). Nutritional epigenetics- impact on metabolic syndrome. Review in book chapter *"Nutrition, Epigenetic Mechanisms and Human Disease"*. CRC Press. Taylor and Francis Group, LLC, pp 259-286.
- Singh S. Intech Open Access Publisher- Drug Development, Chapter on Therapeutic Approaches to Membrane Permeability in Biological Systems: A Systems Biology Perspective.
- Singh S. Chapter on Systems Biology for Infectious Diseases for Bentham Science Publisher E-book on Cheminformatics, Bioinformatics and Drug Discovery.
- Saraswati S, Sanyal M, Bulbule A, Ramdasi A, Kumar D, Behera R, Ahmed M, Chakraborty G, Kumar V, Jain S, Soundararajan G, Ghosh P and Kundu GC (2011). Therapeutic Targeting of Osteopontin in Breast Cancer Cells (Book Chapter).
- Singh S. Quantum pharmacology for infectious diseases using hybrid QM/MM in Current Computer Aided Drug Design (Special Issue)

Patents filed / sealed

Dr. Vaijayanti P. Kale

1. Creation of Artificial Bone-Marrow Environment and Uses Thereof: International patents have been filed in various countries. Granted and sealed in New Zealand.
2. Preservation of Human hematopoietic stem/progenitor cells using mannose binding lectins of plant origin. Indian patent sealed. International applications being defended by DBT

Memberships/ Awards/ Fellowships

Vaijayanti P. Kale

- "Associate Editor" of the international journal "Stem Cells and Development", published in USA from June 2005
- "Associate Editor" of the journal "Annals of Neurobiology" from March 2009.
- "Senior Editor" for the international journal "InSciences
- Member of International Society of Experimental Hematologists
- Member of International Society of Stem Cell Research (ISSCR)
- Life member of Indian Association of Cell Biology
- Life member of Indian Association of Biotechnology
- Life member of Indian Society of Microbiologists
- Member of Ethical committee for clinical research, Sahyadri Hospital
- Member of Animal Ethics committee, Raj Biotech

Lalita S.Limaye

- Annual member of
  - International Society of experimental Haematology
- Life member of
  - Indian society of cell biology
  - Biotechnology society of India
  - Indian women scientists association
  - Indian association of Microbiologists of India

Nibedita Lenka

- Membership
  - Life Member, Indian Academy of Neuroscience.
  - Active Member, International Society for Stem Cell Research (2005 - continuing).
  - Life Member, Stem Cell Research Forum of India (SCRFI).
  - American Society of Microbiology (ASM) (2010).
- Honors/Awards
  - Member Editorial Advisory Board, Annals of Neurosciences (2009-continuing).

Mohan Wani

- Awards and Honours
  - Elected Member of Guha Research Conference 2010.
  - Chancellor nominee as a Executive Council (Governing Body) member, Maharashtra Animal and Fisheries Sciences University (MAFSU), Nagpur (2009-2012).
  - Special Invitee for 46th Meeting of Research Council of Institute of Genomics and Integrative Biology (IGIB), New Delhi.
  - Member of Building and Finance Committee of MAFSU, Nagpur.

- Member, Project Monitoring Committee (PMC) of SBIRI project, DBT.
- Special Invitee for Staff Research Council Meeting of PGI of Veterinary and Animal Sciences, Akola.

- **Memberships**
  - Member of the American Society for Bone and Mineral Research, USA.
  - Member of International Chinese Hard Tissue Society.
  - Member of Molecular Immunology Forum.
  - Life Member of Indian Society of Cell Biology.
  - Member of CPCSEA for NIV, Raj Biotech, NTC, BAIF and Pune University.

Jomon Joseph

- Member - Indian Society of Cell Biology

Sharmila Bapat

- R.M. Tiwari Oration Award at International Federation of Head and Neck Oncology, Bangalore 13th October, 2010; Title of oration – Cancer Stem Cells and their Clinical Significance
- Fellow, National Academy of Sciences, Allahabad
- Active Member of American Association of Cancer Research (AACR)
- Member - International Epigenetics Society (earlier DNA Methylation Society)
- Elected Executive Member - Indian Association of Cancer Research (IACR) 2009-2012
- Member - Indian Women Scientists Association

Manoj Kumar Bhat

- Indian Association for Cancer Research
- Indian Society of Cell Biology

Anjali Shiras

- Member of the Editorial Board for International Journal: Journal of Clinical Rehabitative Tissue Engineering Research
- Member of the Editorial Board of World Journal of Stem Cells
- Member of the International Society of Stem Cell Research (ISSCR), USA
- Member of Indian Association of Cancer Research (IACR), India
- Member of the Neuro-oncology Society of India (NOSI), INDIA

S. Singh

- Life Member -Biotechnology Society of India (BSI)
- Life Member-Society of Biological Chemists, India
- Life Member-Association of Microbiologists of India (AMI)
- Life Member-Association for DNA Fingerprinting and Diagnostics, Membership No. 781
- DST Young Scientist Award
- International Travel Award by CSIR and DBT

R. Srikanth

- Life Member of Indian Society for Mass Spectrometry
- Life Member of Proteomics Society, India
- Member of American Society for Mass Spectrometry

Girdhari Lal

- Memberships
  - Life member, Biological Chemists (SBC), India (since 2011).
  - Life member, Indian Society of Cell Biology (ISCB) (since 2010).
  - Life member, Association of Microbiologist in India (AMI) (since 1999).
  - Life member, Indian Immunology Society (IIS) (since 2003).
  - Trainee member of The Transplantation Society (TTS), USA (2006-2007).
  - Member of American Association for the Advancement of Science (AAAS), USA (2007-2008).
  - Member of American Society of Transplantation (AST), USA (2007-2008).
- Awards
  - Innovative Young Biotechnologist Award 2010 (IYBA-2010) from Department of Biotechnology, Government of India.
  - Young Investigator Award in "American Transplant Congress", held at San Diego, USA, 1st May 2010.
- Fellowships
  - Ramalingaswami fellowship (2010), DBT, Government of India.

Debashis Mitra

- Member, Infectious Disease Biology Task Force, Department of Biotechnology, Govt. of India.

Bhaskar Saha

- Fellow, National Academy of Sciences, India (2009)
- Member of the International Complement Society (since 1993)
- Member of the Molecular Immunology Forum (since 2005)
- Member of the American Society for Microbiology (since 2005)

Samit Chattopadhyay

- Fellow of Academy of Science (**FASc**), Bangalore, 2011
- Fellow of National Academy of Science (**FNASc**), Allahabad
- Fellow of Maharastra Academy of Science (**MAS**)
- Member, Guha Research Conference (**GRC**), 2006 onwards
- Member, Molecular Immunology Forum (**MIF**), 2002
- Member, Indian Society for Development Biologists (**ISDB**)
- Member, Society for Biological Scientists (**SBC**)
- Member, Research Advisory Board at Dr. D Y Patil Vidyapeeth, Pune
- Member, Indian Association for Cancer Research (**ICAR**)
- Task Force Member, CSIR inter-agency project, IICB, Kolkata, 2010
- Member, Society of Indian Cell Biology
- Member, Asian Transcription and Chromatin Biology

Sanjeev Galande

- Recipient of the 'Shantiswaroop Bhatnagar Award-2010' from the Council of Scientific and Industrial Research.

Extramural Funding

Vaijayanti P. Kale

1. Effect of hematopoiesis-regulatory factors/signaling molecules expressed in the stromal cells on the fate of the HSCs interacting with them - (DBT)
2. Identification of signaling mechanisms involved in the regulation of hematopoietic stem cells (HSCs) - (DBT)
3. Creation of an *in vitro* model of the BM niche and studies on its impact on the Hematopoietic Stem Cells - (DBT)
4. Detection of Minimal Residual Disease in acute Leukemia by Molecular Methods - (DBT)

Lalita S.Limaye

1. *Ex-vivo* and *In-vivo* studies on Megakaryocytes and Platelets generated in cultures from hematopoietic stem cells with special reference to role of nutraceuticals as supplement in the culture media. DBT
2. "Functional characterization of the in vitro generated dendritic cells from cord blood derived hematopoietic stem cells." DBT

Vasudevan Seshadri

1. Functional assessment of adult human pancreatic islets following autologous transplantation June 2010-Dec 2011. DBT

Mohan Wani

1. Studies on understanding the role of IL-3 in regulation of human osteoclast and osteoblast differentiation 2010-2012 DBT.

Jomon Joseph

1. Molecular characterization of the interaction between the tumour suppressor Adenomatous Polyposis Coli (APC) and the nucleoporin Nup358. 2008-2011 DBT.
2. Regulation of β-catenin function by the nucleoporin Nup358 in Wnt signaling. 2008-2011 DBT.

Sharmila Bapat

1. Epigenetic Mechanisms in Ovarian Cancer Progression. 2008-2011 DBT.
2. Prognostic Evaluation of the E-box binding Transcription Factors in Ovarian, Breast, Prostate and Head and Neck Cancers. 2010 – 2013 DBT & Finish Academy of Science, Finland.
3. Role of CARF and Mortalin in ovarian cancer and cancer stem cells. 2010 – 2013 DBT & AIST, Japan.

Padma Shastry

1. Role of Prostrate Apoptosis Response-4 (Par-4)and its interactive proteins in chemo resistance/ sensitivity in gliomas using multi-cellular spheroids (MCS) as model. 2010-2013 ICMR.
2. Exploring the potential of TF antigen binding property of lectin from Sclerotium rolfsii for Tumour suppressive activity. 2009-2012 DBT.

Anjali Shiras

1. Unravelling the role of miRNAs in self-renewal and tumorigenicity of brain tumor stem cells derived from Neuroepithelial tumors of the Central Nervous System (CNS). 2008-2011 DBT.
2. Identification of biomarkers for diagnosis and prognostication by Next Gen sequencing of oligodendroglial tumour exome. 2011-14. DBT.

Gopal C. Kundu

1. "Role of Small Molecule Inhibitor(s) as Targeted Therapy in Pancreatic and Prostate Cancers using in vitro and in vivo Models", 2010-2013 DBT.
2. "STAT3 a Key Regulator and Novel Therapeutic Target in Osteopontin-induced Tumor Growth and Angiogenesis in Breast Cancer", 2010-2013 DST.
3. "Silencing Osteopontin and its Downstream Oncogenic Molecules Suppress the Tumor Growth and Angiogenesis in Breast Cancer", 2008-2011 DBT
4. "Role of Splice Variants of Osteopontin in Regulation of PI 3 Kinase Dependent/Independent ILK-mediated VEGF Expression Leading to Breast Tumor Growth and Angiogenesis", 2010-2013 CSIR.

Yogesh S Shouche

NATIONAL:

1. DNA barcoding of butterflies from Western Ghat. 2007-2010. DBT
2. DNA Barcoding of Amphibians from Western Ghat 2007-2010. DBT
3. Screening for Bio-molecules from microbial diversity collected from different ecological niches, funded by 2007-2010. DBT.
4. Characterization of Hox complex and regulatory elements from Anopheles stephensi and Aedes aegypti 2008-2011
5. Establishment of Microbial Culture Collection and Biological Research Centre, funded by 2009-2013 DBT

INTERNATIONAL:

1. Methanotrophic communities in a meteor impact crater lake in India, funded by UK India Research and Educational Initiative 2008-2011 UKIERI.
2. Microbial Diversity & Development of Antibiotic resistance associated with industrial waste water treatment, 2009-2011 funded by Swedish Research Council
3. Mid gut bacteria in Aedes aegypti and vector competence, Forgarty International Research Collaboration Award

Girdhari Lal

1. Innovative Young Biotechnologist Award grant. 2011-2014. DBT.

Debashis Mitra

1. Characterization of anti-HIV activity of Acaciaside-B and pre-clinical studies towards its development as a potential microbicide-spermicide formulation. 2009-2012. DBT Co-Principal Investigators: Debashis Mitra, NCCS, Pune and Syed N. Kabir, IICB, Kolkata.
2. Studies on modulation of Heat Shock Proteins during HIV-1 infection and their functional role in virus replication and pathogenesis. 2010-2013. DBT
   Principal Investigator: Debashis Mitra
3. Structure based discovery of novel antiviral molecules with potential to inhibit drug resistant viruses. 2010-2013 DBT
   P.I: Dr. Ashoke Sharon, BITS, Mesra. Co-PI: Debashis Mitra, NCCS Pune and Dr. D. Chattopadhyay, ICMR Virus Unit, Kolkata.

Arvind Sahu

1. Role of vaccinia virus complement control protein in the viral pathogenesis. 2007-2010. DBT Co-Investigator: Dr. D. Mitra
2. Studies on species specificity in poxviral complement regulators. 2011-2014. DBT

Samit Chattopadhyay

1. Molecular switch in Th1-Th2 response by SMAR1: Its implications in Mycobacterium tuberculosis infection. 2007-2010. DBT.
2. Regulation of HIV-1 LTR mediated transcription by MAR binding protein SMAR1. 2007-2010. DBT.
3. Coordinated role of SMAR1 and p53 in tumorigenesis and apoptosis. 2008-2011. DBT

Seminars

Seminars delivered by Visiting Scientists

1. **Dr. Sriparna Majumdar,** PhD, Department of Ophthalmology, University of California San Francisco School of Medicine, San Francisco, California, Inhibitory Neurotransmission in the Retina, February 9, 2011.
2. **Dr. Uttamkumar Samanta,** School of Biotechnology, KIIT University, Bhubaneswar, LpPLA2 – Its role in Human Health and Structural Insight, December 10, 2010
3. **Prof Vivek Mittal,** Director, Lehman Brothers Lung Cancer Laboratory, Weill Cornell Medical Center, USA has agreed to deliver the talk on "Tumor Microenvironment and Metastasis", Dec 14, 2010.
4. **Dr. Vishal S. Vaidya,** Mechanisms of Kidney Exposure Biology, Brigham and Women's Hospital, Harvard Medical School, Boston, USA, May 20, 2011.

Seminars delivered by NCCS Scientists

Nibedita Lenka

- Factors Governing Neurogenesis from Embryonic Stem Cells *in vitro*. The Federation of Asian-Oceanian Neuroscience Societies (FAONS) Satellite Symposium on "Glial cells in health and disease" organized by School of Studies in Neuroscience, Jiwaji University, Gwalior (Invited Speaker and Poster Evaluation Committee Member).
- Notch involvement during cell fate specification and Neurogenesis. 79th meeting of Society of Biological Chemists (India) on "Regulation of biochemical and cellular processes in diverse systems", organized by Indian Institute of Science, Bangalore, India (Invited Speaker and Poster Evaluation Committee Member).
- Ligand and stage specific influence of Notch during cell fate specification and early neurogenesis from Embryonic Stem Cells. 25th Annual Meeting of Society for Neurochemistry, India (SNCI) and International Conference on "Metabolic Signalling in Brain in Health and Disease", organized by School of Life Sciences, University of Hyderabad, India (Invited Speaker).
- Involvement of both permissive and instructive cues during early Neurogenesis. National Seminar on Cellular and Molecular Neurobiology in Health and Diseases, organized by Dept. of Biochemistry, University of Madras, Chennai, India (Invited Speaker & Session Chair).
- Stem Cells from Bench to Bedside. National Conference on Emerging Trends in Biological and Information Sciences, Udgir, Maharashtra, India (Invited Speaker & Session Chair).

Mohan Wani

- "Surgical techniques in laboratory animals" at National Institute of Virology, September, 4, 2010.
- "Recent advances in bone cell biology and medicine" at Dept. of Zoology, University of Pune, Pune, September 18, 2010.

- "Applications of stem cells in the field of veterinary medicine" at Shirval Veterinary College, Shirwal, Satara, December 6, 2010.
- "Animal cell culture techniques, principles and troubleshooting" Dept. of Zoology, Shivaji University, Kolhapur, December, 7, 2010.
- "Role of Interleukin-3 in regulation of bone remodeling" at Guha Research Conference, Aurangabad, December 16-21, 2010.
- "Alternatives to animal experiments" Smt. Kashibai Navale College of Pharmacy, Pune, January 8, 2011.
- "Cross-talks between bone and immune systems" guest lecture in "Eminent Scientist Seminar Series" at IBB, University of Pune, January 20, 2011.
- "Integrated approach for strengthening research and development in the field of veterinary sciences" in National Symposium on Veterinary Public Health" at Bombay Veterinary College, January 29, 2011.
- "Alternatives to laboratory animal experiments for pharmacological research" Sinhgad College of Pharmacy, Pune, March 7, 2011.
- "Role of interleukin-3 in prevention of collagen-induced arthritis" at Padmashree Dr. D. Y. Patil Institute of Pharmaceutical Science and Research, Pune, March 11, 2011.

Vasudevan Seshadri

- Translational regulation of insulin by glucose: characterization of the 5'-UTR binding proteins, CSHL Translation Control meeting at Cold Spring Harbor laboratories in Sept 2010,

Jomon Joseph

- "Dishevelled: Need to shovel more to unearth its functions". May 10, 2010, National Centre for Biological sciences, Bangalore, India.
- "Dishevelled, a critical player of wnt signaling, reveals an unexpected RNA connection". May 18, 2010, Department of Biochemistry, Indian Institute of Science, Bangalore, India.
- "Nup358 plays a negatively role in Wnt signaling through regulation of □ -catenin localization". International Meeting "WNT2010", Oct 27-30, 2010, Karolinska Institutet, Stockholm, Sweden.
- "Dishevelled, a critical player of Wnt signaling, reveals an unexpected RNA connection". Young Investigators Meet (YIM), February 12-16, 2011, Hotel Mayfair, Bhubaneswar, India.

Sharmila Bapat

- Invited speaker at National Brain Research Centre, Manesar, 5th May, 2010; Title of talk - Comprehensive Approaches towards understanding Ovarian Cancer Stem Cell Biology
- Invited speaker at Turku, Finland 29th August, 2010; Title of talk - Ovarian Cancer Stem Cell Biology
- Invited speaker at the 7th Indo-Australian Biotechnology Conference, Brisbance - 24th - 31st October, 2010; Title of talk - Evaluation of Ovarian Cancer as a Stem Cell Disease

Gopal C. Kundu

- In Vivo Imaging of Tumors in Mice using Bioluminescence and Fluorescence Technologies. Indo-US Workshop on Molecular Imaging, CCMB, Hyderabad, 8-10th November, 2010
- Therapeutic and Diagnostic Significance of Osteopontin in Cancers, ISI, Kolkata, 25th November, 2010
- Therapeutic and Diagnostic Significance of Osteopontin and Integrins in Cancer and Cancer Stem Cells. Intl. Conf. on Stem Cell and Cancer, IIIT, Pune, 11-14th December, 2010
- Role of Stroma and Tumor-derived Osteopontin, a Matricellular Protein in Inflammation, Angiogenesis and Cancer. International Cancer Conference, RGCB, Trivandrum, 20-22nd December, 2010
- Transcriptional regulation of human osteopontin promoter by histone deacetylase inhibitor in cervical cancer cells. 14th Transcription Assembly Meeting, Hyderabad, 19-22nd January, 2011
- Application of Nanomedicine in Cancer: Current Strategies and Future Prospects. DBT Nano Bio Meeting, Amrita Cancer Institute, Kochi, 28th February, 2011
- Understanding the Mechanism of Tumor Progression and Angiogenesis by Osteopontin in Cancer. BITS, Goa, 21st March, 2011
- Mechanism of Tumor Progression and Angiogenesis in Breast and other Cancers: Implication of Osteopontin as Cancer Diagnostics and Therapeutics. IICT, Hyderabad, 13th April, 2011
- Potential Role of Osteopontin as Cancer Diagnostics and Therapeutics. Hokkaido University, Sapporo, Japan, 23rd May, 2011

Yogesh S Shouche

- Gut Microbes Interaction and Its Potential in Disease Management at French-Indian Inter-Academic Symposium on Infectious Disease' from 30th November to 3rd December 2010 at National Institute of Immunology, Pune.
- Genomics of *Anopheles stephensi*: Expressed Sequence Tag analysis and further" International Symposium on Recent Advances in Ecology and management of Vectors and Vector Borne Diseases at DRDO Gwalior 1-3rd December 2010.
- Genome based taxonomy: Unique case of Wolbachia' at International workshop on 'Computational Aspects of Working with Genomes' 28-30th March, Institute of Microbial Technology, Chandigarh India.

S. Singh

- Invited Talk on "Molecular Modeling" at NBAII, Bangalore, 8th-12th November 2010.
- Invited Talk on "Targeting Liposomal Drug Delivery for Anti-Protozoal Drugs: A Systems Biology Approach" at World Congress of Biotechnology, Hyderabad, 21st-23rd March 2011.
- Invited Talk on "Lipid metabolism of *L. major* in Systems Biology Context" at ICABIS, Udgir, Maharashtra, 4th-5th March 2011.

Girdhari Lal

- Epigenetic regulation of foxp3 gene in development and function of regulatory T cells" at International conference on stem cells and cancer 2010 (ICSCC-10): Proliferation, differentiations and apoptosis held at International Institute of Information Technology, Pune, India, on 11-14th December, 2010. (Invited talk)
- Epigenetics: An approach for molecular diagnosis and treatment" at seminar on Biotech 2010: Trends in rDNA Technology, held at Dr. Babasaheb Ambedkar Marathwad University, Subcampus at Osmanabad, Maharashtra, India on 14-15th November, 2010. (Invited talk)
- Importance of epigenetic approaches to induce transplantation tolerance and prevent autoimmunity" at 2nd Annual Biotechnology Conference for Students (ABCS-2010) held at International Institute of Information Technology, Pune, India, on 13th November, 2010. (Invited talk).

Debashis Mitra

- Indo-US conference on Translational Research in HIV/AIDS in India, Panaji, Goa, January 12-14, 2011., Novel anti-HIV lead molecules and their potential as microbicide candidates.
- 14th Transcription Assembly Meeting 2010, Centre for DNA fingerprinting and diagnostics, Hyderabad, January 20-22, 2011, Cyclin K interferes with CDK9-Cyclin T interaction and inhibits HIV-1 LTR mediated gene expression in Nef dependent manner.
- International Conference on Emerging Frontiers and Challenges in HIV/AIDS Research, National Institute of Reproduction and Reproductive Health, Mumbai, February 5-8, 2011., Heat Shock Factor-1 induces HIV-1 gene expression and replication by two different mechanisms.
- 2nd Molecular virology meeting, Indian Institute of Science, Bangalore, April 29-30, 2011, Cyclin K inhibits HIV-1 gene expression and replication in Nef dependent Manner.Sanjeev Galande
- "Ectopic expression of global regulator SATB1 in transgenic Drosophila reveals its role in the Wnt signaling pathway" at the Functional Genomics conference, October 2011, Banaras Hindu University, Banaras.
- "Role of chromatin organizer SATB1 in regulation of transcriptionally poised state of genes" at the International Meeting on Chromatin, December 4-6, 2011, Bangalore.
- "Chromatin organizer SATB1 and its newly indentified partner β-catenin mediate T_H2 differentiation through Wnt signaling" SBC annual meeting, December 13, 2010, Bangalore.
- "Unfolding the chromatin 'loopscape': integrating higher-order chromatin architecture with gene regulation" at the University of Hamamatsu, Japan, May 13, 2011.
- "From Sequence to Consequence" at the Department of Biochemistry, University of Cambridge, UK, October 15, 2010, And at Kyoto Institute of Technology, May 17, 2011.
- "From Genome to Epigenome" lecture was delivered at several colleges in Pune city as well as outside Pune during the period October 2010-April 2010.

Conferences / workshops attended/ Poster Presentations

Vaijayanti P. Kale

- Monika Sharma, Lalita Limaye and Vaijayanti P. Kale 2010 Three-dimensional hydrogel-based cultures of mesenchymal stem cells closely mimic the in vivo hematopoietic stem cell regulatory-niche. Annual meeting of International Society for Hematology and Stem cells, held at Melbourne, Australia. Oral presentation
- Kale V. P. Stromal cell biology: Creation of in vitro niche to regulate stem cell functions. Invited speaker in "InStem-CIRM collaborative meeting" Bangalore, March 21-22, 2011
- Kale V.P. DBT-organized workshop on Good Laboratory Practices (GLP) at NII, New Delhi Aug 2010

Lalita S.Limaye

- Sangeetha VM, Kale V.P. and Limaye L.S. The addition of anti apoptotic compounds to the expansion cultures lead to an improved adhesion and migration properties of CB derived hematopoietic stem/progenitor cells. Poster + Oral presentation by Sangeetha V.M. in ISEH conference held in Melborne, Australia in Sept. 2010

Vasudevan Seshadri

- CSHL Translation Control meeting at Cold Spring Harbor laboratories in Sept 2010, and presented a podium talk on "Translational regulation of insulin by glucose: characterization of the 5'-UTR binding proteins"

Mohan Wani

- Guha Research Conference 2010 at Aurangabad, December 16-21, 2010. Molecular Immunology Forum 2010 at National Institute of Immunology, New Delhi, February 19-20, 2011.

Jomon Joseph

- International Meeting "WNT2010", Oct 27-30, 2010, Karolinska Institutet, Stockholm, Sweden.
- Young Investigators Meet (YIM), Feb 12-16, 2011, Hotel Mayfair, Bhubaneswar, India.

Manoj Kumar Bhat

- Training programme on R&D Project Management 20-24 September 2010. Held at Human Resource Development Centre, Central Government Enclave, Ghaziabad, U.P.
- Translational Cancer Prevention and Biomarkars Workshop, 13-17 February 2011,. Held at Mazumdar Shaw cancer Centre, Bangalore.

Padma Shastry

- Invited talk- "International cancer research symposium" held in Thiruvanthapuram between 20-22 Dec 2010. New insights into tumor invasion – Role of Rictor-mTORC2 component in invasion through Raf-1-MEK-ERK signaling pathway in gliomas.
- Resource person for the Refresher Course in Life Sciences to be held from 9-29 March 2011. Deregulating the survival pathways: The games cancer cells play - Focus on Neuroblastomas &Glioblastomas and Animal Cell and Tissue Culture- Basic Techniques and applications.

Yogesh S. Shouche

- French-Indian Inter-Academic Symposium on Infectious Disease" from 30th November to 3rd December 2010 at National Institute of Immunology.
- International Symposium on Recent Advances in Ecology and management of Vectors and Vector Borne Diseases at DRDO Gwalior 1-3rd December 2010
- International workshop on "Computational Aspects of Working with Genomes" 28-30th March 2011, Institute of Microbial Technology, Chandigarh India

S. Singh

- Nidhi Sanghrajka and Shailza Singh (2011) Interactome Modeling for *L.major*: A Systems Biology Perspective, Bangalore India Bio, BIEC, 4th-6th May 2011. (Poster)

R. Srikanth

- Workshop on "Protein Identification & Characterization", February 23-24, 2011, organized by Agilent Technologies at its center of Excellence in Bangalore.
- The Indian proteomics conference (IPCON 2011), Jawaharlal Nehru University, New Delhi, April 03-05, 2011.

Girdhari Lal

- Lal G, Yin N, Xu J, Ding Y and Bromberg J (2011) "Inflammatory signals induce epigenetic modifications that control natural regulatory CD4 T cells" in American Transplant Congress 2011, held at Philadelphia, on 2nd May. (Poster)
- Lal G, Nakayama Y, Burrell BE, Ding Y and Bromberg J (2011) "Non-humoral function of B cells is required for tolerance" in American Transplant Congress, held at Philadelphia, on 1st May.

Debashis Mitra

- Indo-US conference on Translational Research in HIV/AIDS in India, Panaji, Goa, January 12-14, 2011.
- 14th Transcription Assembly Meeting 2010, Centre for DNA fingerprinting and diagnostics, Hyderabad, January 20-22, 2011.
- International Conference on Emerging Frontiers and Challenges in HIV/AIDS Research, National Institute of Reproduction and Reproductive Health, Mumbai, February 5-8, 2011.

- 19th Molecular Immunology Forum 2011, National Institute of Immunology, New Delhi, February 19-20, 2011.
- 2nd Molecular virology meeting, Indian Institute of Science, Bangalore, April 29-30, 2011.

Arvind Sahu

- "Viral complement regulators: from structure to function" at the Indo-French Seminar on Host Pathogen Interactions in Respiratory Infectious Disease (HOPE IN RED) organized by Indo-French Centre for the Promotion of Advanced Research at Bengaluru, October 12, 2010.
- "Viral complement regulators: from structure to function" at the 79th meeting of Society of Biological Chemists (India) at the Indian Institute of Science (IISc), Bengaluru, December 14, 2010,
- "Domain swapping reveals complement control protein modules critical for imparting cofactor and decay acceleration activities in vaccinia virus complement control protein" at the 19th Molecular Immunology Forum, National Institute of Immunology, New Delhi, February 19, 2011.
- "The complement system as a viral target for immune evasion" at the Symposium on Recent Trends in Biology, Department of Zoology, University of Pune, Pune, March 25, 2011.

Samit Chattopadhyay

- Chattopadhyay S Regulation of T cell development and differentiation. International conference on stem cell and cancer, 15-18th October, at I²IT, Hinjewari, Pune
- Chattopadhyay S. Global gene regulation by tumor suppressor SMAR1, Chromatin-Asia, Chromosome/Chromatin Dynamics: Epigenetics and Disease" December 4-6, 2010, JNCASR, Bangalore
- Chattopadhyay S. Regulation of chemokine gene expression mediated by tumor suppressor SMAR1. Annual Convention of Indian Association for Cancer Research for the year 2010-2011; December 13-15, 2010, SBC, IICB. Kolkata
- Chattopadhyay S. When and how a cell decides to die? Regulation of cellular apoptosis by tumor suppressor p53, Special Guest Lecture at GeneRations-2011, IIT, Mumbai, 8th February, 2011 arranged by IIT students.
- Kiran K Nakka, SRF,ICMR Awarded Best Poster Award at "3rd meeting of Asian Forum of Chromosome and Chromatin: Epigenetics and Disease. JNCASR, Bangalore (4th Dec-6th Dec, 2010). Abstract title: Signal Dependent Regulation of CD44 alternative splicing by SMAR1.
- Kiran K Nakka SRF, ICMR and Chattopadhyay S, Mr. Nakka is selected for Oral Presentation at RNA - 2011, International Conference on Sixteenth Annual meeting of the RNA Society,14th June-18th June, 2011, Abstract title: Regulation of pre-mRNA Splicing by Nuclear Matrix Protein SMAR1. He received Travel Award from the Organizers to attend this prestigious meeting.

Sanjeev Galande

- Senior Fellows' Meeting, November 11-12, 2011, Wellcome Trust, London, UK.
- Chromatin Asia meeting, December 4-6, 2011, JNCASR, Bangalore.
- Guha Research Conference, December 16-20, Aurangabad.
- Annual meeting of the Society of Biological Chemists, December 2010, IISc, Bangalore.
- 14th Transcription Assembly, January 20-22, 2011, CDFD, Hyderabad
- Young Investigator Meeting, February 8-12, 2011, Bhubaneshwar.
- InStem-CIRM meeting on Stem cells, March 21-22, 2011, NCBS-InStem, Bangalore.
- Japanese Society of Developmental Biology Annual Meeting, May 18-21, Okinawa, Japan.

Conferences / workshops attended by students

- Supinder Kour attended "Basic course on digital flow cytometry (LSR II)" organized by BD at Gurgaon, September, 20-22, 2010.

- Supinder Kour attended 37th Annual Conference of the Indian Immunology Society at Shere-Kashmir Institute of Medical Sciences, Jammu, February 7-9, 2011.

- Manasa Gayatri attended 37th Annual Conference of the Indian Immunology Society at Shere-Kashmir Institute of Medical Sciences, Jammu, February 7-9, 2011.

- Snehal Joshi attended "International conference on stem cells and cancer" at I^2IT, Pune, December 11-14, 2010

- Snehal Joshi attended "36th Mahabaleshwar seminar on infection and pathology" at Mahabaleshwar, January 8-13, 2011.

- Rupesh Srivastava presented a paper entitled "IL-3 promotes TGF-β induced development of Foxp3+ regulatory T cells and attenuates collagen-induced arthritis" at the "2nd International Conference on Regulatory T Cells and Th17 Cells and Clinical Application in Human Diseases" Shanghai, China, July 17-20, 2010.

- Priyanka Chaudhary participated in 36th Mahabaleshwar seminar on Infection and Pathology' held from January 8-13, 2011 at Mahabaleshwar, India

- Rupesh Srivastava received the prestigious "G. P. Talwar Young Scientist award" for the work entitled "IL-3 modulates Treg-Th17 cell development and attenuates collagen-induced arthritis" presented at the 37th Annual Conference of the Indian Immunology Society organized by Shere-Kashmir Institute of Medical Sciences, Jammu, February 7-9, 2011.

- Rupesh Srivastava received Second Prize for oral presentation of the paper entitled "IL-3 promotes TGF-β induced development of Foxp3+ regulatory T cells and attenuates collagen-induced arthritis" at the "Graduate Students Meet-2010" of

Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Mumbai, December 17-18, 2010.

- Sohrab Zafar Khan received Best Poster Award at International Conference on Emerging Frontiers and Challenges in HIV/AIDS Research, NIRRH, Mumbai, held on February 5-8, 2011.Title: "Cyclin K interacts with Nef and inhibits HIV-1 gene expression and replication"

- Neeru Dhamija was selected for oral presentation at International Conference on Emerging Frontiers and Challenges in HIV/AIDS Research, NIRRH, Mumbai held on February 5-8, 2011. Title: "Tat mediated regulation of c-Rel gene expression in HIV-1 infected cells"
- Pratima Rawat received the Best Poster Fellowship at 2nd Molecular virology meeting, Indian Institute of Science, Bangalore, April 29-30, 2011. Title: "Regulation of HIV-1 replication and gene expression by Heat Shock Factor 1"

- Sudeep Sabde presented Poster at 2nd Molecular virology meeting, Indian Institute of Science, Bangalore, April 29-30, 2011. Title: "Novel Caffeoyl anilide molecules as dual inhibitors of HIV-1"

- Neeru Dhamija received the Best oral presentation award at 2nd Molecular virology meeting, Indian Institute of Science, Bangalore, April 29-30, 2011. Title: "Genome wide recruitment of Tat in HIV-1 infected T cells and its functional relevance".

Students awarded Ph.D.

Anuradha Vaidya

Title of the Thesis: Identification of Biochemical pathways involved in the Hematopoietic Stem Cell (HSC) Regulation,

Guide: Dr. Vaijayanti P. Kale

Balan Sreekumar

Title of the Thesis: "In Vitro Generation of Dendritic Cell from Hematopoietic Stem Cell: Characterisation, Cryopreservation and Evaluation of Their Role in Cancer Immunotherapy"

Guide: Dr. Lalita S.Limaye

Baskar B.

Title of the Thesis: "Role of structure and organization of mRNA during translation",

Guide: Dr. Vasudevan Seshadri

Prayag Murawala

Title of the Thesis: Functional characterization of the interaction between the nucleoporin Nup358 and Dishevelled in Wnt signaling

Guide: Dr. Jomon Joseph

Neeti Sharma

Title of the Thesis: Studies on Rubinstein Taybi Syndrome

Guide: Dr. Sharmila Bapat

Vimal Pandey

Title of the Thesis: Role of Metabolic Disorders on Neoplastic Development and Outcome of Cancer Chemotherapy.

Guide: Dr. Manoj Kumar Bhat

Reeti Behera

Title of the Thesis: Studies on role of Osteopontin in Regulation of Jak2/Stat3 Dependent Cell Survival and Tumor Growth in Breast Cancer

Guide: Dr. Gopal C. Kundu

Mansoor Ahmed

Title of the Thesis: Studies on role of osteopontin (OPN) in regulation of MTOR D\dpendent transcription facter-mediated ICAM-1 expression in breast cancer

Guide: Dr. Gopal C. Kundu

Surajit Sinha

Title of the Thesis: "Role of MAR binding protein SMAR1 in apoptosis".

Guide: Dr. Samit Chattopadhyay

Sreenath K

Title of the Thesis: "Regulation of viral transcription and signal transduction by a MAR binding protein".

Guide: Dr. Samit Chattopadhyay

Sandeep Singh

Title of the Thesis: "To study SMAR1 interacting proteins and role of SMAR1 in cellular differentiation and tumorigenesis".

Guide: Dr. Samit Chattopadhyay

Sunil K Malonia

Title of the Thesis: "Role of MAR binding proteins in the regulation of cytokine genes."

Guide: Dr. Samit Chattopadhyay

Sulabh Kharbanda

Title of the Thesis: Identification of SMAR1 binding regions in human genome and to study its transcription control by EGF signaling pathway in context to breast cancer.

Guide: Dr. Samit Chattopadhyay

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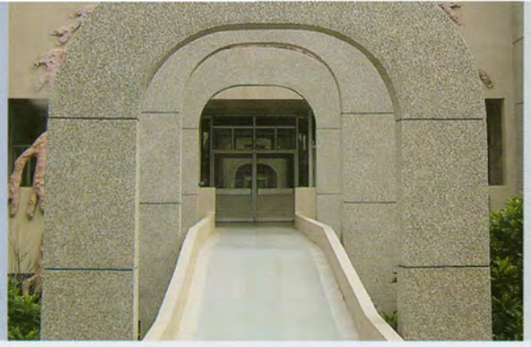

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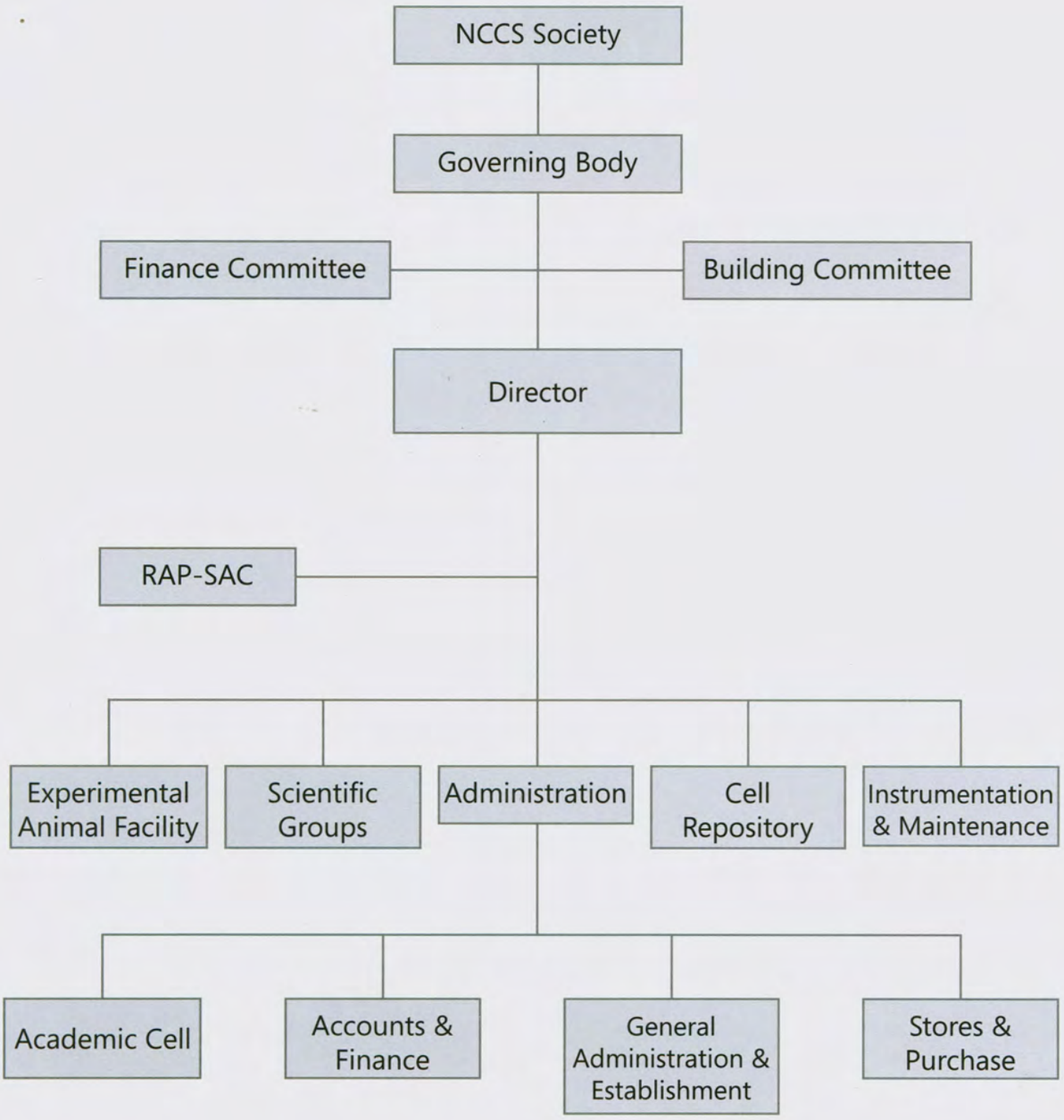

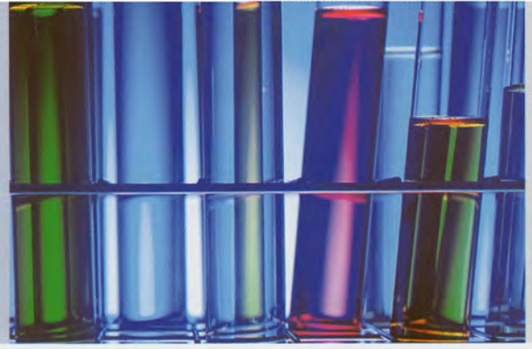

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   G-Block, P-73,
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3. **Dr. Satish Kumar Gupta** — Member
   Staff Scientist VII and Chief,
   Reproductive Cell Biology Laboratory,
   National Institute of Immunology,
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   National Institute of Virology,
   Dr. Ambedkar Road,
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   Director,
   Institute of Life Sciences,
   Nalco Square, Chandrasekharpur,
   Bhubaneswar – 751 023

3. **Dr. V.S. Rao** — Member
   Ex-Director,
   Agharkar Research Institute, Pune

4. **Dr. J. Nagaraju** — Member
   Staff Scientist,
   Centre for DNA Fingerprinting
   and Diagnostics,
   Bldg. 7, Gruhakalpa, 5-4-399/B,
   Nampally, Hyderabad – 500 001

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   Department of Biotechnology,
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   Lodi Road, New Delhi 110 003

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   New Central Building, Pune 411 001

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   National Centre for Cell Science,
   Ganeshkhind, Pune 411007

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9.	**Shri P.Y. Bhusnale** Tech. Officer 'C' (I&M) NCCS, Pune 411007	Convener
10.	**Shri B.G. Acharya** Officer 'D' (Sr. Officer-Admin) NCCS, Pune 411007	Special Invitee
11.	**Dr. M.S. Patole** Scientist 'F' and In-charge -Accounts NCCS, Pune 411007Scientific	Special Invitee

1.	**Dr. Kanuri Rao** International Scientist International Centre for Genetic Engineering & Biotechnology Behind JNU Campus, Aruna Asaf Ali Marg New Delhi 110 067	Chairman
2.	**Dr. B. Ravindran** Director, Institute of Life Sciences, Nalco Square, Chandrasekharpur Bhubaneshwar 751 023	Member
3.	**Dr. N. K. Mehra** Head, Dept of Transplant Immunology & Immunogenetics, All India Institute of Medical Sciences, New Delhi – 110 029	Member
4.	**Dr. Rajesh S. Gokhale** Director, Institute of Genomics and Integrative Biology Mall Road, Delhi-110 007	Member
5.	**Dr. Soniya Nityanand** Professor and Head, Dept of Hematology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Immunology Division, Raebareli Road, Lucknow 226 014	Member
6.	**Dr. Jyotsna Dhawan** Dean, Institute of Stem Cell Biology & Regenerative Medicine, National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bellary Road, GKVK, Bengaluru, Karnataka 560065	Member

7. **Dr. Samit Adhya** — Member
Head,
Molecular and Human Genetics Division,
Indian Institute of Chemical Biology,
4, Raja S. C. Mullick Road,
Kolkata- 700 032, West Bengal

8. **Dr. Amitabha Mukhopadhyay** — Member
Scientist,
National Institute of Immunology,
Aruna Asaf Ali Marg,
New Delhi – 110 067

9. **Dr. V. Nagraja** — Member
Professor,
Microbiology & Cell Biology,
Indian Institute of Science ,
Bangalore 560 012

10. **Dr. Kumarvel Somasundaram** — Member
Associate Professor
Microbiology & Cell Biology,
Indian Institute of Science ,
Bangalore 560 012

11. **Dr. J. Gowrishankar** — Member
Director,
Centre For DNA Fingerprinting & Diagnosis,
ECIL Road, Nacharam,
Hyderabad- 500 076

12. **Dr. R. Varadarajan** — Member
Professor,
Molecular Biophysics Unit,
Indian Institute of Science ,
Bangalore 560 012

13. **Shri. S. Sinha** — Member
Advisor,
Department of Biotechnology,
Block 2, 7th Floor,
CGO Complex, Lodi Road,
New Delhi 110 003

Administration

The NCCS Administration consists of General Administration & Establishment, Civil maintenance, Accounts & Finance, and Stores & Purchase sections. The centre has its Instrumentation & Maintenance unit as well. All these Sections are providing support services to the main scientific activities of the centre.

As on date the centre is having the following staff strength.

Scientists	:	28
Administrative	:	41
Technical	:	66
Total	:	135

RESERVATION POLICY

NCCS is following Govt. of India orders on reservation matters. For the direct recruitment we follow respective rosters; with reservation as 15% to SC, 7.5% to ST and 27% to OBC on All India Basis by Open Competition. The Centre is also following Govt. of India reservation policy for physically handicapped candidates. Liaison officers have been nominated to ensure compliance of the reservation orders issued in favour of SC/ST/OBC.

RIGHT TO INFORMATION ACT 2005

As per the requirement of the RTI Act 2005, NCCS has nominated Shri. B.G. Acharya as CPIO for Administrative matters and Dr. D. Mitra as CPIO for Scientific matters. Shri A.D. Patil has been nominated as ACPIO and Dr. G.C. Mishra has been nominated as the Appellate Authority.

IMPLEMENTATION OF OFFICIAL LANGUAGE

NCCS has constituted Official Language Implementation Committee, which meets quarterly and tries to pursue Govt. of India orders in the matter of implementation of Official Language in day to day official work. Maximum staff members have passed Hindi Pragya Exam conducted by Hindi Teaching Scheme Office. Most of the forms have been made bilingual. Noting/Drafting work on many of the files is done in Hindi. On every Monday, all the work in the Library is carried out in Hindi and the same is made mandatory for all library users. Unicode Encoding System has been enabled in most of the computers so that Hindi work can be carried out easily anywhere in the Institute. The centre also observes Hindi Saptah every year. Essay and Circular writing competitions were held and winners were given cash awards and certificates. Guest lecture was also arranged on Hindi Day. Official language activities are strongly supported by the Director.

VIGILANCE MATTERS

Dr. Bhaskar Saha, Scientist 'F', is the nominated Chief Vigilance Officer of the centre. Vigilance reports are being sent regularly to the nodal ministry i.e. Department of Biotechnology, Government of India, New Delhi.

SECURITY

NCCS has engaged a private Security Agency for providing security services on contractual basis. All important places in the complex have been manned by the security personnel throughout 24 hours. As on date, there is no security related problem at the centre.

DISCIPLINARY MATTERS

The centre follows CCS (Conduct) rules 1965 and CCS(CCA) rules 1965, and NCCS Bye-laws for monitoring the disciplinary matters at the centre.

COMMITTEES

The centre has formed the following committees as required under various statutes and guidelines for smooth functioning of the institute

1. Grievance Committee
2. Committee for prevention of sexual harassment of working women employees
3. Animal Care and Use Committee
4. Biosafety Committee
5. Institutional Ethics Committee