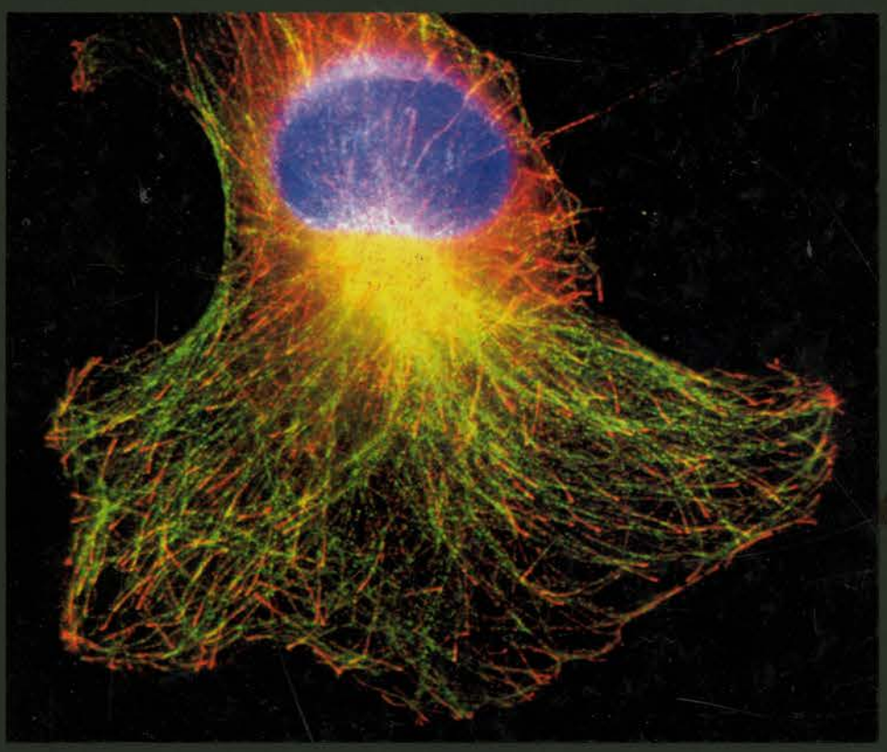
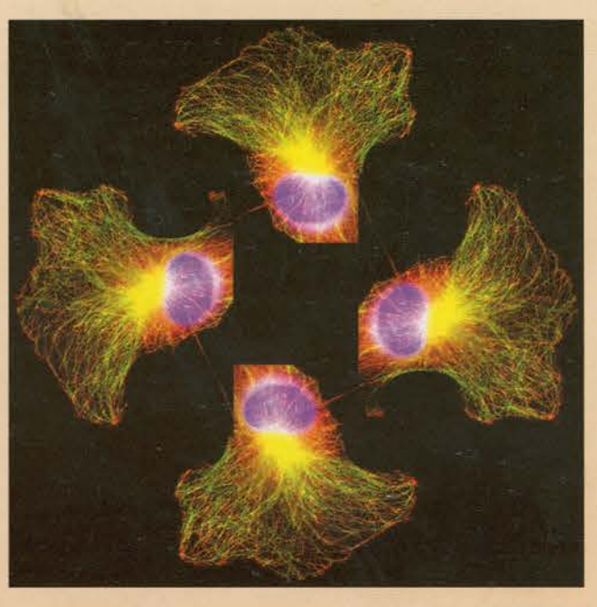
nccs

National Centre for Cell Science



Annual Report 2005 - 2006

National Centre for Cell Science



Annual Report 2005-2006

Contents

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The terms of reference

- 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.

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

- Research and development in the above and cell cultures related materials and products.

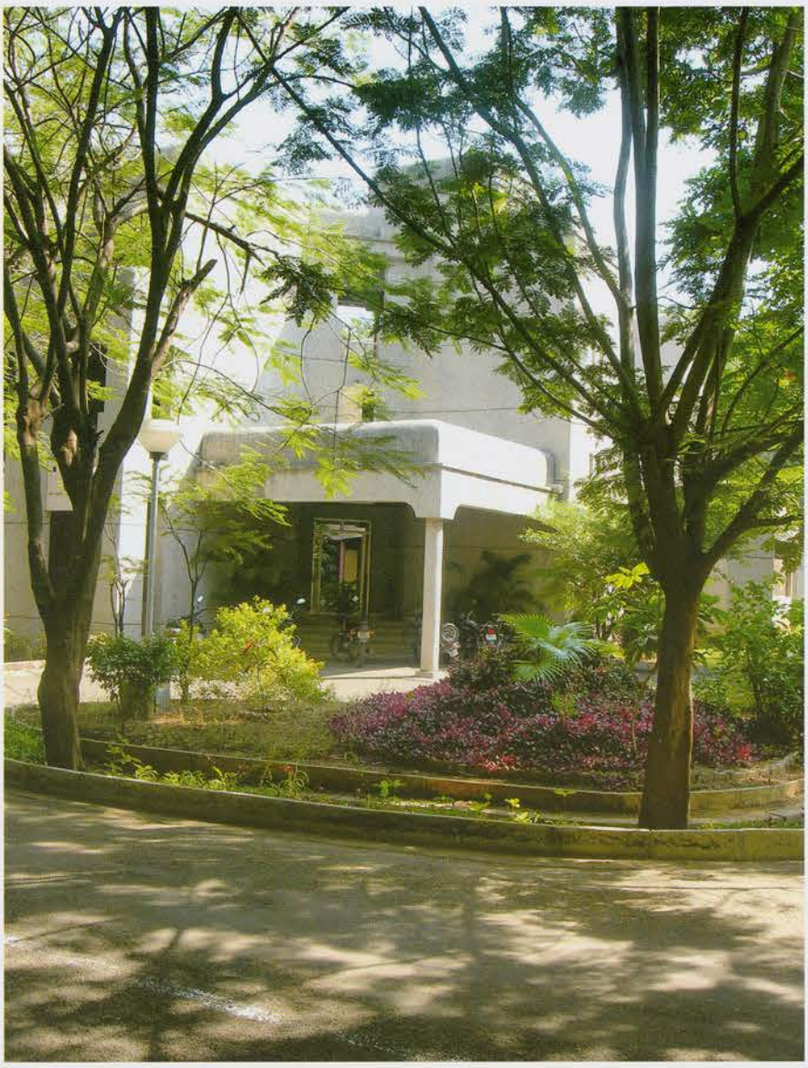
- To establish and conduct postgraduate courses, workshops, seminars, symposia and training programs in the related fields.

- 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 continuous basis between various scientific and research agencies/laboratories and other organizations including industries working in the country.

- To participate in such a program as required in the country for the betterment of society and advancement of science and technology.

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



From the Director's Desk...

It is a pleasure as always, to present the Annual Report of the National Centre for Cell Science (NCCS) for the year 2005-2006. The mission encompasses the tripartite functioning of the institute as a National Cell Repository, in Human Resource Development, and in Research & Development. Over the last year, the NCCS Repository has supplied 1154 cell lines comprising of 148 different cell types to 128 scientific institutions in India. A major move has been in the establishment of an Academic Cell at the institute that facilitates the smooth functioning of matters pertaining to Research Scholars. Further, under our "Teaching and Training" programs, we have conducted workshops on "Basic Techniques in Animal Tissue Culture" on site for 30 researchers at Bangalore and Goa.

In our studies in Cell Biology, we report for the first time that a nuclear pore protein (Nup358) associates with interphase microtubules. Currently we are investigating its potential implication in regulating cell polarity. Further, interaction of Nup358 with β-catenin, a critical molecule in Wnt signaling that goes awry in many cancers, prompts us to study a possible role of the nucleoporin in this signaling pathway. We have also identified a protein molecule from the perivitelline fluid (PVF) of Indian Horse Shoe Crab that has cardiac promoting activity during early vertebrate development.

Increased bone resorption by osteoclasts is a major pathological factor in arthritis, perodontitis and orthopedic implant loosening. We identified RANKL (receptor activator of NF-κB ligand) as a crucial factor for osteoclast differentiation and bone resorption. We have also investigated how TNF-α in association with proinflammatory cytokines such as IL-1 and IL-6, and TGFβ enhances bone resorption, and show that IL-3 has an inhibitory effect on osteoclast differentiation. In another study, the mechanism of activation of insulin mRNA translation upon higher glucose levels is being investigated. The findings indicate that a 60-80 kDa protein binds to the 5'UTR in an insulin-dependent manner and regulates translation.

NCCS has been a pioneer of stem cell research in the country. In understanding the fundamental issues of stem cell biology, we found that the inhibition of stress kinases in primitive hematopoietic cells results in increased proliferation by making them more responsive to growth conditions. We also show that FRIL, a mannose binding lectin has stem cell preservation activity. Optimization of *in vitro* expression and cryopreservation of hematopoietic stem cells is another area of interest where, we have identified additives such as Arachidonic acid (Omega6) and its metabolites that may reduce apoptosis in $CD34^+$ cells. Further, the conditions for enrichment, freezing and *ex vivo* expansion of cord blood megakaryocyte and dendritic cells have been optimized. This has potential application in thrombocytopenia therapy and dendritic cell tumor therapy.

Embryonic stem cell (ESC) systems, which have the unique property to differentiate into all cell types, are being used to understand developmental events during neuro- and cardiomyogenesis. We have achieved differentiation of mouse ESCs into neurons, in particular the dopaminergic neurons. Our studies also indicate that retinoic acid has a role in increasing the neural progenitor population; we are further investigating a potential role of Wnt signaling in cardiomyogenesis.

In the area of ovarian tumor stem cells, we identified that a distinctive nuclear-mitochondrial mutational profile and varying stem cell dynamics are associated with the emergence of a specific lineage on a trajectory towards tumorigenesis. The human homologue of the mouse melanoma gene (M3TR) earlier identified by us was shown to be involved in induction and maintenance of stemness; its functioning as a microRNA also triggers off a cascade of events that culminate in cellular transformation. Further, the deciphering of TNF-α mediated signaling pathway(s) in monolayer and spheroids generated from gliomas revealed that while the two Cyclin dependent kinase (CDK) inhibitors p21 cip/waf1 and p27 kip1 are both expressed on stimulus with TNF-α, only p27 might be important in growth arrest in the spheroids. Our recent studies in prostrate cancer reveal that osteopontin regulates the cyclooxygenase-2 (COX-2) - mediated PGE_2 production and MMP-2 activation by tumor cells, that in turn, mediate tumor progression and angiogenesis. These data suggest that blockade of OPN and COX-2 is a promising therapeutic approach for the inhibition of tumor progression by suppressing prostate tumor growth and angiogenesis.

In exploring the sensitivity of HPV E6 positive cancer cells to chemotherapeutic drugs, we identified that though upregulation of the DNA damage-inducible gene alpha (Gadd45α) occurs as a consequence of apoptotic response to genotoxic stress, induction of apoptosis is solely dictated by the nature of stress and cell type. A potential therapeutic application of the cholesterol depleting agent, methyl-β-cyclodextrin (MCD) in combination with other conventional cytotoxic drugs to facilitate reduction of drug dosage that offers a better chemotherapeutic approach with low toxicity is being evaluated.

In the area of diabetes, our studies indicated that insulin protects cardiomyocytes from oxidative and nitrative stress-induced apoptosis by inhibiting reactive oxygen and reactive nitrogen species generation. Similarly, curcumin pretreatment was found to protect islets against streptozotocin and cytokine induced damage through scavenging cellular ROS and augmenting cellular defense responses. These findings indicate a therapeutic potential of multiple anti-oxidants on oxidative and nitrate stress in diabetes. In another study, we report for the first time that chick pancreatic β islets could be used as excellent alternative *in vitro* model for physiological and pharmacological studies in diabetes research.

Differential membrane dynamics mediated by various lectins indicate that lectins with similar carbohydrate specificity evoke different cellular responses depending on the cell line lineage, through differential protein-lectin interactions. An example in point is that, the jacalin lectin exerts reversible stress on A431 cells through the induction

of phosphorylation of caveolin-1 and p38 but not JNK, whereas another lectin viz. PNA, which has a very similar specificity to that of jacalin, did not induce the same effect. Further studies suggest that jacalin cytotoxicity is mediated through the caveolin-c-src p38 pathway and involves an impairment of the functionality of ORP150 – a novel ER chaperon.

In our studies with the Leishmania housekeeping genes, we have cloned the selenophosphate synthetase [selD] gene and are in the process of characterizing the role of the protein with respect to its cellular localization and stage-specific expression in the parasite. Our studies on the mechanism of immuno-suppression in leishmaniasis suggest that *in vivo* suppression of ERK-1/2 or exaggeration of p38MAP kinase might result in the amelioration of Leishmania infection. We have also identified that anti-IL-2 treatment is effective in the early phase of infection while IL-10 blockade is effective at a later stage of infection. Taken together, these findings could lead to the development of prophylactic and therapeutic principles for the dreaded disease. During our studies on the biology of malarial parasite *Plasmodium falciparum*, we established and characterized a novel population of extracellular erythrocytic parasite culture derived from noninvasive merozoites. We now show that these extracellular forms require RBC membranes for their normal maintenance.

The modulation and induction of CTL responses, by different antigen presenting cells can provide key information in studies of CD8+ T cell mediated immunity. We have been successful in the isolation and characterization of dendritic cell types 1 and 2 (DC1 & DC2). Further, activation of these through the T-independent and T-dependent modes reveals that the DC1 cells have a stimulatory effect while the DC2 cells are regulatory in nature.

Genome sequencing of poxviruses and herpesviruses have shown that members of these families encode structural homologs of human regulators of the complement activation (vCCP) to mask themselves against the host's complement attack. Thus, the Herpesvirus saimiri homolog (HVS CCPH) was seen to possess all the complement regulatory activities present in kaposica and VCP, and possesses 14-fold higher factor I cofactor activity against C3b. Site-directed mutagenesis revealed that R118 contributes significantly to the factor I cofactor activity of HVS CCPH.

We have also shown for the first time that Hsp40 is crucial for Nef-mediated enhancement of HIV gene expression and replication. Anisotropy studies using fluorescein labeled DNA suggests that Tat binds to NFκB enhancer DNA as a dimer with binding affinity in nanomolar range. Further, the Tat:NFκB interaction follows a two site sequential binding model and could be responsible for Tat mediated modulation of cellular genes. Our data also provides preliminary evidence for the importance of IFN-γ in inducing expression of CTL effector molecules perforin and granzyme, an important finding as rescue of impaired CTL functions may help devise an immunotherapeutic strategy to control HIV replication or boost existing strategies.

Understanding the role of a MAR binding protein, SMAR1, in many cellular processes has been another area of interest. This tumor suppressor activates p53 through phosphorylation that in turn modulates global transcription from various promoters. PGA2 mediated repression of Cyclin D1 transcription and cell cycle arrest requires SMAR1. SMAR1 also inhibits TGFβ signaling and its downstream target genes that are involved in tumor cell migration and metastases. In another study, phosphorylation of the T-cell specific transcription factor SATB1 was seen to determine its association with either HDAC1 or PCAF. Further, recruitment of HDAC1 or PCAF to the *IL*-2 promoter *in vivo* is dependent on the phosphorylation status of SATB1 at S185. We have also shown that phosphorylation and acetylation of SATB1 have contrasting effects on gene expression at a global level.

We are interested in understanding the microbial community structure of unique ecosystems like insect gut, human colon and some extreme ecosystems. In the case with *Aeromonas culicicola*, a microbe from the midgut of mosquito, we used the distribution of its toxin genes that are implicated in its virulence to assess the extent of pathogenicity in these organisms. We also describe for the first time the presence of *Ochrobacterium intermedium* in the antrum of a non-ulcer dyspeptic patient diagnosed with *H. pylori*, a view to asses the correlation between infections by these two pathogens in the acidic environment.

Over the last year, NCCS has 46 publications in reputed peer-reviewed journals to its credit, and 3 chapters have been contributed to books. We have filed 7 patents. In addition to the institutional research funds, our scientific endeavours are further substantiated by peer reviewed funding from various national and international funding agencies like DBT, DST, DRDO, Wellcome Trust, etc.

Our research activities over the last year have evinced an increase in the impetus towards translational biology that reflects the commitment of our scientists to societal needs.

G.C. Mishra
Director

Human Resource Development

Workshops on Animal Tissue Culture

One of the main objectives of the centre is to enhance human resources by way of conducting symposia, workshops and tailor made programmes for individuals.

Conducted by Dr. R R Bhonde & Dr. M S Patole
August 22–25, 2005.
'Connexin', Bangalore
10 participants

Conducted by Dr. R R Bhonde
May 22–27, 2006.
Dept. of Zoology, Goa University, Goa
20 participants

In addition, to cater the increased needs of Research Fellows joining the institute, a separate section of "Academic Cell" was established in June, 2005. This Cell monitors all the activities relating to recruitment, placement, and facilitation of the students registering for Ph.D. in various Universities. In addition the cell also looks after the M.Sc Project Training, Summer Training etc.

During 2005-2006, 25 students joined for pursuing Ph.D. under various Scientists. Out of the above, presentation of 24 students were completed and their admission is confirmed by University. The total number of Ph.D. students as on 31.03.06 was 142.

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 - 09
Summer Training - 09.

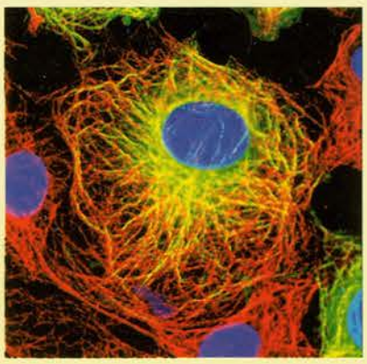
During the year 28 Research Fellows attended seminars/ conferences/symposium conducted by various reputed organizations.

Repository

National Centre for Cell Science serves as National Cell bank for animal cell lines. The work done at repository mainly involves cell line procurement, expansion, cryopreservation and distribution. In this year we have procured 35 different cell types from different repositories. The list of cell lines with details such as media requirements, growth conditions and its use is available now on demand. In 2005-2006, have supplied 1154 cell lines comprising of 148 different cell types to 128 research institutions in the country.

Research Reports

Cell Biology



Role of nucleoporins in cell polarity and Wnt signaling

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Background

Nucleoporins (Nups) are protein components of the nuclear pore complex (NPC) that makes the channel for transport of macromolecules across the nuclear envelope. Nucleoporins play roles in regulating the nucleo-cytoplasmic transport. Recent evidences indicate that Nups are actively involved in other functions including microtubule-kinetochore interactions during mitosis, regulation of transcription and DNA repair. Here we show that Nup358 (also called RanBP2), a nucleoporin residing on the cytoplasmic side of the NPC, associates with interphase microtubules. We further like to extend our understanding of this unexpected observation and hypothesize that Nup358 contributes to interphase microtubule functions. As cell polarization, a process of coordinated reorganization of cellular structures to achieve specific functions, requires microtubules, we are examining if Nup358 is involved in different aspects of polarity. Additionally, we identified an interaction between Nup358 and β-catenin. β-catenin is a critical player of Wnt signaling, which is a conserved pathway important for embryogenesis and development. This pathway often goes awry in cancers, particularly in colon cancer. We are investigating if Nup358 is involved in Wnt signaling and if yes, how?

Aims and Objectives

1. Characterization of Nup358-microtubule interaction

2. Understanding the functional significance of interaction between Nup358 and β-catenin

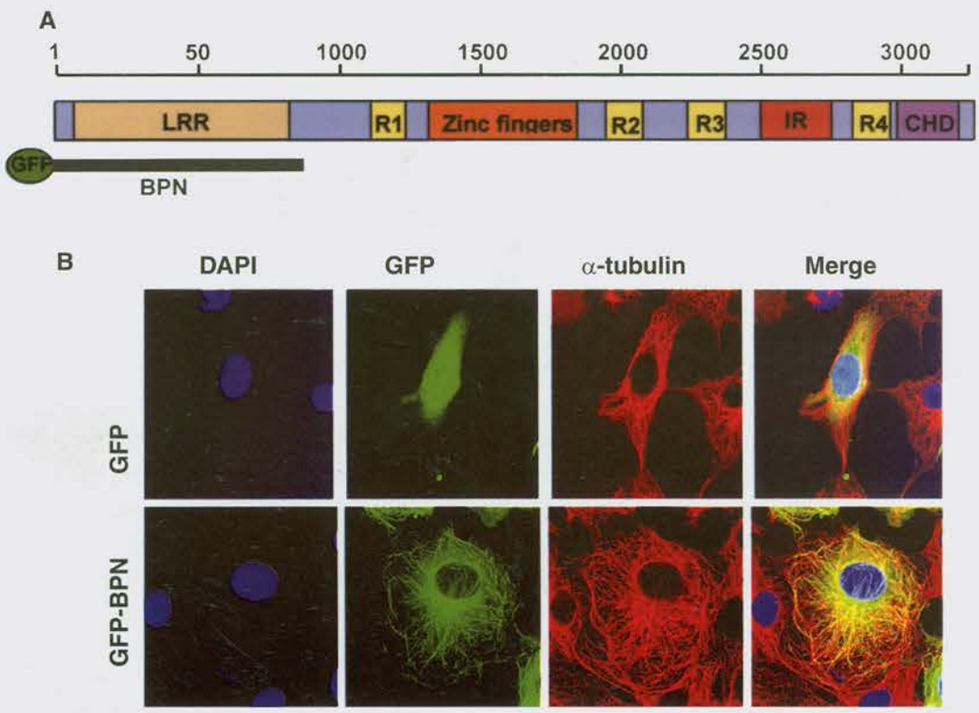

Fig. 1: The N-terminal region (1-900 amino acids) targets Nup358/RanBP2 to Interphase microtubules. (A) Schematic representation of Nup358 showing different domains. It is a large nucleoporin (3224 amino acids) present at the cytoplasmic side of the nuclear pore complex. LRR, Leucine Rich Repeat; R1, R2, R3 and R4, RanGTP binding domains; IR, internal repeats; CHD, Cyclophilin Homology Domain. BPN represents the N-terminal (900 amino acids) region used for expression in mammalian cells as GFP-fusion protein. (B) Localization of GFP-BPN to interphase microtubules. COS7 cells were transfected with either GFP alone (upper panel, green) or GFP-BPN (lower panel, green) and 24 hours later were fixed with formaldehyde and co stained for microtubules with monoclonal anti-α-tubulin antibodies (red). As evident, whereas GFP shows a diffused localization in the cytoplasm and nucleus, GFP-BPN shows association with interphase microtubules. DNA was stained using DAPI (blue).

Work Achieved

Characterization of Nup358-microtubule interaction: Nup358 is a large nucleoporin with many distinct domains (Fig. 1A) and is present on the cytoplasmic face of the NPC. Earlier studies identified Nup358 as a nucleoporin that localizes to kinetochores during mitosis in a microtubule-dependent fashion. To further explore the mode of microtubule interaction, we tagged different regions of Nup358 with green fluorescent protein (GFP) and transiently expressed in COS7 cells. Surprisingly, the N-terminal 900 amino acids (BPN) showed colocalization with microtubules in interphase (Fig. 1B). This indicates that Nup358 could interact with interphase microtubules *in vivo*. Pull down experiments using cell extracts and polymerized microtubules confirmed the *in vivo* interaction of Nup358 with microtubules. Further, BPN expression significantly elevated levels of acetylated tubulin, a marker for stable microtubules, in transfected cells. This suggests to us that Nup358 regulates interphase microtubule dynamics.

Experimental Hematopoiesis

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Background

Steady state production of blood cells in a regulated manner throughout the life of an individual necessitates a precise control of this process at various levels. However, the intricacies of this process are still not clearly understood. Though the role of various growth factors and their mechanism of action on various progenitors have been worked out fairly well, it is still unclear as to how a stem cell decides to take up a particular option like self-renewal, commitment, migration etc. We have been interested in some of these fundamental issues and are trying to collect experimental evidence to address them.

Various experiments carried out by several workers over the years, especially using long-term cultures, have underscored the crucial role of stromal cells in the development of stem cells. However, the exact mechanism(s) involved in the effective cross talk is not yet known and the players involved in this process remain to be identified. We have earlier shown that TGFβ1 released from marrow mononuclear cells in response to erythropoietin stimulus is one such molecule which has the capacity to bring about the cross talk between stromal cells and stem cells. We have also shown the involvement of nitric oxide signaling pathway in the process of stem cell proliferation.

During the course of these studies we made an interesting observation that endothelial nitric oxide synthase (eNOS), the enzyme involved in the generation of NO in cells, was localized to the nuclei of stromal cells in addition to its usual known localization in the plasma membrane caveolae and endoplasmic reticulum-Golgi. This was a novel finding and, therefore, we decided to examine the physiological significance of this observation.

I) Studies on nuclear localization of eNOS:

We first examined the issue as to whether the presence of nuclear eNOS is a specific property of marrow derived stromal cells or a general phenomenon using a panel of different cell lines such as HEK293, CHO, MCF-7, K562 and KG1a. We observed that the nuclear eNOS was present in all the cell lines we examined. These results indicated that nuclear eNOS may be a general property of mammalian cells and may have a role in cellular physiology in a much wider context including hematopoiesis.

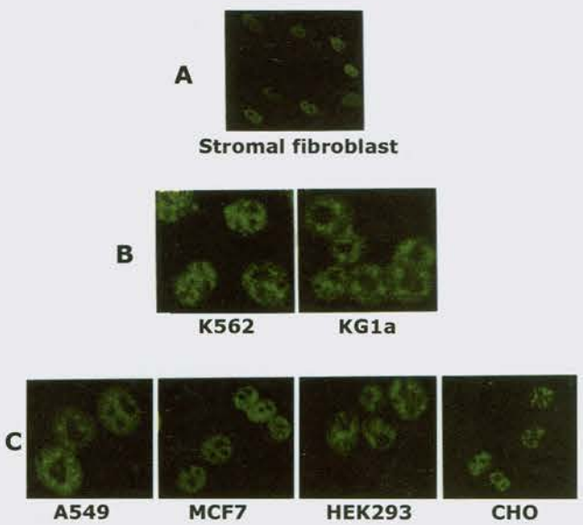


Fig. 1: Immunofluorescence staining of various cell lines with anti eNOS antibody; (A) represents whole cells and (B & C) represent isolated nuclei respectively

Aims and Objectives

Studies on nuclear eNOS (NOS III), especially the mechanisms responsible for its targeting to nucleus and to specific sub nuclear regions using plasmid constructs.

Work achieved

Localization of eNOS protein:
We used both Immunofluorescence and western blotting approaches for studying the localization of eNOS in the cells. Using Immuno-fluorescence studies for eNOS protein in various cell lines, we show that it is present in the nucleus and is seen specifically in the nuclear speckles (Fig. 1). We also found that eNOS colocalized with other important molecules like B23, spliceosomes, splicing factor and c-Src in the nucleus. Immuno-precipitation experiments of eNOS and SC-35 in MCF7 nuclear lysate confirmed the colocalization between eNOS and splicing factor.

Studies with plasmid-constructs containing wild type or mutant nuclear localization sequences (NLS):
A series of eNOS-NLS (nuclear localization signal) plasmid-constructs, wild type and mutated, were designed and prepared. The L1-L7, WT (wild type), MT (scrambled) constructs were with pEGFPC as the vector. These are small synthetic nucleic acid stretches having minimal NLS sequence (predicted by software) which were expressed as fusion protein with GFP tag. By carrying out transfections in MCF-7 cells we observed that the construct indeed drives the GFP to the nucleus. The expression of fusion protein was confirmed by western blotting experiment, with nuclear lysates.

Then we PCR amplified a stretch of eNOS-cDNA which included the putative NLS sequence (700bp plasmid) and cloned it in EGFPC vector. When transfected in MCF-7 cells it was observed that the putative NLS sequence drives the GFP to the nucleus confirming the earlier observations with synthetic constructs. When the cells were transfected with another plasmid containing an additional stretch of the cDNA spanning the Threonine 495 site a strong nuclear and nucleolar localization was seen with out any cytoplasmic leakage as seen with 700 bp construct. A mutation at T495 site by substituting alanine in place of threonine (T495 A) resulted in cytoplasmic localization of the fusion protein. These results indicated that the T495

phosphorylation may be serving as a nuclear and nucleolar retention signal. These results were commensurate with our IF data wherein we had observed that the nuclear eNOS was predominantly phoshorylated at T495 site.

The results suggest the following:

1. The putative sequence identified as NLS in eNOS-cDNA was found to drive GFP to nucleus indicating that it indeed acts as a nuclear localization sequence.

2. The double mutant where in both S1 and S2 serine were mutated, the protein did not go to the nucleolus indicating that the flanking serine residues of NLS especially S1 and S2 may play an important role in its nucleolar localization.

3. Phosphorylation at Threonine 495 is important for the nuclear retention of eNOS.

In summary, we have so far demonstrated that a functionally active and heavily threonine-phosphorylated (position 495) eNOS is present in the nuclei of mammalian cells including marrow stromal cells. It is associated with spliceosomes and colocalizes with splicing factor.

The mutant construct where T495 site has been replaced by alanine results in cytoplasmic localization of eNOS (T495A). Experiments with other constructs are in progress.

Future work

We propose to investigate the mechanism of nuclear localization of eNOS, its role in cellular physiology and its regulation by cellular signaling machinery.

II) Signaling in Stem cells

In addition to the stromal cell mediated signaling we are also examining the signaling mechanisms in stem cells in response to TGFbeta1. We have earlier reported that TGF-β1 which is a well known inhibitor of hematopoiesis stimulated HSC proliferation at low concentrations and inhibited it at high concentrations. On studying the molecular mechanisms behind this dose-dependent effect of TGF-β1, we found out that the low stimulatory doses predominantly induced the p44/42 MAPK pathway while the higher inhibitory doses predominantly induced the p38 MAPK pathway (Kale, 2004). We further examined whether the effect was also reflected in the downstream effector molecules. We found that low doses of TGF β1 enhanced the phosphorylation of the STAT proteins while high doses caused the phosphorylation of the stress kinase transcription factors (Kale and Vaidya 2004). By using a panel of myeloid hematopoietic cell lines we showed that the p44/42 and p38 MAPK pathways were being inversely modulated only in primitive type of hematopoietic cells (Manuscript in preparation).

Aims and Objectives

To identify the role of signaling pathways in the regulation of certain key aspects of HSC physiology such as proliferation, differentiation, migration and self-renewal.

Work Achieved

Inhibition of p38 stress kinase results in induction of proliferative responses in primitive cells:
KG1a cells were serum starved for 24 hours and then were treated or not with PD169316 or U0126 for 4 hours, after which cytospin smears were prepared and the cells were fixed. Indirect immuno-fluorescence staining was carried out using antibodies to Ki67 and B23. It was observed that the treatment of KG1a cells with PD169316 resulted

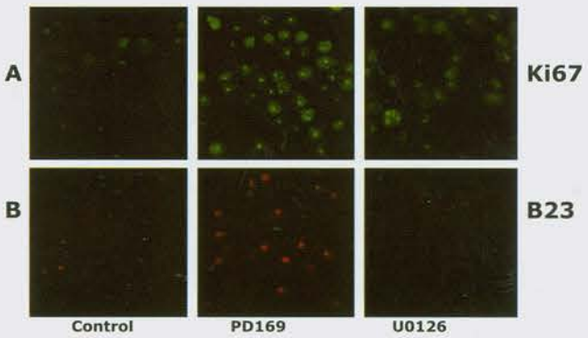


Fig. 2: Inhibition of p38 pathways results in proliferative responses: KG1a cells were serum starved, treated or not with inhibitors for 4 hours and the fixed cytospin smears were immuno-stained with (a) Antibody to Ki67 and (b) Antibody to B23.

in a very strong nucleolar expression of the proliferative markers Ki67 and B23 in the cells (Fig. 2). This indicated that the inhibition of p38 pathway in these cells resulted in induction of proliferative responses in them.

Inhibition of p38 MAPK pathway results in increased cell yield:

KG1a cells treated with PD169316 showed a strong expression of Ki67 and B23. In order to examine whether these cells show a growth advantage we carried out a growth curve experiment. The KG1a cells were serum starved and then were treated or not with inhibitors PD169316 or U0126 for 4 hours. After 4 hours the cell suspension was spun, supernatant was decanted and the cells were seeded in fresh growth medium with or without continuation of the respective inhibitors in the medium. Cell counts were taken after every 24 hours up to 72 hours. It was observed that the yield of PD169316 treated cells was much higher as compared to Control and U0126 treated cells. The effect was more pronounced when the inhibitors were continued in the growth medium.

These data indicate that the inhibition of stress kinases in primitive hematopoietic cells results in proliferative responses in them and makes them more responsive to growth conditions leading to a better cell yield.

Future Work

We propose to identify signaling pathways in various aspects of HSC physiology. Our experimental approach is to examine the effect of modulation of signaling pathways using pharmacological reagents on the behavior of cell lines and then carry out experiments with their counterparts from the primary tissues to validate our findings.

III) Identification and characterization of lectins having stem cell preservation activity

Hematopoietic Stem Cells (HSC) have the ability to self renew as well as to differentiate into all lineages of the blood. Since most mature blood cells have a limited lifespan, the ability of HSC to self-renew and replenish the mature cell compartment in a well regulated continuous manner is a critical factor in maintenance of steady state hematopoiesis. Therefore, efforts to maintain the HSC in a culture system have formed a forefront area in the hematopoietic research due to the immense clinical importance of such system in autologous transplantation and genetic engineering protocols.

Recently, a mannose binding lectin, FRIL, was shown to have stem cell preservation activity. It was shown that this lectin could preserve stem cells in a suspension culture up to 30 days in the absence of growth factors. We, therefore, initiated the work to screen lectin preparations having well defined binding characteristics, for stem/progenitor cell (HSPC) preservation activity. Our contention was that if such lectin molecules could be identified then they would form a well defined tool to identify the molecular interactions and signaling processes involved in the stem cell maintenance, under culture conditions.

Aims and Objectives

To identify lectins having *in vitro* stem-cell preservation activity and investigate the molecular mechanisms involved in the process.

Work Achieved

In the present study, we have selected four mannose specific plant lectins viz. *Dolichos lablab* lectin (DL), *Artocarpin integrifolia* lectin (AL), Banana lectin (BL) and Garlic lectin (GL). $CD34^+$ cells isolated from cord blood were incubated in serum free medium with different concentrations of lectins. Cells without the addition of lectins were kept as controls. The cells were incubated for different time intervals like 10, 20 and 30 days. At each time point the cells were harvested and Colony Forming Unit (CFU) assay was carried out. The colonies were scored as BFU (E), CFU-GM and CFU-GEMM using standard morphological criteria. The numbers of progenitors preserved were calculated by comparing the number of colonies formed by cells incubated with lectin versus colonies formed by cells incubated without lectins.

It was found that all four lectins showed HSPC preservation activity and the optimal concentration range was 100-200pg/ml. These concentrations were used in subsequent long term experiments.

Long Term Culture Initiating Cell (LTC-IC) Assay:
The LTC-IC assays are known to detect the presence of very primitive progenitors present in a given test population. After initial screening using CFU assays we examined whether the lectins had an ability to preserve LTC-IC units which represent primitive progenitors.

$CD34^+$ cells incubated with different lectins for various time points were seeded on an irradiated M210B4 feeder layer. The cultures were maintained for 5 weeks with weekly demi defoliation. After 5 weeks the cultures were harvested and CFU assays were carried out. The colonies were scored at the end of 14 days incubation and the number of primitive cells present in the initial inoculums was estimated.

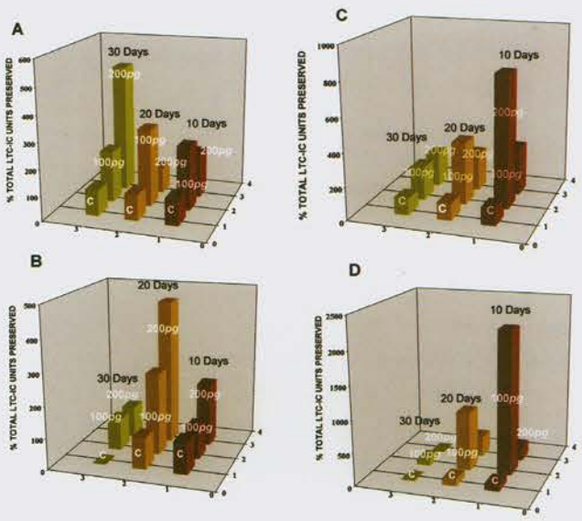


Fig. 3: LTC-IC assay of CD34+ cells pre-incubated with BL (A), GL (B), DL (C) and AL (D) for various time intervals.

It was found that cells incubated with lectins gave rise to more number of colonies as compared to those formed by control cells at various time intervals tested indicating that all four lectins had an ability to maintain primitive HSPCs for 30 days in culture without addition of growth factors (Fig. 3).

Future Work

1. We plan to carry out E-LTC-IC assay to detect very primitive HSPC after incubation with all four lectins.

2. Using mouse system as an *in vivo* model we propose to examine the effect on repopulating ability of the cells incubated with various lectins.

3. Using these lectins as tools we propose to examine signaling pathways induced by these lectins in hematopoietic stem cells.

IV) Caveolae and signal transduction

Caveolae are plasma membrane invaginations which are known to harbor various signaling molecules. Association with Caveolin 1, the structural protein of Caveolae, especially through its scaffolding domain maintains these molecules in inactive state. eNOS is also located in the caveolae and its activity is regulated by interaction with Caveolin1. Since our work pointed towards the role of eNOS in the stromal cell mediated growth promotion of stem cells we initiated experiments to examine the regulation of Caveolin 1 expression under the influence of various signaling processes. A very prominent signaling molecule that is related to Caveolin expression is estrogen receptor signaling. Therefore, we decided to examine regulation of Caveolin 1 expression by estrogen mediated signaling. We have chosen a standard estrogen receptor positive cell line, namely MCF 7, as a starting point to examine the issue.

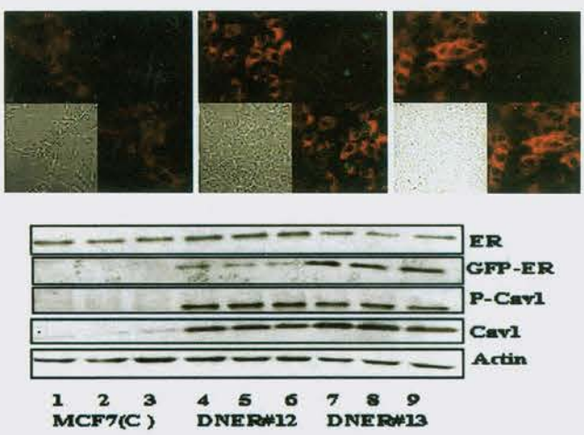
Aims and Objectives

Specific aim of the project is to examine the regulation of Caveolin1 expression by estrogen receptor signaling.

Work Achieved

Effect of expression of dominant-negative ER on Caveolin-1 expression:

We generated stable cell lines od MCF 7 breast cancer cells expressing dominant negative construct of estrogen receptor (DNER cloned in EGFPCN1).Several independent G418 resistant (600ug/ml) clones were selected and characterised for Caveolin 1 expression. Especially two clones, DNER #12 and DNER #13, were found to express very high high level of caveolin-1 protein as examined by immunofluorescence and western bloting (Fig. 4).



Expression of Cav1,ER,GFP,After Transfection of DNER in MCF7 Cell-Line.

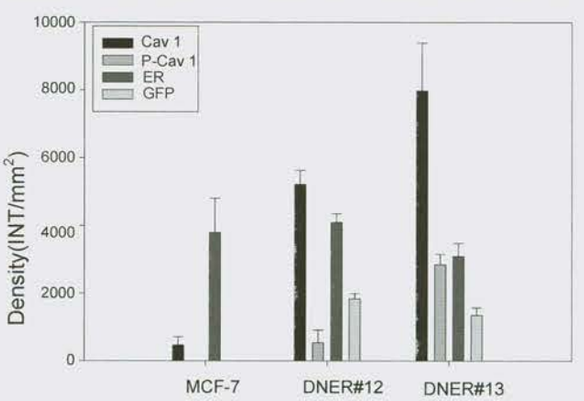


Fig. 4: Increased expression of caveolin-1 by stable clones of MCF-7 cells expressing dominant negative ER.

Studies on expansion and differentiation of haematopoietic stem cells

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Background

In vitro expansion and cryopreservation of hematopoietic stem cells:

In vitro expansion of $CD34^+$ cells is being extensively attempted to overcome the limitations of the small sample size especially of cord blood and pediatric marrow harvests. Optimal storage of these expanded cells is also crucial. There is a possibility that during such *in vitro* manipulations the cells undergo apoptosis. There are reports that functional expression of Fas Ag is induced on $CD34^+$ cells expanded *in vitro* in presence of haematopoietic growth factors. The role of Arachidonic acid (Omega6) and its metabolites in regulation of haematopoiesis is well documented. We plan to use them as supplements in media for *in vitro* expansion of $CD34^+$ cells to see whether they reduce apoptosis. To date a body of evidence has been compiled which attests to the hypothesis that cold shock can induce apoptosis. Thus detection of the level of apoptosis during preservation and expansion and to develop ways and means to prevent it is an important aspect of haematopoietic stem cell research.

Megakaryocyte generation:

Umbilical cord blood (CB) provides an alternative source of haematopoietic progenitor cells for transplantation. However, prolonged thrombocytopenia remains a major obstacle due to the lower numbers of megakaryocyte progenitor cells and their subsequent delayed engraftment. Efforts currently are underway to expand the number of CB stem cells and MK progenitor cells *ex vivo*, which may facilitate platelet production during post transplant nadir and decrease the time of thrombocytopenia. The optimal conditions for *ex vivo* expansion and cryopreservation of MK progenitor cells have not been established. Here we propose to define the conditions of *ex vivo* expansion of MK progenitor cells from umbilical cord MNCs and $CD34^+$ cells using various cytokines and serum free media and nutrient supplements. Attempts will be made to optimize the freezing protocols for the cryopreservation of these *ex vivo* generated cells. Thus our aim is to develop a clinically applicable improved system for enriching, freezing and *ex vivo* expanding CB MK progenitor cells.

Dendritic cell generation:

Dendritic cells (DCs) form a heterogeneous population of cells capable of stimulating naïve T cells and initiating primary immune response. This well known function of DCs has offered the possibility of developing clinical protocol for their use in immunotherapy to tumors. DCs may also play a critical role in the induction of peripheral immunological tolerance, which could have important implications in the treatment of autoimmunity or in the outcome of clinical transplantation. In recent clinical studies DC were used as a vaccine for cancer patients and showed induction of their anti tumor effect. The total number of DCs available for immunotherapy remains limited. DCs have been shown to be generated from CD34+ cells from BM, UCB and GCSF mobilized PBSC. Cryopreservation of $CD34^+$ cells is important to extend the availability of cellular therapy with DC. However, little is known about the effect of cryopreservation on the functional maturation of DCs. *Ex vivo* generation of this cell type and its efficient cryopreservation will have direct application in the clinics.

Our objective here is to explore a culture method to generate a large number of functional and mature DCs from human $CD34^+$ hematopoietic progenitor cells and standardize methods for their efficient freezing.

Aims and Objectives

1. *In vitro* expansion of haematopoietic stem cells:

The aim is to expand and cryopreserve $CD34^+$ haematopoietic cells by preventing apoptosis. We plan to use a class of compounds like antiapoptotic agents, nutraceuticals, antioxidants etc. in the expansion and freezing media.

2. Harnessing the potential of stem cells: *In vitro* generation of megakaryocytes and dendritic cells

The aim is the *in vitro* generation, characterization, and cryopreservation of the two cell types, i.e., megakaryocytes and dendritic cells from cord blood cells.

Work Achieved

***In vitro* expansion of haematopoietic stem cells**

$CD34^+$ cells isolated from cord blood samples were cultured *in vitro* in serum free medium with expansion cocktail of growth factors with and without antiapoptotic agents, Z-VAD FMK and calpain inhibitor .The level of apoptosis was quantitated by caspase activity detection, annexin V–FITC-PI staining, DNA ladder formation and BCL 2 expression. CD34 expression was quantitated on FACS both before and after expansion. CFU assay was done to estimate the progenitor content of expanded cells. The results depicted in Fig. 1 show that both the additives improved the expansion probably by preventing apoptosis.

Megakaryocytes

CD34+ cells isolated from cord blood MNCs were cultured *in vitro* in serum free medium with TPO and SCF. The megakaryocytes generated after 10 days culture were identified by morphology and phenotype. Ploidy levels in the uncultured and cultured samples were estimated by FACS. Expanded cells were cryopreserved and revived, and revival efficiency was detected by CFU-Meg assay as well as by expression of phenotypic markers CD41 and CD61. The revived samples were expanded with growth factors for 10 days and these cells were again characterized for megakaryocyte lineage. Results are summarized in Fig. 2. Results show that cultured expanded cells do show megakaryocyte morphology and phenotype. It is also possible to freeze the cells and further expand the frozen cells.

Dendritic cells

Culture conditions for generating dendritic cells from Cord blood $CD34^+$ cells are being standardized. We used different combinations of various growth factors like GMCSF, FLT3, IL-4, TNFalpha etc. The cells were cultured in serum free or serum containing media. Dendritic cells generated after 12 days of incubation were identified by morphology, phenotype and antigen uptake assays. The results in Fig. 3 show that dendritic cells are indeed generated in cultures

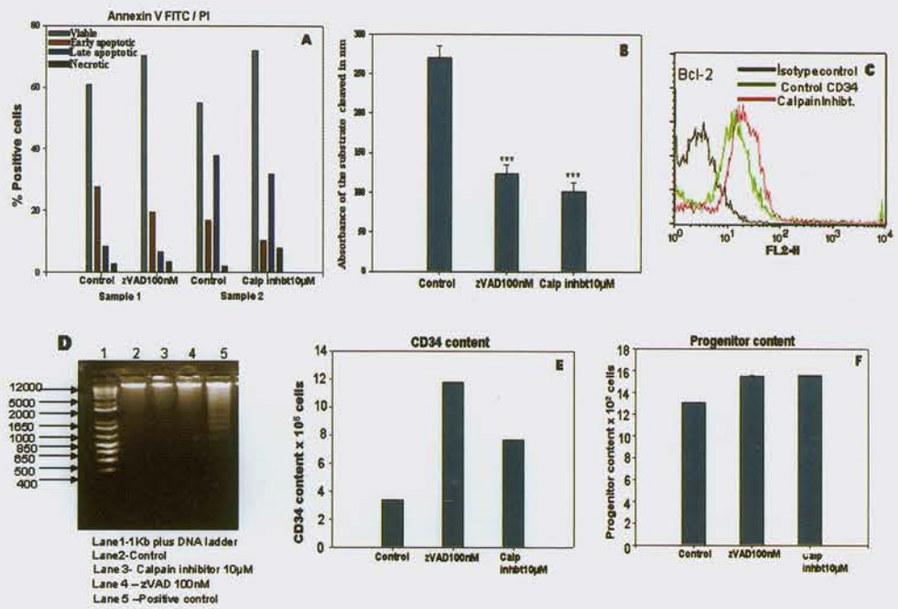

Fig. 1: Expansion of UCB CD34⁺ cells with and without anti-apoptotic agents: Detection of apoptosis by A) Annexin VFITC/PI staining B) Caspase-3 assay C) BCl-2 expression and D) DNA fragmentation. Quantitation of E) CD34 content and F) Progenitor content of the expanded cells.

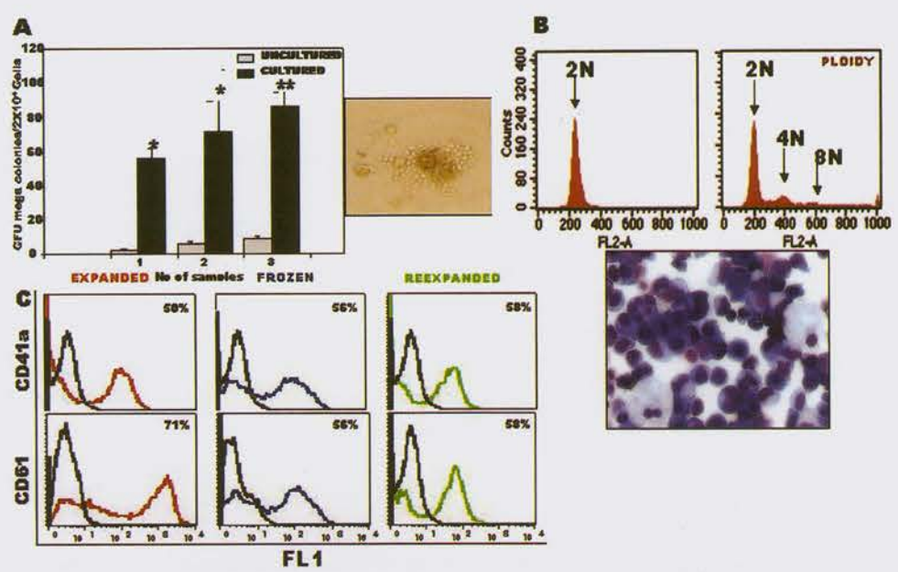

Fig. 2: Megakaryocytes from UCB CD34⁺ cells. A) CFU Meg colonies formed in methyl cellulose by uncultured and cultured cells B) FACS histogram showing ploidy of uncultured and cultured cells and Wright,s Giemsa image of cultured cells. C) Phenotypic expression on cultured cells before freezing, after freezing and after re-expansion.

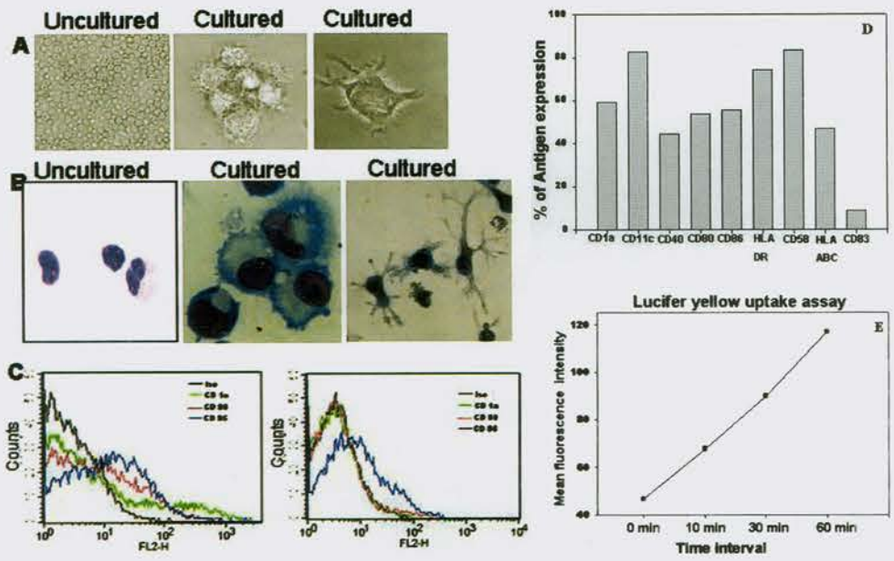

Fig. 3: Generation of dendritic cells from cord blood CD34⁺ cells. A. Phase contrast and (B) Wright-Giemsa images of uncultured and cultured cells. C. Comparison of surface antigen expression in serum containing and serum free medium. D. Surface antigen expression of dendritic cells in serum containing media. E. Antigen uptake by dendritic cells.

and now our efforts are on way to further optimize the culture conditions and characterize the cells by functional assays.

Future Work

In vitro expansion of hematopoietic stem cells

1. Cryopreservation of expanded cells with and without the selected additives.

2. Expansion of cryopreserved CD34+ cells will be undertaken

Megakaryocytes

1. Standardisation of functional assays like platelet activation, chemotaxis of expanded cells, ploidy estimation etc. will be undertaken.

2. Having standardised the culture conditions we will now use nutraceuticals as additives in the culture medium and study their effect on megakaryocyte expansion.

3. Optimization of Cryopreservation and revival protocols for *in vitro* expanded megakaryocyte progenitors.

Investigation of neurogenesis and cardiomyogenesis using ES cell model

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Background

Embryonic stem cells (ESCs) have the unique property to differentiate into all the cell types of the body and recapitulate the same developmental pathway during *in vitro* differentiation as it is followed during *in vivo* development. We have used this extraordinary property of ESCs and aimed at understanding the developmental events during early neuro- and cardiomyogenesis.

Aims and Objectives

The major focus of our group has been,

1. To establish stable transgenic ES cell clones using live reporter gene expression under the regulatory control of neural specific promoters/enhancers.

2. To differentiate the ES cells into neural lineages and understand the underlying molecular basis of lineage commitment and specification.

3. Manipulate extrinsic factors for the efficient generation of proliferative neural progenitors, differentiated neurons with special reference to the dopaminergic neuronal subtypes from ES cells *in vitro*.

4. To explore the efficacy of these *in vitro* generated cells in cell replacement therapy using animal models.

5. To investigate the role of Wnt signaling during ES cell maintenance.

6. To investigate the role of Wnt signaling during ES cell differentiation into cardiac lineage.

Work Achieved

Neurogenesis:

Our group's interest lies in investigating the early neurogenic proceedings and generating an enriched population of dopaminergic neuronal subtypes using the murine ES cell model *in vitro*. Simulating the *in vivo* conditions, these ES cells in the neural precursor cell stage express nestin and they become nestin-negative upon differentiation into mature neurons. Thus, ES cells make an excellent model system to unravel the role played by nestin during neurogenesis. However, the question arises whether a different set of neuronal progenitors specific for specific neuronal subtypes exists during development. If this is true whether we could identify and characterize the midbrain specific dopaminergic neurons and their progenitors that would provide insight into the signaling cascade(s) operational during dopaminergic neuron generation. Accordingly we have used the dopaminergic neuron specific promoter/enhancer to demarcate the stated population and tried to address these issues.

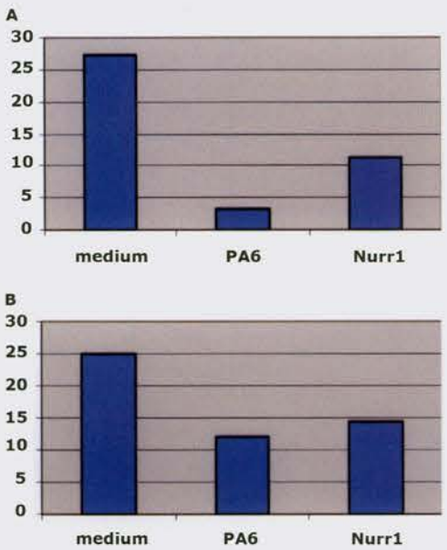

Fig. 1: Graphical representation of FACS analysis showing % of dopaminergic neurons generated in the medium versus co-culturing on PA6 and with Nurr1 over-expression.

Enrichment of ES cell derived dopaminergic neurons:

As reported earlier, we have been successful in differentiation of mouse ES cells into neurons, in particular the dopaminergic neurons. Enrichment of the dopaminergic neurons have been a prime requirement for transplantations. In this regard we have used an already established transgenic ES cell line having the mouse Tyrosine Hydroxylase [mTH] promoter driving the EGFP live reporter expression in these cells to identify and purify these dopaminergic neurons. The FACS quantitation at d7 (Fig. 1A) and d10 (Fig. 1B) revealed enrichment in these population in a growth factor devoid medium that was better than that done by overexpression of the Nurr1 cDNA or growing on PA6 stromal cell line. Previous studies by a couple of investigators indicated that, co-culturing with a stromal cell line helps enriching the dopaminergic neuron generation. However, that would result in a carry over of the co-cultured cell line as contaminants which might have adverse effect during transplantation or genetic manipulation studies. Hence, our strategy without any of these contaminants would be ideally suited for enrichment and transplantation experiments and also help investigating the cellular signaling cascades underlying the differentiation of dopaminergic neurons from ES cells.

We have also investigated the influence of various factors on further enrichment of these neuronal subtypes. Our study indicated that retinoic acid has a role in increasing the neural progenitor population (nestin positive) some of which are Nurr1 positive indicating their dopaminergic neuronal precursor status (Fig. 2A, B). On the contrary, the FACS analysis after 2 weeks revealed a decrease in the generation of these neurons on treatment with retinoic acid. This ascertained the inhibitory role of retinoic acid on the differentiation of the dopaminergic neurons while increasing the generation of Nurr1 expressing precursors. We are also in the process of exploring the existence of specific dopaminergic progenitors, specifying and characterizing

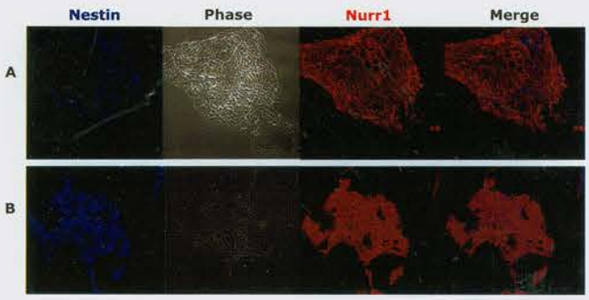

Fig. 2: Effect of medium with (A) or without (B) RA on the Nurr1 and Nestin expression on d4.

those. The transplantation studies in parkinsonian rat model are also underway. Our investigation would thus help us in understanding basic developmental events operational during early neurogenesis and simultaneously would help exploring the therapeutic potential the ES cells research would deliver.

Cardiomyogenesis:

Highly complex and tightly regulated cardiogenesis begins with the commitment of cells to cardiac fate at early gastrulation. A number of studies have indicated the role of BMP and FGF signaling in cardiogenesis and many mutant and knockout phenotypes have established their indispensable role in heart development. However, the role of Wnt signaling in cardiogenesis is poorly described. The wingless protein of Drosophila, highly related to mammalian Wnt, is required for heart development, but the Wnt proteins of vertebrates are known to inhibit cardiogenesis in heart field and its inhibition can induce heart formation. Endogenous Wnt antagonists (Crescent, Dickkopf) are secreted from organizer or anterior endoderm, the layer adjacent to cardiogenic mesoderm, and required for heart field specification. On the contrary Wnt signaling is also required for mesoderm specification and development. The matter becomes more complex because both positive and negative regulation of cardiogenesis by Wnt has been described. Still it is elusive whether Wnt signaling plays a role in committing cells to adopt a cardiac fate or it is inducing some other cell types which in turn give signals for cardiogenesis.

We have established earlier stable ES cell lines that express fluorescent protein under the regulatory control of cardiac specific promoters. To understand the role of Wnt at different developmental stages of cardiac differentiation we have activated the Wnt signaling by Wnt3a conditioned media (WCM) at different time points with the readout being the percentage number of beating EBs as well the beating clusters per EB. While the cardiac differentiation was not hampered by CCM (control conditioned media), the continuous exposure of canonical Wnt signaling totally inhibited the cardiomyogenesis indicating the negative influence of canonical Wnt signaling on the cardiac differentiation process. We have also monitored the cardiac differentiation by examining the EGFP expression. The EGFP is expressed under the regulatory control of cardiac specific beta myosin heavy chain promoter thus allowing us to monitor the cardiac differentiation in live culture. The EGFP expression was also totally blocked by WCM continuous treatment but not by CCM (Fig. 3). The strategy has also helped us dissecting out the critical time window of Wnt action during cardiomyogenesis.

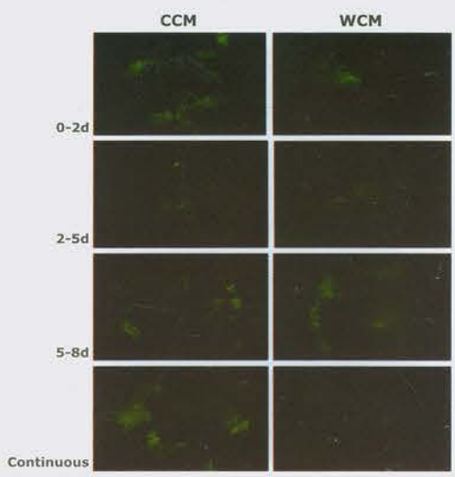

Fig. 3: EGFP expression profile of CCM/WCM treated EBs at d10 of differentiation.

To delineate further whether Wnt activation is keeping the cells remain undifferentiated and hence affecting cardiomyogenesis we conducted the experiments on ES cells prior to differentiation and investigated the role of Wnt signaling during ES cell maintenance. As expected, the colonies were compact and round when grown in presence of LIF, while in its absence cells were not maintained as undifferentiated and formed flattened colonies with some cell death in 6 days. Similar results were obtained when the medium was supplemented with CCM. However, WCM alone was able to maintain ES cell phenotype even in the absence of LIF for 6 days. This was further corroborated with OCT4 immunostaining on these cells. To assess further if Wnt could substitute LIF for long term maintenance of ES cells in an undifferentiated state, we passaged the ES cells using the stated conditions. Our data suggested that LIF is a mandatory requirement for ES cells sustenance. Interestingly, the cells grown in presence of WCM and without LIF also could maintain the undifferentiated status even after four passages (Fig. 4A), however, with lower cell count compared to LIF alone (Fig. 4B). Hence, we could deduce that, Wnt to certain extent could help the ES cells to maintain the undifferentiated state even in the absence of LIF, and thus addition of Wnt to differentiating culture might be blocking the differentiation. However, for long term cultures Wnt might not be potent enough to displace the requirement of LIF.

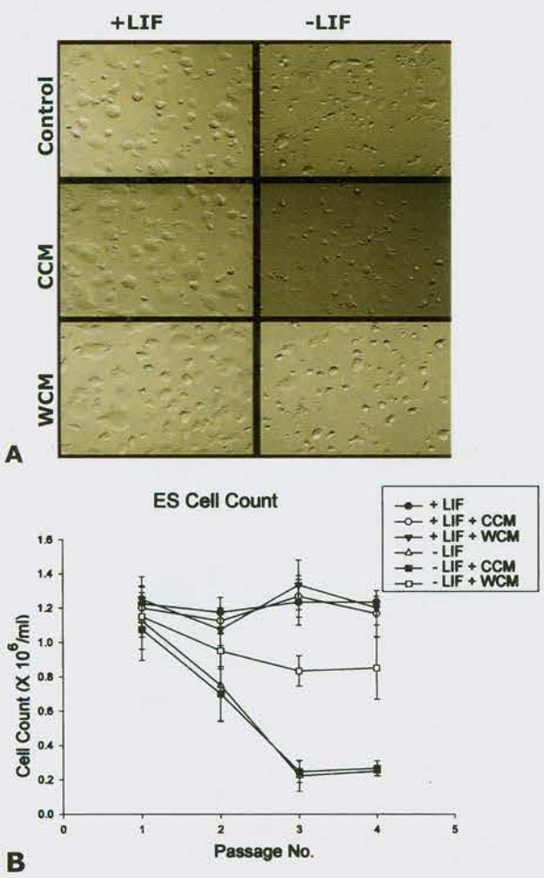

Fig. 4: ES cells growth characteristics in presence or absence of CCM/ WCM.

Future Work

1. Identification and characterization of dopaminergic neuronal specific progenitors.
2. Notch profile during the complete neural differentiation and its relation to the regulation of nestin expression in the progenitor state.
3. Dissecting the signaling cascades involved during neurogenesis.
4. Electrophysiological characterization and transplantation of the neural progenitors and dopaminergic neurons into Parkinsonian animal model and exploring their potential in functional recovery by the behavioral studies.
5. To study the molecular basis of Wnt signaling underlying ES cell differentiation into mesoderm and in particular the cardiac lineage.

Characterization of differentiation factor(s) in the perivitelline fluid of the Indian Horse Shoe Crab embryos

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Background

Our preliminary studies on perivitelline fluid (PVF) of Indian Horse Shoe Crab (Tachypleus gigas) indicated that PVF influences early vertebrate development. Chick embryo explants cultured *in vitro* were used as a model for this purpose. We found that fraction 7 of the PVF contains cardiac development promoting activity. We have sequenced and identified the active protein molecule. Due to possible relationship between cardiac development and angiogenesis, we have carried out preliminary experiments to assess the angiogenic potential of PVF.

Aims and Objectives

1. To evaluate fraction 7 of Perivitelline fluid of Indian horse Shoe Crab for angiogenic activity.

2. Study the expression of various cardiac developmental markers in embryos treated with 7th fraction of perivitelline fluid.

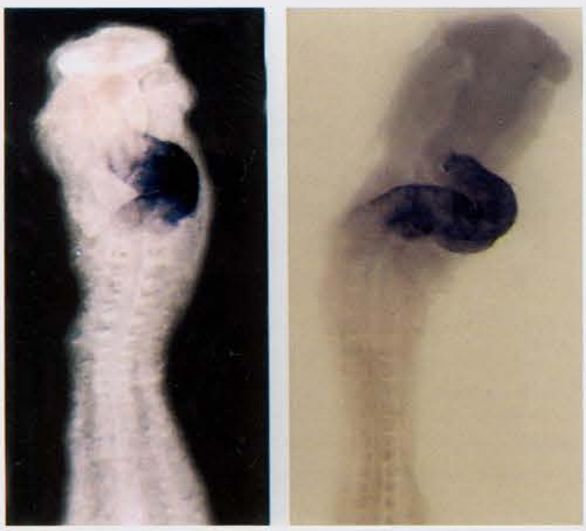

Fig. 1: VMHC (Ventricular Myosin Heavy Chain) transcripts in developing heart of control (left) and PVF fraction VII-treated (right) chick embryo. Note advanced development of heart due to PVF Fraction VII.

Work achieved

Elucidation of molecular mechanism by transcriptional studies

Transcriptional studies were carried out by quantitative real-time PCR for the markers Noggin (Signaling molecule, antagonistic to BMP2, recently shown to be involved in cardiomyogenic differentiation), GATA5 and Nkx2-5 (myocardial transcription factors governing commitment to the cardiomyogenic lineage) and aMHC (atrial Myosin Heavy Chain specific protein) and vMHC (ventricular Myosin Heavy Chain specific protein) both of these being cardiac muscle specific proteins. There was overall increase in the expression of these markers in stage 5 of chick development. Stage 7 of development showed a decrease in their overall expression and this trend was reversed in stage 10 of development of chick embryo.

Future work

To evaluate fraction 7 of Perivitelline fluid of Indian horse Shoe Crab for angiogenic activity.

Translational Regulation of Insulin mRNA

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Background

The pancreatic β-cell contains a large pool of cytoplasmic insulin mRNA (10–15% total mRNA), which is translationally quiescent at hypoglycemic (<3 mM) glucose concentrations. Recruitment of this mRNA to polysomes and the activation of translation occur in response to higher glucose levels, leading to a 50-fold increase in biosynthesis. It has been shown that together the 5'- and 3'-UTRs act cooperatively to markedly increase glucose-induced insulin biosynthesis. The mechanism by which this regulation occurs remains unclear. Translational regulation mostly occurs at the initiation step due to the interaction of specific proteins that bind to the 5'UTR with the translation initiation machinery. It is likely that elements within the 5'- and 3'-UTRs of insulin mRNA interact with specific proteins, to confer glucose-regulated insulin synthesis. Our hypothesis is that protein(s) bind to the UTR and regulate the translation by increasing the rate of translation initiation. The mechanism by which these proteins interact with the UTRs needs to be described to gain an understanding of the glucose stimulated translational regulation of insulin mRNA. We synthesized RNA corresponding to the 5'-UTR of rat insulin mRNA and have shown by RNA gel-shift assays, that a complex is formed upon incubation with RIN cell extract. We have also observed that a similar complex is formed by the protein extracts prepared from Rat pancreatic islets, and the complex formation is increased when the extracts are prepared from the islets treated with high glucose levels. Our preliminary RNA secondary structure analysis identified conserved structural elements including a stem loop structure in the 5'UTR of Rat insulin mRNA, that is important for the complex formation. We have characterized the protein that binds to the 5'UTR by UV-cross-linking studies. Further purification and identification of proteins binding to the UTR are under investigation.

Aims and Objectives

1. To identify the sequence and secondary structure elements in the 5'UTRs that is necessary for the RNA-Protein complex formation.

2. To Functionally characterize the RNA structural elements by *in vitro* translation assay.

3. Purification of the insulin mRNA UTR binding protein or protein complex by RNA affinity chromatography.

4. To understand the basic mechanism of translation regulation of insulin and the role of the 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 but the mechanism of this induction in translation is not completely understood. There have been suggestions that the un-translated regions (UTR) of the insulin mRNA are essential for this regulation. Rat insulin mRNA has 57 base

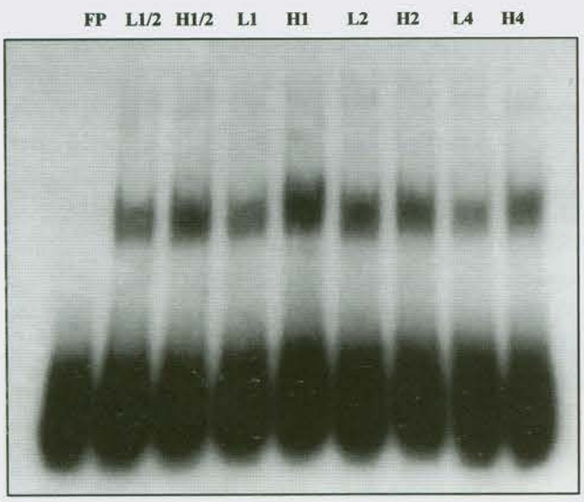
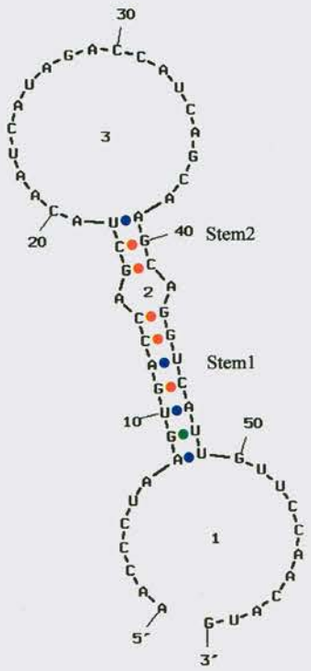


Fig. 1: Activation of RNA binding protein(s) in Rat pancreatic beta cells. Extracts were prepared from beta cells treated with low glucose (L) or high glucose (H) for ½, 1, 2 or 4 hours. 4µg of the protein was incubated with the radioactively labeled 5'UTR of the rat insulin gene1 RNA. The Complexes were resolved on 6% PAGE.



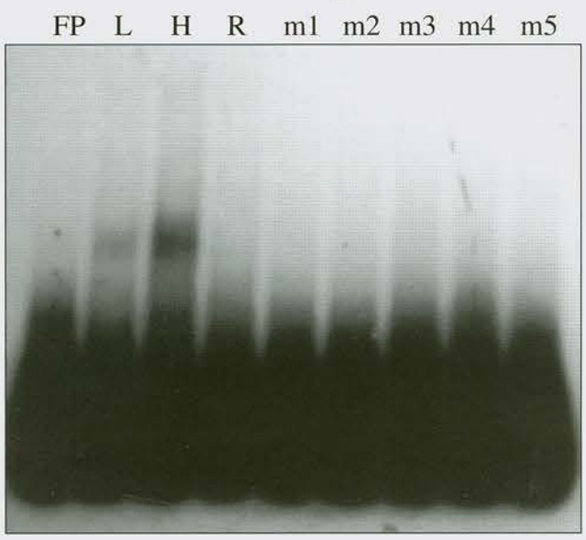


Fig. 2: Mutational analysis of the stem structure of the 5'UTR. RNA mobility shift experiments were performed with the radioactive rat insulin 5'UTR probe, and the beta islet extracts in the presence of unlabelled 5'UTR mutants. The mutants are m1: The stem1 sequence is changed to its complementary sequence, m2: The stem 2 sequence is changed to its complementary sequence, m3: both stem1 and stem 2 are altered, m4: the middle bulge between the two stem is altered to form a perfect long stem (A15 to U) and m5: conserved A19 to G in the loop region.

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 is also formed with extracts from rat pancreatic islets. The complex formation is induced by high glucose treatment of the islets (Fig. 1). We further synthesized 5'UTR with various deletions, and used them as cold competitors in the gel-shift assay to identify the sequences that are essential for the formation of the complex. Our analysis shows that the predicted stem loop structure is important for the complex formation. We further performed mutational analysis of the stem region to show that the stem structure is important for the formation of the complex but the sequence per se does not seem to be important (Fig. 2). We performed *in vitro* translation assay, using the luciferase reporter gene flanked by the rat 5' and 3'UTR, to show that the 5'UTR is functional as a translational activator in presence of the high glucose treated extracts (Fig. 3). UV-cross linking experiments reveal that a 60-80 kDa protein binds to the 5'UTR. Further characterization of this protein is in progress.

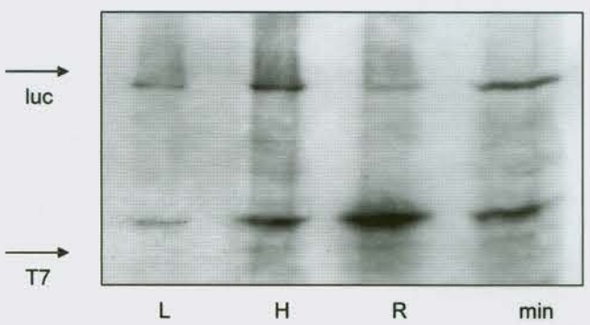


Fig. 3: Functional analysis of the rat insulin 5'UTR. RNA coding for luciferase reporter gene flanked by 5' and the 3' UTR of insulin mRNA was translated *in vitro* using the rabbit reticulocyte lysate, in the presence of low glucose (L) or high glucose (H) beta islet extracts. The induced translation was competed by the 5'UTR RNA but not by the non specific RNA (min). RNA coding for the T7 gene 10 was used as a marker for translation control for the retic lysate.

The mechanisms that underlie nutrient-induced translational regulation of insulin biosynthesis are likely to be crucial in 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 contributes to cell dysfunction and decreased availability of insulin.

Future Work

We would be characterizing the 5' UTR complex by gel filtration chromatography to identify the molecular weight of the complex. We have biotinylated the 5'UTR of the insulin mRNA and we are in the process of identifying the proteins that bind to the 5'UTR specifically.

Regulation of TNF-α-induced bone resorption by IL-3

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Background

Osteoclasts that have vital role in physiological bone remodeling also function in the local bone destruction that occurs in inflammatory bone diseases. Increased bone resorption by osteoclasts is a major pathological factor in arthritis, perodontitis, and orthopedic implant loosening. The differentiation and activation of osteoclasts are under the aegis of a variety of cytokines. The crucial factor for osteoclast differentiation and bone resorption is receptor activator of NF-κB ligand (RANKL). TNF-α also induces osteoclast differentiation and bone resorption *in vitro* independent of RANKL, and induces bone resorption *in vivo* by activation of osteoclasts. It is the key mediator for pathological bone loss in rheumatoid arthritis and other inflammatory diseases, and in association with accumulated proinflammatory cytokines such as IL-1, IL-6, PGE_2 and TGF-β, enhances bone resorption. Therefore, it is important to investigate the factors that control TNF-α-induced bone resorption.

IL-3, a cytokine secreted by T-helper cells, stimulates the proliferation, differentiation and survival of pluripotent hemopoietic stem cells. Although osteoclasts differentiate from hemopoietic stem cells, the role of IL-3 in osteoclast differentiation and bone resorption is not fully delineated. Recently, we have shown that IL-3 inhibits TNF-α-induced osteoclast differentiation (Yogesha *et al.* 2005, *J Biol Chem* 280: 11759-11769). These results indicated the potent inhibitory nature of IL-3 on osteoclast differentiation. However, the role of IL-3 on bone resorption, and cartilage and bone destruction *in vivo* is not yet known.

Aims and Objectives

1. To examine the role of IL-3 in TNF-α-induced bone resorption.

2. To investigate the mechanism(s) by which IL-3 regulates pathological bone resorption.

Work Achieved

IL-3 potently and irreversibly inhibits TNF-α-induced bone resorption

Effect of IL-3 was examined on TNF-α-induced bone resorption and osteoclast formation on bone slices. TNF-α induced bone resorption in a dose-dependent manner, which was inhibited by anti-mouse TNF-α antibody, and not by osteoprotegerin, the soluble decoy receptor for RANKL, suggesting that TNF-α induces bone resorption independent of RANKL (Fig. 1A). IL-1α, which is a strong stimulator of bone resorption, enhanced TNF-α-induced bone resorption in a dose-dependent manner. IL-3 inhibited both osteoclast formation (Fig. 1B), and bone resorption in a dose-dependent manner (Fig. 1C). Photomicrographs of bone slices in Fig. 1D show the significant inhibition of tartarate-resistant acid phosphatase (TRAP)-positive multinuclear cells and bone resorption by IL-3. These results suggest that IL-3 inhibits pathological bone resorption. Interestingly, the inhibition of TNF-α-induced bone resorption by IL-3 was irreversible. After removal of IL-3, TNF-α was unable to induce bone resorption even in the presence of IL-1. TNF-α and RANKL are present at suboptimal concentrations at the sites of inflammatory bone erosions, and minimal levels of one markedly enhance the bone resorption capacity of other.

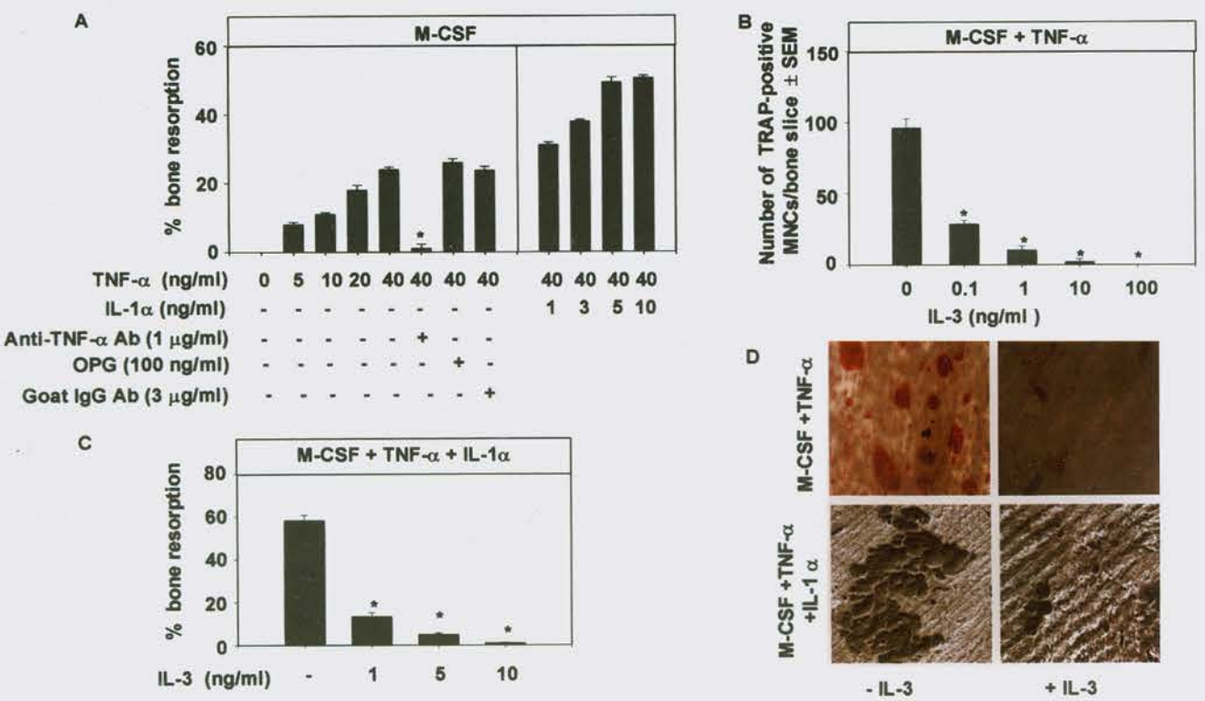


Fig. 1: IL-3 inhibits TNF-α-induced bone resorption. (A) TNF-α-induced bone resorption independent of RANKL and IL-1a enhanced bone resorption. (B and C) IL-3 inhibited both osteoclast formation and bone resorption in a dose-dependent manner (D) Osteoclasts on bone slices and resorption pits (magnifications, x20 and x250). Data are expressed as means ± SEM for six cultures. *$p < 0.01$ vs. control.

This synergy involves enhanced activation of intracellular signaling pathways necessary for osteoclastogenesis. In this study IL-3 significantly inhibited the synergistic effect of RANKL and TNF-α on bone resorption. Proinflammatory cytokines such as TGF-β, PGE_2, IL-1 and IL-6 are present at the site of inflammation and induces bone resorption directly or indirectly leading to more bone destruction. Increased bone resorption was seen when these cytokines were added to the cultures in the presence of TNF-α. To our surprise, IL-3 significantly inhibited bone resorption in the presence of these cytokines. These results indicate the potent inhibition of TNF-α-induced bone resorption by IL-3.

IL-3 inhibits expression of $\alpha_v\beta_3$ integrins and MMPs

Integrins α_v and β_3 are the principal cell/matrix attachment molecules and are most essential for osteoclasts to accomplish their bone destructive mission. A progressive increase in bone mass has been shown in β_3 knockout mice that occur due to dysfunctional osteoclasts, suggesting a crucial role of $\alpha_v\beta_3$ in bone resorption. Integrins α_v and β_3 also plays a critical role in the pathogenesis of rheumatoid arthritis. In this study, TNF-α-induced expression of both α_v and β_3 was significantly down-regulated by IL-3 (Fig. 2A). IL-3 also down-regulated the expression of both α_v and β_3 integrins on authentic bone slices (Fig. 2B). Osteoclastic bone resorption requires demineralization of bone followed by degradation of organic matrix by proteinases, which include cystein proteinases such as cathepsin K and MMPs. Recent evidences suggest that gelatinases MMP-2 and MMP-9 are expressed in osteoclasts, and play a major role in bone destruction in rheumatoid arthritis. IL-3 significantly inhibited the expression of MMP-9 at both mRNA and protein level (Fig. 2C). Also, our experiment using conditioned medium confirmed the inhibition of MMP-9 and MMP-2 enzyme activity (Fig. 2D). These results suggest that IL-3 inhibits important molecules necessary for bone resorption.

IL-3 inhibits TNF-α-induced AP-1 activation

To further investigate the molecular mechanism by which IL-3 inhibits TNF-α-induced bone resorption, effect of IL-3 was examined on activation of NF-κB and AP-1 transcription factors. NF-κB and AP-1 activated by TNF-α play a key role in osteoclast differentiation and bone resorption, and regulates the expression of many genes. NF-κB knockout mice are osteopetrotic because of arrest in bone resorption. In this study IL-3 did not affect TNF-α-induced NF-κB binding activity and nuclear translocation of p65, suggesting that IL-3 inhibits bone resorption without affecting NF-κB.

TNF-α is released into inflamed joints, and activates c-fos, an integral component of AP-1. Interestingly, IL-3 significantly inhibited TNF-α-induced AP-1 DNA binding activity, and specificity of AP-1 DNA binding was confirmed using excess unlabeled wild type and mutant oligonucleutides specific to AP-1 (Fig. 3A). These results suggest that IL-3 affects TNF-α-induced bone resorption by inhibition of AP-1 activity. Because IL-3 inhibited AP-1, we next examined whether IL-3 inhibits nuclear translocation of c-fos and c-jun, the key players of AP-1 complex. c-Fos, a member of the AP-1 family is essential for osteoclastogenesis, and mice deleted of this molecule are osteopetrotic. IL-3 inhibited TNF-α-induced nuclear translocation of c-fos on bone slices, however, showed no effect on c-jun (Fig. 3B). Our results strongly suggest that IL-3 inhibit TNF-α-induced bone resorption by abrogating c-fos/AP-1 activation. c-Fos/c-jun heterodimer (AP-1) is also important in regulating the expression of IL-1, IL-6, TNF-α, and collagenase, which are essentially important in rheumatoid arthritis, and overexpression of c-fos gene leads to joint destruction. Thus, inhibition of c-fos/AP-1 activation appears essentially important in arthritic joint destruction.

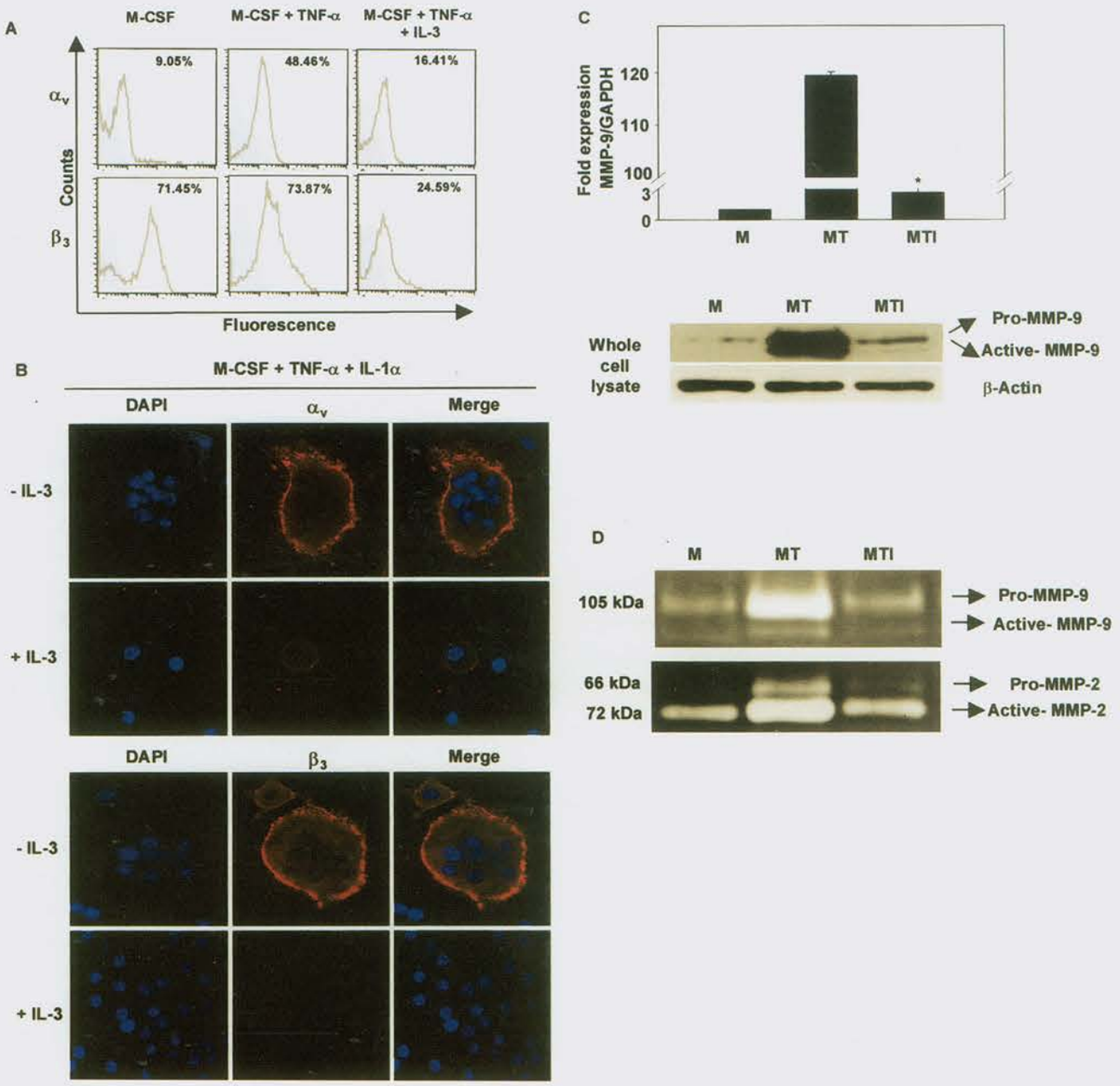


Fig. 2: IL-3 inhibits TNF-α-induced $\alpha_v\beta_3$, MMP-9 and MMP-2 expression. Cells were incubated with (A) M-CSF or M-CSF and TNF-α with or without IL-3 (10 ng/ml). Stained for α_v or β_3 (open profiles), or control IgG (filled profiles). (B) Cells on bone slices were incubated with M-CSF, TNF-α and IL-1α with or without IL-3, and analyzed for α_v and β_3 by immunofluorescence. Magnification, x63. (C) Total RNA was extracted and subjected to Real-time RT-PCR analysis for MMP-9 expression. Expression of MMP-9 was also analyzed by immunoblotting. (D) Enzyme activity of MMP-9 and MMP-2 by zymography. *$p < 0.01$ vs. control.

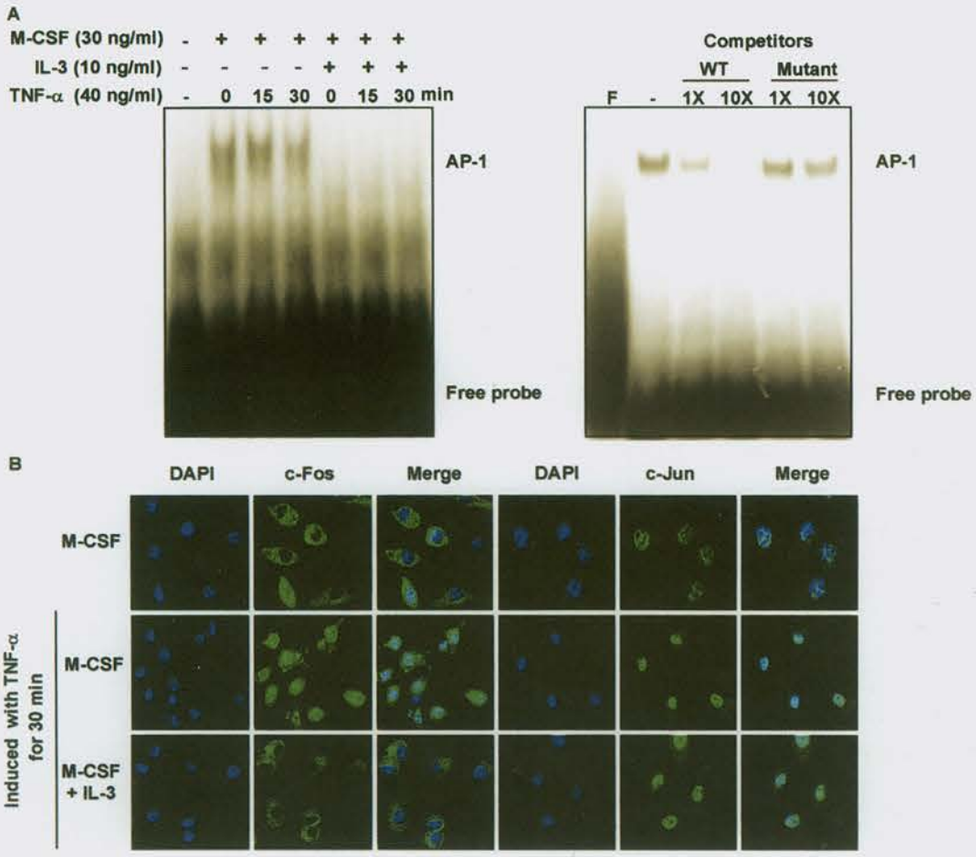
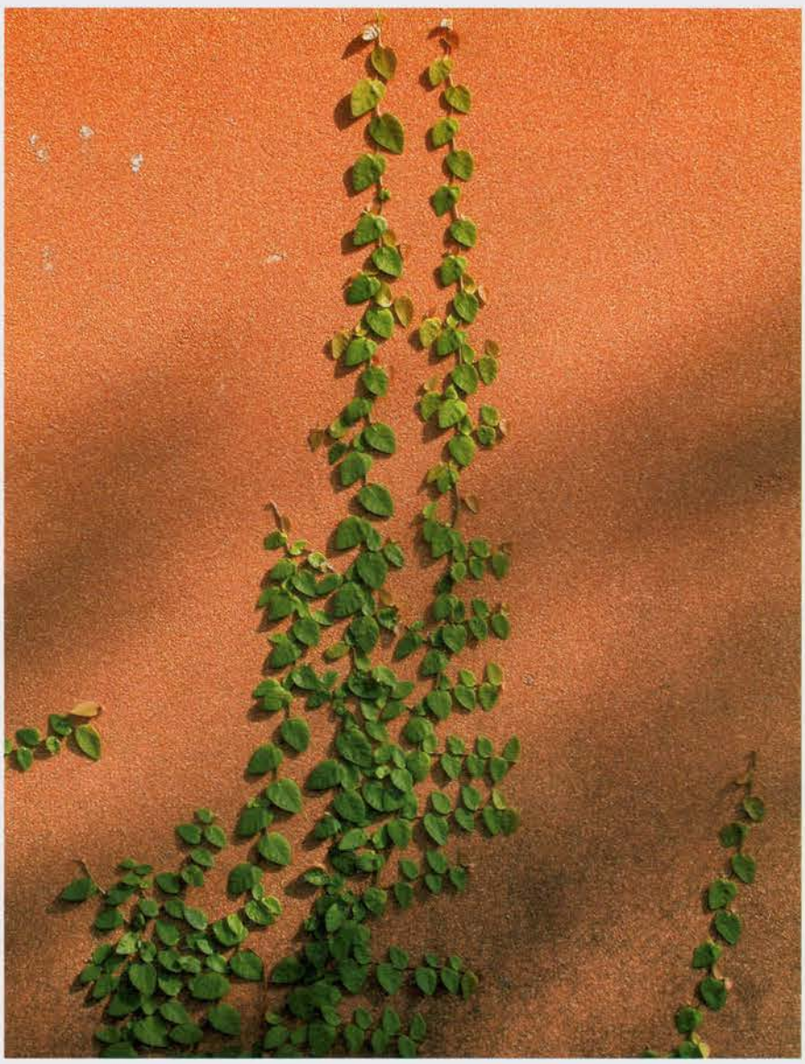


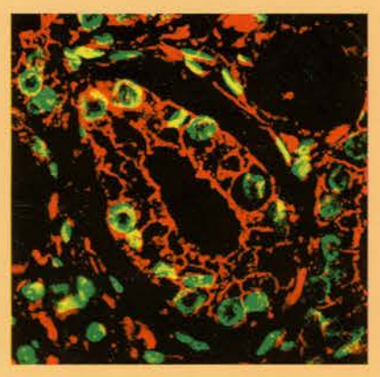
Fig. 3: IL-3 inhibits TNF-α-induced AP-1 activation. (A) Cells were incubated with M-CSF with or without IL-3 and starved for 8 hours before stimulation with TNF-α. Nuclear extracts were subjected to EMSA for AP-1 binding activity. Specificity of AP-1 binding was confirmed using cold competition with 1- and 10-fold excess wild type and mutant AP-1 oligonucleotides. (B) Nuclear translocation of c-fos and c-jun. Magnification, x63.

Future Work

Since IL-3 potently inhibited TNF-α-induced bone resorption even in the presence of other proinflammatory cytokines, we hypothesize that IL-3 may show *in vivo* inhibitory effect on pathological bone and cartilage loss in arthritis. We are currently investigating the *in vivo* role of IL-3 in prevention of inflammatory arthritis.



Cancer Biology



Mutational Profiling of Ovarian Tumor Stem Cells

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Background

Over the last few years, cancer is being viewed as a disruption of stem cell hierarchical organization brought about through transformation of stem cell populations. Such tumor stem cells (TSCs) were initially isolated from hematologic malignancies, and more recently, also in solid cancers including breast, lungs, neural and colon tumors. We have recently shown that human ovarian cancer may be a result of stem cell dysfunction. This was achieved through the establishment of a novel, comprehensive *in vitro* model system consisting of a single tumorigenic clone, yet another clone that underwent spontaneous transformation in culture, providing a model of disease progression, and 17 untransformed clones. On this background, we asked whether it would be possible to identify the heterogeneity within tumors as a cumulative effect of genetic drift and amplification of specific stem cell lineages.

Aims and Objectives

1. To trace the genetic heterogeneity between various stem cell lineages within a tumor, and

2. To understand the molecular differences between normal and tumor cells that could possibly define specific events within a stem cell lineage that places it on a trajectory towards tumorigenesis.

Work Achieved

Identification of a specific mitochondrial mutation profile associated with the Tumor Stem Cell population

Mitochondrial genome (mtDNA) analyses through PCR amplification and direct sequencing revealed that the primary tumor cells and ascites derived cells expressed identical mtDNA profiles, consisting of thirty-seven nucleotide variations distributed throughout the genome in the protein coding as well as non-coding genes. The same mutational profile was also associated with fourteen immortalised clones. We further conducted a chase-back study towards tracing the origin of these variations. Normal tissue sample of the same patient was unavailable; hence, a peripheral blood sample from the son was obtained - valid since mtDNA is almost exclusively maternally inherited. A comparison of profiles thus revealed that the above variations could be germline polymorphisms. Further, identification of a variant mtDNA profile in a small group of five clones (viz. A2, A3, A4, B2 and C4 – Fig. 1a) was achieved. This group includes the two tumorigenic clones A2 and A4, indicating that the profile defines the TSC lineage as a distinct identity amongst the others within the tumor. The mutant profile is characterized by twelve sequence variations that are distinct from the germline profile – (Fig. 1b). Acquisition of the mutant profile was accompanied by reversal of thirty-two variations expressed

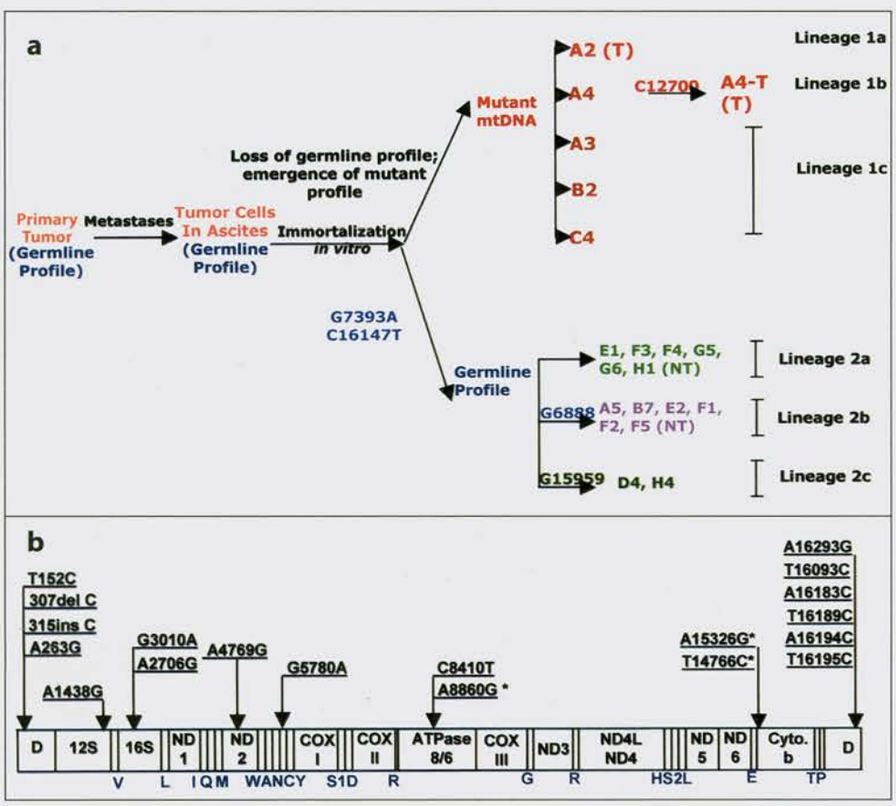

Fig. 1: a) mtDNA genome evolution in ovarian tumor samples and cell clones. b) Schematic representation of the mutant mtDNA profile marked on a linearized mitochondrial genome; * - sequence variations causing non-synonymous amino acid changes in coding genes.

in the germline profile (to reported sequences in the CRS – Cambridge Reference Sequence). The heteroplasmic state of some of these variations suggests that acquisition of the mutant profile and loss of some of the wild-type polymorphisms is a continuous ongoing process that ultimately would culminate in fixing of mutations to a homoplasmic state.

Sub-lineage demarcation within the germline and mutant mitochondrial profiles

Within the germline profile clones, lineage demarcation was evident. Six clones (E1, F3, F4, G5, G7 and H1) express the germline profile that may be surmised to define at least one stem cell lineage, six others (A5, B7, E2, F1, F2 and F5) show a variance through acquisition of an additional mutation – G6888R in the COX I gene resulting in a termination codon. The remaining 2 wild-type clones (D4 and H4) express another heteroplasmic mutation in the tRNA proline gene – G15959R, defining yet another lineage/sub-line emerging from the common, wild-type profile. Similarly, within the mutant profile lineage, demarcation into three different sub-lineages is also possible despite their similar mitochondrial profiles. A2 is functionally distinct due to its tumorigenic potential. A4 cells acquired an additional heteroplasmic, non-synonymous mutation (C12700T) in the ND5 gene: leucine to isoleucine that coincided with acquisition of tumorigenicity, while the remaining three clones (A3, B2 and C4) retained the profile with no change either in mtDNA sequence or tumorigenicity.

CREBBP exon mutations in the mutant mtDNA profile

From the above analyses in the mutant mtDNA profile clones, it became quite evident that acquisition of a particular mutant mitochondrial profile, although highly implicative of a signatorial association with the TSCs, cannot be the sole determinant of tumorigenicity. To resolve this, we further carried out mutational analyses of a nuclear tumor suppressor gene – CREBBP.

Significantly, no exon mutations were evident in the H4 genome that retained its wild type CREBBP profile. Within the five mtDNA mutant profile clones, 4 mutations in the nuclear hormone receptor domain (NHRD) of the gene, 8 in the CREB binding domain (KIX), 3 in exons 12-17, 3 in the bromodomain (BD) and 2 in the histone acetyl transferase (HAT) domain were identified (Fig. 2). The tumorigenic clones A2 and A4-T showed an identical non-synonymous mutation in the HAT domain (asparagine to isoleucine); another non-synonymous mutation (aspartate to asparagine) was also detected in this domain in the non tumorigenic clones B2 and C4. These mutations could be significant as the HAT domain mediates a key acetylation function of the protein and triggers off several downstream signal transduction pathways. In this particular instance,

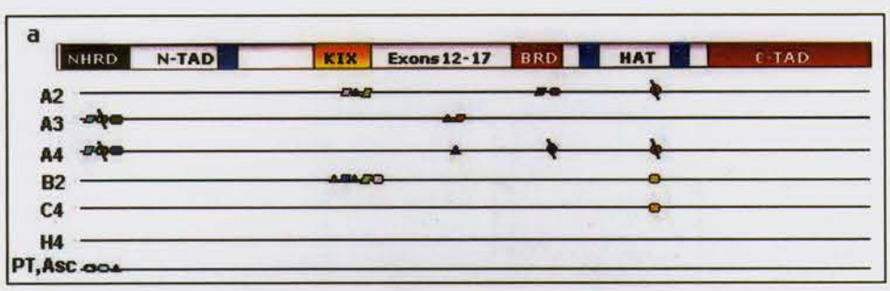

Fig. 2: Mutations in the coding region of the CREBBP gene. Functional domains are represented as boxes and include: NTAD (NH2-terminal Transactivation domain), KIX (CREB binding domain), BRD (Bromo domain), HAT (histone acetyl transferase domain) and CTAD (COOH Terminal transactivation domain)

the presence of a specific mutation coincidentally in the two tumorigenic/mtDNA mutant group clones is indicative of an altered protein with implied association of a predisposition or definitive role in tumorigenicity. Mutations in the bromodomain were also expressed exclusively by the two tumorigenic clones. Clones A3 (non- tumorigenic) and A4-T (tumorigenic) expressed two identical and one differential mutation each in the NHRD domain; one differential mutation was seen in each of the A2 (tumorigenic) and B2 (non-tumorigenic) clones in the KIX domain (KIX is the region where CREB binds and mediates the genomic effects of cAMP. The mutations in A2 and A4-T in HAT and bromodomains are suggestive of being responsible for the tumorigenic functions of these clones; the differential mutations between these clones in the NHRD and KIX domain could contribute to the qualitative differences during tumor formation and progression from these clones.

CREBBP Intron/Exon boundary (I/Eb) mutation analyses

The most striking intronic mutation was expressed in the five mutant clones within a polyA tract (A13) located at the tail end of intron 18 (IVS18). A successive deletion was expressed at this site in the five clones, with B2 (non-tumorigenic) having a single adenine deletion (delA), C4 and A3 (non-tumorigenic) showing an AA deletion and the two tumorigenic clones showing a triple AAA deletion (Fig. 3). These successive mutations, due to an attribute of being a part of the normal acceptor splice site, could have some significance in splicing; alternatively, they may serve as a definite marker for delineating the mutant clones within the tumor or cause dosage effects of the gene. The tumorigenic clone A4 expressed two more splice site variants in the acceptor site of intron 13 and 18 respectively. The latter may complement the earlier mutation (del AAA in IVS18), contributing further to the differential tumorigenicity between the A2 and A4-T clones. Similar kind of intronic mutations causing splicing defects has been reported earlier in several cancers.

Our study thus, clearly shows that TSCs have a distinctive mitochondrial – nuclear signature within a tumor, and gives a rare glimpse of changing mutational patterns accompanying varying stem cell dynamics and turnover within the organ during tumor evolution. These findings will be critical in addressing several themes that could lead to better optimization of therapeutic regimes for ovarian cancer. Further developments on the identification of continuing mutagenesis in subsets with the mutant phenotype could also provide a mechanism for monitoring minimal residual disease.

Future Work

To explore the functionality/relevance of the association of the mitochondrial germline profile clones with the tumor stem cell clones.

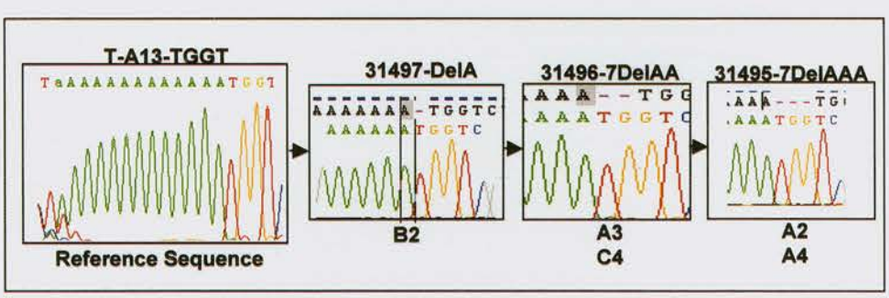

Fig. 3: Electrophoretograms depicting a putative hotspot at the Intron17/Exon18 boundary

Chemo sensitivity of cancer cells to drugs and mechanism of cell death

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Background

The importance of apoptosis induction in cancer therapy has been recognized and the ongoing discovery of numerous apoptosis-regulating proteins provides new potential targets for a molecular cancer therapy. DNA-damaging drugs are commonly used chemotherapeutic agents for the treatment of various cancers including HPV-positive cancers. In general, these drugs inhibit proliferation of sensitive tumor cells by the induction of apoptosis. Numbers of key factors are involved in the regulation, coordination and execution of apoptotic response by the cells. Drug sensitivity may depend on the relative intactness of these pathways in individual tumor cells. The necessary information about the proteins and the pathways involved in the cytotoxic action of a given drug may provide a better and more rational approach for the treatment of individual tumor type. Moreover, these studies may further provide the understating of the molecular basis of drug resistance in non-responsive tumors. Because of E6 viral – oncogene expression, HPV-positive cells are deficient in endogenous p53 function, which plays central role in DNA damage response of the cells. In these cell types, the status and role of p53 as well as other key regulators, in response to chemotherapeutic drugs are poorly understood. Present study is focused on investigating the involvement of Gadd45a, a key regulator of apoptosis, in Carboplatin or 5-fluorouracil induced apoptosis in HPV-positive cancer cells. Gadd45a is shown to be induced by a wide spectrum of DNA-damaging agents and implicated in negative regulation of cell growth.

Ideally chemotherapeutic drugs should specifically target only cancerous cells by inducing cytotoxic or cytostatic effects thereby decreasing the tumor growth without affecting normal cells. The effectiveness of chemotherapy has suffered due to the lack of specificity, rapid drug metabolism and both intrinsic and acquired drug resistance as well as induction of side effects due to high dosage. This produces transient decline in quality of life of the patients. These problems could be overcome by a low doses treatment of drugs in combination of other compounds/drugs, which show additive or synergistic cytotoxic effects. In addition, effectiveness could also be increased by enhanced uptake of these drugs. To increase the uptake and to improve efficacy of chemotherapeutic drugs,

plasma membrane is one of the most promising targets. It plays important role in drug influx or efflux and in cellular signaling. One of the integral components of cell membrane is cholesterol, which is essential for execution of these functions. Cholesterol depletion from plasma membrane shows increased permeability to both ions and small non-electrolytes. This makes various cholesterol depleting agents, a well-accepted tool to modify the structure and function of plasma membrane. Among these depleting agents, methyl-β-cyclodextrin (MCD) is reported as most effective agent for removal of cellular cholesterol.

Aims and objectives

1. To understand mechanisms of chemotherapeutic drugs mediated cells killings.

2. Investigate the molecules and molecular events that contribute or enhance the drugs induced cell death.

Work done

Susceptibility to chemotherapeutic drugs-Investigations on HPV E6 positive cells as a model

Understanding and unraveling the mechanisms of anticancer drugs-induced apoptosis is of prime importance not only for designing effective therapeutic interventions and development of novel cancer therapeutic strategies but also for monitoring cancer responses to chemotherapeutic drugs. In response to DNA damage, various molecular and cellular processes are activated as a part of the cellular stress response that result in cell cycle arrest and induction of the DNA-repair machinery to restore the damaged DNA or to activate cell death program. The tumor suppressor p53 has been demonstrated to play an important role in these biological events, in part through its downstream target genes. Among various downstream genes regulated by p53, the growth arrest and DNA damage-inducible gene alpha (Gadd45αacts as an important player in the control of G2-M checkpoint, repair and apoptosis. In addition, the potential roles of Gadd45α in malignant transformation and tumor progression have also been described. Studies so-far suggest that induction as well as function of Gadd45α is directly or indirectly regulated by p53. Since, in HPV-positive cells p53 function is abrogated by endogenous E6 expression; the involvement of p53 in Gadd45α expression as well as the function of Gadd45α in response to DNA damage is not very clear. We demonstrate that Gadd45α promoter is partially regulated by p53-functions in HPV-positive cells. Importantly, the functions of induced Gadd45α in response to DNA-damage stress may vary with the cell type, because in the present study, apoptosis induced by Carb or 5-FU was found to be independent of Gadd45α functions in HPV-positive HEp-2 and HeLa cells. Therefore, we propose that even though Gadd45α up-regulation occurs as a consequence of apoptotic response to genotoxic stress, its significance in the induction of apoptosis is solely dictated by the nature of stress and probably by the cell type differences.

Investigate the compound/Drug and related molecular events that contribute or enhance drugs induced cell death

The response rates of extensively used chemotherapeutic drugs, carboplatin (Carb) or 5-fluorouracil (5-FU) are relatively disappointing because of considerable side effects associated with their high-dose regimen. In the present study we determined whether treatment with a cholesterol depleting agent, methyl-β-cyclodextrin (MCD) enhances the weak efficacy of low doses of Carb or 5-FU in human breast cancer cells. Data demonstrates that pretreatment with MCD significantly potentiates the cytotoxic activity of Carb and 5-FU in both MCF-7 and MDA-MB-231 (Fig. 1). Further we explored the molecular basis of enhanced cytoxicity and our data revealed that low-dose treatment with these drugs in MCD pretreated cells exhibited significantly decreased Akt phosphorylation, NF-κB activity and down-regulation in expression of anti-apoptotic protein Bcl-2 (Fig. 2). In addition MCD

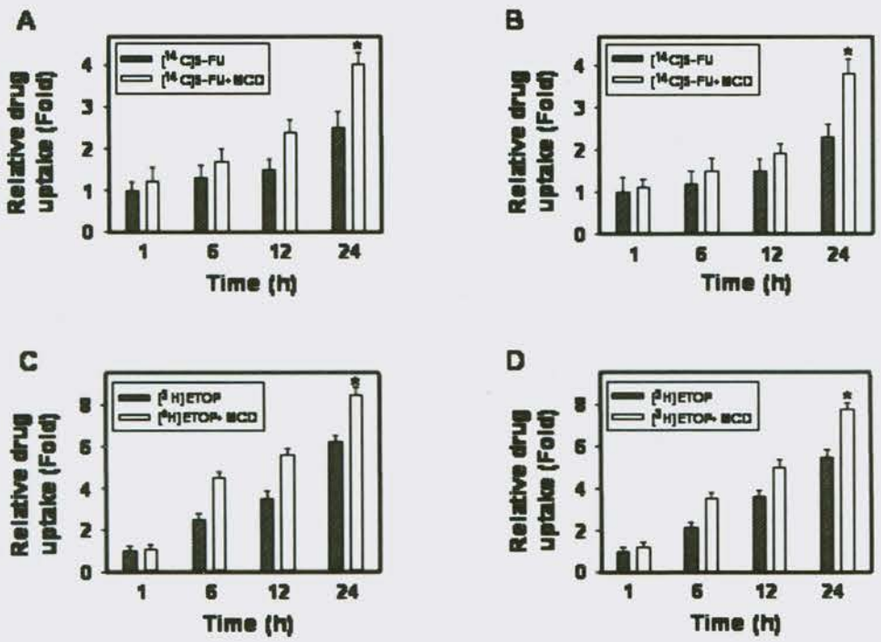

Fig. 1: MCD enhances intracellular drug accumulation in MCF-7 and MDA-MB-231 cells. (A) MCF-7 or (B) MDA-MB-231 cells were incubated with 5-FU (C14) for indicated time either in presence or absence of pretreatment with 5 mM MCD. (C) MCF-7 or (D) MDA-MB-231 cells were incubated with etoposide [ETOP (H^3)] for indicated time either in presence or absence of pretreatment with 5mM MCD Intracellular drug accumulation was quantified by a Top Count Micro plate Scintillation Counter (Pacard, Albertville, USA) and results represent the mean ± S.D. of at least three independent experiments. The * indicates that mean value is significantly different ($P<0.05$).

pretreated cells demonstrated an increased intracellular drug accumulation as compared to cells treated with drugs alone. Taken together, our data provides the basis for potential therapeutic application of MCD in combination with other conventional cytotoxic drugs to facilitate reduction of drug dosage that offers a better chemotherapeutic approach with low toxicity.

Future work

Studies on HPV E6 positive cancer cells will be continued and these investigations will be expanded to other cell lines derived from various cancers.

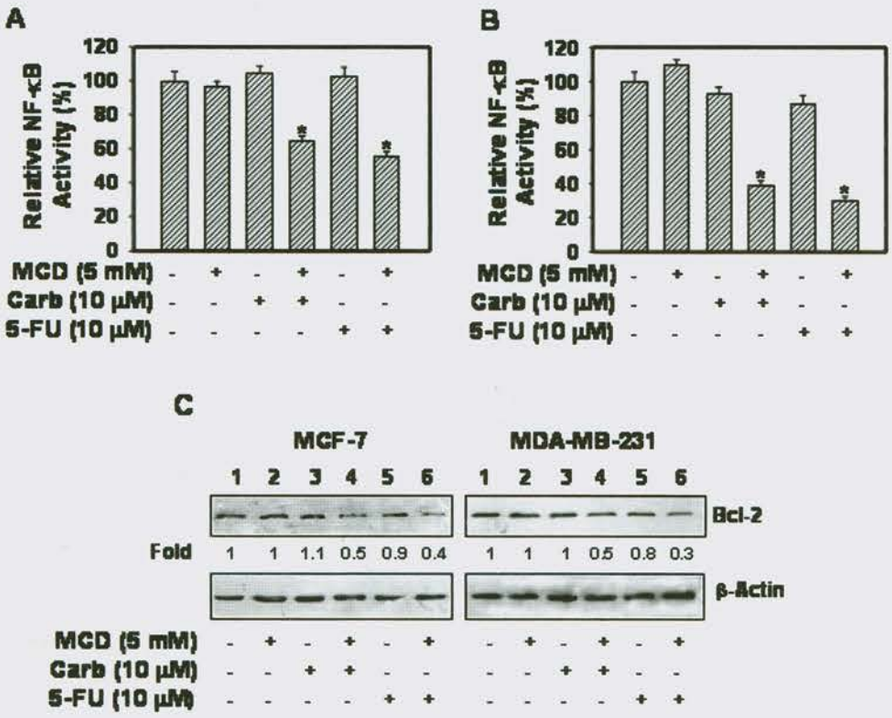

Fig. 2: Drug treatment down-regulates the transcriptional activity of NF-κB and Bcl-2 protein levels in MCD pretreated cells. (A) MCF-7 or (B) MDA-MB-231 cells were transfected with pNF-kB-Luc construct and treated with low-dose (10 μM) of Carb or 5-FU either in presence or absence of MCD pretreatment as indicated. Equal amount of protein was taken for NF-κB luciferase activity. Luciferase activity (normalized to EGFP expression) is reported as % of arbitrary relative light units by taking control as 100%. Results represent the mean ± S.D. of at least three independent experiments. The * indicates that mean value is significantly different ($P<0.05$). (C) MCF-7 and MDA-MB-231 cells were treated with (μM) of Carb or 5-FU either in presence or absence of MCD pretreatment as indicated. Cells were harvested and processed for western blot analysis. Expression levels of Bcl-2 protein were determined by quantitation of bands using densitometry scanner analysis (Phosphorimager, Bio-rad, California, USA). Fold expressions are with reference to untreated cells (control=1).

Signaling pathways in receptor mediated cell death in human glioma cells

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Background

Gliomas are the most common of the primary brain tumors in adults. In gliomas, macrophages are recruited and remain at the site of tumor constituting a major proportion of the tumor mass. The activated macrophages secrete a wide variety of molecules that affect the survival, growth and proliferation of the tumor. Factors released by the activated macrophages such as cytokines contribute largely to the effect of the macrophages residing in the tumor. While some cytokines have cytocidal effect and inhibit tumors, others can promote their growth and proliferation. Studies towards understanding the specific role(s) of the factors involved in manipulation of tumors will be important in development of novel chemotherapeutic agents.

Aims and Objectives

1. To study the role of cell death receptor ligands in gliomas with reference to proliferation and cell death using monolayers and spheroids as models and

2. To decipher the TNF-α mediated signaling pathways in monolayer and spheroids generated from gliomas.

Work Achieved

The Cyclin dependent kinase inhibitors (CDK) p21 cip/waf1 and p27 kip1 are important in cell cycle progression in gliomas. p21 is a universal inhibitor of cell cycle progression and is up-regulated in G0/G1 as well as G2/M arrest. We examined the effect of TNF-α on the expression of these CDKI proteins in the monolayer and spheroids generated from wild type and IkBa mutant LN18 cells. The expression of p21 was below the level of detection in the cells and on TNF-α treatment the levels in monolayers was marginally increased while the expression was strongly enhanced in the spheroids from wild type cells. In contrast, p21 was not detected in the mutant cell line and TNF-α treatment did not induce the protein p27 was expressed in the monolayers and increased drastically with spheroidogenesis and activation with TNF-α resulted in further up regulation of p27 protein expression in both the culture systems. Experiments with mutant cell line yielded similar results (Fig. 1).

Time kinetics for expression and localization of p21 and p27 was done by Confocal Laser scanning Microscopy (CLSM). For detection of p21, an antibody that recognizes p21 in the nucleus as well as cytoplasm was used. The staining pattern showed that p21 was localized in the nucleus and was induced from 3 hr post-treatment with

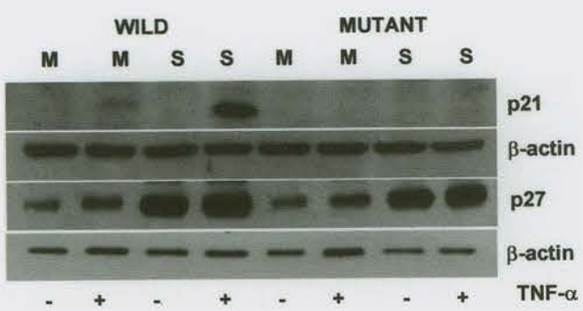

Fig. 1: Expression of p21 and p27 in LN-18 wild and mutant cell lines. Cells from monolayers (M) and spheroids (S) derived from in LN-18 wild and mutant cell lines were treated with TNF-α (10 ng/ml) for 3hr and lysates were analyzed by Western blotting for p21 and p27 expression.

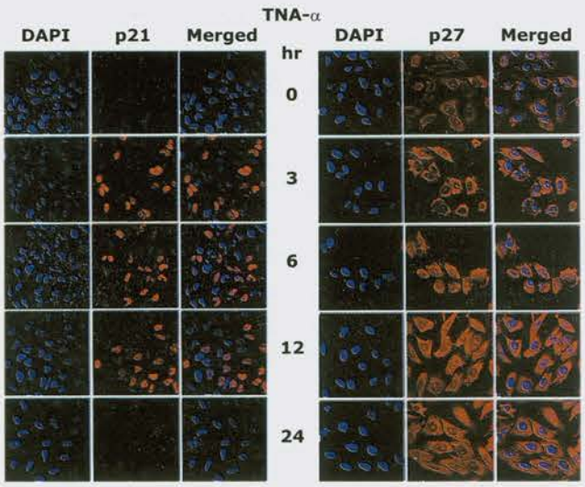

Fig. 2: Time dependent expression and localization of p21 and p27 in LN-18 cell line. Cells were treated with TNF-α (10 ng/ml) for 3, 6, 12, and 24 hr and analyzed by Confocal Laser scanning Microscopy (CLSM).

TNF-α and the expression was lost by 24 hr. The untreated LN-18 cells stained positive for p27 with predominantly cytoplasmic staining, and increase in the intensity was seen with TNF-a treatment from 3 hr onwards in wild type cell line (Fig. 2). The results suggest that p27 might be important in growth arrest in the spheroids and on TNF-α treatment. Localization of p21 in the cytoplasm and its phosphorylation has been reported to correlate with its anti-apoptotic function. In this study while p21 was inducible on stimulus with TNF-α, it was not found to have antiapoptotic function suggesting that it might not contribute to the resistance to TNF-α mediated cell death in glioma cells.

Future Work

Further will be focused on identifying the survival pathways that might contribute to resistance to TNF-a mediated cell death in gliomas.

Studies on a novel non-coding RNA – M3TR

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Background

The melanocytes are the neural crest derived pigment producing cells. Melanoma, the most fatal cancer of all, arises due to malignant transformation of melanocytes. In an endeavor to study novel genes in melanoma genesis, an expression cDNA library was generated from mouse melanoma cells – Clone M3. From the Clone M3 cDNA an entirely new kind of molecule – M3TR was identified that was unconventional in the sense that it was a non-coding RNA and yet displayed the ability to transform cells. The ncRNAs constitute RNAs that are not translated into proteins. The ncRNAs are present in organisms ranging from bacteria to mammals, and affect a large variety of processes including plasmid replication, phage development, bacterial virulence, chromosome structure, DNA transcription, RNA processing and modification, dosage compensation, genomic imprinting, and development control.

Aims and Objectives

1. Molecular characterization of the non-coding RNA-M3TR
2. Study of the biological function of the M3TR RNA
3. Understanding the mechanism of gene regulation and cell signaling mediated by M3TR RNA

Work Achieved

The M3TR gene identified by us from mouse melanoma cells was a non-coding RNA and was characterized as a partial cDNA clone by the Northern hybridization data. The human homologue of the mouse M3TR was identified from human Neuroblastoma cells, cloned and sequenced. The gene sequence was found to be conserved across evolution in species ranging from human to zebrafish. The human gene was found to possess similar transforming potential as the mouse homologue. When expressed ectopically it could induce anchorage independent growth *in vitro* and tumorigenicity *in vivo*. Expression studies with real time PCR showed its restricted expression to fetal and adult brain, embryo and tumor cell-lines of neuroectodermal and lymphoid origin. Tumors generated from *M3TR* displayed rapid tumor progression and high grade anaplastic growth. Interestingly, the cells cultured from the tumor showed growth of two kinds of cells – one was the melanocyte-like population and second was the fibroblast population growing from the explants. This finding suggested an important role of *M3TR* in induction and maintenance of stemness. Experimental data indicated the transforming function of *M3TR* was mediated by it acting as a microRNA or siRNA. We provide experimental evidence to show that the M3TR SiRNA upon exogenous expression induced creation of DNA double stranded breaks, followed by constitutive activation of ATM-Gamma H2AX1/p53 pathway, failure of checkpoint control, induction of replicative stress, genomic instability and eventually tumorigenesis. The following mechanistic model is proposed to delineate the steps involved in transformation mediated by *M3TR* (Fig. 1).

Future Work

We plan to study expression and role of M3TR in human brain tumor biopsies like gliomas and tissue samples from neurodegenerative cases during surgeries to provide us with an additional insight to arrive at a definitive role of *M3TR* in disease, development and normal homeostasis.

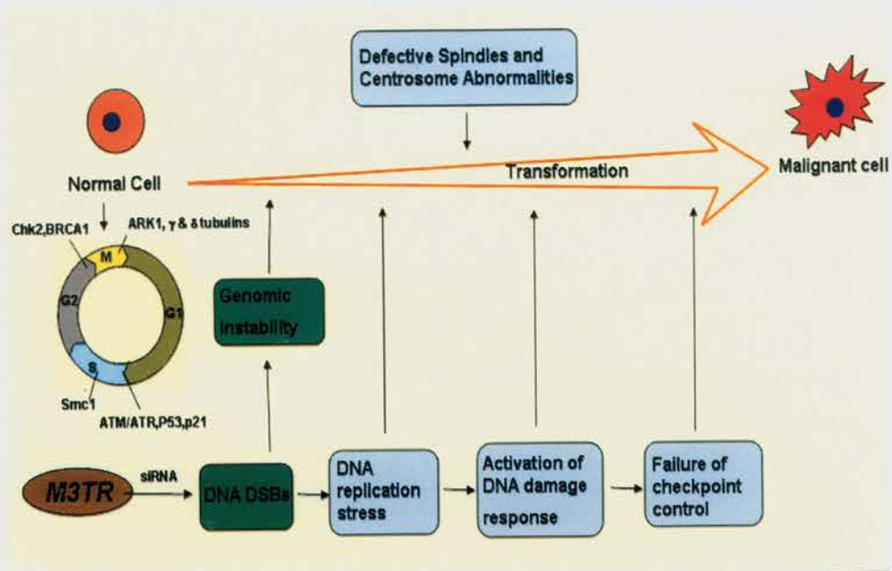
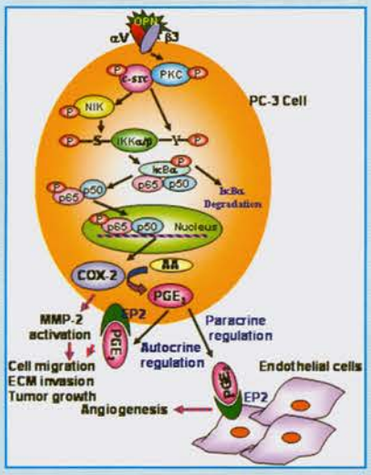


Fig. 1: Genomic instability contributes to transformation induced by M3TR

Signal Transduction



Cyclooxygenase-2: a key regulator in Osteopontin-induced Prostate Tumor Progression and Angiogenesis

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Background

Prostate cancer is one of the major causes of death in men especially in western world. Prostate cancer progression is a series of complex events, which require crosstalk between several oncogenic molecules, and enable the cancer to spread and evoke angiogenesis. High-level of constitutive cyclooxygenase-2 (COX-2) expression has been detected in colorectal, gastric, pancreatic, head and neck, lung, breast, and in other cancers. COX-2 is induced in many cell types by mitogens, growth factors, cytokines and tumor promoters and its increased expression is associated with cancer progression through prostaglandin-dependent manner. Osteopontin (OPN), a member of SIBLING (small integrin binding ligand N-linked glycoprotein) family of chemokine like protein that plays significant role in determining the oncogenic potential of various cancers and is recognized as a key marker in the processes of tumorigenicity and metastasis. Previous studies have indicated that c-Src and PKCα play crucial roles in COX-2 expression and COX-2 dependent prostate tumor progression. We have recently reported that OPN induces uPA secretion and MMP-2/-9 activation through c-Src/PI 3-kinase/MAPK signaling pathways in breast cancer and melanoma cells. However, the molecular mechanism by which OPN regulates PKCα/c-Src dependent IKK mediated NFκB activation, which ultimately regulates tumor progression, and angiogenesis through induction of COX-2 expression in prostate cancer and signaling cascades underlying these processes are not well defined.

Aims and Objectives

1. To examine whether OPN induces $\alpha_v\beta_3$-integrin mediated PKC/c-Src/IKK dependent NFκB activation in prostate cancer (PC-3) cells

2. To study whether OPN regulates PKC/c-Src dependent COX-2 expression and COX-2-mediated PGE_2 production and MMP-2 activation in prostate cancer cells

3. To delineate whether OPN-induced COX-2 regulates cell motility, angiogenesis and tumorigenesis of prostate cancer through both autocrine and paracrine mechanisms and whether the increased expressions of OPN and COX-2 correlate with enhanced MMP-2 expression and angiogenesis in prostate cancer specimens of various grades.

Work Achieved

In this study, we have shown that OPN regulates PKCα/c-Src/IKK/NFκB signaling cascades leading to COX-2-mediated PGE_2 production and MMP-2 activation in prostate cancer (Fig. 1). Furthermore, we have demonstrated that OPN-induced COX-2 regulates cell motility, angiogenesis and tumorigenesis of prostate cancer through both autocrine and paracrine pathways. However, suppression of COX-2 activity by nonsteroidal anti-inflammatory drug (NSAID)

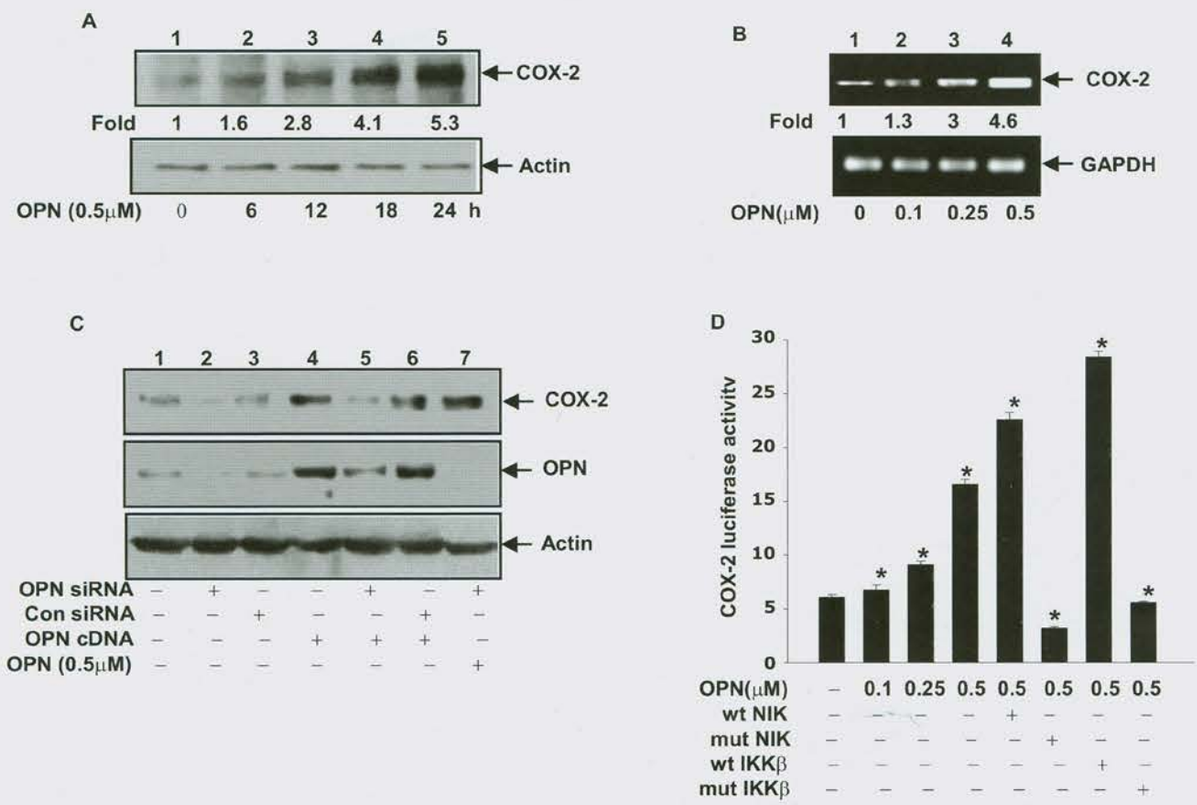

Fig. 1: OPN-induces COX-2 promoter activity and expression in PC-3 cells. A, PC-3 cells were treated with OPN for 0-24 h. The level of COX-2 was analyzed by western blot. B, cells were treated with OPN for 6 h and total RNA was isolated and RT-PCR analysis was performed. C, PC-3 cells were transfected with OPN cDNA, OPN specific siRNA or nonsilencing scrambled siRNA. In separate experiments, OPN siRNA transfected cells were either cotransfected with OPN cDNA or treated with OPN. COX-2 and OPN were analyzed by western blot. D, OPN enhances NIK/IKK-mediated COX-2 promoter activity. Cells were transfected with luciferase reporter construct (COX-2-Luc) and then treated with OPN or cotransfected with wt and mut NIK or wt and dn IKKβ and then treated with OPN and luciferase activity was measured.

celecoxib or blocking the interaction between PGE_2 and its receptor EP2 by using specific anti-EP2 blocking antibody significantly suppressed OPN-induced *in vitro* cell motility, invasiveness and *in vivo* tumor growth. Moreover, the clinical data indicated that the increased expressions of OPN and COX-2 correlates with enhanced MMP-2 expression and angiogenesis in prostate cancer specimens of higher grades (Fig. 2A). Consequently, OPN plays important and essential role in two key aspects of tumor progression: (i) COX-2-mediated PGE_2 production and MMP-2 activation by tumor cells (ii) COX-2/PGE_2-mediated tumor progression and angiogenesis (Fig. 2B). These data suggest that blockade of OPN and COX-2 is a promising therapeutic approach for the inhibition of tumor progression by suppressing prostate tumor growth and angiogenesis.

Future Work

The roles of COX-2 and PGE_2 in OPN regulated prostate tumor growth and skeletal metastasis will be studied.

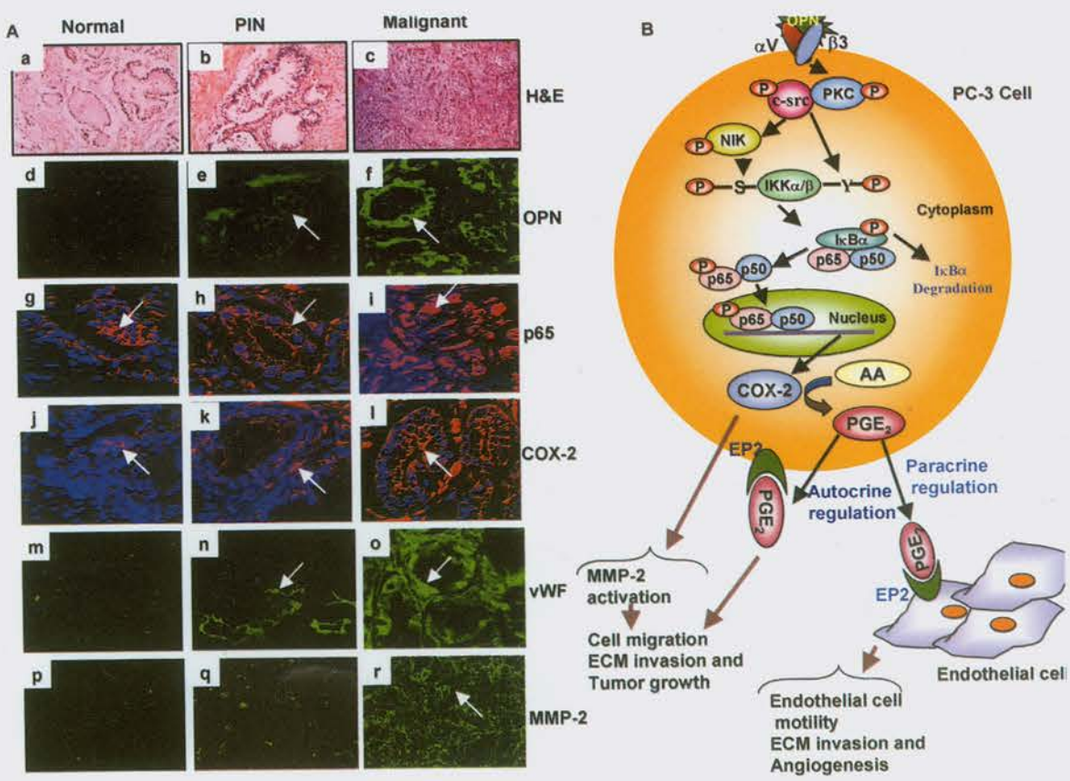

Fig. 2: Expressions of OPN, COX-2, MMP-2 and NFκB, p65 localization and their correlation with human prostate cancer progression and angiogenesis in different pathological grades. A, prostate tumor specimens were stained with H&E (a-c). The levels of OPN (d-f), COX-2 (j-l), vWF (m-o) and MMP-2 (p-r) expression and cellular localization of p65 (g-i) were detected by immunohistochemical studies using their specific antibodies. B, Schematic representation of OPN-induced PKCα-c-Src-IKK-NFκB-mediated COX-2 expression leading to enhanced PGE_2 production and MMP-2 activation that further induces tumorigenesis and angiogenesis via autocrine and paracrine mechanisms.

Stress-induced phosphorylation of caveolin-1 and p38, and downregulation of EGFr and ERK by dietary lectins

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Background

To date, several plant lectins have been exploited to understand the nature of glycosylation on cell surface molecules due to their distinct carbohydrate binding property, while the consequences of their binding to mammalian cell surfaces is beginning to be understood in detail. It has been noted that plant lectins also affect the proliferation of cells, probably through binding to specific carbohydrate moieties, thereby affecting the function of mammalian cells. Although several lectins have been shown to be mitogenic to T cells, or B cells, or both, the effect of the lectins on cells of other lineage vary. For example, native jacalin is mitogenic to T cells and has been shown to up-regulate extracellular signal regulated kinase (ERK) phosphorylation in CD4+ T cells, although it is a noncytotoxic inhibitor of proliferation of HT29 cells and it also down-regulates ERK phosphorylation in the same cells. If one compares the different cellular responses of PNA (proliferation of HT29 cells), ABL (behaves like jacalin), and nJacalin on HT29 cells, even though these lectins have nearly the same sugar specificity i.e., toward the human malignancy-associated Thomsen-Friedenreich disaccharide (TF disaccharide: Galβ1-3GalNAcα]), their biological effect on the same cell differs. This suggests that the effect of plant lectins on the cellular response may be governed by other factors in addition to their sugar-binding property. In this regard, the study of plant lectins with respect to understanding mammalian cell signaling pathways appears to be necessary, because plant lectins are an abundant part of the human diet. In summary, these observations raise several important questions: Why do the same cells respond differently to different lectins despite similar carbohydrate specificity? Is there any role for a protein backbone in the elicited cellular response? Is the cellular response merely due to stress, and if it is, what are the pathways and molecules involved? A detailed understanding of cellular stress orchestrated by plant lectins on mammalian cells is required to delineate the pathways that will shed light on cellular stress and its eventual consequences (i.e., to survive, proliferate, or die).

We examined the effects of all three lectins on cell proliferation, membrane integrity, and phosphorylation status of stress markers such as caveolin-1 and p38, and JNK along with epidermal growth factor receptor (EGFr) phosphorylation. Our studies indicate that the

jacalin lectin exerts reversible stress on A431 cells (i.e., it induces the phosphorylation of caveolin-1 and p38 but not JNK, whereas PNA, which has very similar specificity to that of jacalin, did not induce the same). Our results suggest that the jacalin-modulated effects might be due to manipulation of cell surface chaperones whose role/function is yet to be understood.

Aims and Objectives

Initial investigation and identification of cellular stress pathways modulated by proteins that bind to TF disaccharide containing proteins.

Work Achieved

We specifically chose TF disaccharide–binding lectins for the present study because TF disaccharide is an oncofetal carbohydrate antigen known to result in an increased expression in malignancy and hyperplasia. In the present study, jacalin was found to be a noncytotoxic inhibitor of proliferation of HT29 colon cancer cells. The same lectin is, interestingly, cytotoxic to A431 epidermoid carcinoma cells. However, both HT29 and A431 recover after removal of the lectin. An interesting observation that emerged from this experiment is the cellular responses evoked by recombinant single-chain jacalin (rJacalin). We recently reported that this single-chain rJacalin has 1000-fold less affinity for TF disaccharide in comparison to the two-chain nJacalin. However, both native as well as recombinant jacalin evoked a nearly identical magnitude of cytotoxic responses as far as their effects on A431 and HT29 cell proliferation are concerned. Hence, it is possible that factors apart from the carbohydrate binding property of jacalin also contribute to the observed cellular responses, because both nJacalin and rJacalin with 100-fold differences in affinity (overall difference for carbohydrates in general) evoked the same magnitude of cytotoxic response.

Jacalin treatment results in morphological changes, and changes in the phosphorylation status of essential signaling molecules like EGFr, ERK, caveolin-1 and p38 (see Fig. 1). These changes are apparently due to cellular stress which is similar to the observations on NIH3T3 cells after treatment with 600 mM sucrose. We could not observe any detectable level of caveolin-1 phosphorylation upon PNA treatment of A431 cells. These observations once again suggest a differential mode of interaction between jacalin and PNA to target cells despite similar carbohydrate specificity.

To investigate the putative proteins that jacalin or PNA can bind on the A431 cell surface, we passed the A431 cell lysate through jacalin- and PNA-immobilized sepharose columns and the bound proteins were analyzed by mass spectrometry. Mass spectrometric analysis of the jacalin eluate exhibited the presence of ORP150, a novel ER chaperone that contains the TF disaccharide along with several proteins that belong to heat shock family. These data suggested that jacalin-induced cytotoxicity is mediated through ORP150, and that impairment of ORP150 functions with the help of jacalin, makes the cells more susceptible to death due to stress. In view of the same magnitude of cytotoxicity evoked by rJacalin, it is possible that the initial recognition between jacalin and ORP150 could be due to carbohydrate binding, which might be further strengthened by protein-protein interactions. These data also suggest that jacalin might interact with ORP150 and this could be one of the proteins that might have played a role in the jacalin mediated cytotoxicity of A431 cells.

Four important points have emerged from this study. First, lectins with similar carbohydrate specificity evoke different cellular responses, especially stress, depending upon the lineage of the cell line (see Fig. 1). Second, this cellular response could not be entirely due to high affinity interaction between lectin and carbohydrate, but other factors such as lectin-protein interactions may also contribute to the observed cellular responses as

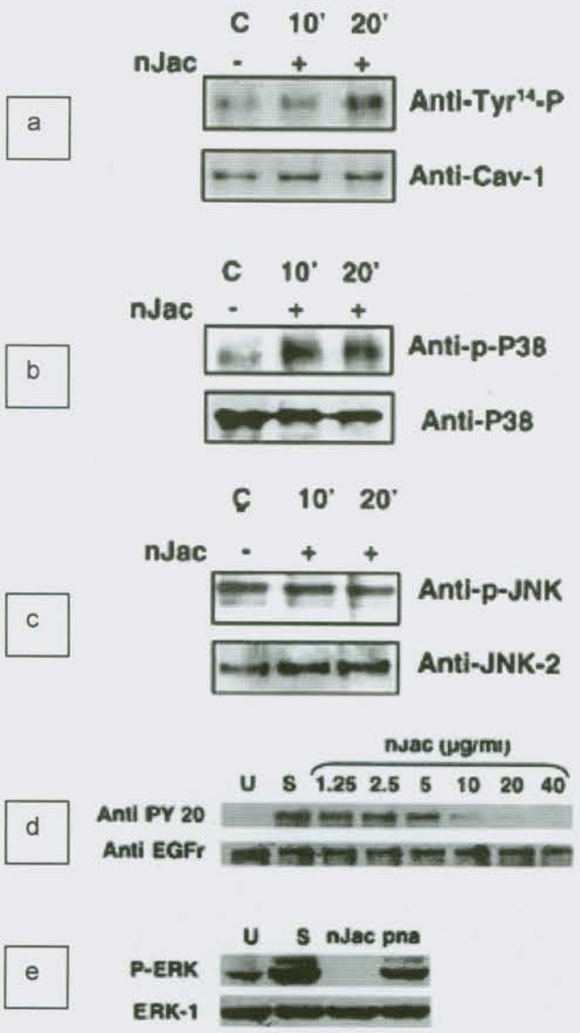

Fig. 1: Phosphorylation status of caveolin-1, p38, JNK, EGFr and ERK after jacalin treatment. (From top) a) Time-dependant phosphorylation of caveolin-1 after treatment of nJacalin. b) Time-dependant phosphorylation of p38 after treatment of nJacalin. c) Stress-induced phosphorylation of JNK after treatment of nJacalin. d) Concentration dependence of phosphorylation on EGFr after the indicated concentration of jacalin treatment followed by stimulation with TGFα. Panels lanes designated with a U and an S indicate unstimulated cells and cells stimulated with TGFα respectively. The top and bottom panels were obtained by probing with antiphosphotyrosine and anti-EGFr, respectively. e) Phosphorylation status of ERK of A431 cells after lectin treatment was carried out as described above for EGFr. Cells stimulated with nJacalin or PNA for 30 min followed by stimulation with TGFα.

nJacalin, and PNA have elicited different stress responses. Hence, the involvement of the lectin backbone in the elicited responses cannot be completely ruled out. Third, it appears that jacalin induces the novel cellular stress pathway on A431 cells, probably through the caveolin-1 >c-src >p38 pathway. Fourth, our data suggest that jacalin-induced cytotoxicity is probably mediated through the ER chaperone ORP150, because overexpression of ORP150 in A431 cells abolished jacalin-induced cytotoxicity.

Hence, lectins or other proteins that bind/interact with cell surface chaperones such as ORP150 and their role in inducing cytotoxicity or proliferation will be of considerable importance to understand the nature of interactions and their functional ramification inside the cell.

Table 1: Peptide sequences obtained by mass spectrometry of Jacalin-sepharose elutate. ORP150 is an ER chaperone which protects cells from hypoxic stress.

Sequence	Mass	Residues in ORP150
TVLSANADHMAQIEGLMDDVDFK	2520.2	318–340
AEAGPEGVAPAPEGEK	1508.7	670–685
EKAANSLEAFIFETQDK	1941	737–753
LPATEKPVLLSK	1295.8	878–889
EVQYLLNK	1006.6	901–908

Future Work

We wish to study the role of ER chaperone in protection of cancer cells under hypoxic stress with the help of its unnatural ligands.

Diabetes

Divergent approaches for control and management of experimental diabetes

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Background

Reactive oxygen species (ROS) is known to play an important role in beta cell death and is the proposed mediator of type I diabetes. Several studies have shown that exposure of islets to cytokines or Streptozotocin (STZ), a known beta cell toxin, greatly increases ROS production and leads to oxidative damage to islet cells. Since pancreatic cells contain very low levels of ROS scavenging/anti-oxidant enzymes, they may be more susceptible to toxic actions of pro-oxidant compounds. In agreement with the proposed role of ROS, over-expression of anti-oxidant enzymes conferred protection to several insulinoma cell lines against the toxic effects of ROS. We proposed that pre-treatment of islets with an anti-oxidant compound, like curcumin, might be effectual in reduction of cellular ROS and increment in levels of cellular anti-oxidant defense status, thereby reducing islet cell death on exposure to STZ or cytokines.

Most of the studies in diabetes are carried out using mammalian models and there are hardly any reports on the use of other vertebrates. Amongst aves, chick embryo has a distinguished history as a major model system in the fields of developmental biology, toxicology, virology, cancer research etc. Hamburger and Hamilton in 1951 staged the chicken embryos in 46 stages based on morphological events. The milestones of development of chick embryo during 21 days incubation period have been correlated with gestation period of different mammals. This allows specific developmental stages to be seen and studied with experimental manipulations providing developmental key to this model organism. However, potential of this model for diabetes studies has not yet been explored. Hence, we undertook present work to evaluate developing chick embryo and chick pancreatic islets as a model for diabetes research.

Isolated islets from pancreata of rodents provide an attractive *in vitro* model to study the action of insulin secretagogues and screening hypoglycemics. Similarly, isolated pancreatic islets from several mammalian species have been employed for functional and transplantation studies. However, there are very few reports on isolation of islets from chick pancreas. Since aves and mammals are closer phylogenically we were interested in

examining the status of chick islets as an *in vitro* model against that of mouse islets. Unlike mammals, the avian endocrine pancreas is characterized by two basic islet types confined almost exclusively to different lobes of pancreas viz. glucagon or A-islets (spleenic lobe) and insulin or B-islets (dorsal lobe).

Aims and Objectives

1. To investigate the role of anti-oxidants in cyto-protection of islets for possible prevention of diabetes.

2. Development of alternative *in vitro* models for diabetes research.

Work Achieved

Prevention of islet cell death by pretreatment with curcumin: Earlier we have shown that pretreatment of islets with 10 μM curcumin for 24 hrs imparted significant protection against STZ induced islet death. We have now been able to show that pretreatment of islets with curcumin at the same concentration and for the same time interval imparts protection to islets from cytokine insult (TNF α, IL-1 β and IFN γ) as evidenced by ROS generation and concomitant viability studies. Levels of insulin secretion in control and experimental islets were determined by insulin secretion assay. Determination of Nitric Oxide (NO) levels revealed that islets exposed to STZ and cytokines exhibited high levels of NO while islets pretreated with curcumin did not show this increase. Concomitantly, levels of inducible

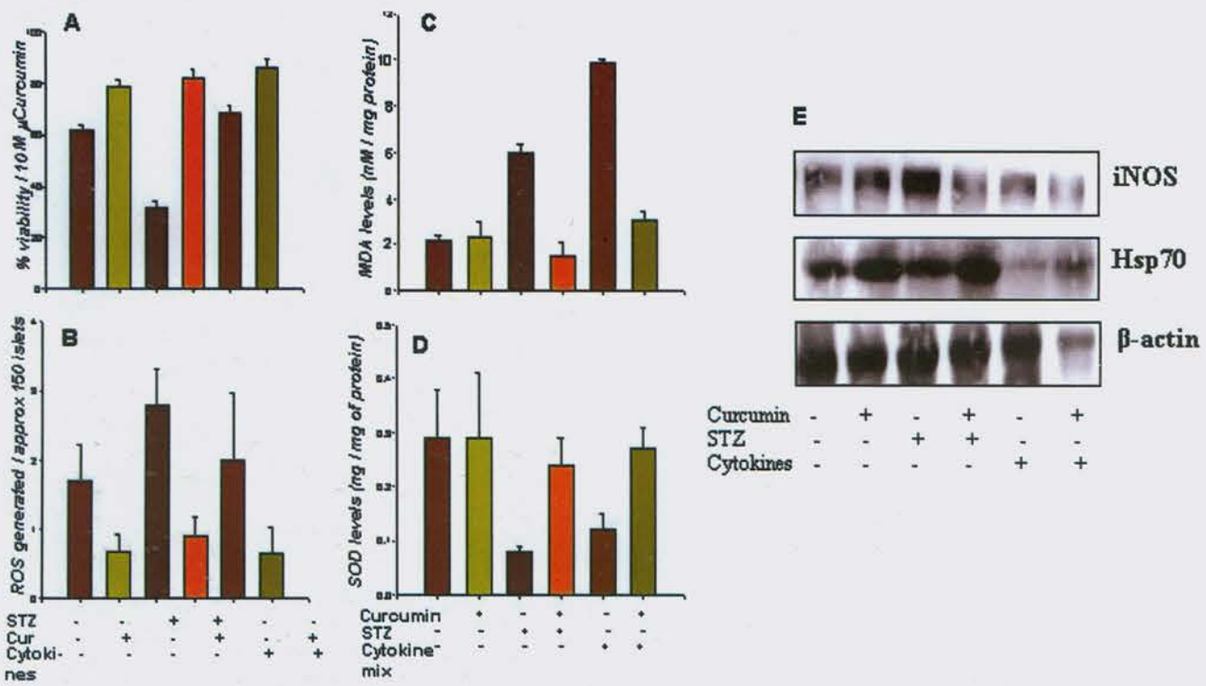

Fig. 1: A and B depict % viability and concomitant ROS levels, C and D represent Superoxide Dismutase and Malonydialdehyde levels respectively while E depicts expression of inducible nitric oxide synthase (iNOS) and Hsp70 in curcumin pretreated and control islets on exposure to Streptozotocin and combination of cytokines (TNF α, IL-1 β and IFN γ).

Nitric Oxide Synthase (iNOS) in STZ and cytokine treated islets were higher as compared to islets pretreated with curcumin. It was also observed that Hsp70 levels increase due to application of stress (STZ and cytokines), as it is a cellular defense mechanism. We found that curcumin treated islets had higher levels of Hsp70 than untreated islets. It is known that antioxidant enzymes play an important role in reducing oxidative damage to islets. We found that there was a marked depletion of Cu/Zn SOD in STZ and cytokine treated islets while islets pretreated with curcumin showed near normal levels of this primary cellular ROS scavenging enzyme. Malonyl Dialdehyde (MDA) levels, an indicative of Advanced Glycation End products (AGE) related cellular damage, showed significant increase in islets samples exposed to STZ and cytokine, while islets pretreated with curcumin showed near normal levels of MDA. Together these findings clearly indicate that curcumin pretreatment protects islets against STZ and cytokine induced damage through increasing cellular ROS scavenging enzyme potential and augmenting the cellular defense responses.

Development of shell-less chick embryo culture: A simple shell-less chick embryo culture model has been developed for *ex ovo* studies. This model involves the culturing of chick embryos from second to fifth day of incubation, outside the eggshell in a glass bowl thus permitting visualization and manipulation of embryonic development. This model was used to investigate whether high doses of glucose induce malformations in developing chick embryos similar to those reported in mammalian embryos. It was observed that treatment of 50 and 100 mM glucose resulted in more than 70% mortality for younger embryos and a variety of malformations in older embryos. The data obtained also confirmed that disturbances of growth and extent of malformations are concentration and stage dependent. Since the use of chick embryos in stages up to 10 days of incubation does not evoke severe ethical issues its usage for the study of glucose-induced malformations during embryonic development would be an appropriate alternative to other animal experiments. Thus we demonstrate suitability of this model to depict glucose-induced malformations reported in mammals.

Chick pancreatic islets as a model for screening hypoglycemics: We developed a simple technique for isolation of B-islets, based on the sequential collagenase

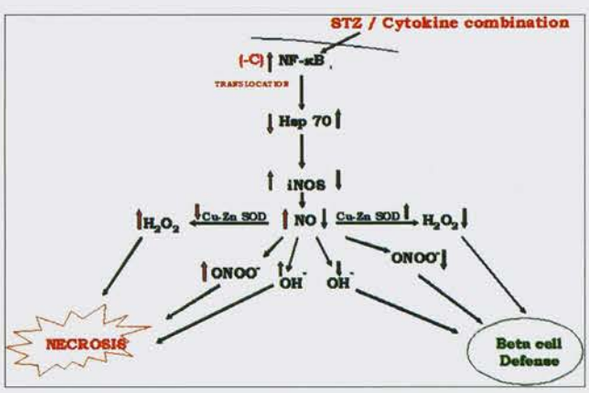

Fig. 2: Schematic summary depicting increase or decrease in levels of various proteins involved in islet cell defense/death pathway and its retardation upon curcumin treatment.

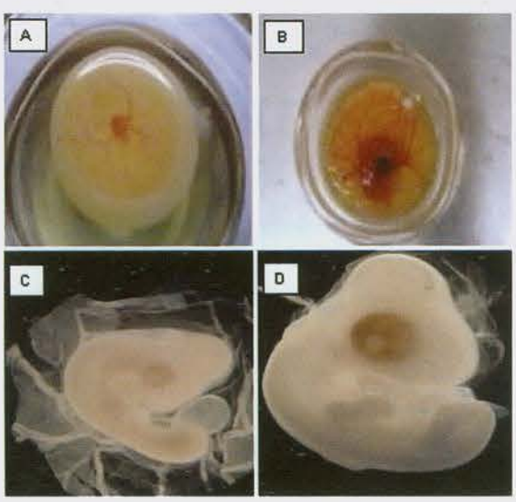

Fig. 3: A (Day 5) and B (Day 15) show growing chick embryo in shell less culture. Figure C depicts untreated chick embryo while Figure D shows Glucose treated embryo, Showing marked macrosomia

digestion. We showed that it is possible to isolate and culture more than 6000 viable and functional B-islets from dorsal and ventral lobe of a 5-6 day old chick pancreas. Our insulin secretion data further showed that contrary to the earlier reports B-islets from 5-6 d old chick do respond to secretagogues like glucose, arginine and tolbutamide stimulation. Thus, we demonstrate for the first time that chick pancreatic B islets could be used as an excellent alternative *in vitro* model for physiological and pharmacological studies in diabetes research.

Future Work

We plan to check levels of eNOS and cNOS both of which are related to nitric oxide generation. We also plan to check levels of nitro-tyrosine, an indicative of peroxynitrite damage, during STZ and cytokine exposure.

Mitochondrial dysfunction, oxidative damage and apoptosis in hyperglycemia induced cardiomyocyte death

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Abstract and background

Diabetic cardiomyopathy affecting the cardiac system is among the most frequent complications of diabetes. The etiology and the underlying mechanisms are as yet unclear. Recent studies showed that the incidence of apoptosis increases in the heart of patients with diabetes and STZ-induced diabetic animals i.e. loss of cardiomyocytes by apoptosis is proposed to cause heart failure.

Mitochondria play an important role in apoptosis under a variety of pro-apoptotic conditions, such as oxidative stress. Reactive oxygen species (ROS e.g. superoxide – O_2^-, Hydrogen peroxide – H_2O_2) and reactive nitrogen species (RNS e.g. nitric oxide – NO and peroxynitrite – ONOO-) have been proposed to participate in the high glucose-induced cardiac apoptotic cell death. Among apoptotic stimuli, ROS have been shown to cause mitochondrial cytochrome c release and activation of caspase-3. A correlation between ROS generation and the pathogenesis of various diabetic complications has been observed. However, the source and mechanism of ROS induction are unclear. There are no studies on the direct measurement of ROS generation during high glucose treatment. Such direct measures are needed to clarify important questions that remain regarding the role of ROS as inducing agents, including their source, where they are metabolized, and the relative contributions of different oxidant species to the high glucose-induced cardiac cell death.

The purpose of our study is therefore to investigate the role of mitochondrial ROS and/or RNS in the induction of high glucose-induced cardiac cell death, to clarify which ROS and/or RNS are required for the cell death response. Clinical studies established a significant correlation between the control of patient's blood glucose level (Glycemic control) and the severity of the damage to the cardiac system and because insulin has been reported to have protective effects, we examined whether insulin prevents cardiomyocytes from oxidative and nitrative stress–induced apoptotic death. For this study, we used the embryonic rat cardiac muscle cell line, H9c2.

Aims and Objectives

1. To investigate the role of mitochondrial ROS and RNS in hyperglycemia-induced myocardial apoptosis.

2. To clarify which ROS and RNS are required for the cell death response (signaling pathways) including their source using STZ diabetic rat/mice and H9c2 rat cardiac myoblast cells.

3. To characterize the mitochondrial Ca^{2+} influx and efflux mechanisms in cardiac muscle cells and determine how these mechanisms regulate excitation-contraction coupling ultimately leading to new insights for therapy. The involvement/role of Ca^{2+} channels and the various subtypes of voltage-sensitive calcium channels (VSCCs) viz L-type (nifedipine-sensitive), N-type (omega-conotoxin GVIA-sensitive), and N- and P/Q-type (omega-conotoxin MVIIC-sensitive) will be studied using specific inhibitors.

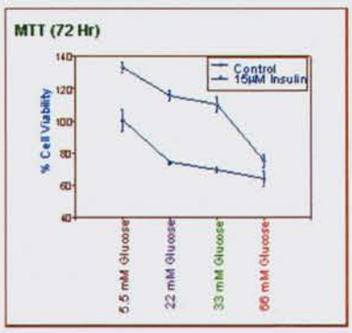
Work achieved

During the last year we observed that treatment with high glucose induces apoptotic cell death by inducing mitochondrial generation of ROS and RNS in H9c2 cells. Furthermore, mitochondrial anion channels and cytosolic dismutation to H_2O_2 as well as peroxynitrate formation may be important steps for oxidant induction of high glucose-induced cardiac cell death.

During the current year we have studied the protective effects of insulin on oxidative and nitrative stress. H9c2 cells were cultured in euglycemic (5.5 mM) and hyperglycemic (22, 33 mM) conditions for varying time periods. Exposure of H9c2 cells to high-glucose resulted in cell death in a

Protective effect of insulin on High Glucose induced cell death

(a) In Vitro



(b) In Vivo

Table 1. Whole Blood Glucose levels in Wistar rats (14 months)

Gr. No	Experimental details	Whole Blood glucose (mg/dL)
I	Control	108.5 ± 9.983
II	Vehicle Control	108 ± 8.406
III	STZ treated (55mg/kg body weight)	409 ± 37.64
IV	STZ Treated Followed By Good Glycemic Control (Insulin 2U/day)	113.3 ± 5.859
V	STZ Treated Followed By Poor Glycemic Control (Insulin 0.5 to 1 U 2-3 times a week)	245.33 ± 29.006
VI	STZ Treated Followed By Poor Glycemic Control with Insulin 0.5 to 1 U 2-3 times a week for 8 months) + Good Glycemic Control with Insulin 2U/day for 6 months	123.0 ± 8.485
VII	STZ Treated Followed By Good Glycemic Control (8 months with Insulin 2U/day) + Poor Glycemic Control (6 months with Insulin 0.5 to 1 U 2-3 times a week)	245.667 ± 9.074

Fig. 1: Protective effect of insulin *in vivo* and *in vitro*. a) Cells were treated with 5.5 mM glucose, 33 mM glucose, 33 mM Glucose + 15 μM Insulin for 72 h and cell viability was assessed by MTT. (b) Effect of insulin on good glycemic control (GC) and poor glycemic control (PC) was studied in male Wistar rats for 14 months. Whole blood glucose levels were expressed as mg/dl at the end of the experiment.

dose- and time-dependent manner as seen by MTT assay (Fig. 1a). Addition of insulin significantly inhibited cell death. Positive controls were ascertained by addition of 20 μM H_2O_2 (control for H_2O_2) Xanthine +Xanthine Oxidase (control for O_2-), SIN-1 (control for ONOO-) and 100μM tert-butyl hydroperoxide (t-BH) to the cells also induced cell death.

Exposure of H9c2 cells to high glucose resulted in increase in ROS oxidation of the probe dichlorofluorescein (sensitive to H_2O_2), and a similar increase in the oxidation of dihydrorhodamine 123 (sensitive to ONOO-) but moderate oxidation of dihydroethidine (sensitive to O_2-) as studied by FACS analysis and confocal images. All the DCF, DHE and DHR 123 oxidation signals during hyperglycemia were attenuated with insulin. However, best protection as offered for DCF oxidation (Figs. 2 and 3). These results suggest that insulin protects cardiomyocytes from oxidative and nitrative stress-induced apoptosis by inhibiting ROS and RNS generation.

Further to study the effect of insulin, antidiabetic pharmaceutical preparations e.g. metformin, rosigitazone, pioglitazone, glibenclamide and glimepride on poor glycemic control (PC) and good glycemic control (GC) as well as therapeutic potential of multiple anti-oxidants on

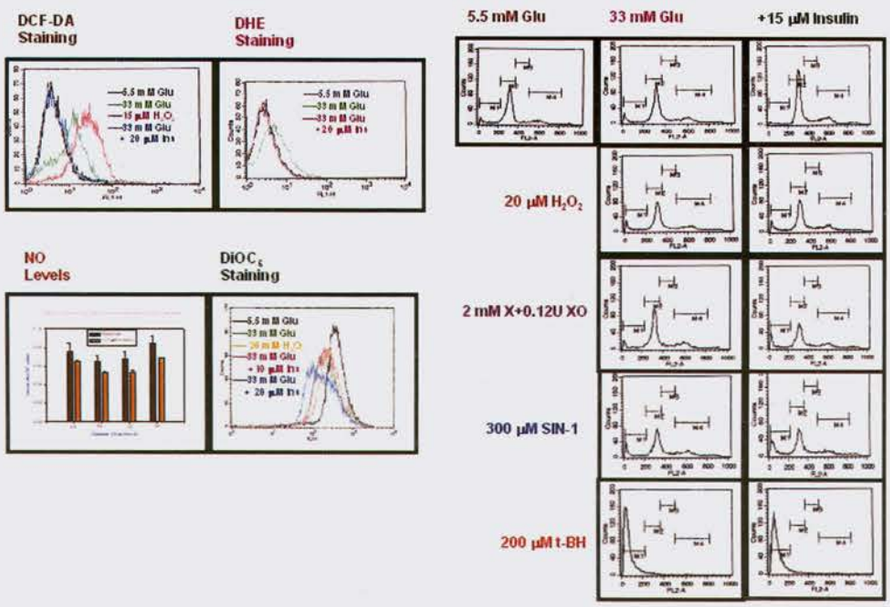


Fig. 2: Intracellular ROS, RNS Generation, Change in Mitochondrial Membrane Potential (MMP) and Apoptotic cell death during High Glucose Treatment : Protective role of Insulin – (a) Cells were treated with 5.5 mM glucose, 33 mM glucose, 33 mM Glucose + 10 and 20 μM Insulin and 15 μM H_2O_2 for 30 min. Role of ROS ad RNS generation was studied by measuring the change in fluorescence intensity by FACScan using DCF, DHE and DHR 123 fluorescence during high glucose treatment. Change in mitochondrial trans-membrane potential was examined using $DiOC_6$ staining. (b) Cells were treated with 5.5 and 33 mM D-Glucose for 72 h and cell cycle analysis was done by PI staining. Positive control was ascertained by addition of 20 μM H_2O_2 (control for H_2O_2) Xanthine +Xanthine Oxidase (control for O_2-), SIN-1 (control for ONOO-) and 100 μM tert-butyl hydroperoxide (t-BH).

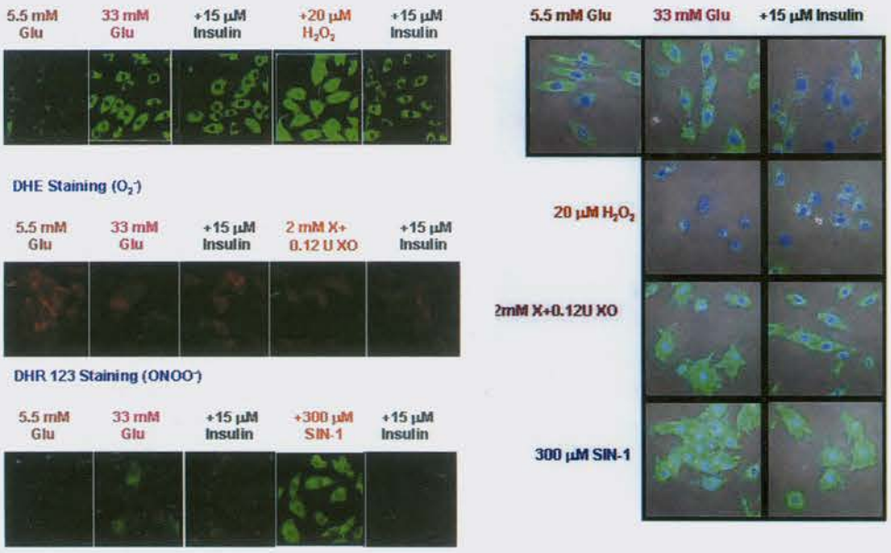


Fig. 3: Intracellular ROS, RNS Generation, Change in Mitochondrial Membrane Potential and Apoptotic cell death during High Glucose Treatment: Protective role of Insulin – (a) Cells were treated with 5.5 mM glucose, 33 mM glucose, 33 mM Glucose + 20 µM Insulin and 15 µM H_2O_2 for 30 min. Role of ROS ad RNS generation was studied by measuring the change in fluorescence intensity by confocal microscopy using DCF, DHE and DHR 123 fluorescence during high glucose treatment. Change in mitochondrial trans-membrane potential was examined using DiOC6 staining. (b) To study change in mitochondrial membrane potential and apoptotic cell death, cells were treated with 5.5 and 33 mM D-Glucose for 72 h and co-stained with $DiOC_6$ and DAPI using standard protocols. Positive control was ascertained by addition of 20 µM H_2O_2 (control for H_2O_2) Xanthine +Xanthine Oxidase (control for O_2^-) and SIN-1 (control for ONOO-).

oxidative and nitrative stress in diabetic rat, rats are made diabetic by STZ injections and insulin in one group and multiple antioxidant mixture is being given to these rats for 12-14 months. Cardiac function using Millar transducer catheters and role of oxidative and nitrosative stress in these conditions is being studied.

Future work

1. Characterization of high glucose induced mitochondrial ROS, RNS and Ca^{2+} influx and efflux mechanisms in cardiac muscle cells, and determine how these mechanisms regulate excitation-contraction coupling ultimately leading to new insights for therapy using various specific calcium anion channel blockers.

2. Study signaling pathways involved both *in vitro* and *in vivo* using specific inhibitors.

3. Study the effect of good glycemic control and therapeutic potential of multiple anti-oxidants on oxidative and nitrative stress in diabetic mice/rat.

Biodiversity

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3730 DNA Analyzer

Molecular taxonomy and diversity studies using 16S rRNA and other tools

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Abstract and background

Microbial organisms occupy a peculiar place in the human view of life. The workings of the biosphere depend absolutely on the activities of the microbial world. However, the pure-culture approach to the study of the microbial world seriously constrained the view of microbial diversity because most microbes defy cultivation by standard methods. Moreover, the morphological and nutritional criteria used to describe microbes failed to provide a natural taxonomy, ordered according to evolutionary relationships. Molecular tools and perspective based on gene sequences are now alleviating these constraints to some extent.

Carl Woese proposed the use of comparative sequence analysis of small-subunit ribosomal RNAs (16S and 18S rRNAs for determining evolutionary relationships between organisms and thereby 'quantifying' diversity as sequence divergence on a phylogenetic tree. Norman Pace and colleagues further outlined a molecular approach that bypassed the need to cultivate a microorganism in order to determine the sequence of its 16S rRNA gene (16S rDNA). Since its inception in early 90s, many researchers have applied this approach to a wide variety of environmental samples and, this has added to our understanding of these ecosystems.

In our laboratory we have used these techniques to understand microbial community structure of unique ecosystems like insect mid gut, human colon and some extreme ecosystems.

We isolated *Aeromonas culicicola*, from the midgut of the mosquito, *Culex quinquefasciatus*. Since then, isolation of this species has been reported from diverse environments all over world. It also contains virulence factor and hence could be a potential pathogen. Hence we have also initiated studies on regulation of gene expression of virulence factors.

Aims and Objectives

1. To understand the "uncultured" microbial diversity with long-term aim of utilizing it for the biotechnological purpose.

2. To study the diversity and evolutionary relationships in different organisms.

3. To study the genetic regulatory circuits and virulence factors in *Aeromonas culicicola*.

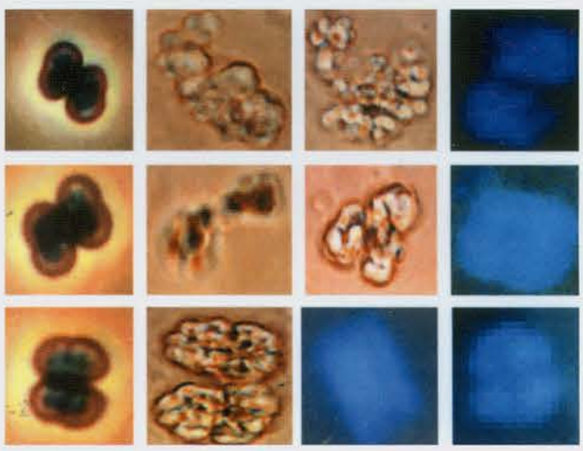
Work achieved

For studies on understanding of "unculturable microbial flora", PCR amplification of a "molecular chronometer" gene from total DNA extracted from environmental sample, followed by cloning and sequencing is the most commonly used strategy for studies of microbial community structure. We have adopted this strategy for some systems of either academic or applied interest.

A) Microbial Diversity of Anaerobic Digesters

Energy crisis due to unassured and short supply of petroleum products, has given an impetus to the use of biogas technology. Stable biogas plant operation requires that these bacterial groups must be in dynamic and harmonious equilibrium.

In order to asses the microbial diversity of biogas plants PCR amplification of 16S rRNA gene was carried out with primers targeting prokaryotic 16S rRNA. Partial sequencing of cloned 16S rRNA genes was conducted for phylogenetic analysis. To detect specifically the methanogens and their phylogenetic diversity, methanogen – specific *mcrA*, clone libraries were also established from biogas plants. Biogas plant performance was also monitored by analyzing the VS, TS, VFA, biogas production rate and methane content of it. In biogas plant running at ambient temperature, the most abundant species was *Methanosaeta. concilli*, followed by



(A) (B)

Fig. 1: a) Amplification of pmoA and mxaF genes were carried out using primer set pmoAf/pmoAr and mxaf/mxar from biogas plant running at ambient temperature. b) Methanogens present in stored dry dung cakes (A) 2 months (B) 8 months. 1, 2, 3 represent phase contrast pictures of *Methanosarcina*. 4. represent fluorescent pictures of *Methanosarcina* under UV light. (C) 2 years. 1, 2 represents phase contrast pictures of *Methanosarcina*. 3 and 4 represents fluorescent pictures of *M. concilli* and *Methanosarcina* respectively under UV light. (Magnification 400X).

Methanospirillum hungatei, Methanocorpusculum parvum, Methanobacterium formicicum, Methanobrevibacter ruminantium, Methanocorpusculum labreanum, Methanosphaera stadtmanae and *Methanocorpusculum* sp. Microbial community structures of methanogens associated with dry cattle dung cakes of 8 and 24 months old ages were investigated by PCR amplification, cloning and sequencing of "functional" gene methyl coenzyme-M reductase A (*mcrA*). Molecular approaches used in this study provided useful descriptions of the methanogenic community involved in biogas production process using dry dung cakes stored for prolonged period of time.

B) Studies on regulation of virulence genes in *A. culicicola*

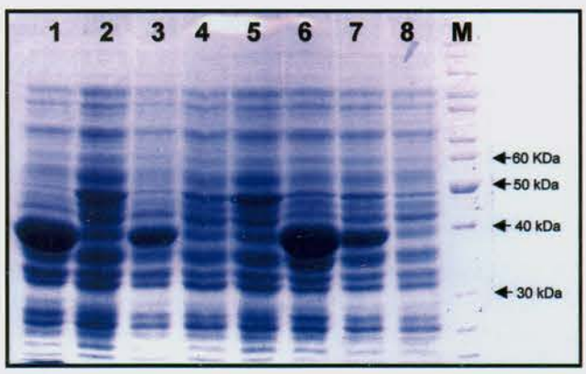
Aeromonas spp. is associated with intestinal and extra-intestinal infections. *Aeromonas* are now considered to be emerging pathogens. *Aeromonas* spp. produces an array

of virulence factors enterotoxin (ent), cytotoxin (cyt) and haemolysin (hae). Toxins secreted by *Aeromonas* helps the organism to evade the host cells and survive in harsh environments. The virulence of *Aeromonas* is related to the presence of these toxin genes. However, the distribution of toxin genes in *Aeromonas* doesn't correlate to species and source of isolation. Detection of these genes has been used widely to assess the extent of pathogenicity in these organisms.

The complete gene sequences of probable virulence genes were determined by using inverse PCR method. The virulence genes aerolysin, two superoxide dismutase gene (Mn-SOD and Fe-SOD) and Ferric Uptake Regulator (FUR) were cloned by using inverse PCR method. The sequencing of 2554 bp DNA fragment containing Manganese superoxide dismutase gene (*sodA*) revealed an open reading frame (orf) which codes for 22.7 KDa protein. The Iron superoxide dismutase gene (*sodB*) consisted of 1483 bases with an orf coding for 21.5 KDa protein. Both the superoxide genes were cloned and expressed in *E. coli*. 1810 bp fragment of Ferric Uptake Regulator encoded for 16.2 kDa protein. Further, the functionality of superoxide dismutase and FUR genes were determined by the complementation studies in *E. coli* mutants. We have also determined the transcription initiation site for the Aerolysin gene by primer extension and determined the promoter region using Electrophoretic Mobility Shift Assays.

C) Molecular characterization of *Ochrobactrum* spp. from the non-ulcer dyspeptic patients from India

Human stomach is being colonized by diverse groups of bacteria from ages ranging from pathogenic to non-pathogenic, irrespective of their role in the survival, health and disease. However, there is a little known information regarding the role of presence of *Ochrobactrum* species in the human stomach. Here, we describe for the first time presence of *Ochrobactrum intermedium* (*O. intermedium*) in the antrum of non-ulcer dyspeptic patient from India, who was diagnosed positive for the *H. pylori*. 75 isolates of *Ochrobactrum* spp. were isolated from Indian populations, which were suffering with stomach-associated problems. The isolates were Metronidazole resistant and Clarothromycin sensitive. Some of the patients were positive for *H. pylori*. All the isolates were urease positive and facultative anaerobic. All the isolates were biochemically characterized by using traditional techniques and API20NE approach revealed the genus *Ochrobactrum*. At the same time, antibiotic susceptibility profiles of the majority of



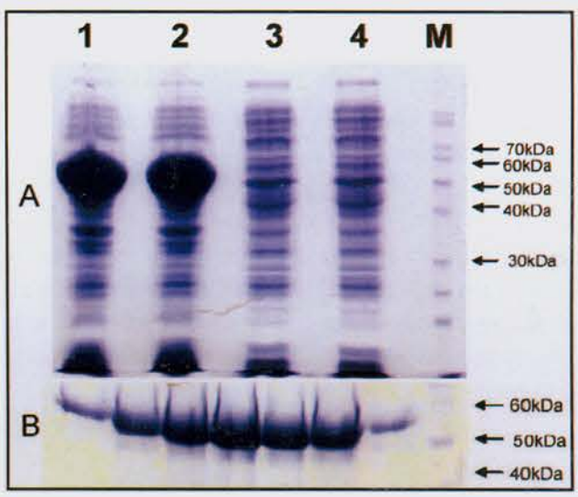


Fig. 2: a) Expression of superoxide dismutase genes in *E. coli*. b) Over expression of His(6)-aerolysin in *E. coli* BL21A1 as inclusion bodies. Lane1, 2-inclusion bodies (pellet); 3, 4-supernatant; M- Molecular Weight Marker. B. His(6)-aerolysin purified using Ni-NTA resin.

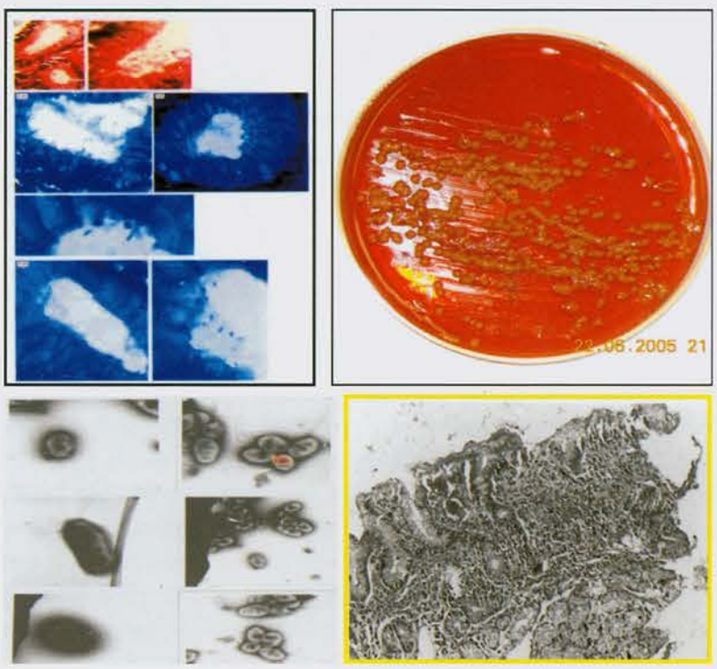


Fig. 3: Histopathological image from the patient SP86 from which the first case of *Ochrobactrum* was found to occur, the isolates were grown on MacConkey's agar, Transmission electron micrograph indicates the morphology of these.

isolates were studied by standard diffusion techniques using standard disc diffusion methods. Molecular phylogenetic affiliation of all the isolates were studied by gene sequencing and typing approaches targeting 16S rRNA, 23S rRNA, Gyrase B and RNA polymerase gene subunit D. Isolates were also studied by using fragment length amplified polymorphism approach and found to have clonal ability. *In vitro* and *in vivo* experiments are in progress to check the correlation and the exact role-played between *Ochrobactrum* and *H. pylori* in the acidic environment.

D) Molecular phylogeny, systematic studies on Western ghat Indian Amphibians (Ranids and Bufonids) and Caecilians (Ichthyophis and Gegeneophis), Fresh water fishes (Cichlids and economically important fish species) and Shield tail Uropeltid snakes

Indian Western ghats are one of the 25 hotspots of the world and well known for species richness and diversity. Genetic diversity studies of Western Ghat vertebrates and invertebrates belonging to various phyla were studied using mitochondrial and nuclear genes. More than 130 amphibian species belonging to Ranidae and *Bufonidae* were studied for their genetic diversity using mitochondrial (1500 nucleotides) and nuclear genes (500 nucleotides). We found that Ranids are genetically highly diverse,

whereas Indian *Bufo melanostictus* are monophyletic this genus was originated in the Northeast part of India and subsequently migrated in Asia including Indonesia and other hotspots of the world. Another group of caecilian amphibians was also evaluated for genetic diversity studied by using mitochondrial genes like 12S, 16S rRNA, Cytochrome B and Cytochrome oxidase subunit I, wherein we found the remarkable diversity in Unstriped *Ichthyophis* sp. throughout the western ghat spanning 1500 Km^2 area covering almost entire western ghat hotspot. The rare and ecologically important species like *Gegeneophis* sp. is presently being evaluated for it's evolution in this part of the world. We are also in the process to study the possibility of endemism of North-east Indian amphibians using various phylogenetic markers. More than 110 specimens of fresh water fishes were collected from the localities in and around Pune region and protein coding and non-protein coding genes were utilized for studying their diversity. Shield tail snakes belonging to genus uropeltis were also studied for their DNA based diversity for Cytochrome b and ND4 regions to answer their endemism and phyletic status in Western Ghats of India, which is one of the major hotspot of vertebrates and invertebrates.

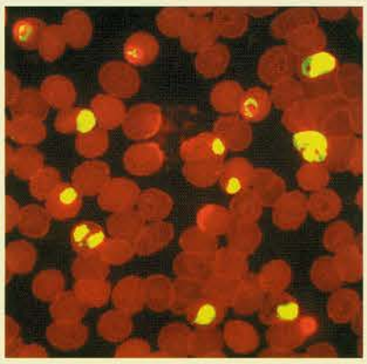
E) Molecular studies on bioactive potential of insect gut microbiota from Western Ghats of India

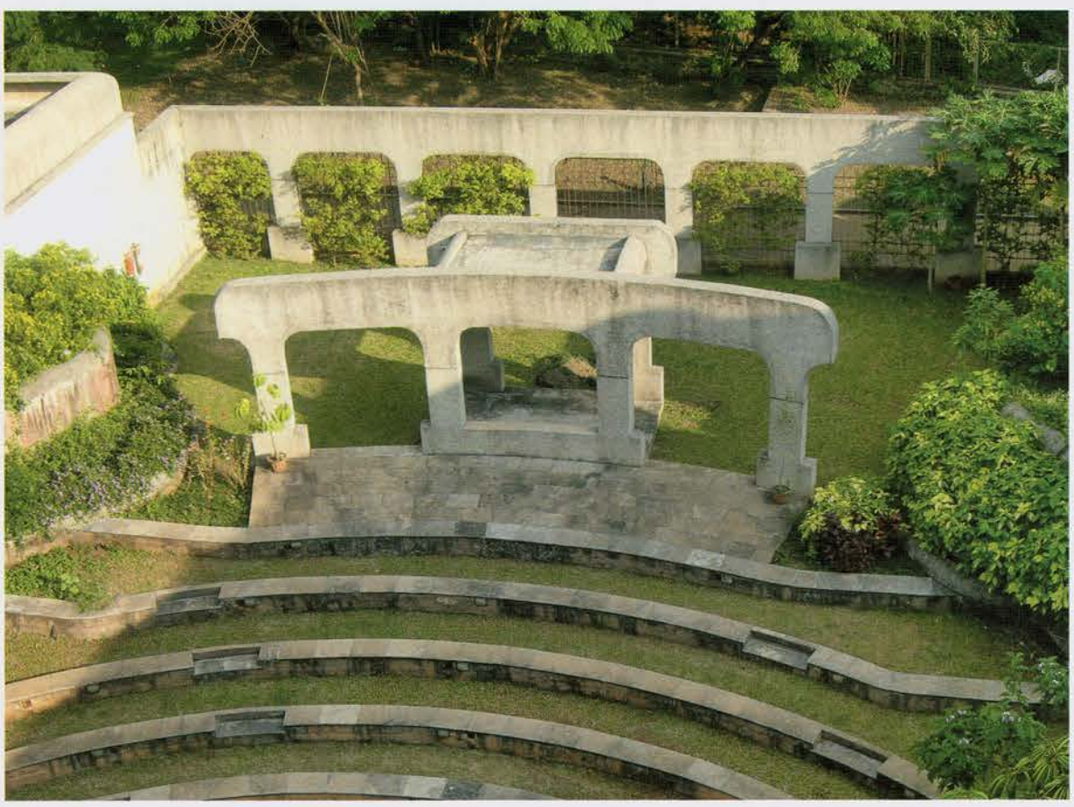
Study is aimed to characterize microbial flora of biodiversity hotspots of Indian Western Ghats insects of India and explore for the production of bioactive molecules. Insect specimens (with different feeding habitats) were collected and identified morphologically in the field from different parts of Western Ghats of India especially from Western Ghat region spanning in Maharashtra state, India. The insects were processed for identification by using 16SrDNA primers specific for insect taxa yielding 600 base pair amplicons. These amplicons were purified and sequenced on 3730 DNA analyzer (ABI, Foster City, USA) following manufacturer's instructions. To explore the bacterial flora, digestive gut samples were removed from individual insects and preserved in saline solution and plated on different culture media with different dilutions.

Fig. 4: Specimens collected from Indian Western Ghat region to study the diversity of Indian Bufos.

The plates were incubated and the colonies appeared on the plates were identified by 16S rDNA primers targeting eubacterial domains. The amplicons were sequenced on DNA analyzer for initial 500 bases to identify the bacteria. Bacterial isolates were further checked for the production of bioactive compounds like Protease, Cellulase and Lipase activities. Furthermore, the isolates were also screened for the presence of nanoparticle like gold (Au) and Silver (Ag) in presence of different salts. Sixteen different insect specimens belonging to different insect families were collected from different feeding and niche habitats. Many bacteria were cultured from digestive tracts of the insects by using different types of nutrient media like Luria- agar, Blood agar, Trypticase soya agar, Brain heart infusion agar, Columbia blood agar etc. These bacterial isolates were identified by polymerase chain reaction targeting eubacterial 16S rDNA primers followed by sequencing. *Wolbachia* endosymbionts and Cockroach endosymbionts were studied based on *ftsZ* gene, wherein lineages belonging to both phyla fall in supergroup F of Termites.

Infection & Immunity





Spontaneous Extracellular Growth of Erythrocytic stages of *Plasmodium falciparum* malaria *in vitro*

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Background

Earlier, we have reported spontaneous development of erythrocytic stages of parasites outside the human host RBC. During prolonged *in vitro* cultures of *FAN5HS* parasite line, some of the merozoites emerged from the intracellular schizont were able to attach to the erythrocytes but failed to invade the host cells. Instead of disintegration, these merozoites transformed into ring stages, then into pigment (hemozoin) containing trophozoites and finally developed into schizonts with merozoites. The parasites were characterized by PCR and immunofluorescence by using *P. falciparum* specific primers and antibodies, respectively.

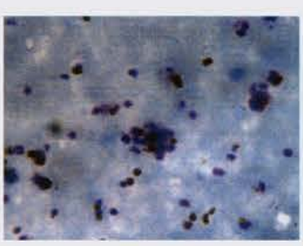
Aims and Objectives

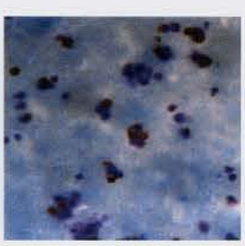
1. Growth of extracellular erythrocytic stages of *P. falciparum* in RBC extract.
2. 35S-methionine up take by these parasites
3. Characterization of parasites by RT-PCR.

Work Achieved

Growth of extracellular erythrocytic stages of *P. falciparum* in RBC extract: As reported earlier, we have seen spontaneous extracellular growth of erythrocytic stages of *P. falciparum in vitro*. The parasite growth was examined in total RBC extract containing HB and RBC membranes. Spontaneously growing extracellular parasites cultures, with about 7.5% parasites outside the host RBC, were washed twice with culture medium and the culture pellet (200 µl) was treated with 0.1% saponin solution for 15 seconds. The content was then diluted with 5ml culture medium with shaking. The parasite pellet was separated by centrifugation (3000 rpm, 10 min. at 4°C). The resultant pellet was washed twice with culture medium and resuspended in culture medium (2 ml). The culture medium (0.5 ml) containing extra-cellular parasites (2.5 X 10^3/ml) were seeded in 24 well tissue culture plates (0.5ml/well) and supplemented with either HB (2.5%), or total RBC extract (5%). The plates were incubated in candle jar desiccators at 37°C. The culture medium was replenished every 48h. Parasite culture smears were made after 6 days for assessment of parasite growth. The smears were stained either with AO or DAPI. Cultures were also stained with vital dye Rhodamine 123 for viability.

Results: The parasites cultured in the total RBC extract were in young and mature trophozoites with hemozoin (malaria pigment) and schizonts with 7-10 merozoites (Figs. 1 and 2). However, the parasites were deformed in the cultures without RBC membrane.





Figs. 1 and 2: Extracellular parasites cultured (six day old) in the medium supplemented with erythrocyte extract. The picture shows young, mature traphozoite and schizont stages.

^{35}S-methionine incorporation: The extracellular parasites were cultured in the medium containing total RBC extract as described above. Four days old parasite culture suspension were seeded into 96 well tissue culture plate 50 µl/well and exposed to S-35 methionine (50 Ci/ml) for 10 h and the supernatant was removed and the parasite pellet was washed 5 times with sterile 1 x PBS (200 µl/well). The parasite pellet was suspended in 10 µl PBS and the content was transferred to glass filter papers and dried. CPM counts were recorded in the beta liquid scintillation counter. Only RBC membranes along with culture medium were also included in the assay as controls.

Results: the incorporation of ^{35}S-methionine in the parasites indicates – metabolically active nature of the parasites (by CPM counts).

Characterization of these parasites by RT-PCR and sequencing: The extracellular forms of the erythrocytic stages were cultured as described above for a week and total RNA was isolated. The RNA is being used to characterize the parasites using *P. falciparum* specific primers. Use: The spontaneously developed extracellular erythrocytic parasites cultures transformed from noninvasive merozoites will be useful in studying parasite biology.

Future Work

The work is concluded.

Signaling requirements for differentiation of CD8$^+$T-cells and memory generation

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Abstract

Cytotoxic T lymphocyte response plays a critical role in antiviral immunity, tumor rejection and other antimicrobial immunity. Understanding the mechanistic basis of modulation and induction of CTL responses by different antigen presenting cells can provide key information in studies of CD8$^+$T cells mediated immunity.

In our previous study we have found that differentially activated B-cells exhibit different effects on CD8$^+$T cells (Fig. 1). We have used LPS activated B-cells which mimic T-independent mode of activation or anti-IgG + anti-CD40 activated B cells which mimic T-dependent mode of activation, to provide accessory signals to the naïve CD8$^+$T cells. Our results show that in comparison to anti IgG + anti-CD40 activated B-cells, LPS activated B cells failed to induce significant level of proliferation, cytokine secretion and cytotoxic ability of CD8$^+$T cells. The LPS activated B cells express significantly high TGF-β on its surface which was responsible for hyporesponsiveness of CD8$^+$T cells. The observed hyporesponsiveness of the CD8$^+$T cells was significantly rescued by anti-TGF-β neutralizing antibody. Moreover these hyporesponsive CD8$^+$T cells activated by LPS-B cells undergo a state of anergy. The addition of IL-2 completely reversed the hyporesponsiveness of CD8$^+$T cells, whereas IL-6 and TNF-α could partially rescue the phenomenon.

Based on the above observation we have extrapolated our hypothesis in examining the role of differentially activated dendritic cell type I (derived from myeloid origin) and DC2 (derived from lymphoid origin) on naïve CD8$^+$T-cell activation and differentiation. It has been known that DC1 induces a generation of stimulatory CD8$^+$T-cells, whereas DC2 is involved in driving it towards regulatory CD8$^+$T cell, inducing tolerance or suppression. Thus the molecular mechanistic study of CD8$^+$T-cell activation by different dendritic cell subsets may provide useful information on the induction and modulation of CTL responses.

A possible model of Ag specific CD8⁺T–cell development during an immune response.

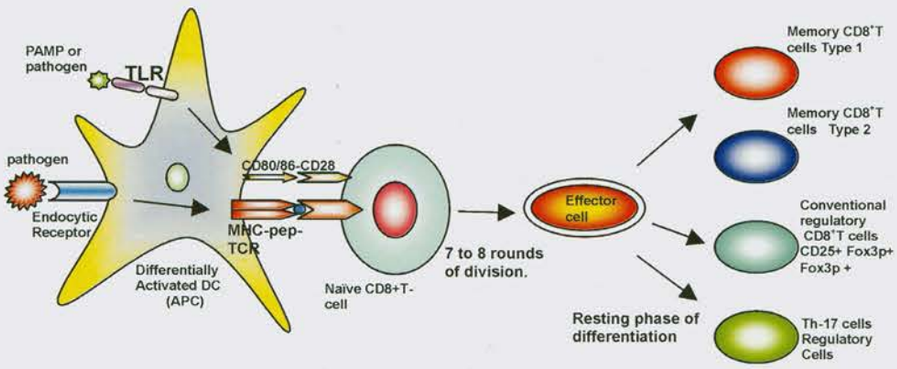

Fig. 1: Schematic presentation of possible fates of naïve Ag specific CD8⁺T cell development during immune response. The CD8⁺T cell development occurs at two stage program. At the first stage, the antigenic stimulation differentially activates the dendritic cell (Ag induced APC program) which leads to effector cells generation. This effector cells will be qualitatively different depending upon encounter with differentially activated APC. At the second stage the effector cell undergo successive changes in gene expression and other cellular process depending upon the cytokine and growth factors in the environment that occur over the next several weeks and gradually transform into different cell lineages performing vital roles in the regulation of immune response.

Objective

To understand the molecular mechanism of CD8⁺T-cell activation and differentiation *in vitro* and *in vivo* using antigen specific system.

Work achieved

We have been successful in establishing the isolation and characterization of DC1 and DC2 cells. We differentially activated these cells with microbial products viz. unmethylated CpG repeats, LPS or anti-CD40 antibody to mimic the T-independent and T-dependent modes of APC activation respectively. Combinations of these modes were also used to activate the APCs. DC1 and DC2 were activated by the above signals to ascertain their ability to differentiate CD8⁺T-cells. Naïve murine CD8⁺T-cells were cultured in the presence of anti-CD3e as a source of the primary signal. Differentially activated DC1 and DC2 were used to provide the secondary co-stimulatory signal. The differentially activated DCs differed qualitatively as well as quantitatively in terms of cytokine production and costimulatory molecule expression to induce naïve CD8⁺T-cell activation. It was found that the expression of CD86, CD80 and CD40 were maximally increased on the surface of DCs, activated by anti-CD40, CpG, LPS, anti-CD40+CpG and anti-CD40+LPS (Fig. 2a).

However, the increase in the expression of co-stimulatory molecules was significantly less on DC2. Prominently, anti-CD40+CpG activated DC1 and DC2 showed the same extent of expression of CD80, CD86 and CD40, and also IL-12 and IL-6 cytokine synthesis (Fig. 2b). Importantly, differentially activated DC2 expressed higher levels of T-cell regulatory molecules like TGF-β, FAS-L and PDL1 in comparison to DC1. DC1 cells produce high IL-12 and IL-6 but no IL-10, which DC2 cells showed more IL-10 secretion (Fig. 3). Thus DC1 cells were found to be more stimulatory and DC2 cells to be regulatory in nature. Further characterization of CD8⁺T-cells stimulated by differentially activated DCs in terms of surface markers and cytokine production is needed to mechanistically reveal the signal requirements involved in the process of differentiation.

Co-stimulatory molecules expression by differentially activated DC1 and DC2

	B7.1 (CD80)		B7.2 (CD86)		CD40		M150		gp96	
	DC1	DC2	DC1	DC2	DC1	DC2	DC1	DC2	DC1	DC2
Medium DC	26 % (46) MFI	14% (113) MFI	18 % (61) MFI	8% (85) MFI	41 % (74) MFI	18% (86) MFI	46% (83) MFI	13% (62) MFI	13 % (217) MFI	6% (647) MFI
αCD40 – DC	25% (31)	16% (88)	41% (104)	20% (84)	45% (53)	20% (70)	51% (78)	18% (73)	11% (271)	8% (478)
CpG –DC	36% (37)	26% (81)	42% (74)	20% (74)	56% (100)	30% (74)	60% (97)	23% (94)	11% (305)	8% (494)
LPS-DC	43% (71)	31% (101)	35% (72)	18% (74)	59% (117)	37% (101)	56% (68)	25% (68)	10% (316)	10% (766)
αCD40 +Cp G – DC	30% (56)	36% (221)	55% (93)	36% (85)	47% (62)	40% (179)	49% (80)	21% (66)	11% (309)	9% (734)
αCD40+LPS – DC	38% (49)	28% (90)	43% (74)	32% (87)	56% (58)	32% (80)	54% (63)	27% (64)	10% (318)	10% (547)

Fig. 2a: Bone marrow derived dendritic cells (type I and II) were activated with αCD40, CpG, LPS, acd40+CpG and Acd40+LPS in a 24 well plate for 48 hrs and stained with CD80, CD86, CD40, M150 and gp96 antibodies. The data shown represent one of the three individual experiment.

Co-stimulatory molecules expression by differentially activated DC1 and DC2

	TGF-β_1		FAS-L		PD -L_1		PD-L_2		MHC-II	
	DC1	DC2	DC1	DC2	DC1	DC2	DC1	DC2	DC1	DC2
Medium DC	12 % (209) MFI	22% (108) MFI	26% (118) MFI	34% (86) MFI	17 % (63) MFI	18% (57) MFI	12% (45) MFI	10% (76) MFI	45 % (320) MFI	21% (153) MFI
αCD40 – DC	10% (188)	24% (241)	20% (110)	34% (88)	21% (54)	28% (64)	12% (47)	6% (70)	55% (380)	24% (229)
CpG –DC	10% (245)	34% (350)	25% (122)	47% (170)	24% (47)	32% (72)	12% (58)	8% (103)	60% (485)	27% (204)
LPS-DC	12% (144)	33% (186)	18% (77)	37% (98)	23% (71)	36% (77)	12% (48)	7% (88)	60% (411)	36% (225)
αCD40 +Cp G – DC	12% (113)	35% (233)	18% (88)	43% (112)	33% (59)	42% (61)	9% (47)	7% (108)	56% (343)	30% (239)
αCD40+LPS – DC	11% (145)	31% (187)	17% (89)	28% (73)	27% (65)	41% (66)	10% (45)	8% (90)	57% (374)	38% (274)

Fig. 2b: Bone marrow derived dendritic cells (type I and II) were activated with αcd40, CpG, LPS, αcd40+CpG and αCD40+LPS in a 24 well plate for 48 hrs. and stained with TGF-β1, FAS-L, PD-L1, PD-L2 and IA^b antibodies. The data shown represent one of the three individual experiments.

Cytokine secretion by differentially activated DC1 and DC2

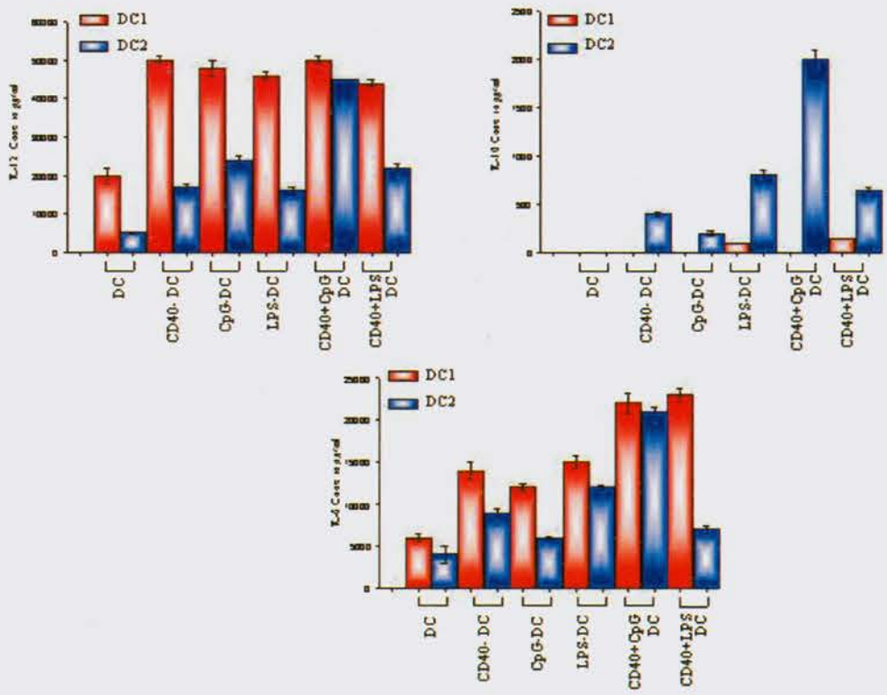

Fig. 3: 5 x 105 Dendritic cells/ml were stimulated with a CD40, CpG, LPS, a CD40+CpG and a CD40+LPS in a 24 well plate. Supernatants were harvested at 48 hrs for IL-12, IL-10, IL-6, and IL-4, after simulation. IL-4 was not detected in the culture medium.

Future Work

1. To examine the ability of differentially activated DC1 and DC2 on the activation, proliferation and differentiation of $CD8^+$T-cells including memory and regulatory phenotyes. This will be analyzed by Granzyme B, IFN-γ quantitative estimation (ELISA), specific cells marker expression and *in vitro* target lysis.

2. The knowledge of *in vitro* studies will be employed to demonstrate the functional characteristics of differentiated $CD8^+$T-cell. For this purpose naive $CD8^+$T-cell from TCR transgenic mice (LCMV and OT-I) will be co-cultured with best candidate DC. This selected DC will be loaded with respective peptides and adoptively transferred to Thy1.1 congenic mice. The generation of differentiated $CD8^+$T-cells will be evaluated quantitatively as well as qualitatively by analyzing the T-cells gated on Thy1.2 cells (the cells which were adoptively transferred) and by specific markers for particular type of differentiated $CD8^+$T-cell. The qualitative evaluation of transferred cells will be done by *in vivo* target lysis and recall responses by re-challenging with the antigen.

Molecular and cellular basis of HIV pathogenesis

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Background

Human immunodeficiency virus is the causative agent of acquired immunodeficiency syndrome (AIDS), which is defined by a reduction in the number of $CD4^+$T cells (less than 200 cells/μl) and the onset of opportunistic infections. The incidence of HIV infection has reached alarmingly high levels worldwide including India. The therapeutic strategies being used at present can reduce the viral load remarkably but is not the ultimate answer to AIDS patients. Our group has been working on three different aspects of HIV, related to viral pathogenesis, immune response and drug discovery. The primary objective is to gain more understanding of the virus and its interaction with the host cell, which may lead us 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. Molecular basis of CTL dysfunction in HIV infection and

3. Identification of novel molecules with anti-HIV activity.

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. 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 for the first time 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 (Fig. 1). The interaction between Nef and Hsp40 is important for increased Hsp40 translocation into the nucleus of infected cells, which seems to facilitate viral gene expression by becoming a part of the cyclin dependent kinase 9 (cdk9) associated transcription complex regulating long terminal repeat (LTR) mediated gene expression. The finding is consistent with the failure of Nef deleted virus to induce Hsp40, resulting in reduced virus production. Our data further shows that while Hsp40 over expression induces viral gene expression, silencing of Hsp40 reduces the gene expression in a Nef dependent manner (Fig. 2). Thus our results clearly indicate that Hsp40 is crucial for Nef-mediated enhancement of viral gene expression and replication.

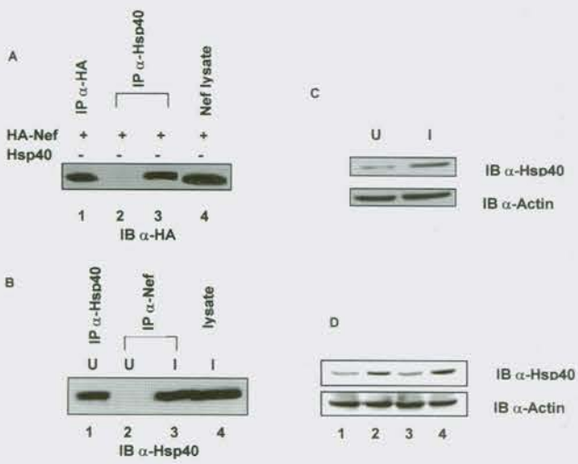

Fig. 1: Hsp40 physically interacts with Nef and is upregulated in HIV-1 infected cells. (A) Hsp40 and Nef co-immunoprecipitates from transfected cell lysate. 293T cells were transfected with HA-NL4-3 Nef expression vector alone or with Hsp40 expression vector. After 48 hrs, the cells were lysed, immunoprecipitated with anti-Hsp40 antibody (lane 2 and 3) and followed by immunoblotting with anti-HA antibody as indicated in the figure. (B) Nef co-immunoprecipitates with Hsp40 in HIV-1 infected CEM-GFP cell lysate. CEM-GFP cells were infected with HIV-1 NL4-3 virus. The cells were lysed on day seven post-infection. The lysates of uninfected CEM-GFP cells (lane-2) and infected CEM-GFP cells (lane-3) were immunoprecipitated with anti-Nef antibody followed by immunoblotting with anti- Hsp40 antibody. (C) Hsp40 is up regulated in HIV-1 infected CEM-GFP cells. HIV-1 NL4-3 infected CEM-GFP cells were lysed on day seven post infection and equal amount of protein from uninfected and infected cells were used for immunoblotting of Hsp40. (D) Nef is required for up regulation of Hsp40 expression. 293T cells were transfected with empty vector (lane 1), HIV-1 NL4-3 molecular clone (lane 2) and Nef deleted NL4-3 molecular clone (lane 3) or pCDNA-Nef along with Nef deleted NL4-3 (lane 4). After 48 hours of transfection, 293T cells lysates were prepared and equal amount of protein was used in immunoblotting for Hsp40. Actin was used as a loading control.

We have earlier shown a direct interaction of Tat with nuclear factor kappa B (NFκB) enhancer, a global regulatory sequence for many cellular genes both *in vitro* and *in vivo*. We have now performed biophysical studies using fluorescence anisotropy and isothermal titration calorimetry (ITC) to identify the strength and nature of binding. Anisotropy studies using fluorescein labeled DNA suggests that Tat binds to NFκB enhancer DNA as a dimer with binding affinity (K_d, dissociation constant) in nanomolar range. Curve fitting of ITC data suggest that Tat:NFκB interaction follows a two site sequential binding model. In addition, we have shown that this interaction could be responsible for Tat mediated modulation of cellular genes.

Studies done till date to elucidate the pathways involved in HIV-1 induced T cell depletion has revealed that apoptosis underlie the etiology; however, a clear molecular understanding of HIV-1 induced apoptosis has remained elusive. Although evidences pointing towards

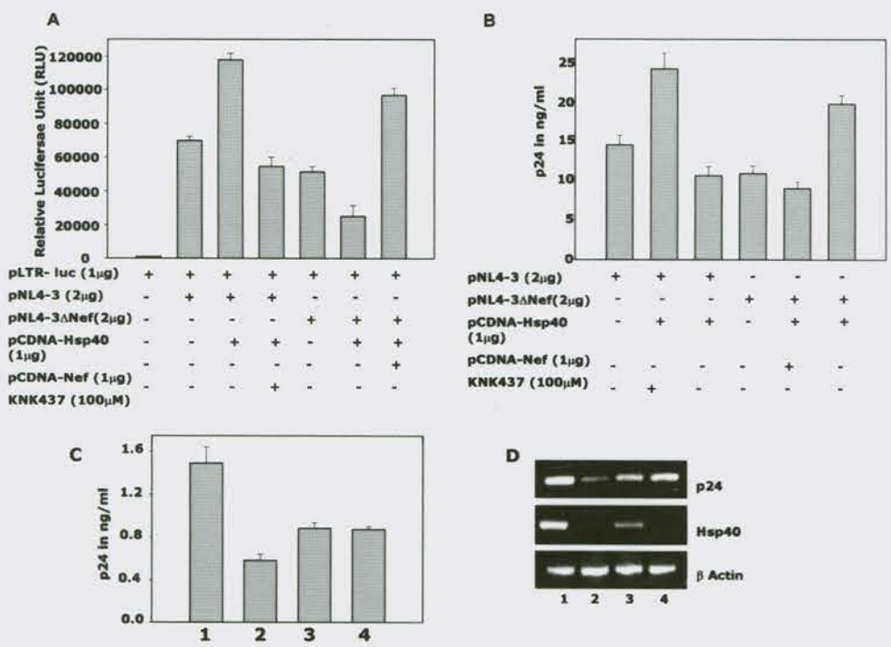

Fig. 2: Hsp40 over expression induces viral gene expression and virus production whereas silencing of Hsp40 reduces virus production in a Nef dependent manner. (A) Nef is required for Hsp40 induced LTR mediated gene expression. 293T cells were transfected with different expression vectors along with LTR-Luc reporter as indicated in the figure. After 4 hrs of transfection some of the transfected cells were treated with 100 mM KNK437 for 32 hours as indicated. The cells were lysed and Luciferase assay was performed. (B) Nef is required for Hsp40 induced increase in virus production. Virus production was determined in culture supernatants of 293T cells transfected in the experiment above (A), by using p24 antigen capture ELISA. (C) Hsp40 down regulation by siRNA reduces virus production in presence of Nef. Control or Hsp40 siRNA transfected 293T cells as described above were again transfected after 24 hours with either NL4-3 molecular clone (lane 1 and 2) or Nef deleted NL4-3 molecular clone (lane 3 and 4). Culture supernatants were collected at 72 hours of siRNA transfection and virus production was determined by using p24 antigen capture ELISA. (D) HIV-1 p24gag expression is down regulated in Hsp40 silenced cells in presence of Nef. RNA was isolated from transfected 293T cells described above (C) after 72 hrs of siRNA transfection and RT-PCR was performed for HIV-1 p24, Hsp40 and human b-actin.

the importance of mitochondrial energy generating system in apoptosis exist but it's exact role remains to be clearly understood. We have previously shown specific down regulation of a complex I subunit NDUFA6 with simultaneous impairment of mitochondrial complex I activity in HIV infection. We are now looking at the role of other complexes of oxidative phosphorylation and their expression during HIV induced T cell apoptosis.

Molecular basis of CTL dysfunction in HIV infection

HIV-1 is a uniquely difficult target to develop immunological intervention against it. Despite a high frequency of HIV-specific $CD8^+$T cells, most HIV-infected patients fail to control viral replication without antiviral drugs suggesting an impairment of the CTLs. Indeed, $CD8^+$T cells are active in containing acute HIV infection but not in chronic infection. The inability is related to the failure of these cells to mature into fully differentiated effector cells. Maturation into competent CTLs may be blocked during

the initial encounter with antigen because of defects in antigen presentation by dendritic cells or HIV-infected macrophages. $CD8^+$T cell dysfunction can partially be corrected *in vitro* with short-term exposure to interleukin-2, suggesting that impaired HIV-specific $CD4^+$T helper function may also play a significant causal or exacerbating role. Based on the data from our previous studies, the present work involves understanding the role of IL-12 and IFN-γ in the elicitation and maturation of CTL against HIV-1 envelope protein gp120 using DNA immunization of mice with HIV-1 subtype C gp120 encoding expression vector. Although wild-type BALB/c mice responded well to pcgp120 immunization, the response was severely compromised in IL-12- and IFN-g-deficient mice. Co-priming with IL-12 vector rescued the CTL activity in IL-12-deficient mice but not in IFN-g-deficient mice, indicating the importance of IL-12 in the elicitation phase of CTL production but probably not in the maturation step. In contrast, IFN-γ, which might be induced by IL-12 in the first phase, might help in the CTL maturation, perhaps associated with the acquisition of cytotoxic properties. Our recent results also show the role of CD40 and IL-12 in priming of $CD4^+$ cells for providing help in $CD8^+$ cells in CTL maturation. Further our data also provides preliminary evidence for the importance of IFN-γ in inducing expression of CTL effector molecules, perforin and granzyme. Finally, finding means of rescuing an impaired CTL functions may help devise an immunotherapeutic strategy to control HIV replication or boost existing strategies.

Identification of novel molecules with anti-HIV activity

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 unable 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 in natural resources. We have performed screening of anti-HIV activity in marine bivalves of Indian coastline. Our studies have revealed the presence of anti-HIV activity in green mussel and clams. The clam lysate have been fractionated and a pure active fraction has been obtained, which is currently being analyzed for identification of the lead molecule.

Contrary to the previous prediction of some cholic acid derivatives to be HIV-1 protease inhibitors, we have observed that these compounds enhance HIV-1 replication with induction of syncytia formation. The fold increase in the viral load was also found to be proportional to the increase in syncytia formation. As syncytium is formed by cell-cell fusion, it will be interesting to study the molecular basis of this induction. The syncytia inducing property of cholic acid analogues may also be useful for screening the efficacy of compounds which inhibit syncytia induction and also for screening the efficacy of the existing and novel anti-retroviral drugs by artificial enhancement of HIV-1 replication and syncytia formation. Finally, these compounds were also found to activate latently infected cells and thus could be potentially used along with anti-retroviral therapy.

Future Work

The future work involves characterizations of some of the other Nef interacting clones and identifying their functional relevance in HIV lifecycle. We are also looking at the chromatin modulation by Tat protein in HIV infection, both in acute and latently infected cells. Studies are also in progress to find a physiological relevance for Tat-NFκB interaction. Identification of differentially expressed genes and their relevance to HIV induced cell death will be continued, with a focus on mitochondrial oxidative phosphorylation system in infected cells. We are continuing our studies on the role of CD40, IL-12 and IFN-γ in elicitation and maturation of CTL in response to gp120 immunization in mice. The study also intends to identify the viral mechanism of inhibition of the IFN-g-induced CTL maturation and how it could be overcome. Finally, studies are in progress to identify novel anti-HIV molecules, both from natural resources and synthetic chemistry.

Characterization of house keeping genes from *Leishmania*

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Background

The parasitic protozoa *Leishmania* belongs to the order Kinetoplastida and is the causative agents of disabling and incurable diseases known as leishmaniasis. Glucose is the major carbon source of energy for the parasite and its metabolism in Kinetoplastida differs from other eukaryotes. In Kinetoplastids including *Leishmania*, the first seven glycolytic enzymes are sequestered in peroxisome-like organelles called glycosomes. Hexokinase is a rate limiting enzyme in glucose metabolism. It plays important role in energy metabolism in flying insects and parasites. Among the kinetoplastids, including species of *Leishmania*, universal regulatory enzymes of glycolysis, including hexokinase, have lost their regulatory properties and therefore it is essential to study this enzyme and its gene from Leishmanial parasite.

Selenium is an essential trace element. Selenium exerts its biological role in the form of selenocysteine that is incorporated co-translationally by a specific tRNA and is directed by a UGA codon in the mRNA. All selenoenzymes described are found in oxidation-reduction reactions and the selenocysteine residue participates in the enzyme catalysis. Intracellular stage of the Leishmania amastigote survives and multiplies within the phagolysosome in the presence of toxic ROS and acidic environment. As selenoenzymes play significant role in metabolism of ROS, study of selenoprotein synthesis and activity is necessary to elucidate the pathogenesis of *Leishmania*. The selenophosphate synthetase enzyme catalyses the first step in elemental selenium metabolism. Hence we have cloned and are in process of characterizing role of SPS protein in *Leishmania*.

Aims and Objectives

1. To find out how *Leishmania* survives in the host macrophage and whether parasite uses selenoproteins for its defense.

2. To study how glycosomal proteins are also used for defense by parasite.

Work Achieved

1. In order to confirm the functionality of the Leishmanial selenophosphate synthetase [*selD*], the gene *selD* was cloned in pET vector. A mutant *E. coli* strain deficient in *selD* was used for the complementation. This mutant can not synthesize selenoenzyme namely formate dehydrogenase. Leishmanial *selD* complemented the loss of bacterial *SelD*, which can be deduced from the positive reaction for the formate dehydrogenase using benzyl viologen dye. The positive complementation is viewed as purple color *E. coli* colonies upon reduction by the formate dehydrogenase.

2. From these positive complementation clones, we observed that the *SelD* protein is not present in the inclusion bodies but is present in soluble fraction. Using nickel affinity column we have purified the *SelD* protein.

3. The expression of the *SelD* gene was detected in amastigote stage with the help of RT-PCR and it was found that the expression of the SPS is increasing with increase in time of infection of macrophage cell line J744.1 with *Leishmania*.

4. Sucrose gradient subcellular fractions of *Leishmania* donovani were collected and total proteins were subjected to western analysis with anti Hexokinase antibodies. The results indicated that hexokinase is present in the glycosomal fraction and that it is "leaky" as it was present more or less in all the different density glycosomes from 30% to 60% sucrose. Hence immunofluorescence was performed to reconfirm and it clearly showed a punctate pattern of staining for hexokinase and it co-localized with two glycosomal markers namely HGPRT and PEX7.

5. Axenic amastigotes were made from *L. donovani* and western blotting was done to check for HK stage specific expression. It was in accordance with the transcriptional level data for *L. donovani* and suggestive of similar hexokinase contents in pro- and amastigotes.

6. Transfections in *L. donovani* were performed with different constructs having hexokinase and GFP sequences. Proteins from the lysates obtained form all the transfectants were passed through PEX7 column. It was found that the PEX7 could pull down both wild type and GFP tagged HK, and also PTS2-GFP. The tag PTS-2 is proved to be very much specific for glycosomal targeting through interaction with PEX7.

Future Work

We would like to perform the kinetic studies with selenophosphate synthetase enzyme purified from recombinant source. The gene inactivation is ongoing project for *SelD* gene in *L. major* to elucidate the function of the gene. Biogenesis of peroxisomes using hexokinase as a model protein will be done using anti-Hexokinase antibodies.

Role of T cells and non-T cells in the resistance and susceptibility to leishmaniasis

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Aims and Objectives

To understand the mechanism of resistance or suspectibility to *Leishmania* infection.

Work done

Leishmaniasis is a disease that kills millions of people worldwide. The disease is caused by a protozoan parasite, *Leishmania* sp. The hall mark of the infection is severe immuno-suppression. We have worked on the mechanism of such immuno-suppression and the development of prophylactic and therapeutic principles.

Mechanism of immuno-suppression: The immuno-suppression is the result of both T cell and non-T cell functions. Among the non-T cells, the macrophages are skewed to the pro-parasitic functions. As we showed previously, macrophages express CD40 as a costimulatory molecule that signals in the macrophages resulting in the modulation of its effector functions. We have shown that the mechanism of immuno-suppression lies in the skewing of CD40 signaling through ERK-1/2 in infected macrophages. However, how the same CD40 molecule signals through either p38MAP kinase or ERK-1/2 remained to be elucidated. Now, we show that CD40 signaling through Ras-Raf-1-MEK-1/2 results in ERK-1/2 activation and in Leishmania-infected macrophages, this pathway is exaggerated including Ras phosphorylation, which is a membrane proximal event. On the other hand, the CD40 signaling through Lyn, PKC-β and MKK-3/6 results in p38MAP kinase activation and in *Leishmania*-infected macrophages, this pathway is suppressed. Therefore, based on these findings, we argued that the *in vivo* suppression of ERK-1/2 or exaggeration of p38MAP kinase might result in the amelioration of *Leishmania* infection.

Relating to the role of CD40 doses in immune response, as we showed previously *in vitro* in macrophages, we explored whether such dose responses are important *in vivo*. We observed that mice expressing different levels of CD40 or CD40-ligand, showed differences in T cell responses against tumor and *Leishmania* infection. In both the cases, low CD40

expression or low CD40-ligand expression resulted in the preferential induction of T-reg-like cells with suppressive effects.

For the role of T cells in the observed immuno-suppression, we observed that interleukin-2 played a crucial role in regulatory T cell development in the periphery. During the initial week of infection, IL-2 production is not impaired and following the second week of infection, immuno-suppression ensues. We observed that the immuno-suppression- measured in terms of DTH, T cell proliferation and parasite killing in macrophages- can be significantly prevented by IL-10 neutralization. Interestingly, blocking IL-2 and IL-2 receptor also prevented the immuno-suppression. We demonstrated that immediately after infection, there was a surge of production of many cytokines, of these, IL-2 induced IL-10 producing T cells that mediated the suppression in the effector phase. Corroborating to this finding, we find that anti-IL-2 treatment is effective in the early phase of infection while IL-10 blockade is effective at a later stage of infection. The observation led us to propose the principle of phase-specific immunotherapy.

MHC class-II as a signaling molecule: It has been the dogma of antigen-presenting cell-T cell interaction that the antigen-presenting cell acts as the servile presenter of antigen and the costimulatory signal to T cells triggering the T cell activation, differentiation and effector functions. Here, we have shown that MHC-II-Antigen-T cell receptor complex, which is formed during the antigen presentation, signals not only through T cell receptor but also through MHC-II. Although such possibilities were indicated earlier, we were the first to show that MHC-II constitutively associates with Platelet activating factor receptor (PAFR), which is a G-protein coupled receptor. We characterized the signaling pathway explaining how Staphylococcal Enterotoxin B (SEB) binding to MHC-II resulted in a biphasic TNF-a production. We have also shown that blocking this pathway prevents SEB-induced fatal toxic shock syndrome in mice.

Dendritic cells in vaccination and in therapy: We have shown that irrespective of the dendritic cell subset, high dose CD40 stimulation and concomitant IL-10 blockade can result in host-protective anti-leishmanial and anti-tumor T cell effectors and memory. Therefore, the relationship between CD40 and IL-10 and T cell memory was elucidated for the first time. We have shown that lower the expression of CD40, higher is the propensity to generate IL-10-dependent T-reg cells. IL-10 neutralization reduces the suppression and consequently, results in amelioration of *Leishmania* infection and regression of tumor.

Future Work

In the coming year, we will work on the mechanism of reciprocal CD40 signaling.

Role of viral complement control proteins in immune evasion

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Background

The complement system is an integral participant in the innate mechanisms of immunity and thus has a burden of performing surveillance in the host and protecting it from all the pathogens including viruses. Earlier studies have decisively demonstrated that both acute as well as latent viruses are susceptible to complement-mediated neutralization. Thus, complement exerts a strong selective pressure on viruses during infection. These data suggest that for their successful survival, viruses must have developed mechanisms to subvert this system. Consistent with this premise, genome sequencing of poxviruses and herpesviruses have shown that members of these families encode for structural homologs of human regulators of the complement activation (RCA) family. Our working hypothesis is that these viruses encode complement regulators (vCCP) to mask themselves against the host's complement attack and are vital for their successful *in vivo* survival and propagation. Our current emphasis is on understanding the mechanism by which the vCCPs inhibit complement and the molecular basis behind it. Studies in our lab primarily focus on vCCP of vaccinia virus (VV), Herpesvirus saimiri (HVS) and Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8). Our long-term goal is to understand how complement controls viral infections and how vCCPs help subverting it.

Aims and Objectives

1. How vCCPs inactivate complement?
2. Which are the determinants of vCCPs important in complement inactivation?
3. What role vCCPs play in viral pathogenesis?

Work Achieved

The vCCPs are formed by four tandemly-repeating bead-like structures termed complement control protein (CCP) domains or short consensus repeats (SCRs) or sushi domains. These are separated by linkers of four residues which impart flexibility to these proteins. Sequence comparison of vCCPs shows that the sequence similarity between the poxvirus homologs exceeds 91% while that amongst the herpesvirus homologs varies from 43%-89%. These data suggest that the herpesvirus homologs are more diverse in structure compared to the poxvirus homologs. Previously, we have performed the functional analysis of vCCPs of vaccinia virus (VCP) and KSHV (kaposica). By utilizing various functional assays we showed that these proteins bind to human complement proteins C3b and C4b and regulate complement by acting on classical/lectin and alternative pathway C3 convertases. The regulation was achieved by two distinct mechanisms: (i) by accelerating the irreversible dissociation of the classical/lectin (C4b, 2a) and to a limited extent alternative (C3b,Bb) pathway C3-convertases and (ii) by serving as cofactors in serine protease factor I-mediated inactivation of C3b and C4b (the subunits of C3-convertases). We also studied the binding mechanism of these vCCPs with the target proteins C3b and C4b. Unlike human regulators which followed complex binding models, viral regulators followed a simple 1:1 binding model and showed transient binding to the target proteins. It seems that this very ability allows them to regulate many target proteins in a relatively short time. Our efforts in mapping the functional domains in VCP and kaposica revealed that different domains are required for different functional activities, which explained why the four-domain structure is highly conserved in different viruses.

Whether structural diversity in herpesvirus homologs is also reflected in their function was not clear until now as amongst the herpesvirus homologs, detailed functional characterization was performed only for kaposica. We, therefore, undertook a study on functional characterization of Herpesvirus saimiri homolog (HVS CCPH). Our data showed that HVS CCPH possesses all the complement regulatory activities present in kaposica and VCP. Thus, it appears that in spite of significant sequence differences between herpesviral complement regulators, the functional activities have been conserved. These data along with previous observations therefore point out that

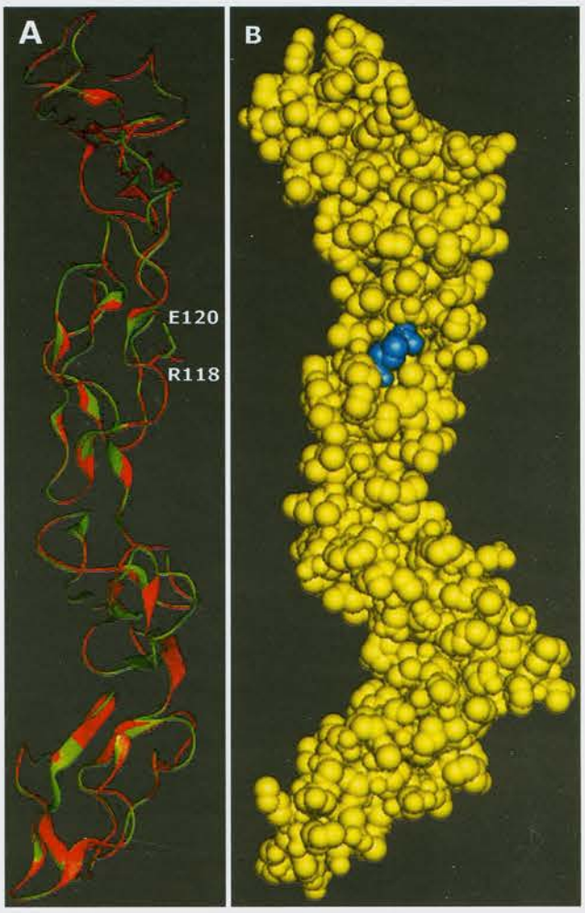


Fig. 1: Model of HVS CCPH depicting R118, the residue important for its cofactor activity. The model was built by utilizing VCP crystal structure as the template using SWISS-MODEL. (A) Overlap of ribbon models of HVS CCPH (red) and VCP (green) structures. The side chains of R118 of HVS CCPH and the corresponding residue E120 of VCP are labeled. (B) Solid surface presentation of HVS CCPH model showing exposed R118 in blue.

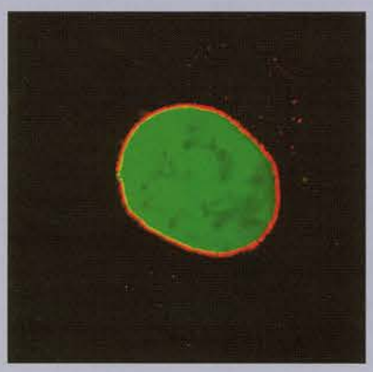
maintenance of various complement regulatory functions must be important to the pox as well as herpesviruses. Whether sequence variations in herpesviral complement proteins have resulted in acquisition of any new functions is not clear at present and requires further analysis. Interestingly, we found that HVS CCPH possesses 14-fold higher factor I cofactor activity against C3b. Site-directed mutagenesis revealed that R118 contribute significantly to the factor I cofactor activity (Fig. 1).

Currently, efforts are being made to delineate the functional sites within the SCR domains and characterization of role of vCCPs in pathogenesis.

Future Work

1. Fine mapping of functional sites in VCP and kaposica.
2. Effect of modulation of electrostatic potential on functional activities.
3. Role of VCP-complement interaction in VV virulence.

Chromatin Architecture & Gene Regulation



Transcriptional regulatory mechanisms by MAR binding protein SMAR1

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Background

Our laboratory is interested in understanding the role of a MAR binding protein, SMAR1 that initially was identified through its direct binding to MARβ sequence at TCRβ locus in T cells (Chattopadhyay *et al.*, 2000). Upon overexpression, SMAR1 along with Cux represses Eβ mediated transcription at TCRβ, implying its role in the chromatin accessibility and control of V(D)J recombination (Kaul-Ghanekar *et al.*, 2004). Interestingly, SMAR1 transgenic mice exhibit defective V(D)J recombination at specific Vβ's (Kaul-Ghanekar *et al.*, 2005). SMAR1 protein sequence shows more than 99% homology with its human counterpart BANP and has been mapped to the human locus 16q24.3. Loss of heterozygosity (LOH) at this locus is frequently reported in breast, colon and prostate cancer along with Cyclin D1 deregulation (Cleton-Johnson *et al.*, 2000). Further we have established that SMAR1 interacts with HDAC1 associated repressor complex at Cyclin D1 promoter and allows histone deacetylation to cause its transcriptional repression (Rampalli *et al.*, 2005). Considering the inverse correlation of SMAR1 and Cyclin D1 levels in breast carcinoma cell lines, we extrapolated this study in different grades of breast carcinoma tissue samples. Based on these observations we hypothesize that SMAR1 might bind to various MARs and regulate various genes. SMAR1 could also cross-talk with other important cell cycle regulatory proteins and govern cell fate decisions. In this context, the most we have shown that SMAR1 interacts and modulates p53 functions. Our recent studies also show that cellular stress may play an important role in regulating SMAR1 expression. We are also interested in identifying the post translational modifications of SMAR1 that could modulate its functions. Our current strategy is to analyze structure function relationship of SMAR1, by various biophysical tools. Since the protein is shown to affect V(D)J recombination, we have extended the study to look into its role in T cell immune response.

Aims and Objectives

1. Regulation of SMAR1 by p53
2. Regulation of transcription by SMAR-p53-MDM2 ternary complex
3. Role of SMAR1 in the regulation of genes pertinent to cellular transformation and metastasis
4. PGA2 mediated regulation of SMAR1 and Cyclin D1 transcription

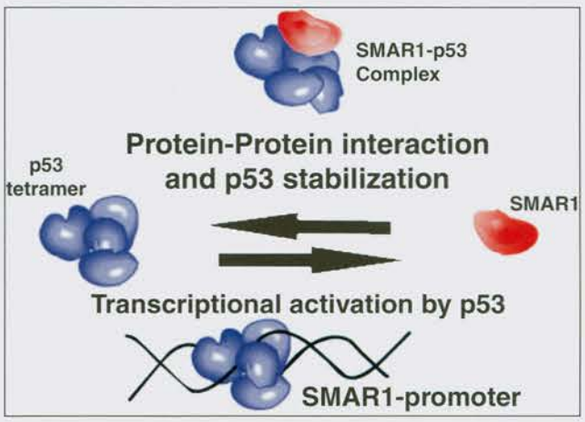

Fig. 1: SMAR1 activates p53 through phosphorylation that in turn modulates global transcription from various promoters

Work Achieved

Regulation of SMAR1 by p53

Earlier we have shown that SMAR1 interacts with p53 and stabilizes it in the nucleus. Further we characterized and cloned SMAR1 promoter and found that promoter activity of SMAR1 is induced upon Doxorubicin treatment in a p53 dependent manner. p53 directly affects SMAR1 transcription through its cognate binding sites on SMAR1 promoter. Thus we established that SMAR1 and p53 exist in a feed forward loop (Fig. 1).

Regulation of transcription by SMAR-p53-MDM2 ternary complex

The intra-cellular levels of tumor suppressor protein p53 are tightly controlled by an auto regulatory feed back loop comprising of p53 and MDM2. MDM2 is a well known ubiquitin ligase, that ubiquitinates and degrades p53. We have observed that SMAR1, a MAR binding protein (MARBP), previously known to interact with p53 also interacts with MDM2. We have evidence for the presence of a ternary complex of SMAR1, MDM2 and p53, contributing to the transcriptional regulation by p53. Our studies on SMAR1 thus provide a link between the masking serine-15 phosphorylation of p53, enabling MDM2 to enhance the deacetylation of p53. This perturbs the sequence specific DNA binding and transcriptional activity of p53. Since SMAR1 binds with much higher affinity to ser 15 phosphorylated form of p53 we hypothesized that this could be a sequestering mechanism for MDM2 to still interact and repress p53 target genes in the presence of SMAR1. Since MDM2 had been shown to bring about repression of apoptotic target genes of p53 in Vm10 cell line, we extended this study that would implicate the presence of SMAR1 in a ternary complex with these proteins. Involvement of MDM2 comes to the fore as SMAR1 binds to ser15 phosphorylated form of p53 often thought to bring about binding of other transcriptional co activators like p300 and acetylate p53 at the lysine residues. We hypothesize that SMAR1 binding to ser15 p form is a masking mechanism for MDM2 to bring about transcriptional repression of certain p53 target genes. Thus we propose that SMAR1, under conditions of over expression, can serve as a transcriptional regulator of p53 and check cell cycle fate (Fig. 2).

Dysregulated/Defective SMAR1-p53 feed forward loop in breast cancer

SMAR1, being a MAR binding protein, acts as a repressor of Cyclin D1. Its repressor activity is governed through the recruitment of HDAC1-mSin3a complex at the MAR

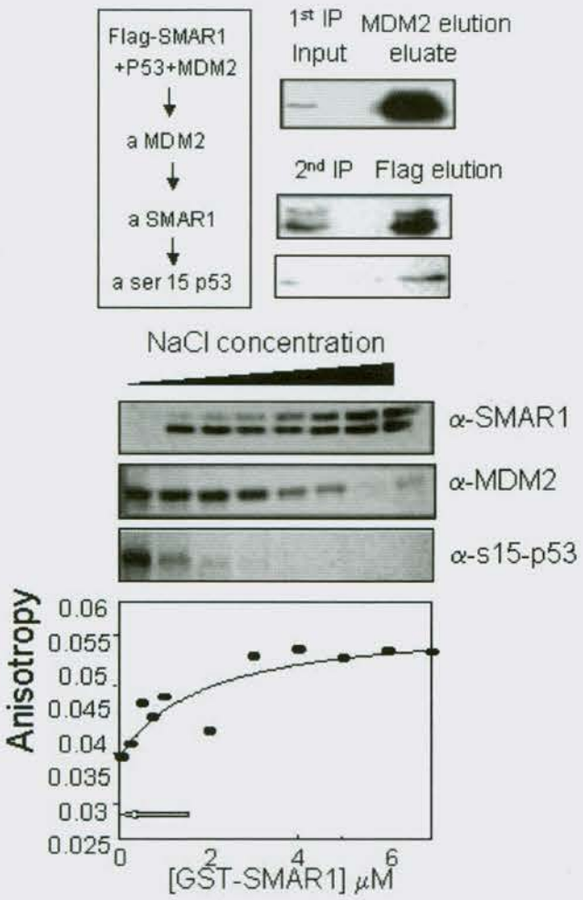

Fig. 2: SMAR1 exists in a ternary complex with p53 and MDM2

site of Cyclin D1 promoter (Rampalli *et al.*, 2003). We have shown that Cyclin D1, upregulation of which is a hallmark in breast cancer, is a direct target of SMAR1 (Rampalli *et al.*, 2005). As an extension of that study, now we also demonstrate that SMAR1 expression is drastically reduced during advancement of human breast cancer due to dysfunctioning of p53, resulting in dysregulated Cyclin D1 expression. SMAR1 expression was checked in sixty breast cancer samples including thirty malignant IDC grade I, II and III along with thirty benign fibro-adenoma samples including three lactating benign fibro-adenoma

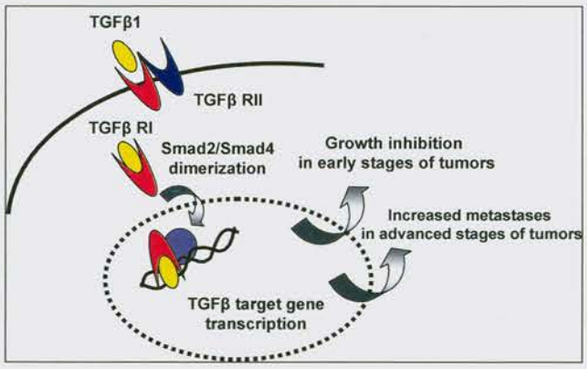

Fig. 3: A model showing the SMAR1 mediated changes of p53 acetylation and consequently changes in cell proliferation. In higher stages of cancers, p53 is more acetylated and sequestered in the nucleolus. The unavailability of p53 in the nucleus reduces SMAR1 expression.

cases. SMAR1 expression in tumors was compared with its expression in nearby normal breast tissue. A basement membrane disruption along with altered tubular architecture was observed which was also associated with calcification, in IDC Grade I, II and III as compared to fibro-adenoma benign sample). In this regard, earlier it was shown that TGFβ upregulation is another hallmark of various cancers. The Malignant transformation of breast carcinoma involves activation of pro-migratory signals including TGFβ. Now we show that ectopic expression of SMAR1 inhibits TGFβ signaling and its downstream target genes that are involved in tumor cell migration and metastases (Fig. 3). Higher Cyclin D1 expression along with activated TGFβ signaling is correlated to reduced SMAR1 expression in human breast cancer. Thus, the newly identified tumor suppressor SMAR1 plays a critical role in cell proliferation and tumor metastases and either directly or indirectly involved in cancer (Fig. 3).

Repression of Cyclin D1 transcription and cell cycle arrest by PGA2 requires SMAR1

Prostaglandin A2 (PGA2), a tumor suppressor chemotherapeutic agent, acts as an antiproliferative agent *in vitro*. PGA2 exerts its growth inhibitory effect

through modulation of expression or activity of several key cell cycle regulatory molecules in various cell lines. For example, it has been shown to affect the Cyclin D1 levels by decreasing the mRNA stability. Recently, we reported that SMAR1, a tumor suppressor protein down regulates Cyclin D1 expression in MCF-7 cells by recruiting HDAC1/ Sin3A complex on the promoter. In the present study, we show that PGA2 can also affect the transcriptional status of Cyclin D1 by stabilizing SMAR1 mRNA. The up regulated SMAR1 in turn binds to Cyclin D1 promoter, deacetylates and methylates the histones at the loci, switching off active gene transcription. Further, the cell cycle arrest function of PGA2 requires SMAR1, as the knock down of SMAR1 results in the release of cells from G1 to S and G2 phase. Collectively, we provide a new mechanism for the transcriptional repression of Cyclin D1 upon PGA2 treatment.

Future Work

To elucidate the structure of SMAR1 using NMR, CD and X-ray crystallography.

Elucidating the role of SMAR1 in T cell immune response with respect to Th1/ Th2 lineage commitment.

Mechanism(s) involved in the regulation of the MAR-binding activity of SATB1

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Background

The T-cell specific transcription factor SATB1 (Special AT-rich binding protein 1) is critical for expression of large number of genes involved in T-cell development. Studies utilizing SATB1 knock out mice indicate that ~2% of the genes were de-repressed at appropriate stages of T-cell development in a spatio-temporal manner. SATB1 was shown to regulate distant genes by selectively tethering BURs forming a distinguished 'cage-like' network in thymocytes that presumably demarcates heterochromatin from euchromatin, and by acting as a 'docking site' for several chromatin modifiers. The N-terminal PDZ-like domain of SATB1 is a putative region for interactions with other proteins and also aids in the formation of SATB1 homodimer that is essential for its DNA binding. The various chromatin modifiers recruited by SATB1 were suggested to alter the gene expression at SATB1-bound MARs. SATB1 also regulates gene expression by directly influencing the promoter activity of globin gene by interacting with CBP, and regulates *IL-2* and *IL-2Rα* expression by recruiting HDAC1. Thus, the ability of SATB1 to regulate transcription at global level presumably stems from its unique ability to bind large number of regulatory sequences including promoters and MARs and recruit regulatory proteins to the binding sites. Although SATB1 was shown to interact with HDAC1 and also with CBP/p300, how these interactions lead to regulation of transcription is not clear. Moreover, whether interaction with HAT causes acetylation of SATB1 is not reported. This prompted us to investigate if SATB1 interacts with any other HATs and its consequence on the acetylation status of SATB1. Protein kinases and phosphatases are among the most important and abundant eukaryotic regulatory proteins. In general, phosphorylation or dephosphorylation acts as a triggering signal for many cellular processes and is catalyzed by kinases or phosphatases, respectively. We therefore monitored if SATB1 is phosphorylated *in vivo* and the effects of such modification on the transcriptional activity of SATB1.

Aims and Objectives

1. To identify proteins interacting with SATB1 through its PDZ-like domain.

2. To study the mechanism(s) by which SATB1 regulates transcription by interacting with other cellular proteins through its PDZ-like domain.

3. To monitor the effect of posttranslational modifications of SATB1 on its MAR-binding activity and on global gene regulation.

Work Achieved

Phosphorylation of SATB1 determines whether it associates with HDAC1 or PCAF

We previously demonstrated that HDAC1 interacts with SATB1 via its PDZ-like domain. We found that SATB1 interacts with PCAF also via the same PDZ-like domain. Since these two proteins exert opposing effects on transcription, it is of interest to delineate the molecular mechanism of how SATB1 can choose to interact with one and not the other. Therefore, we asked if phosphorylation of SATB1 would affect its interaction with HDAC1 and PCAF. Immunoaffinity column chromatography and co-immunoprecipitation analysis revealed that phosphorylated SATB1 preferentially binds HDAC1 whereas unphosphorylated SATB1 preferentially binds PCAF. Phosphorylation status of SATB1 had no effect on its interaction with p300, and that unphosphorylated SATB1 failed to immunoprecipitate with anti-HDAC1. Upon treatment with TSA, SATB1 could be immunoprecipitated efficiently with anti-PCAF and not with anti-HDAC1. These results indicated that SATB1 interacts with either HDAC1 or PCAF depending on its phosphorylation status. To unequivocally demonstrate this, we next performed *in vivo* co-IP using newly raised anti-peptide antibodies against phosphorylated and acetylated SATB1. PCAF was efficiently immunoprecipitated by anti-SATB1 and anti-A-SATB1 (Fig. 1A, lanes 3 and 6) but not by anti-P-SATB1 and anti-HDAC1 (lanes 1 and 2). Similarly, HDAC1 was immunoprecipitated by anti-P-SATB1 and anti-SATB1 (Fig. 1B, lanes 3 and 4) but not by anti-A-SATB1 (lane 1). We also performed *in vitro* His-tag pull down experiment using WT and S185A SATB1. Jurkat lysate was passed through the affinity columns and bound proteins were eluted and analyzed by immunoblotting. Phosphorylated WT-SATB1 pulled down HDAC1 but not PCAF (Fig. 1C, lane 1). The non-phosphorylatable S185A SATB1 displayed efficient interaction with PCAF than the HDAC1 (lane 2). WT-SATB1 interacted with both HDAC1 and PCAF (lane 3). To evaluate the effect of phosphorylation and acetylation of SATB1 on its transcriptional activity, we performed Luciferase reporter assay using MAR-linked reporter construct and cotransfecting constructs expressing SATB1, HDAC, PCAF, S185A and K136A mutants in various combinations. Expression of SATB1 together with HDAC1 resulted in downregulation (Fig. 1D, lane 8), and K136A behaved similarly (lane 10). However, S185A not only failed to downregulate, but showed activation of MAR-linked reporter (lane 12). In presence of PCAF, SATB1 mediated repression was abolished (lane 7), but K136A failed to show similar kind of derepression (lane 9). The non-phosphorylatable S185A mutant led to upregulation of luciferase activity in the presence of PCAF in a manner similar to, but two-fold higher than, WT SATB1 (lane 11) and failed to achieve downregulation even in presence of HDAC1 (lane 12). These results demonstrated that phosphorylated SATB1 acts as a repressor by associating with HDAC1. Furthermore, dephosphorylated SATB1 interacts with PCAF and is acetylated by it as a result it loses its MAR-binding ability leading to derepression of linked gene expression.

Phosphorylation and acetylation of SATB1 have contrasting effects on gene expression at a global level

We wished to test the effect of the SATB1 posttranslational modifications on its ability to function as a global repressor at its targets. We therefore overexpressed the WT SATB1 and the S185A and K136A mutants in HeLa cervical epithelial

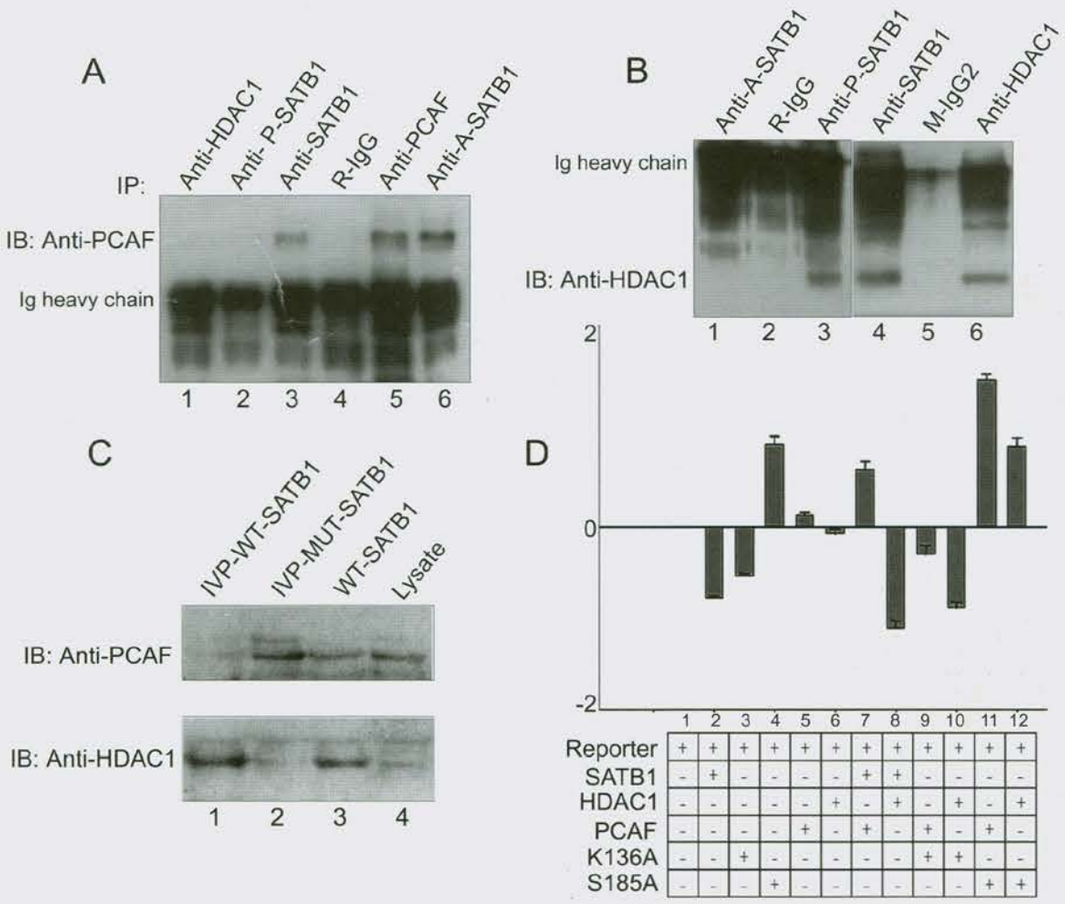

Fig. 1: Phosphorylation-dependent association of SATB1 with HDAC1 and PCAF. (A) and (B) *In vivo* Co-IP analysis. PCAF (A) or HDAC1 (B) were immunoprecipitated from Jurkat cell extract using anti-P-SATB1, anti-A-SATB1, anti-SATB1, anti-PCAF, and anti-HDAC1 and detected by immunoblot analysis using corresponding antibodies. (C) *In vitro* phosphorylation (IVP) of WT and S185A SATB1was performed in the presence of PKC. Jurkat cell lysate was incubated with these proteins and bound proteins were analyzed by immunoblotting with anti-PCAF (top panel) and anti-HDAC1 (bottom panel). (D) The effect of phosphorylation on the transcriptional potential of SATB1 was monitored by *in vivo* Luciferase reporter assay. The IgH-MAR driven reporter construct was cotransfected with various combinations of WT, S185A, K136A, HDAC1 and PCAF constructs in 293T cells and reporter assay was performed. Each error bar depicts standard deviation calculated from triplicates.

cells that do not express SATB1 and Jurkat T lymphoblastic cells that express SATB1. The mRNA expression profiling was performed using microarray chips containing 19,000 human cDNAs. Surprisingly, greater than 10% of genes showed significant differences upon overexpression of WT and mutant SATB1. The fold difference in expression of genes showed two distinct profiles. Genes which were downregulated by WT and K136A were upregulated upon S185A overexpression. We randomly picked 22 different genes including GAPDH as control and analyzed their

Fig. 2: Cluster analysis of gene expression in HeLa cells. Normalized microarray datasets were analyzed by unsupervised hierarchical clustering, using average linkage algorithm using 'Cluster', and the results of clustering were visualized by 'Tree View' using the softwares available at http://www.microarrays.org/software.html. Only fraction of the dysregulated genes are represented in this figure. Due to dye swap colors are represented in opposite manner than the usual as follows: Green color indicates upregulation, dark green corresponds to maximum upregulation. Red color indicates downregulation, dark red corresponds to maximum downregulation. The columns are: HM. Overexpression of S185A mutant SATB1, HA. Overexpression of K136A mutant SATB1 and HWT. Overexpression of WT SATB1.

transcriptional activity in HeLa cells in the presence of WT, K136A, and S185A SATB1. We revalidated these results by quantifying the transcripts using real time RT-PCR. Additionally, cluster analysis of gene profiling data revealed that WT SATB1 and K136A mutant clustered together (Fig 2). Strikingly, S185A clustered away from the WT and K136A cluster as evident by distinct upregulation of the targeted genes (Fig. 2). RNA-interference (RNAi) mediated silencing of endogenous SATB1 in 293T cells resulted in reduction in transcription of many of the above genes, confirming the direct role of SATB1 in their transcriptional upregulation. Taken together, these results unequivocally demonstrated that phosphorylation of SATB1 downregulates gene expression at global level whereas acetylation leads to upregulation.

Recruitment of HDAC1 or PCAF to the *IL-2* promoter *in vivo* is dependent on the phosphorylation status of SATB1 at S185

We previously showed that *IL-2* is a direct target of SATB1. We therefore monitored the occupancy of factors at the *IL-2* promoter during activation using ChIP assay. Time course analysis of *IL-2* expression following ionomycin treatment showed gradual increase that peaked at 6 hr of treatment (Fig. 3A). Immunoblot analysis indicated that SATB1 level was constant during the entire time course experiment (Fig. 3B, top panel). Interestingly, a time-dependent decline and increase was observed with respect to its phosphorylated and acetylated forms respectively (Fig. 3B). These results prompted us to monitor the *in vivo* occupancy of SATB1, HDAC1 and PCAF

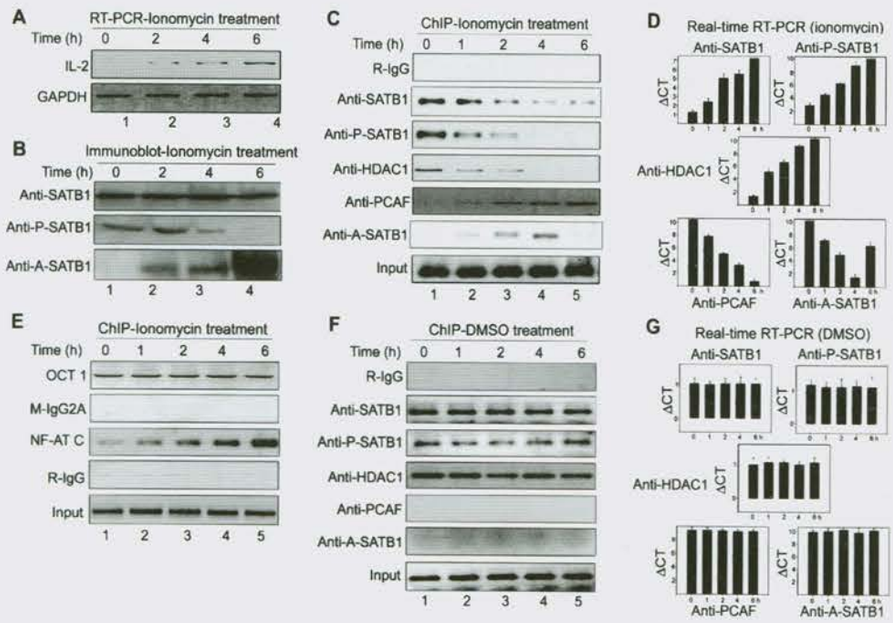

Fig. 3: Phosphorylation-dependent association of HDAC1 or PCAF with SATB1 and recruitment to the *IL-2* promoter *in vivo*. (A) Jurkat T cells were activated by treatment with ionomycin. RT-PCR analysis of IL-2 expression was performed using RNA isolated from cells at indicated times following activation. (B) Content of total SATB1, phosphorylated and acetylated SATB1 during first 6 hr of T cell activation was monitored by immunoblot analysis using anti-SATB1, anti-P-SATB1 and anti-A-SATB1. (C) and (F) ChIP analysis of *IL-2* promoter region by anti-SATB1, anti-P-SATB1, anti-HDAC1, anti-PCAF and anti-A-SATB1 antibodies during ionomycin and DMSO treatment. (E) ChIP analysis of IL-2 promoter region by anti-OCT1 and anti-NF-AT C upon activation. (D) and (G) Quantification of immunoprecipitated chromatin was performed by the real-time PCR analysis and Δ Ct values were plotted as bar graphs. Each error bar depicts standard deviation calculated from triplicates.

on *IL-2* promoter during T-cell activation. ChIP analysis using anti-SATB1, anti-P-SATB1, anti-A-SATB1, anti-HDAC1 and anti-PCAF demonstrated that phosphorylated SATB1 occupied the *IL-2* promoter upto 2 hr (Fig. 3C, third panel) and recruited HDAC1 (fourth panel). Strikingly, PCAF gradually occupied the promoter (fifth panel) during the later time points of activation. As a consequence of this the acetylation status of SATB1 was enriched (sixth panel) and after 6 hr acetylated SATB1 showed reduced occupancy on *IL-2* promoter region presumably due to its dissociation. These results corroborate with that of anti-SATB1 immunoprecipitated chromatin and suggest that SATB1 dissociates from the *IL-2* promoter region upon activation (second panel), leading to upregulation of *IL-2* transcription. These results were quantified and verified by real time PCR analysis (Fig. 3D). Interestingly, OCT1, which constitutively binds to the *IL-2* promoter, does not show significant change in its occupancy while the activation-induced binding of NF-AT C is apparent (Fig. 3E). In presence of DMSO which is the vehicle for ionomycin, we do not observe any significant changes in the occupancy of SATB1, phospho-SATB1, HDAC1 and therefore we also do not observe any occupancy by PCAF and acetyl-SATB1 at the *IL-2* promoter (Fig. 3F and G). These results prove that phosphorylation acts as a molecular switch determining the association of SATB1 with HDAC1 or PCAF, which in turn have contrasting effects on expression of individual target genes via differential recruitment of factors at the level of promoters.

Future Work

1. To investigate the role of the PDZ-mediated interactions on regulation of transcription of cytokines and their receptors by SATB1 in T cells.

2. To study SATB1-mediated dynamic organization of chromatin at its genomic binding sites including regulatory regions of genes important for T cell development.

3. To study phosphorylation-dependent global gene regulation by SATB1.

Support Units

Experimental Animal Facility (2005-2006)

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The Team
Mr. Md. Shaikh
Mr. A. Inamdar
Mr. P.T. Shelke
Ms. Vaishali Bajare
Mr. Sanjay Gade
Mr. Dilip Thorat

The Experimental Animal Facility is an infrastructural service department of the Institute to provide husbandry, veterinary care and research technical support facilitating animal experimentation. 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
129/SvJ
FVB/NJ
NOD/LtJ
SWISS
BALB/c*
Nude Mice
Genetically engineered mutant mice (knock-out and transgenic mice-27 lines)

Rats: WISTAR
LEWIS

Rabbits: NEWZEALAND WHITE

Mastomys: MASTOMYS COUCHA

Defined barrier practices are followed scrupulously without any exception or allowance, with access to a select few personnel, to minimize the risk of microbial infection to the animals housed in the facility.

The breeding program for the propagation of the two different inbred lines viz. BALB/c and C57BL/6 is structured in a two-tier format, i.e. the Foundation colonies (FC) and the Production colonies (PC). The remainder inbred and mutant (gene knock-out) mice are maintained as foundation colonies. Strict full-sib pairing only propagates the animals in the FC. These colonies are the nuclear colonies for the long-term propagation of an inbred strain.

During the reporting period the facility has added a further 17 different gene knock-out/transgenic mice lines to the existing ones, taking the total now to 27 different mutant mice lines. With this the total number of mice strains, both inbred and mutant being maintained at the Experimental Animal Facility rose to a phenomenal **37 (thirty seven)**. Attempts made to establish and breed these mice for supply and use in the ongoing R & D projects have been mostly successful. These mice are housed in Individually Ventilated Caging systems. An aseptic/sterile routine has been standardized for the housing, breeding and handling of these mice. Already these mice have been supplied in reasonable numbers for use in ongoing research projects.

The complete technical support and advice has been extended regularly to Scientists/Research Scholars in the various aspects of animal experimentation namely,

handling of laboratory animals, collection of blood and other samples, immunizations, surgical procedures 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. The details of the animals bred in the facility, procured from various sources, and supplied for various R & D activities are given below.

1-4-2005 to 31-03-2006

Sr. No.	Strains/ species	Animals Bred	Animals Supplied
1	**Rats**		
	Wistar	194	158
	Lewis	72	–
2.	**Mice**		
	BALB/c	4349	4292
	C57BL/6	1733	953
	SWISS	1000	310
	DBA/2	272	78
	DBA/1	10	04
	Nude (nu/nu)	90	248
	BALB/c*	293	68
	FVB/NJ	1417	414
	NOD/LtJ	287	–
	Mutant Mice (27 strains/lines)	6167	959
3.	**Mastomys**	59	–
4.	**Rabbit (NZW)**	18	15

* BALB/c with cataract mutation.

Library

Rameshwar Nema, *Sr. Lib and Info. Assistant*
Aparna Panse, *Office Assistant B*
Madhukar Randive, *Helper A*

The NCCS Library has collection in the frontier areas of biotechnology having relevance to NCCS research activities. The Library holds approximately **six thousand five hundred bound journals, two thousand books,** and subscribe to **fifty nine scientific journals** and **twenty five other periodicals**. The Library information and important scientific links are available on the library Webpage and online searching of all the library documents is available using the Web-OPAC.

In the development of its collections, the Library's priority is to support NCCS research activities. The Library collection is expanded in consultation with the NCCS scientists. The Library's print collections are growing by approximately **seven hundred and fifty volumes** per year. During the period of 2005-2006, the Library has added **eighty two books** and **six hundred and sixty nine volumes of journals** to its collection. From the current year, the library has started online access to a few key journals and links to these and to all the open access journals have been provided on the library webpage.

Additional documentation facilities include local area network for library activities, PubMed database access and a number of CD-ROM database including, full text and factual database. The Library continues to be a part of the Pune Library Network and Medical Library Association of India.

Computer Section

Mr. Rajesh J Solanki
Mr. Shivaji S Jadhav
Mrs. Rajashri C Patwardhan
Mrs. Kirti S Jadhav

- **Installation of New 1 Mbps Broad band Internet Connectivity**
  A new 1 Mbps (1:1) internet connectivity based on licensed band Radio frequency has been installed and configured for browsing. The process is on to configure it as an alternate link for mail/dns.

- **Upgradation of 4 new Servers**
  The four HP-Compaq Proliant ML330servers were upgraded (i.e. addition of one more CPU, RAM and Hard disk respectively) and regular data backup of server is taken on the new parallel connected Hard disk.

- **Installation and Configuration of new Desktop PCs**
  35 new HP desktops PC were configured/installed and given to all scientists for scientific use.

- **Computer Support Services provided**
  To increase the quality of scientific presentation of NCCS staff and students attending national and international conferences/seminars, computer centre is helping in DTP work, CD writing, scanning images and transparency printing on color LaserJet printer.

- **Administration and management of NCCS Server (mail/Web/DNS Servers)**
  WEB/Mail/DNS server administration and management, which involves user addition/deletion, checking logs, hard disk space management and taking regular backups.

- **Administration, maintenance and updating of NCCS website**
  Routine updating of NCCS website for new contents like Project post advertisement, Project Training/ Summer training, Tenders, individual scientists web pages, etc.

- **Computer System and LAN Management, Maintenance and Up gradation**
  Computer Centre is providing technical support to More than 100 computers and 45 printers which includes installing Operating System, Software's and Drivers, Anti-Virus package, Installation and configuration of new computers, CDROM/RAM/Network card installation and connecting to LAN, Server operating system upgradation and maintenance.

FACS facility

Ms. Hemangini Shikhare, *Technician*

We have a BD FACS Vantage machine in our FACS facility. The laser is a water cooled dual wavelength Enterprise II from Coherent. Laser life was over and a new laser was purchased in Sept. 2005 and installed in Dec. 2005. During the period under consideration a total of 10161 samples were acquired on the machine (Surface labeling 8662 + cell cycle PI staining 1499). Sorting of samples is done once in a week. A total of 19 samples were successfully sorted this year.

Confocal Microscopy

Mrs. Ashwini Atre, *Technician*

In the current year 1 Apr 2006 to 31 Mar 2006, the confocal facility has scanned about 2,640 samples originating from the NCCS scientists and about 27 samples outside NCCS.

DNA Sequencing Facility

Mr. Sarang Satoor, *Technician*

Ours is the core facility providing solutions to high throughput processing of the DNA samples for sequencing, sequencing applications and post sequencing analysis. This service is extended to many individuals and institutes like National Institute of Virology, Microbial Containment Complex, National Chemical Laboratory, Agharkar Research Institute, Dept. of Microbiology and Zoology Dept. – Univ. of Pune, North Maharashtra University depts., Serum Institute, Central Institute of Fisheries Education, Mumbai, Gulbarga University depts., Karnataka, Chennai University Depts, Chennai and A.F.M.C., Pune departments with a nominal fee. In this year we have processed as many as 50,000 samples for sequencing alone.

New Additions

1. For the first time in NCCS we have introduced Valrack system for laboratory instruments, which has traditionally been used for computing systems. To accommodate growing number of PCR machines, save space, non cumbersome power supply unit and proper heat dispensing outlet.

2. A new Real Time PCR System 7300 was added to this facility.

3. The Eppendorf epMotion 5070, a robotic liquid handling workstation was made operational this year. This workstation has a robotic hand which dispenses from 1µl to 50µl in row of 8 wells at a time in a 96 well or 384 well plates. All the sequencing reactions are set using this machine.

4. This unit currently has three dual block machines and one new gold block GeneAmp 9700 PCR machine to take the PCR load.

Publications and Patents

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3. Salunke, DB, Ravi DS, Pore VS, Mitra D and Hazra, BG. Amino functionalized novel cholic acid derivatives induce HIV-1 replication and syncytia formation in T cells. **J. Med. Chem**. 2006; 49: 2652-2655,.
4. Joseph, J. Ran at a Glance. **J. Cell Sci**. 2006; 119: 3481-3484.
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6. Prunuske AJ., Liu J, Elgort S, Joseph J, Dasso M and Ullman KS. Nuclear envelope breakdown is coordinated by both Nup358/RanBP2 and Nup153, two nucleoporins with zinc finger modules. **Mol. Biol. Cell**, 2006; 17: 760-769.
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9. Bapat SA and Mishra GC. Stem cell pharmacogenomics: a reality check on stem cell therapy. **Curr. Opin. Mol. Ther**. 2005; 7: 551-6.
10. Kale VP. MAP Kinase: A switch in Fate Determination of Stem Cells. **Stem Cells and Dev**. 2005; 14: 248-251.
11. Sasnoor LM, Kale V and Limaye L. A combination of catalase and trehalose as additives in the conventional freezing medium results in improved cryoprotection of human hematopoietic cells with reference to *in vitro* migration and adhesion properties. **Transfusion**, 2005; 45: 622-633.
12. Sasnoor LM, Kale V and Limaye L. Cryopreservation of mouse bone marrow: Prevention of apoptosis as a possible mechanism of improved protection of cells by catalase and trehalose as additives in conventional freezing medium. **Transplantation**, 2005; 80: 1251-1260.
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15. Singh, S, Upadhyay, AK, Ajay, AK and Bhat, MK. Gadd45alpha does not modulate the carboplatin or 5-fluorouracil induced apoptosis in human papillomavirus-positive cells. **Journal of Cellular Biochemistry** 2006; in press.
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17. Rangaswami H, Bulbule A and Kundu GC. Nuclear factor inducing kinase: A key regulator in osteopontin-induced MAPK/IκB kinase dependent NFκB-mediated promatrix metalloproteinase-9 activation. **Glycoconjugate J**. 2006; 23: 223-234.
18. Chakraborty G, Rangaswami H, Jain S and Kundu GC. Hypoxia regulates crosstalk between Syk and Lck **leading to breast cancer progression and angiogenesis. J**. *Biol. Chem*. 2006; 281: 11322-11331.
19. Shalini J, Chakraborty G and Kundu GC. The Crucial Role of cyclooxygenase-2 in osteopontin-induced PKCα/c-Src/IKKα/β dependent prostate tumor progression and angiogenesis. **Cancer Res**. 2006; 66: 6638-6648.
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a microscopic overview. **Langmuir**. 2005; 21: 10644-10654.

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22. Lakra WS, Sivakumar N, Goswami M and Bhonde RR. Development of two cell culture systems from Asian seabass *Lates calcarifer* (Bloch), **Aquaculture Res**. 2006; 37: 18-24.
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29. Murugaiyan G, Basak S and Saha B. Reversal of tumor-induced dendritic cell paralysis: A treatment regime against cancer. *Curr. Rev. Immunol*. 2006; **2**: 261-272.
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34. Sarkar A, Kulkarni A, Chattopadhyay S, Mogare D, Sharma KK, Singh K and Pal JK. Lead-induced upregulation of the heme-regulated eukaryotic initiation factor 2 alpha kinase is compromised by hemin in human K562 cells. **Biochem. Biophys. Acta**. 2005; 1732: 15-22.
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39. Wani AA, Devkar N, Patole, MS, and Shouche YS. Description of two new Cathepsin C gene mutations in patients with Papillon Lefevre Syndrome. **J. Periodontology**. 2005; 77: 233-237.

40. Lakshmikanth S, Manohar M, Patnakar J, Vaishampayan P, Shouche Y and Lalitha J. Optimization of culture conditions for the production of extracellular agarases from newly isolated Pseudomonas aeruginosa AG LSL-11. **World Journal of Microbiology & Biotechnology** 2006, 22; 5: 531-537.
41. Wani AA, Prasad VS, Siddharth J, Raamesh GR, Patole MS, Ranade DR and Shouche YS. Microbial diversity of Lonar Soda Lake, India: An impact crater in a basalt area. *Extremophiles*. 2006; in press.
42. Prakash D, Fesel C, Jain R, Cazenave PA, Mishra GC, Pied S. Clusters of Cytokines Determine Malaria Severity in *Plasmodium falciparum*-infected Patients from Endemic Areas of Central India. **J. Infect. Dis**. 2006; 15; 194: 198-207.
43. Sahasrabuddhe A, Ahmed N and Krishnasastry MV. Stress-induced phosphorylation of caveolin-1 and p38, and downregulation of EGFr and ERK by the dietary lectin jacalin in two human carcinoma cell lines. **Cell Stress & Chaperones**, 2006, 11: 135–147.
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45. Kumar PP, Purbey PK, Sinha CK, Notani D, Limaye A, Jayani RS and Galande S. Phosphorylation of SATB1, a global gene regulator, acts as a molecular switch regulating its transcriptional activity *in vivo*. **Mol. Cell**. 2006; 22: 231-243.
46. Kumar PP, Bischof O, Purbey PK, Notani D, Urlaub H, Dejean A and Galande S. Functional interaction between PML and SATB1 regulates chromatin loop architecture and transcription of the MHC class I locus. 2006. **Nature Cell Biology** (Accepted).

Book Chapters

N. Lenka. Derivation and Characterization of Neural Cells From Embryonic Stem Cells Using Nestin Enhancer. 2006. Methods in Molecular Biology Vol. 330, Embryonic Stem Cell Protocols, Ed: K. Turksen, Humana Press, Second Edition, 2: 33-54 (Invited Chapter).

Samit Chattopadhyay and Lakshminarasimhan Pavithra. MARS and MARBPs: Key modulators of gene regulation and disease manifestation. Book chapter in Chromatin and Disease (Invited Chapter).

V.V. Agte, S.S. Mengale and R.R. Bhonde. "Potential of Indian spices for their Antidiabetic and Nutraceutical Potential" in press with Volume 19 of the Series "Recent Progress in Medicinal Plants" (In press)

Patents filed/sealed

Dr VP Kale

Creation of Artificial Bone-Marrow Environment and Uses Thereof: Indian and PCT applications filed

Adipogenic Differentiation of human hematopoietic cells Induced by Mannose Binding Dietary Lectins of plant origin. Indian patent application filed

Preservation of Human hematopoietic stem/progenitor cells using mannose binding lectins of plant origin. Indian and PCT applications filed.

Dr. SA Galande

"A novel protein expression system" Indian patent filed # 105/MUM/2005 US patent filed # 11/347,717/2006

Dr. PB Parab

A new molecule for cardiac development promoting activity. Application No. 435/MUM/05 Filing Date 06/04/2005

Awards and Honours

Dr MR Wani

Awards

- B.M. Birla Science Prize in Biology for the year 2004 by B.M. Birla Science Centre, Hyderabad.
- Prof. B.K. Bachhawat International Travel Grant for Young Scientists for the year 2006 by Christian Medical College, Vellore, India.
- Best poster presentation award for the work "Regulation of TNF-α-induced Osteoclast Differentiation and Bone Resorption by IL-3 and GM-CSF". Yogesha, S.D., Shruti M. Khapli, and Mohan R. Wani, at National Symposium on Molecular Mechanism of Diseases and Drug action held at Saha Institute of Nuclear Physics, Kolkata, November 16-18, 2005.

Membership

- Member of the American Society for Bone and Mineral Research, USA, 2005 onwards.
- Member of International Chinese Hard Tissue Society, 2005 onwards.
- Member of Molecular Immunology Forum, 2004 onwards.
- Life Member of Indian Society of Cell Biology, 2002 onwards.

Dr AS Shiras

Travel Grant from ISSCR; USA for participation in the ISSCR Meeting held at San Francisco, USA.

Dr AK Sahu

Awards

Awarded International Senior Research Fellowship, Wellcome Trust, UK (2001-2006)

Membership

- Member of the International Complement Society (since 1999)
- Member of the Molecular Immunology Forum (since 2005)
- Member of the American Society for Microbiology (since 2005)

Dr S Chattopadhyay

Nominated member of Guha Research Conference, Goa, 2005

Dr SA Galande

Member of American Society for Microbiology (ASM), 2005 onwards

Dr N Lenka

Awards

Member (Expert), Faculty Selection Committee, All India Institute of Medical Sciences (AIIMS), New Delhi (2005).

International Society for Stem Cell Research (ISSCR) travel award (2005).

Membership

- Indian Academy of Neuroscience: Life Member
- International Society for Stem Cell Research (2005 – continuing).

Dr B Ramanamurthy

Awarded DBT Overseas Associateship 2004-Long term from Jan. 2005 to Jan. 2006, for research training at the "Functional Genomics Section, National Institute of Dental and Craniofacial Research (NIDCR), National Institutes of Health, Bethesda, USA.

Extramural Funding

Dr V Seshadri

DBT funded project titled "Dual role of insulin in regulating the expression of IGFBP-1". Funding of 22.8 lakhs for three years

Dr VP Kale

- Identification of biochemical pathways involved in the Hematopoietic Stem Cell (HSC) regulation – DBT
- Modulation of signaling pathways in hematopoietic cells as a possible tool to improve the efficiency of transfection – DBT
- Identification of molecular mechanisms involved in the adipogenic conversion of hematopoietic cells by mannose specific dietary lectins namely, banana lectin and garlic lectin, and exploration of differentiation inducing capacity of lectins having defined binding characteristics – DBT
- Assessment of Caveolin-1 Mutations as Possible Risk Factors in the Development of Human Breast Carcinoma – DST

Dr LS Limaye and Dr VP Kale

- *Ex vivo* expansion and cryopreservation of haematopoietic cells: Prevention of apoptosis to improve the outcome." – DRDO(LSRB), Period 2004-2007, Rs. 53.99 lakh.
- Harnessing the potential of stem cells: *In vitro* generation of megakaryocytes and dendritic cells – DBT, Period 2005-2008, Rs. 37 lakh.

Dr MR Wani

Received research grant from the Department of Biotechnology, New Delhi for the project "Studies on *in vitro* differentiation of osteoblasts from human adult stem cells". Duration 2005-2007.

Dr GC Kundu

- Received Funding from Department of Biotechnology, Government of India on "Role of osteopontin, a chemokine like protein in regulation of vascular endothelial growth factor dependent tumor growth and angiogenesis in breast cancer", (2006-2009).
- Received Funding from Department of Science and Technology, Government of India on "Studies on role of Osteopontin in regulation of transcription factor-mediated matrix metalloproteinase-9 activation, cell motility, tumor growth and metastasis", (2006-2009).

Dr P Shastry

DBT – Differentiation of human Mesenchymal stem cells (MSC) towards neural lineage, 2005-2008

Dr AS Shiras

DBT funded projects

- Identification and Characterization of Brain Tumor Stem Cells (BTSC) from a novel human cell line – HNGC-2 and elucidation of pathways for its differentiation.
- Harnessing the potential of Adult Stem cells – An Institutional Stem Cell Project;
- Identification, Development and Characterization of long term Neural Stem cell-lines from adult brain tissue.

Dr D Mitra

- DBT project No. BT/PR5131/Med/14/590/2004 Molecular basis of CTL dysfunction in HIV infection. Debashis Mitra, Bhaskar Saha and GC Mishra, NCCS, Pune
- DBT project No. BT/PR7020/Med/14/930/2005 Identification of anti-viral compounds with potential for development of Microbicides to prevent HIV infection and transmission. Debashis Mitra, NCCS, Pune; KK Bhutani and IP Singh, NIPER, Mohali

Dr AK Sahu

International Senior Research Fellowship, Wellcome Trust, UK (2001-2006)

Dr S Chattopadhyay

- Altered expression of tumor suppressor SMAR1 in breast cancer causes destabilization of Cyclin D1. Funded by Department of Biotechnology, New Delhi. (BT/PR/6589/MED/14/854/2000). Three years project 2006-2009.
- Indo-foreign Research project "Spatio-temporal expression of SMAR1 and Cux in the CNS (5/4-5/11/ Indo-Foreign/2004-NCD-I). Indo-French project from ICMR, New Delhi. 2006-2009.

Dr N Lenka

DBT (2003-2006); (2004-2007) and (2005-2008: Programme Support).

Dr PB Parab

Ongoing DBT project "Identification, isolation and characterisation of growth promoting and differentiation factors from the Perivitelline fluid of developing embryos of the Indian Horse Shoe Crab.

Dr MV Krishnasastry

Quantum Dots: Development of new generation diagnostics against HIV and Malaria from Department of Biotechnolgy, India for one year for 32.67 lakh.

Seminars

Seminars at NCCS by Visiting Scientists

Dr Girish Ratnapati
Gene regulation in early development in Drosophila
University of California, LA, USA
Date: 6th January, 2006

Neerja Kaushik-Basu
Molecular Modulators and Inhibitors of HCV and SARS replicase".
Department of Biochemistry and Molecular Biology
MSB E-673, UMDNJ – New Jersey Medical School
185 South Orange Avenue
Newark, New Jersey 07103, USA
2nd March, 2006

Prof. Vivek Ragnekar
A paradigm for cancer selected apoptosis
University of Kentucky, USA
Date: March 13, 2006

Dr Phillippe Bouvet
Structure and function of nucleolin, a major nucleolar protein
Ecole Normale Superieure de Lyon
LBMC/UMR 5161, Lyon Cedex, France
Date: March 16, 2006

Dr Sebastian Joyce
Immunological Functions of the Invariant Natural T (iNKT) Cells
Professor of Microbiology and Immunology
Vanderbilt University
Tennessee, USA
14th June, 2006, Wednesday, 3 pm

Dr Christian Dimaano
The multivesicular body pathway: a protein sorting network in eukaryotic cells.
Department of Biology
University of Utah, USA
5th July, 2006

Seminars by NCCS Scientists

Dr SA Bapat

- "Ovarian Tumor Stem Cell Biology" at LNCIB (Laboratorio Nazionale CIB, AREA Science Park, Padriciano, 99, 34012 Trieste, Italy – 25th May, 2005.
- "Cancer Stem Cells" – A talk delivered at:
  (i) International work shop on Biotechnology at Modern College, Pune, 16th Oct 2005.
  (ii) "Innovations in Pharmaceutical Biotechnology", Pune College of Pharmacy, Bharati Vidyapeeth, 28th February 2006.

Dr LS Limaye

- Workshop for journalists on biotechnology from May 26-28, 2005 in NCCS, Pune.
  Topic: Stem cell banking.
- International work shop on Biotechnology in Modern college, Pune in Oct 05.
  Topic: Stem cell Banking.
- Biotechnology day 14th Nov.05 in Fergusson College, Pune.
  Topic: Haematopoietic stem cell banking.

Dr MR Wani

- "New Molecular Targets for the Treatment of Osteoporosis and other Degenerative Diseases of Bone" in 3rd Indo-Australian Conference on Biotechnology held at Centre for DNA Fingerprinting and Diagnostics, Hyderabad, March 6-8, 2006.
- "Role of Osteoclasts in Bone Metastasis: Suppression of Increased Osteoclast Number and Activity by Interleukin-4" in 25th Annual Convention of the Indian Association for Cancer Research held at ACTREC, Mumbai, February 15-18, 2006.
- "Role of IL-3 in the Regulation of Bone Resorption" in Molecular Immunology Forum-2006 held at Regional Medical Research Centre, Bhubaneswar, January 27-30, 2005.

- "Animal Cell Culture, Stem Cells and its Applications" in UGC sponsored regional seminar on Recent Trends in Biotechnology and Bioinformatics held at Karmaveer Bhaurao Patil Mahavidyalaya, Pandharpur, January 19-20, 2006.

Dr GC Kundu

- Metastatic gene osteopontin: deciphering the molecular mechanism involved in tumor progression and metastasis. May 5th, 2006, Special Centre for Molecular Medicine, JNU, New Delhi.
- Osteopontin, a member of SIBLING gene family: role in cell signaling and tumor progression. August 8th, 2005, JNCASR, Bangalore.
- Role of Osteopontin in regulation of cell signaling and tumor progression, September 11-16th, 2005, Gordon Research Conference, Big Sky Resort, Montana, USA.
- Metastatic Gene, Osteopontin: Deciphering the Molecular Mechanism of Tumour Progression and Metastases. September 28th, 2005, SSB Function, Vigyan Bhavan, New Delhi.
- Osteopontin, a member of SIBLING gene family: role in cell signaling and tumor progression. October 28th, 2005, IICB, Kolkata.
- Molecular Mechanism of Tumor Progression and Metastasis by Osteopontin, a Member of SIBLING Gene Family. November 16-18th, 2005, Molecular Mechanism of Disease and Drug Action (MDDA), SINP, Kolkata.
- Role of osteopontin in regulation of VEGF expression and angiogenesis. November 24-28th, 2005, Guha Research Conference, Goa.
- Osteopontin, a member of SIBLING gene family: role in tumor progression and angiogenesis. December 8-9th, 2005, Annual Meeting of The National Academy of Sciences, India, Pondichery, University, Pondichery.
- Metastatic gene, Osteopontin: deciphering the molecular mechanism of tumor progression and metastasis. December 18-21st, 2005, International Symposium on Translational Research: Apoptosis and Cancer, Trivandrum.
- Osteopontin, a member of SIBLING gene family: role in cell signaling and tumor progression. February 14th, 2006, National Centre for Biological Sciences, Bangalore.
- Deciphering the molecular mechanism of tumor progression and metastasis by chemokine like protein, Osteopontin. February 24th, 2006, ICGEB, New Delhi.
- Transcription factors: its role in regulation of cell signaling, tumor progression and angiogenesis. February 25th, 2006, Biotechcellence, Centre for Biotechnology, Anna University, Chennai.
- Osteopontin, a member of SIBLING gene family: role in cell signaling, tumor progression and angiogenesis. March 13th, 2006, DBT-SAC(O), New Delhi.
- Osteopontin, a member of SIBLING gene family: it's role in cell signaling, tumor progression and angiogenesis. May 8th, 2006, Serono Pharmaceuticals, Rome, Italy.

Dr RR Bhonde

- Poster presented at the 3rd Annual Meeting of the International Society for Stem Cell Research (ISSCR) held at San Francisco, CA, USA. June 23-25, 2005.
- International Conference on Biosciences, Biotechnology and Biodiversity held at Modern College, Pune during Oct 15-17, 2005 – organized one day symposium on stem cells. (Invited speaker)
- Symposium on "Recent Advances in reproduction Endocrinology and Development and associated disorders" January 30-31, 2006. (Invited speaker)

Dr D Mitra

- Molecular Biology in the new millennium, V National Molecular Biology Workshop 5th to 19th August 2005, Department of Microbiology, Armed Forces Medical College, Pune. Molecular Biology of Human Immunodeficiency virus-1

- Ninth Transcription Assembly, 17th to 19th September 2005. CCMB, Hyderabad Nef Modulates HIV-1 gene expression by interacting with cellular heat shock proteins
- National Symposium on Molecular Mechanism of Diseases and Drug Action (MMDDA-2005) 16th to 18th November 2005, Saha Institute of Nuclear Physics, Kolkata. New insights into Human Immunodeficiency Virus-1 Tat mediated regulation of viral and Cellular gene expression.
- Third Symposium on 'Frontiers in Molecular Medicine', 19th-20th Jan. 2006, Special Centre for Molecular Medicine, JNU, New Delhi. Role of cellular heat shock protein 40 in HIV-1 gene expression and replication induced by Nef
- Molecular Immunology Forum-2006, 27th to 30th January 2006, RMRC, Bhubaneswar Impairment of mitochondrial complex I activity during HIV-1 induced T cell apoptosis
- 2nd CSIR-NSFC Workshop on Genome Informatics, 22nd to 26th February, 2006 Institute of Genomics & Integrative Biology, New Delhi. Modulation of host proteins during HIV infection
- VII Sir Dorabji Tata Symposium, HIV/AIDS – Research Issues, March 10-11, 2006 National Institute of Advanced Studies, IISc Campus, Bangalore. Indian marine bivalves as a source of anti- HIV activity

Dr AK Sahu

March 31, 2005, Department of Biotechnology, University of Pune, Pune, "Viral evasion of the host complement system".

Dr S Chattopadhyay

- Invited talk presented at Ninth Transcription Assembly Meeting, CCMB Hyderabad; 17-19th September, 2005.
- Invited talk presented at National Symposium on Molecular Mechanism of Diseases and Drug Action (MMDDA) 2005, Kolkata, 16th-18th November, 2005
- Invited talk presented at Guha Research Conference, Goa, 24th-28th of November, 2005.
- Invited speaker of International Symposium on Translational Research on Apoptosis and Cancer, Trivandrum, December 18-21, 2005.

Dr SA Galande

- "Post-translational modifications of SATB1, a global gene regulator, act as a molecular switch regulating its MAR-binding activity *in vivo*" Transcription Assembly IX meeting, CCMB, Hyderabad, September 29, 2005.
- "Phosphorylation of SATB1, a global gene regulator, acts as a molecular switch regulating its association with HDAC1 and PCAF *in vivo*" Society of Biological Chemists (SBC) meeting, Lucknow, October 9, 2005.
- "Phosphorylation of SATB1, a global gene regulator, acts as a molecular switch regulating its transcriptional activity *in vivo*". Asian conference on Transcription ACT IX, December 14, 2005, Taiwan.
- "Bridging signal transduction and chromatin architecture in T cells: Role of PDZ-like domain of SATB1 in regulation of global gene expression". This talk was delivered at:
  i. Indian Institute of Technology, Kanpur on October 9, 2005
  ii. Institute of Life Sciences, Bhubaneshwar on February 5, 2006
  iii. Centre for DNA Fingerprinting and Diagnostics, Hyderabad, on March 24, 2006.
  iv. Life Sciences Dept., University of Delhi South Campus, on February 22, 2006.
- "Molecular profiling". Moving Academy of Medicine and Biomedicine, on January 17, 2006.

Dr N Lenka

- Embryonic Stem cells and Cardiomyogenesis *in vitro*. The Third Conference of the Biotechnology Society of India "BIOTECH-2005", Gurgaon, India (Invited Plenary Speaker).
- Embryonic Stem Cells, the niche and the lineage diversification *in vitro*. Meeting on Stem cells: From Laboratory to Treatment of Diseases, Imgenex India Pvt. Ltd, Bhubaneswar, India (Invited Plenary Speaker, Session Chair).
- Intrinsic control and lineage diversification during embryonic stem cell differentiation. Indo-Danish workshop on stem cell research, National Centre for Biological Sciences, Bangalore, India sponsored by Department of Biotechnology, Ministry of Science and Technology, Government of India (Invited Speaker).

Dr PB Parab

Medical Applications of Monoclonal Antibodies. Seminar delivered at "Innovations in Pharmaceutical Biotechnology, Poona College of Pharmacy. Bharati Vidyapith. 1st March 2006.

Dr YS Shouche

- International Conference Microbial Diversity: Current Perspectives and Potential Applications (April 2005) at University of Delhi South Campus.
- "Molecular Taxonomy of genus Aeromonas" at Recent Trends in Computational Biology (November 2005) at C-DAC, Pune.
- International Conference on Insect Molecular Biology (Januray 2006) at Centre for DNA Fingerprinting and Diagnostics, Hyderabad

Conferences/workshops attended

Dr SA Bapat

- EMBO Workshop and IRCC Conference on "Invasive Growth: A Genetic Program for Stem Cells, Cancer and Cancer Stem Cells" from 26th-29th May, 2005 at Candiolo, Torino, Italy
- Invited to participate in DBT Stem Cell meeting at New Delhi from March 8-9, 2006.

Dr VP Kale

- Co-ordination of Workshop of Press Trust of India and DBT for journalists. (26th-28th May 2005).
- Reciprocal Regulation of MAPK Pathways in Primitive Hematopoietic Cells: A Switch for Proliferation International Society for Stem Cell Research 3rd Annual Meeting June 23-25, 2005 in San Francisco, California USA. Abstract accepted for presentation.
- International Workshop on Stem Cell Therapy In India. June 28th to June 31st 2005 at CMC, Vellore.
- Invited to deliver a talk on "Plasticity of marrow derived stem cells" in "Platinum Jubilee Symposium on Stem Cell Research" (part of International Conference on Biosciences, Biotechnology and Biodiversity)" organized by NCCS, National Academy of Sciences, India and Modern college. 15th-17th October 2005.
- Invited speaker in symposium on stem cell research, 26th-27th Nov 2005 AIIMS, New Delhi to give a talk on "Hematopoietic stem cell regulation through microenvironment mediated signaling".
- Invited speaker in "International Symposium on Stem Cells and Regenerative Medicine" at Reliance Life Sciences. 27-28 Feb 2006 "Preservation of Cord Blood Derived Stem Cells in Culture".
- Invited participant in DBT organized Indo-Danish Work shop on stem cell research. 21st-22nd of February, 2006, NCBS, Bangalore.
- Invited to participate in DBT stem cell meeting Delhi 8-9 March 2006.

Dr MK Bhat

- 74th Annual Meeting of Society of Biological Chemists (India). Central Drug Research Institute, Lucknow, UP, November 7-10, 2005
- Title of the presentation: *Abrogation of p53: Its implications in Cell Growth, Cellular Signaling and Chemosensitivity*. Rishi Raj Chhipa and Manoj Kumar Bhat
- Invited as an Examiner in The Department of Biochemistry, University of Mysore, Mysore. May 29-31, 2006

Dr P Shastry

- Signaling pathways in death receptors mediated responses in multicellular spheroids of human glioblastoma cells – Role of NFκB, Akt and p21 Invited speaker-International symposium on Translational Research – Apoptosis and cancer. Trivandrum December 18-21 Dec. 2005

Dr AS Shiras

- Participated in the International Society for Stem Cell Research (ISSCR) Meeting held at San Francisco, USA, June 2005.

Dr RR Bhonde

- Poster presented at the 3rd Annual Meeting of the International Society for Stem Cell Research (ISSCR) held at San Francisco, CA, USA. June 23-25, 2005.
- International Conference on Biosciences, Biotechnology and Biodiversity held at Modern College, Pune during Oct 15-17, 2005 – organized one day symposium on stem cells. (Invited speaker)
- Symposium on "Recent Advances in reproduction Endocrinology and Development and associated disorders" January 30-31, 2006. (Invited speaker)

Dr D Mitra

- National Symposium on Molecular Mechanism of Diseases and Drug Action (MMDDA-2005) 16th to 18th November 2005, Saha Institute of Nuclear Physics, Kolkata. HIV-1 Nef interacts with Hsp40 to induce viral gene expression and replication. Manish Kumar and Debashis Mitra
- 32nd Annual Conference of Indian Immunology Society (IIS), 24th to 27th November 2005, Chandigarh, Immune response to HIV-1 subtype C gp120 and Tat expressing bicistronic vector in mice. Shalini Gupta, Bhaskar Saha and Debashis Mitra
- International Symposium on Translational Research: Apoptosis and Cancer (ISTR-2005), 18th to 21st December 2005, Trivandrum, Impairment of mitochondrial energy generating system in HIV-1 induced T-cell apoptosis. Manoj K. Tripathy, Jayashree S. Ladha and Debashis Mitra

Dr AK Sahu

- Mullick, J., Singh, A.K., Panse, Y., Yadav, V, Bernet, J. and Sahu, A. Identification of domains important for complement regulatory activity in viral complement regulators VCP and Kaposica. 10th European Meeting on Complement in Human Diseases, Heidelberg, Germany, (2005), September 9-13.
- Singh A.K., Mullick, J. and Sahu, A. Mechanism of complement inactivation by the Herpesvirus saimiri ORF4 protein. 8th FIMSA/IIS Advanced Immunology Course: Focus on clinical Immunology, New Delhi, (2006), March 1-5.
- Mullick, J., Singh, A.K., Panse, Y., Yadav, V., Bernet, J. and Sahu, A. Identification of domains important for complement regulatory activity in viral complement regulators VCP and Kaposica. 8th FIMSA / IIS Advanced Immunology Course: Focus on clinical Immunology, New Delhi, (2006), March 1-5.

Dr S Chattopadhyay

DBT-NCBS Cancer Meet at NCBS, Bangalore, August 6-7, 2005.

Dr SA Galande

- Cold Spring Harbor conference on 'Gene Regulation and Signaling in the Immune System', April 26-30, 2006, Cold Spring Harbor, NY, USA.
- "Asian conference on Transcription ACT IX", December 12-15, 2005, Taiwan. (Chaired a session and also delivered oral presentation).
- "Wilhelm Berhard Workshop on Cell Nucleus", September 1-9, 2005. Germany.
- "Transcription Assembly IX" meeting, CCMB, Hyderabad, October 28-30, 2005.
- "Molecular Immunology Forum" meeting, Bhubaneshwar, February 2-4, 2005.
- DBT meeting on "Systems Biology-New Initiatives and Perspectives", Manesar, Gurgaon, April 3-5 2006.

Dr N Lenka

N. Lenka, Verma M.K. 2005. Generation, characterization and purification of chamber specific cardiomyocytes from ES cells *in vitro*. International Society for Stem Cell Research (ISSCR) 3rd Annual Meeting, San Francisco, USA.

Dr PB Parab

XXIX All India Cell Biology Conference held at Lucknow from 17-20 Jan 2006. A novel protein from a marine living fossil shows potential cardiac development promoting activity.

Dr YS Shouche

- Poster selected for the oral presentation at the 'International conference on Emerging Infectious diseases (ICEID)' held at Atlanta, Georgia, USA, during March 19-22, 2006, entitled as "Comparative genomics of Helicobacter pylori from Western and Northern India: Insights into virulence and population diversity", by M.S. Dharne, S.P. Misra, M.S. Patole, L. Kakrani, V. Misra, M. Dwiwedi and Y.S. Shouche.
- Mahesh Dharne, Milind Patole, Yogesh Shouche. Microbiology of Insect guts; tale from mosquitoes and bees. (Accepted for Publication as a clipboard article in Journal of Biosciences)
- Poster selected for the oral presentation at the 'International conference on Emerging Infectious diseases (ICEID)' held at Atlanta, Georgia, USA, during March 19-22, 2006. Identification and Characterization of Type Four Secretory System and Multidrug Efflux Protein from Aeromonas culicicola MTCC3249T. A.Y. Rangrez, Kannayakanahalli M. Dayananda, Aijaz A. Wani, Milind S. Patole and Yogesh S. Shouche.

NCCS students participation at Workshops/ Collaborative Work

- Mr. P. Pavan Kumar, SRF was invited to deliver lectures at Rockfeller University; Harward Medical School, University of Massachusetts Medical School, USA.
- Mr. Umasankar, SRF, has been invited for collaborative research work to be carried out at Institute of Parasitology of McGill Laboratory, Canada for 6 months.

Oral and Poster presentations by students

- Poster selected for the oral presentation at the 'International conference on Emerging Infectious diseases (ICEID)' held at Atlanta, Georgia, USA, during March 19-22, 2006, entitled as Presence of Virulence Genes in Aeromonas culicicola MTCC3249T. K.M. Dayananda, A.Y. Rangrez, A.A. Wani, M.S. Patole, Y.S. Shouche.
- Participated in oral presentation at Graduate Students Meet – "Trends in Life Sciences" held at Advanced Centre for Treatment, Research & Education in Cancer (ACTREC), Kharghar, Navi Mumbai on 24th September 2005.
- Gurdeep Rastogi: Participated in International Conference on Microbial diversity: Current prospetives and potentials from 16-18 April, 2005 at Department of Microbiology, University of Delhi, South Campus Delhi, India.
- Dayananda KM, Ashraf Yusuf Rangrez, Aijaz Ahmed Wani, Santosh Atanur, MS Patole and Yogesh S Shouche. 2005. *Aeromonas culicicola*: a possible pathogen? Indo-Australian Conference on Biotechnology in Infectious Diseases, Manipal, India.

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