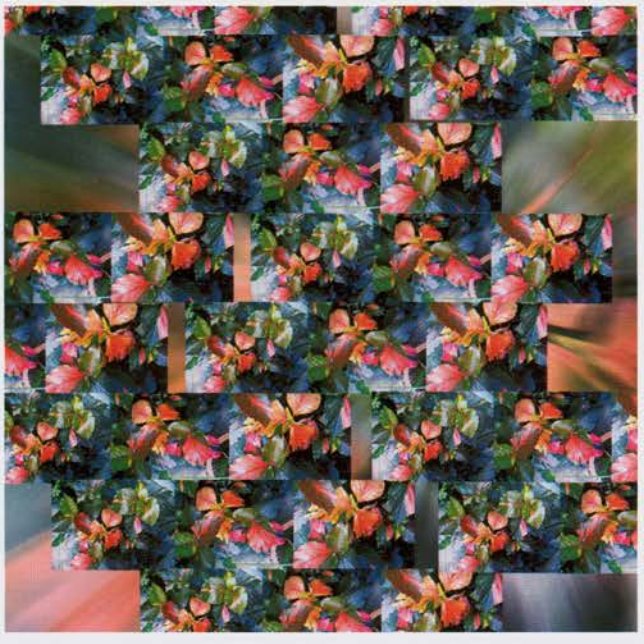
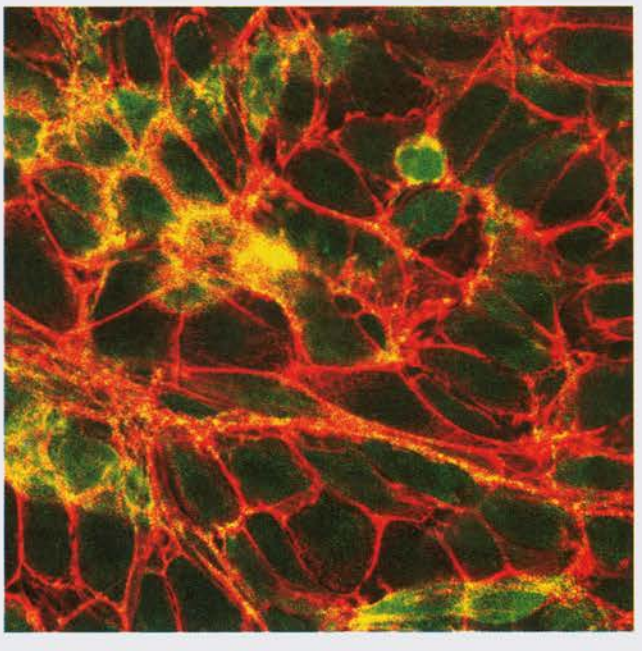
National Centre for Cell Science

Annual Report 2002-2003

Common thread of life . . .





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From Director's Desk

I am delighted to present the Annual Report of the National Centre for Cell Science (NCCS) for the year 2002-2003. Our mission is to perform research and development in various areas such as Stem Cell Biology, Cancer Biology, Cell Biology, Immunology, Diabetes and HIV Biology, Teaching and Training. I am proud to state that we have achieved significant progress in all our endeavours. We also serve as National Cell Repository and this year we have supplied 625 cell lines to about 120 scientific institutions and have continued our efforts towards the establishment and characterization of new cell lines.

Cell migration plays a critical role in tissue formation, angiogenesis and metastatic tumor growth. In this connection we have shown that osteopontin (OPN) induces NF-κB activity and urokinase type plasminogen activator (uPA) secretion. OPN also induces PI 3'-kinase dependent Akt phosphorylation and NF-κB-mediated uPA secretion that ultimately controls motility of breast cancer cells. We observed enhanced migration and invasion by SiHa, cervical carcinoma cells when treated with EGF that was synergistically enhanced in presence of serum. Migrating cells showed a multifold increase in the amount of pro-MMP-9 and in presence of ECM gel matrix proteins also revealed an active MMP-9. We also modified the Boyden chamber assay to check invasion of live cells, first of its kind. Osteoclasts, the only cells responsible for bone resorption play a crucial role in bone remodeling. We identified molecular mechanisms for the action of IL-3 in osteoclast differentiation. IL-3 inhibits RANKL and TNF-α-induced osteoclast differentiation and prevents RANKL-induced nuclear translocation of NF-κB by inhibiting the phosphorylation and degradation of IκB. Thus, our results first time reveal that IL-3 acts directly on early osteoclast precursors and irreversibly block RANKL-induced osteoclast differentiation by diverting the cells to macrophage lineage. We employ embryonic stem cells as a developmental paradigm to explore early neuro- and cardiogenic proceedings. Because of the therapeutic prospects the stem cells envisage, we intend to investigate the differentiation and transdifferentiation potential of fetal and adult stem cells. The early onset intermediate filament gene nestin transgenic ES cell clones express EGFP at the undifferentiated state itself, however, remained confined to the neural lineage upon differentiation.

We have identified and characterized *M3TR*, a non-coding RNA from Cloudman melanoma cells that inducing cellular transformation and tumour formation. We have established a novel model system from human malignant glioma comprising of two human cell lines – Human Neural Glial Cell-line HNGC-1 and HNGC-2, which express neural stem cell-like characters. Our studies on mechanisms of cell death indicated the involvement of GADD45 and Bcl-2 in

the cell death pathway of cells possessing mutant p53. We found that a novel p53 interacting tumour suppressor protein SMAR1 directly interacts and phosphorylates p53 at it's serine-15 residue. We have isolated and purified a 5 kDa protein named BGPI3 from seeds of *Momordica charantia*, Linn. that resulted in significant tumour regression. Our studies regarding the role of cell cycle proteins in apoptosis revealed the overexpression of Survivin in G2/M but not G1 cells. Staurosporine treatment induced significant drop in G1 but not in cells synchronized in G2/M phase. Thus, in G2/M cells survivin may confer resistance to STS induced apoptosis.

We are studying the signal transduction pathways induced by direct action of various important modulators of hematopoiesis especially TGFβ1. We have shown that $CD34^+$ cells are the direct targets of stimulatory action of TGFβ1. The differential activation of MAPK pathways by TGFβ1 as a function of its concentration perhaps forms the molecular basis of its dose dependent bi-directional effect on hematopoiesis. Our continued effort on bone marrow cryopreservation and revival of stem cells has led to successful transplantation. Various stresses stimulate a number of receptors through inhibition of protein tyrosine phosphatases. We have focused on the identification of the PTPase involved in the process. We show for the first time that endogenous rPTPσ has been shown to be responsible for the dephosphorylation of EGFr and its downstream recipients. In another study we have shown Syk, a protein tyrosine kinase suppresses the cell motility and inhibits the PI 3'-kinase activity and uPA secretion by blocking the NF-κB activity through tyrosine phosphorylation of IκBα. These data further demonstrate a functional molecular link between Syk regulated PI 3'-kinase activity and NF-κB mediated uPA secretion, all of these ultimately control the motility of breast cancer cells.

NCCS has ongoing scientific programmes to address mechanisms as well as treatment alternatives for both diabetes type I and II. Our interests include the identification of new factors affecting the growth of pancreatic β cells and islet neogenesis. Elucidation of the regenerative potential in experimentally induced diabetes is of interest as an alternative therapy for diabetes. We show for the first time the potential of trace elements like vanadium to induce islet neogenesis in vitro without supplementing any other growth factor. We have also gathered evidence to demonstrate regeneration of islets from ductal cells. The identification and characterization of autoantigens in a rat autoimmune diabetes model is underway.

Ribosomal RNAs are essential components of all living cells that are functionally and evolutionarily conserved. This has made them a valuable tool in molecular taxonomy, phylogeny and diversity studies. We employ ribosomal RNA and other markers for the diversity studies including those on "uncultivable" microbes. We have delineated the consensus DNA sequence for the regulation of expression of housekeeping genes such as hexokinase in fruit-fly.

Basic research in various aspects of cellular immunology has been a major thrust area at NCCS. Our focus is to understand the basics of immune responses towards Mycobacterium, Leishmania, viruses and Plasmodium. Our results prove that even though LPS-B expresses sufficiently high surface costimulatory molecules, when used as accessory cells, their ability to induce $CD8^+$ T cells responses were severely compromised as compared to the ability of CD40-B. Furthermore, we show that TGF-β1 but not IL-10 is responsible for hyporesponsiveness of LPS-B. These results thus illuminate the immune evasion strategies adopted by both gram negative bacteria and retroviruses that specifically target TLR-4 signaling in B cells. In our studies in HIV biology, our interest was to identify differentially expressed molecules in cells undergoing apoptotic cell death as compared to non-apoptotic cells in the HIV infected T- cell population and to elucidate the interaction of those molecules in the signaling cascade leading to cell death. We have initiated screening of anti-HIV activity from marine bivalves of Indian coastline and also from plants and trees of medicinal importance in western region. Our initial study reveals the presence of anti-HIV activity in some marine bivalves, which are being fractionated to identify the active components. Nitric oxide is an important reactive oxygen species that kills the Leishmania parasite. It is observed that during *Leishmania* infection, CD40 signaling through p38MAP kinase is impaired resulting in unrestricted parasite growth. We have shown that CD40, a

costimulatory molecule on macrophages, signals through p38MAP kinase to induce inducible nitric oxide synthetase 2 (iNOS2) that catalyzes nitric oxide formation. Although viruses are small and have relatively simple structure, both acute and latent viruses can be efficiently recognized and neutralized by the complement system. We are interested in unraveling the molecular mechanisms underlying the interaction between host's complement proteins and CCPHs of VV, HVS and KSHV. We have identified and characterized functionally important determinants of viral homologs of complement control proteins. Multiple monoclonal antibodies against VCP are characterized with respect to their binding to VCP and inhibition of functional activities of VCP.

In the past few years the role of chromatin architecture in the regulation of gene expression is emerging. A novel candidate tumor suppressor and MAR binding protein SMAR1 has been shown to play direct role in the control of V(D)J recombination and T cell development. Furthermore, MARs were shown to regulate transcription over distance in vivo. The PDZ signalling domain of the T lineage specific MAR binding protein SATB1 was found to interact with several proteins that play important roles in T cell homeostasis. This year we have initiated a program for generating transgenic mice. The necessary equipment has been procured and we hope to make our first transgenic mice by next year. We have procured 15 different strains of knockout mice that are being used for understanding various immunological phenomena.

This year NCCS has published **42** papers in reputed journals. Several of them are published in high impact factor international journals. We have also succeeded in filing **6** USA patents. This year the scientists at NCCS continued to attract peer reviewed extramural funding. In addition, our library and documentation facility has procured 675 journals volumes and 78 books in the frontier areas of biotechnology.

Our achievements are significant and I am confident that we will continue to set higher standards for ourselves and aim to achieve them.

G.C. Mishra
Director

Repository

The repository of National Centre for Cell Science is the only repository that houses human and animal cells in India. The NCCS repository serves to receive, identify, maintain, store, cultivate and supply animal and human cell lines and hybridomas. The work done at repository mainly involves cell line procurement, expansion, cryopreservation and distribution. The repository has procured cultures from various sources within the country and abroad from 35 animal species. A major bulk of the cell lines stocked in the repository has been procured from the American Type Culture Collection (ATCC) and the European Collection of Animal Cell Cultures (ECACC). In this year we have procured 20 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. During the year 2002-2003, we have supplied 625 cell lines comprising of 148 different cell types to 120 research institutions in the country. The repository has initiated programmes to develop, immortalize and characterize cell lines from different tissue/tumour types.

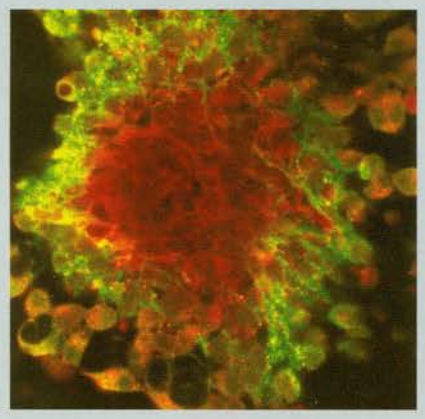
Human Resource Development

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. To achieve this goal, the centre has conducted various workshops on tissue culture techniques. During this year NCCS conducted a workshop on "Animal Tissue Culture: Basic Techniques" from April 15-27, 2002 for scientists from Orissa. A total of 22 individuals and 8 summer students from all over India were trained by NCCS during 2002-2003.

In addition, the centre has also attracted about 10 summer trainees and 6 project M.Sc. students from various universities all over India. The projects were appreciated by scientists of other institutes and many of the trainees showed interest to pursue future Ph.D. programme at NCCS.

Number of NCCS scientists actively participated in various teaching activities and coordinating workshops at various universities, colleges and departments. These include Department of Zoology, Microbiology, Biochemistry and Biotechnology of University of Pune and also local collages.

During this year 8 new Junior Research Fellows joined at NCCS for Ph.D. programme. The total strength of research fellows reached to 75. In addition three Research Associates are currently working on various projects at NCCS.



Research Reports

Cell Biology

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Establishment and characterization of cell lines from human ovarian tissues

Amit Kumar, Avinash Mali *(Technician)*

Collaborators: (i) Dr. C.B. Koppikar, *Cancer Surgeon, Jehangir Hospital and Medical Centre, Pune.*
(ii) Dr. Sanjay Gupte, *Gynecologist, Gupte Hospital, Pune.*

Abstract and background:

Ovarian cancer has been recognized as the most aggressive of all gynecological cancers. The poor prognosis associated with ovarian cancer is attributed to an inability of identifying definitive pre malignant lesions as well as lack of diagnostic tools for early detection. Very few permanent cell lines have been developed from primary ovarian tumors, while none exist from normal ovarian epithelia thereby presenting limiting amounts of research resources.

The establishment and characterization of cell cultures and cell lines that retain the plasticity associated with normal ovarian tissues as well as the benign and transformed phenotypes, which could further be developed as models for studies relating to epithelial-mesenchymal transition (EMT), metastasis and tumorigenesis, stromal-epithelial interactions, etc. constitute the mainstay of this project.

Aims:

1. Establishment of cell lines from ovarian epithelia including normal epithelium and benign and malignant epithelial tumors. Lymphoblastoid cell lines from the same individuals will be established that would serve as isotypic controls in loss of heterozygosity (LOH) studies.

2. Establishment of cell lines from stromal and germ cell tumors of the ovary which are low incidence tumors (5-10% of total ovarian tumors) as well as from normal stroma.

Work achieved:

Several primary cultures have been established in working towards the aims of the project. Of the various samples processed this year, two show promise of establishing into extended life-span cultures (i.e. cultures that can be maintained to around 20 passages) in vitro *viz.*

(i) OT29Asc – an ascietic fluid derived cell culture established from a 60 year old individual who had undergone 3 cycles of chemotherapy prior to surgery. This epithelial-like culture has retained the ability to form free floating spheroids in culture.

(ii) OT32 – an epithelial cell culture derived from a primary tumor (dermoid cyst) that was surgically removed from a 29y old individual.

In addition, three lymphoblastoid cell lines *viz.* OT25LBL, OT26LBL and OT32LBL were established. The latter would find application in LOH studies on dermoid cysts since extended life cultures of the primary tumor from the same individual (ii above) have also been established.

Future work:

(i) Immortalization of the primary cultures: Work been initiated to immortalize primary cultures using expression vectors of hTERT (human telomerase reverse transcriptase). This will be continued.

(ii) Culture Characterization: Karyotyping, tumorigenecity assays, identification of cell specific markers will be undertaken with the established cell lines.

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Human genetic mutant repository

Avinash Mali *(Technician)*

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Abstract and background:

Genetic diversity is the sum of genetic information contained in the genes of individuals. Each species consists of many organisms and virtually no two members of the same species are genetically identical. In conjunction with the Human Genome Project, studies based on genetic diversity could fundamentally contribute to a new era of modern molecular medicine and also transform the current scientific understanding of evolution. A comprehensive study of human genetic diversity in the Indian populations could help in mapping genetic variability in Indian populations. To begin with, there is a need to establish a storehouse of research resources and associated data to base predictions upon. The Human Genetic Mutant project represents such a collection of immortalised cell lines and primary cultures established from individuals and families of interest in view of conservation of Indian Human Genetic Biodiversity.

Aims:

The purpose of this project is to develop research methods and resources for the understanding of genetic variation in Indian populations. These include:

1. Establishment of lymphoblastoid (EBV transformed B lymphocytes) cell lines from peripheral blood samples.

2. Establishment and immortalization of skin fibroblast cell lines.

3. Use of such tissues, cultures and cell lines for study of the various molecular and cellular changes associated with specific genetic anomalies.

Work achieved:

The following cultures and cell lines have been established*:

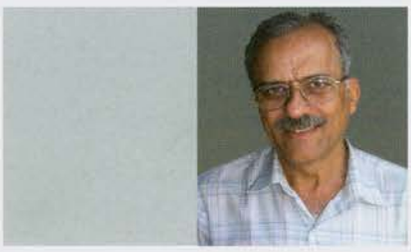
Cell Line/Culture	Syndrome
(i) GM1LBL	Rubinstein – Taybi Syndrome
(ii) GM2aSF & GM2bLBL	Ectodermal Dysplasia (2a – proband, 2b – carrier mother)
(iii) GM3LBL & GM3SF	Rubinstein – Taybi Syndrome
(iv) GM4LBL & GM4SF	Multiple Pterygium Syndrome
(v) GM5SF	Waardenburg Syndrome with phenylketonuria (suspected)
(vi) GM6SF	Cornelia de Lange Syndrome

* **Note:** The suffix LBL refers to a lymphoblastoid cell line; SF refers to a skin fibroblast culture.

CD19 and EBNA (Epstein-Barr Nuclear Antigen) immunofluorescence detection for lymphoblastoid cultures and vimentin expression for skin fibroblasts has been carried out to confirm the identity of the cells.

Future work:

Several studies are required to be undertaken to confirm the clinical diagnosis. Some of these include – karyotyping to detect gross chromosomal anomalities, FISH analyses to detect microdeletions, protein truncation tests to confirm the loss of protein functionality, etc.



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Structure-function analysis of eukaryotic cells: Epithelial-mesenchymal Transition

M.R. Vipra (*Technician*)

Abstract and background:

Epithelial-mesenchymal transition (EMT) plays a central role in many physiological processes such as embryogenesis, wound healing and pathological processes such as tumor invasion and metastasis. Epithelial cells lose their cohesive nature and are converted into individual motile fibroblastic cells. EMT is associated with loss of epithelial features and gain of mesenchymal characteristics.

Role of EMT has been increasingly accepted in the tumor metastasis. EMT has been implicated in metastatic progression of variety of tumors such as bladder carcinoma, breast carcinoma, pancreatic cancer, uterine carcinoma and nasopharyngeal carcinoma. EMT represents the first stage in metastatic progression of tumor cells.

Many factors are thought to be involved in the acquisition of cell motility. Growth factors are also essential for tissue repair and morphogenesis, role in the expansion, invasion and metastasis of the tumor cells. Number of growth factors like scatter factor, epidermal growth factor (EGF), transforming growth factor TGFβ) have been shown to induce EMT both *in vivo* and *in vitro.* Some types of tumor cells are reported to produce growth factor receptors at various stages, thereby increasing their reactivities. Each factor induces a particular transduction system and affects motility of cells but the net effect seen is a consequence of interaction of various transduction systems.

Aims:

1. To look for epithelial-mesenchymal transition like changes in cervical carcinoma cells.

2. To analyze migration and invasion capacity of cells in response to external stimuli.

3. To study the cell cycle pattern of the cancer cells when induced to migrate.

Work achieved:

Our earlier work has reported the enhanced migration and invasion by SiHa, cervical carcinoma cells when treated with EGF. A strong synergistic effect with serum was noted. The cells typically underwent epithelial mesenchymal transition like changes. Epithelial mesenchymal transition is associated with metastasis or acquisition of invasive potential by cancer cells. We continued our studies on EMT for the invasive potential of SiHa cells, using Boyden chamber assay. We modified this assay to check invasion of live cells, first of its kind. The filter was coated with ECM gel. Cells were seeded on the upper chamber in presence of EGF and the lower chamber was filled with FCS and EGF. Cells were stained with vital stain Hoechst 33342 and observed during incubation period to assess the cell invasion. Cells were observed at different levels in the gel as well as the filter. Under phase contrast, cells were not very clearly visible. However, Hoechst 33342 fluorescence images from the nuclei were sharper and clearly defined, locating the cells at various focus levels. This modification also revealed cells that have traversed through the gel but not yet reached the lower side of the filter, a significant fraction not included in the existing methods of visual counting. We further tested this method by image analysis and confocal laser scanning microscope. Fluorescence was detected from the cells at various levels in the gel from upper surface of the gel to the lower surface of the transwell filter. In one such experiment, 16 optical sections were collected over a distance of 321 µm along the z-axis and used for 3-D reconstruction. These optical sections clearly revealed the fluorescing cells at different levels.

In order to check the morphology of the cells during invasion in 3-D culture, we carried out ECM gel assay. This assesses the capacity of cells to form colonies in the ECM proteins and the capacity to degrade the surrounding gel. NIH 3T3 and MDA-MB-231 cell lines were used as negative and positive controls, respectively. SiHa cells formed loose colonies with individual cells seen clearly, indicating a very well cell-cell binding, compared to NIH 3T3 cells, which formed very compact colonies. Similarly, SiHa invaded the surrounding gel and the colonies were seen spread through the length of the gel. Also cell shape of the invading cells was clearly fibroblastoid like similar to cell shape change seen in 2-D assay. This assay further confirmed the invasive behaviour especially so in presence of EGF and the phenotype change in SiHa cells.

Further, to assess the possible role of matrix metalloproteinases in invasive capacity of SiHa cells, we analysed the conditioned medium by gelatin zymography (Fig. 1). It showed presence of pro-MMP-9 in uninduced non-migrating cells. Cells when induced to migrate in the migration assay, showed a multifold increase in the amount of pro-MMP-9. Cells induced by growth factors in presence of ECM gel matrix proteins in the Boyden chamber assay not only showed a considerable increase in pro- form of MMP-9, but also revealed an active MMP-9. The zymograph also showed 5 more gelatinolytic bonds, three of which matched with the pro-MMP-2, intermediate form of MMP-2 and an active form of MMP-2. Additional

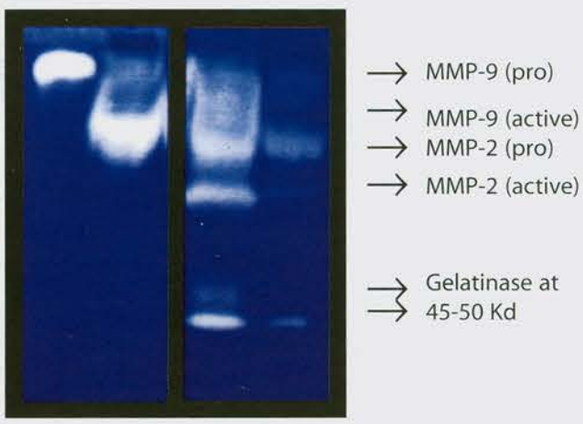

Fig. 1: Detection of matrixmetalloproteinases (MMPs) by gelatin symography. Uninduced cells produced only proform MMP-9, whereas cells induced by EGF and ECM produced both pro and active forms of MMP-2, MMP-9 and a third gelatinase at 45-50 Kd.

two bands (couplet) seen at about 45 kD were not identified. Though the conditioned medium from cells induced to migrate in the migration assay did not reveal any active MMP-2 in the zymography analysis, the cell extracts revealed pro-MMP-2 in the uninduced cells and both pro and active MMP-2 in the cells induced to migrate by western blotting with anti-MMP-2 antibodies.

Future work:

To identify the key molecules involved in induction of EMT by EGF in SiHa cells.

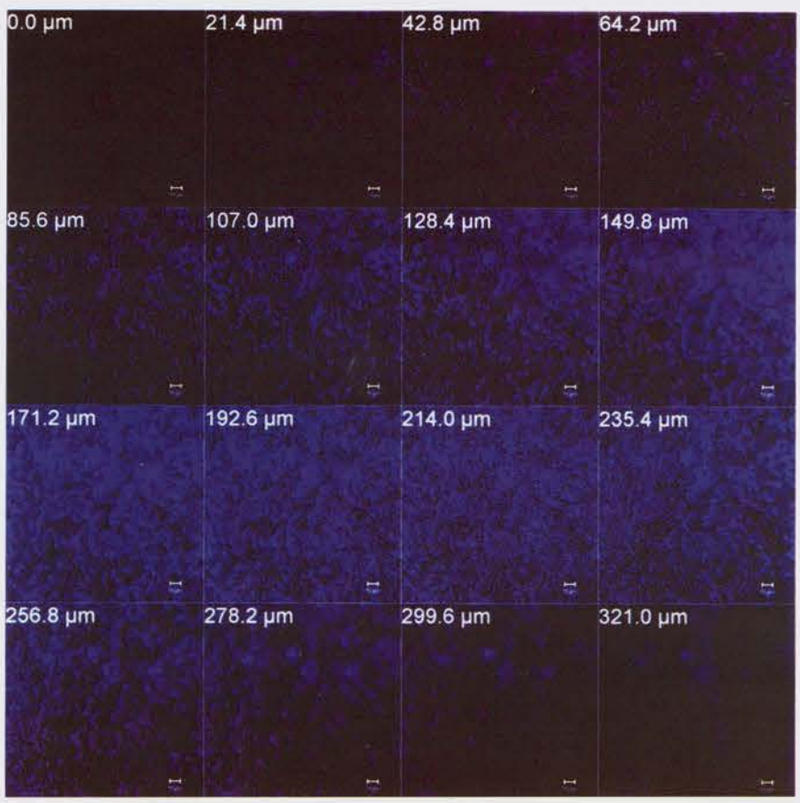

Fig. 1: ECM gel invasion by SiHa. It shows gallery of 16 optical sections collected over a distance of 321 µM along the z-axis, depicting cells stained with Hoechst 33342 vital stain.

Introduction:

Transforming Growth Factor-beta 1 (TGFβ1) is a pleiotropic molecule exerting cell specific effects. It has been shown to have both stimulatory as well as inhibitory effects on various cell types. We have shown earlier that TGFβ1stimulated colony formation from bone marrow derived mononuclear cells (BM MNC) when used at low concentrations while high doses induced inhibition of colony formation (Kale and Limaye, 1999, Stem Cells). We, therefore, examined whether the stimulation of colony formation by TGFβ1 at low concentrations involves a different signal transduction pathway(s) than that mediated by high concentrations.

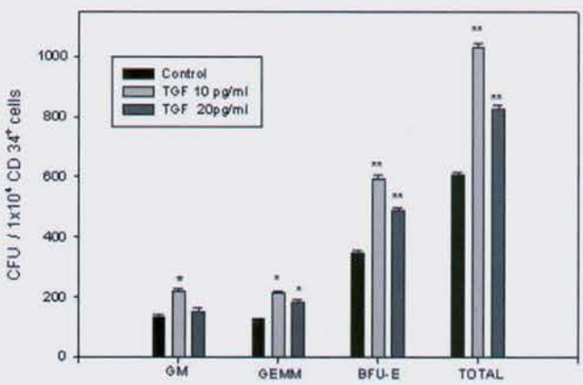

Fig. 1: CD34⁺ cells are the direct targets of stimulatory action of TGFβ1. * Indicates p value < 0.05, ** indicates p value <0.01

Results

Low doses of TGFβ1 induce enhanced colony formation from CD34⁺ cells isolated from adult human bone marrow:

We carried out the experiments on adherence depleted CD34⁺ cells isolated from adult bone marrow (N=5). Clonal cultures were set up with these cells using various concentrations of TGFβ1 in presence of limiting amounts of growth factors. The colonies belonging to various lineages such as BFU-E, GM and GEMM were scored by standard morphological criteria. Since in the first set of experiments, concentrations of TGFβ1 beyond 20 pg/ml led to almost total inhibition of colony formation from CD34⁺ cells, we used 10 and 20 pg/ml concentrations in all subsequent experiments. It was observed that low concentrations of TGFβ1 resulted in significantly higher colony formation (Fig. 1). The stimulatory effect at theTGFβ1 concentration of 10 pg/ml was higher than that at 20 pg/ml.The data indicate that CD34⁺ cells are the direct target of the stimulatory effect of low TGFβ1. The effect was not directed towards any specific type of lineage but was of a general nature, as both erythroid as well as myeloid colonies including GEMM type of colonies were stimulated.

Treatment of KGIa cells with TGFβ1 induces differential activation of signalling pathways:

After establishing the fact that the CD34⁺ cells form the direct target of stimulatory action of low TGFβ1, we initiated the experiments to examine the signaling pathways induced in these cells by low vs. high doses of TGFβ1. The yield of CD34⁺ cells from the BM samples used in our studies was usually low and thus did not yield enough protein for signal transduction analysis. We, therefore, carried out these experiments on a cell line model. We treated serum starved KG1a cells with various doses of TGFβ1 as indicated and prepared the cell lysates. Equal amounts of protein were separated on a 12.5% poly acryl amide gel and were electrically transferred to PVDF membrane. The blots were probed sequentially with antibodies to phosphorylated forms of p44/42 MAPK, p38 and AKT pathways. Levels of native molecules served as the loading controls.

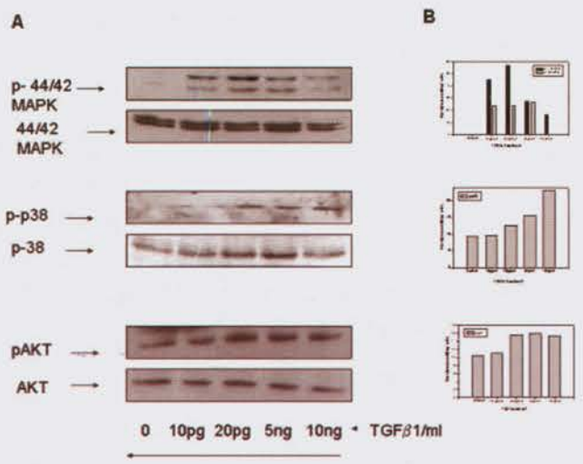

Fig. 2: Dose dependent differential activation of MAPK pathways by TGFβ1

As seen in Fig. 2(A and B) the most striking difference between the low versus high concentrations of TGFβ1 was in p44/42 MAPK and p38MAPK activation respectively. TGFβ1 at low doses (10-20 pg/ml) stimulated p44/42 MAPK while at high doses (5-10 ng/ml) stimulated p38 pathway. AKT was found to be activated in a dose dependent manner till the concentration of 5ng/ml but then showed a slight decrease. These data indicate that TGFβ1 stimulates different signaling pathways especially p44/42 and p38 MAPK as a function of its concentration and this differential MAPK signaling may be the molecular mechanism behind the dose dependent bi-directional effects of TGFβ1 on hematopoietic cells.

Inhibition of MAPK pathway by specific pharmacological inhibitor PD 98059 abrogates the stimulation mediated by low TGFβ1.

Using KG1a as a model system, we observed that TGFβ1 induces different MAPK pathways as a function of its concentration. Low dose of TGFβ1 (10 pg/ml) that was earlier shown to have a stimulatory effect on colony formation (Fig. 1) was found to induce activation of p44/42 MAPK pathway. We, therefore, examined the effect of inhibition of p44/42 MAPK pathway on the stimulation of colony formation mediated by low TGFβ1. We used a pharmacological inhibitor PD 98059, which blocks the activation of p44/42 MAPK at upstream level, for the purpose. PD 98059 at 50 μM concentration was added in the methylcellulose along with TGFβ1 (10 pg/ml). Equivalent amount of DMSO was incorporated in cultures without PD. It was observed that the inhibitor abrogated the stimulation of colony formation in response to TGFβ1 (Fig. 3) indicating that the stimulatory activity of low TGFβ1 was mediated though p44/42 MAPK pathway. Since the base level colony formation nearly equivalent to control plates was observed in presence of PD 98059 it appears that perhaps an independent pathway most probably mediated through growth factor signaling was operative in the system and thus, was not affected by PD98059.

The use of PI3K inhibitor LY 294002 which blocks AKT activation by inhibiting PI3K was found to inhibit the colony formation drastically indicating that this pathway was essential for colony formation in response to growth factors used in our studies (Data not shown).

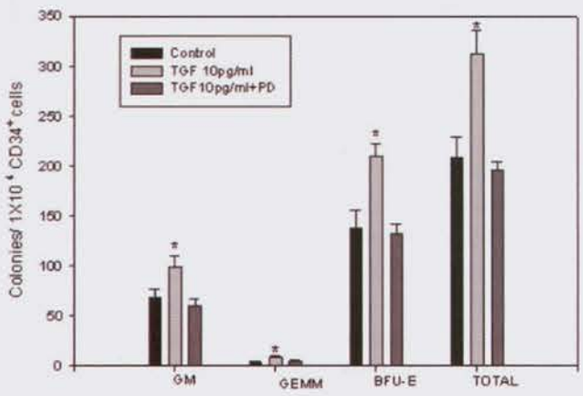

Fig. 3: Abrogation of stimulatory activity of TGFβ1 by pharmacological inhibitor PD 98059: * Indicates p value < 0.05

Pharmacological activation of stress kinases by Anisomycin leads to inhibition of colony formation:

TGFβ1 is known to activate stress kinase pathways namely, p38 and Stress Activated Protein Kinase/c-Jun-N-Terminal Kinase (SAPK/JNK) in hematopoietic cells and has been shown to inhibit colony formation from them. We observed that at high concentrations, TGFβ1 induced p38 activation in KG1a cells. In this set of experiments we examined whether the pharmacological activation of stress kinases leads to inhibitory responses. We used Anisomycin in the colony formation assay at concentrations of < 25 ng/ml as at such low concentrations it does not affect protein synthesis. As seen in the figure 4A, Anisomycin inhibited the colony formation from $CD34^+$ cells in a dose dependent manner in the range of 1-5 ng/ml. At higher concentrations of 10 and 20 ng/ml we observed a complete inhibition of colony formation (data not included). The differential scoring of colonies at 5ng/ml concentration (Fig.4 B) showed that all types of colonies namely BFU-E, CFU GM as well as CFU GEMM were affected. These results indicate that activation of stress kinases in progenitor cells leads to inhibition of their colony forming ability in response to growth factors.

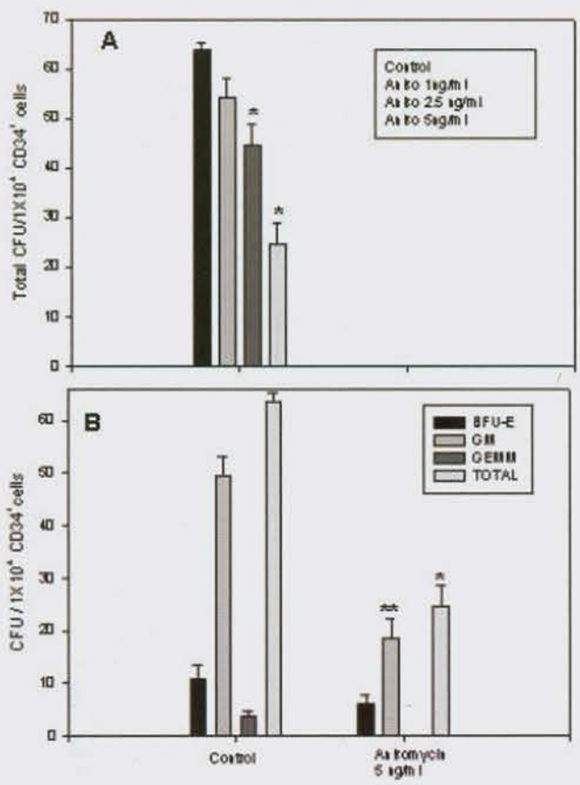

Fig. 4: Activation of stress kinase pathways by Anisomycin leads to inhibition of colony formation. * Indicates p value < 0.05

In summary we have shown that $CD34^+$ cells are the direct targets of stimulatory action of TGFβ1. Using KG1a cell line as a model system we have shown that the low stimulatory concentrations of TGFβ1 induce p44/42 MAPK while high inhibitory concentrations lead to p38 MAPK activation. Thus, the differential activation of MAPK pathways by TGFβ1 as a function of its concentration perhaps forms the molecular basis of its dose dependent bi-directional effect on hematopoiesis.

Future plans:

Further analysis of the signal transduction pathway mediated by TGFβ1 as a function of its concentration and its correlation with stimulatory vs. inhibitory actions on hematopoiesis.

Part 2: Role of nitric oxide in the regulation of hematopoiesis:

We have earlier reported that the treatment of stromal cells with TGFβ1 leads to enhanced expression and Ser 1177 phosphorylation of eNOS in the stromal cells. We have correlated this activity with the enhanced proliferation of stem cells exposed to TGFβ1 treated stromal cells. Our data has given a strong indication that the nitric oxide signaling is involved in the hematopoiesis. We are in the process of generating various expression plasmid vector constructs of specific regulatory sequences from eNOS cDNA. The protein will be expressed with a GFP tag facilitating its direct visualization in the cell under various experimental conditions. We are also standardizing the NO activity assays using fluorescent indicators such as DAF. NO is a free radical and it can have deleterious effects if present in excess amounts. The activity assays will be helpful to quantitate the NO activity in the stromal cells in response to various stimuli. This will help us to define the threshold of NO activity for hematopoietic support vis-à-vis its toxic actions.

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Standardization and transfer of technology of Bone marrow cryopreservation

Nikhat Siddiqui *(Technician)*

Collaborators: R.L. Marathe, *Jahangir Hospital, Pune*, S.G.A. Rao, *(Retired scientist) CRI, Mumbai*

Abstract and background:

This technology was standardized at NCCS and transferred to AFMC. A programmable freezer was purchased and shifted to AFMC under this scheme. They are setting up the laboratory at AFMC and have carried out transplantations using frozen samples. However these samples were frozen in –80 ^{0}C as they do not have liquid nitrogen facility. They are submitting proposal for installation of liquid nitrogen plant.

Aims:

1. Standardization of technology of Bone marrow cryopreservation.
2. Transfer of technology to hospitals that need it.
3. Help the hospital staff in case of any trouble shooting.

Work achieved:

The technology of cryopreservation of bone marrow and Cord blood was standardized by NCCS and CRI and NIV and transferred to AFMC. Using this technology they have frozen 78 CB, 1 BM and 12 MPBL samples. These were frozen and stored at -80^{0}C .Out of these samples 6 MPBL and 1 BM were used for autologous transplantation. These were used for treatment of Multiple myeloma, Hodgkin's disease and Germ cell tumors. Out of the various samples frozen a total of 7 (6 MPBL+1 BM) were used for clinical purpose i.e. for autologous transplantations.

Future work:

AFMC is now planning to equip their laboratory with liquid nitrogen plant so that they can store the cells at -196^{0}C. They are also planning to start HLA typing facility.

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Studies on cryopreservation of haematopoietic cells

Nikhat Siddiqui (*Technician*)

Abstract and background:

This is an ongoing project of NCCS. Here our aim has been to optimize the freezing protocols to get improved recovery in terms of functionality and viability after freezing.

We have used antioxidant and membrane stabilizers as additives in the conventional freezing medium and tested its effect on in vitro and in vivo engraftment.

Aims:

1. To optimize freezing protocols so as to get improved recovery of haematopoietic cells in terms of viability and functionality.
2. To preserve quality of graft as close to the fresh cells as possible.
3. To study the quality of frozen cells by various in vitro and in vivo assays.

Work achieved:

Long-term cultures:

Since there is no in vivo assay to study engraftment potential of frozen human haematopoietic cells, we studied the same by setting up long-term cultures. They mimic the in vivo microenvironment. These assays are laborious and time consuming and require careful tissue culture handling. We set up cultures from frozen revived MNCs as well as CD34 cells isolated from frozen MNCs. These MNCs were obtained from Cord blood and fetal liver haematopoietic cells. Our results indicate that cells frozen with additives showed increased number of colonies in methyl cellulose cultures from LTC as compared to those frozen without additives.

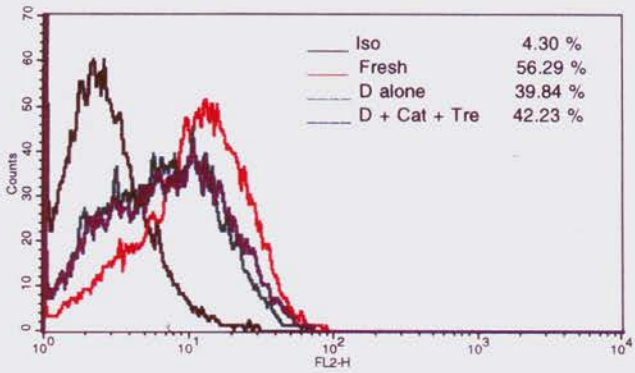

Fig. 1. Expression of CXCR-4 on HL-60 cells

Expression of homing molecules:

This study was done on flow cytometry. The expression of homing molecule CXCR4 was assessed on frozen HL60 cell line (Fig.1). The preliminary results need confirmation.

In vivo engraftment:

Since there is no way to do in vivo experiments using human samples we standardized this assay using mouse bone marrow. Mouse bone marrow cells were isolated from Swiss albino mice and frozen by programmable freezer. The revived samples were infused intravenously in irradiated syngenic mice. Fresh Mouse bone marrow and PBS were also infused as positive and negative controls. The engraftment was assessed by measuring survival, MNC count, neutrophil and platelet count at weekly intervals. The preliminary results are encouraging but need repetition for confirmation.

Frozen MNC and CD34 cells isolated from them show better colony formation in long term cultures when trehalose and catalase are present as additives in the freezing mixture. Expression of the homing molecule CXCR4 is better preserved when additives are present in the freezing mixture.

Future work:

In vivo experiments will be further repeated using C57BL6 mice of Ly5.1 and 5.2 strains as recipients and donors respectively.

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Use of membrane stabilizers and antioxidants in the conventional freezing medium to conserve growth factor responsiveness and engraftment potential of frozen marrow

Lalita Sasnoor

Collaborator: V.P. Kale, *NCCS*

Abstract and background:

This project was sanctioned to us for 3 years in March 2000. It was successfully completed in March 2003. Here the goal was to conserve functionality of frozen haematopoietic cells with respect to expression of adhesion molecules, growth factor receptors, adhesion assays and growth factor responsiveness. It was also proposed to do some in vivo assays to study engraftment potential of frozen marrow.

Aims:

1. Freezing of haematopoietic cells with membrane stabilizer and antioxidant in the conventional freezing medium to conserve growth factor responsiveness and expression of growth factor receptors.

2. To study effect of additives in freezing mixture in conserving expression of adhesion molecules and adhesive properties.

3. To study in vitro and in vivo engraftment potential of haematopoietic cells frozen with additives.

Work achieved:

Growth factor responsiveness:

The responsiveness of frozen MNC/CD34 cells from cord blood and fetal liver haematopoietic cells to 2 early acting cytokines viz IL-3 and SCF was studied by using limiting concentrations of the growth factors in methyl cellulose assay. Results suggest that the cells frozen in the test set (with additives) form more colonies than control set (without additives). Results of responsiveness of CD34 cell are depicted in Fig. 1.

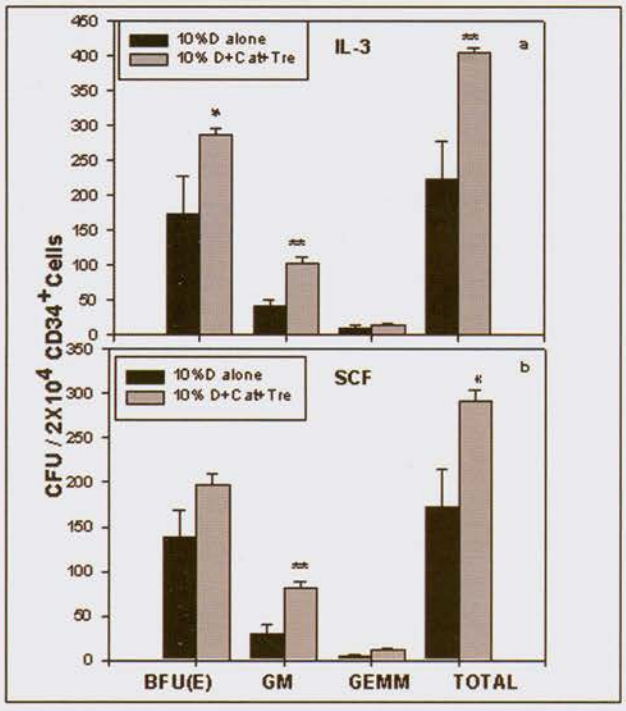

Fig. 1: Growth factor responsiveness of $CD34^+$ cells isolated from frozen MNC. MNC were frozen with (test) or without (control) catalase and trehalose as additives in conventional freezing medium. $CD34^+$ cells were isolated from the revived MNC and plated at a density of $2x10^4$/plate for colony formation assay in methyl cellulose. A coctail of four growth factors were added to each plate viz. EPO, GM-CSF, IL-3 & SCF. The concentration of all the growth factors was optimum except IL-3 (a) & SCF (b). The plates were incubated for 14 days and colonies of various progenitors were counted. The fig shows data from a representative experiment carried out with CB $CD34^+$ cells. *$p \leq 0.05$, **$p \leq 0.01$.

Expression of adhesion molecules and growth factor receptors:

This was studied by flow cytometry. The results show increased expression of these molecules on cells frozen with additives.

Adhesion assay:

This assay was done to study interaction of frozen cells with stromal cells. Frozen cells were labeled with radioactive chromium and recharged on stromal cell line M210B4.They were allowed to adhere for 2 hrs and then the radioactivity in the adherent and nonadherent fraction was measured. The results with KG1a cell line and CD34 cells isolated from frozen revived MNC.

CFU-S assay:

Short term engraftment of frozen mouse bone marrow was assessed by this assay. The results suggest that the engraftment is indeed improved when the cells are frozen with various combinations of additives than the control cells.

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Embryonic stem cells as a developmental paradigm to explore early neuro- and cardiogenic proceedings

Ranju R. Nair, Mahesh Kumar Verma, Kaustubh Gokhale, Aparna Salunkhe *(Technician)*

Collaborator: Anil Chatterji, *NIO, Goa*

Abstract and Background:

The uniqueness of stem cells reside in their potential to self renew and differentiate into wide array of cell types. In this context, the embryonic stem (ES) cells derived from the blastula stage embryo are bestowed with the distinctive characteristic of pluripotency thereby conferring these cells to serve as an ideal model for investigation of early embryonic development and their potential implication in cell replacement therapy. Our interest in fact has been in the exploration of molecular basis of cell commitment and differentiation into various lineages using both the murine and the human ES cell systems. Furthermore, because of the therapeutic prospects the stem cells envisage, we intend to investigate the differentiation and transdifferentiation potential of lineage committed and uncommitted cells derived from embryonic, fetal and adult stem cells. This holds promise for the possible exploitation of these cells in future cell replacement therapies in case of various degenerative diseases.

Aims:

To unravel the complex temporo-spatial cell fate decision machinery from uncommitted ES cells, 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 tissue-specific promoters/ enhancers.

2. To differentiate the ES cells into cardiac and 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 and differentiated neurons with special reference to the dopaminergic neuronal subtypes from ES cells *in vitro*.

Work achieved:

Undifferentiated ES cells:

The maintenance of embryonic stem (ES) cells in undifferentiated state is one of the essential prerequisites in the ES cell research because of its subsequent influence on pluripotency. Self-renewal involves proliferation with a concomitant suppression of differentiation. The prevailing view depicts culturing of ES cells in leukemia inhibitory factor (LIF) supplemented medium or growing on a monolayer of mitotically inactive embryonic fibroblast feeders. In the present investigation we tried to substitute LIF with marine extract (ME) and investigated its influence on murine ES cell maintenance and differentiation. The rational being the proven potential of ME as a potent inhibitor of differentiation of dendritic cells and osteoclasts etc. In fact marine animals are presumed to be a rich source of factors in influencing several cell growth parameters and hence, are considered candidates in drug industries.

The cells cultured with ME supplementation remained undifferentiated depending on the concentration used. Morphologically they were quite compact at least for the investigated regimen of 10-15 passages (Fig. 1). This was

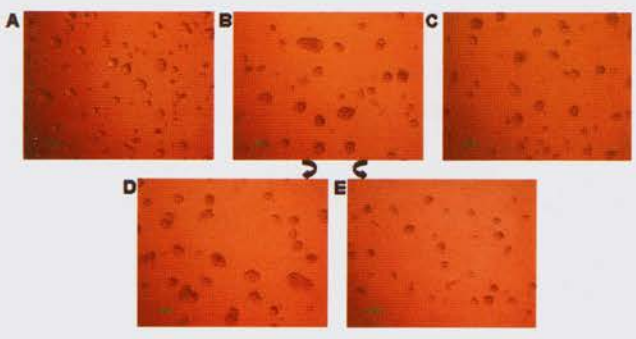

Fig. 1: Murine ES cells maintaining the undifferentiated state with purified fractions of marine extract A: ES cells + LIF; B: ES cells + ME – Fr.1; C: ES cells + ME – Fr.2; D: ES cells + ME – Fr.1a; E: ES cells + ME – Fr.1b

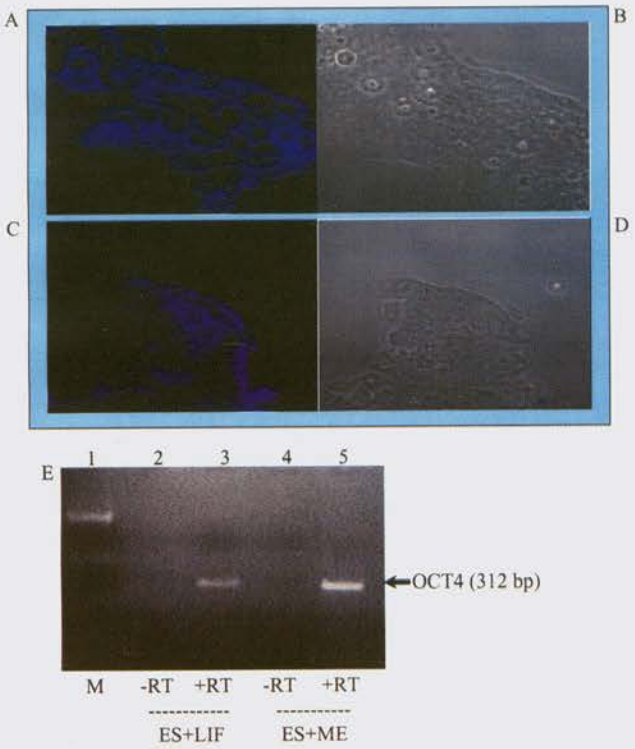

Fig. 2: SSEA-1 & OCT4 expression in undifferentiated ES cells. A: SSEA-1$^+$ undifferentiated ES cells (ES + LIF); B: Bright field view (ES + LIF); C: SSEA-1+ undifferentiated ES cells (ES + ME); D: Bright Field view (ES + ME); E: RTPCR analysis showing OCT4 transcript in undifferentiated ES cells

substantiated by OCT4 and SSEA-1 expression (Fig. 2). To verify whether the ME exposed cells do retain the potential to differentiate into various lineages, we tried to differentiate the ES cells into neural and cardiac lineages. The neural differentiation remained unaffected (Fig. 3C-E). However, the cardiomyocyte differentiation was inhibited with either no or low number of beating areas seen on the EB when compared with LIF exposed cells, even though they did differentiate into mesodermal lineage with a number of alpha actinin positive cells (Fig. 3A, B). In fact, this study sheds light on a LIF independent pathway in the maintenance of stem-cell-ness. Otherwise, the ME could possibly mimic LIF on conferring the self-renewal of ES cells and to some extent might serve as a

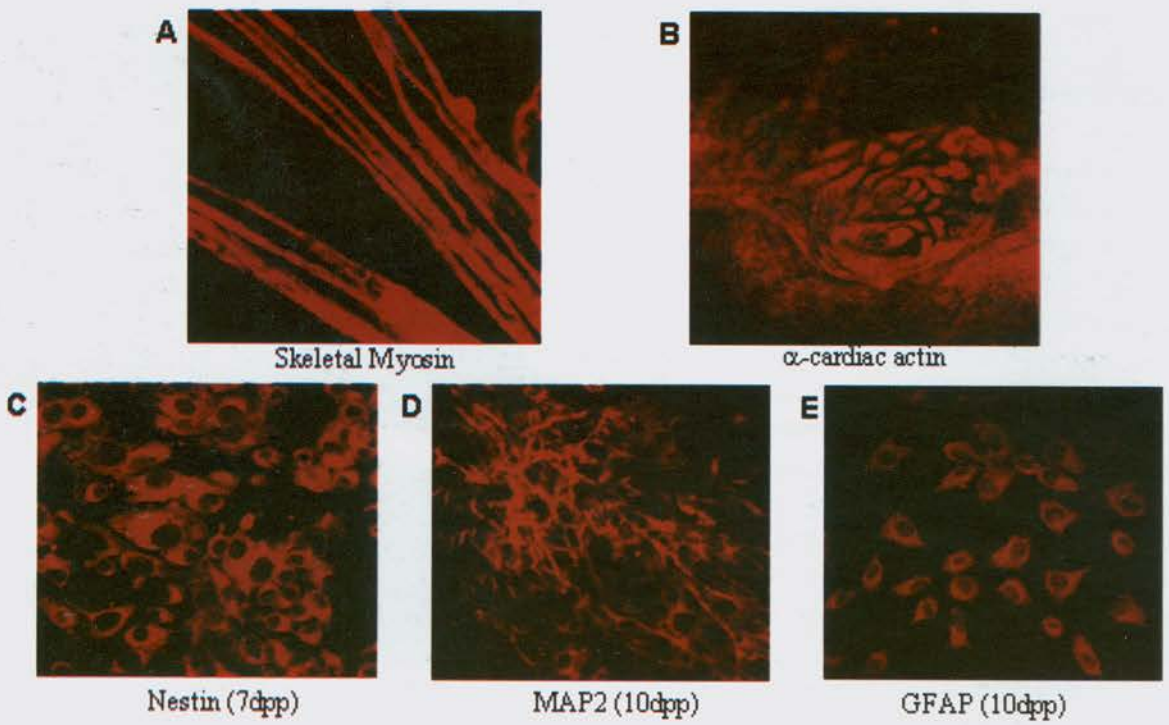

Fig. 3: Differentiation of ME treated ES cells into muscles. A: skeletal; B: cardiac and neural cells (C-E): immunocytochemical characterization

substitute for the same for a limited number of passages, if not for an indefinite period due to its cytotoxic nature.

Moreover, the ME supplementation to the medium during the differentiation of ES cells that resulted in pronounced cytoskeletal structures as evidenced by profuse alpha-actinin staining delineates the fact that the marine animals being a rich source of calcium might help in the build up of stronger cytoskeleton. Further fractionation of ME and narrowing down to the fraction containing the active component would facilitate in demonstrating its nature and functional significance along with its downstream signalling cascade in ES cell self-renewal and differentiation.

Neural Differentiation and specification:

At the outset we tried to differentiate the ES cells into various lineages including the neural one in order to substantiate their pluripotency. The wild type ES cells upon aggregation and plating gave rise to nestin positive neural progenitors (Fig. 4A). Subsequently these progenitors differentiated into more mature neurons (Map 2^+; Fig. 4B) and astroglia ($GFAP^+$; Fig. 4C). Moreover, we could successfully differentiate the murine ES cells into dopaminergic (Th^+; Fig. 4D) and serotonergic ($5\text{-}HT^+$; Fig. 4E) neuronal subtypes.

Never-the-less, as mentioned in our earlier report we have taken advantage of the live reporter based cell trap system

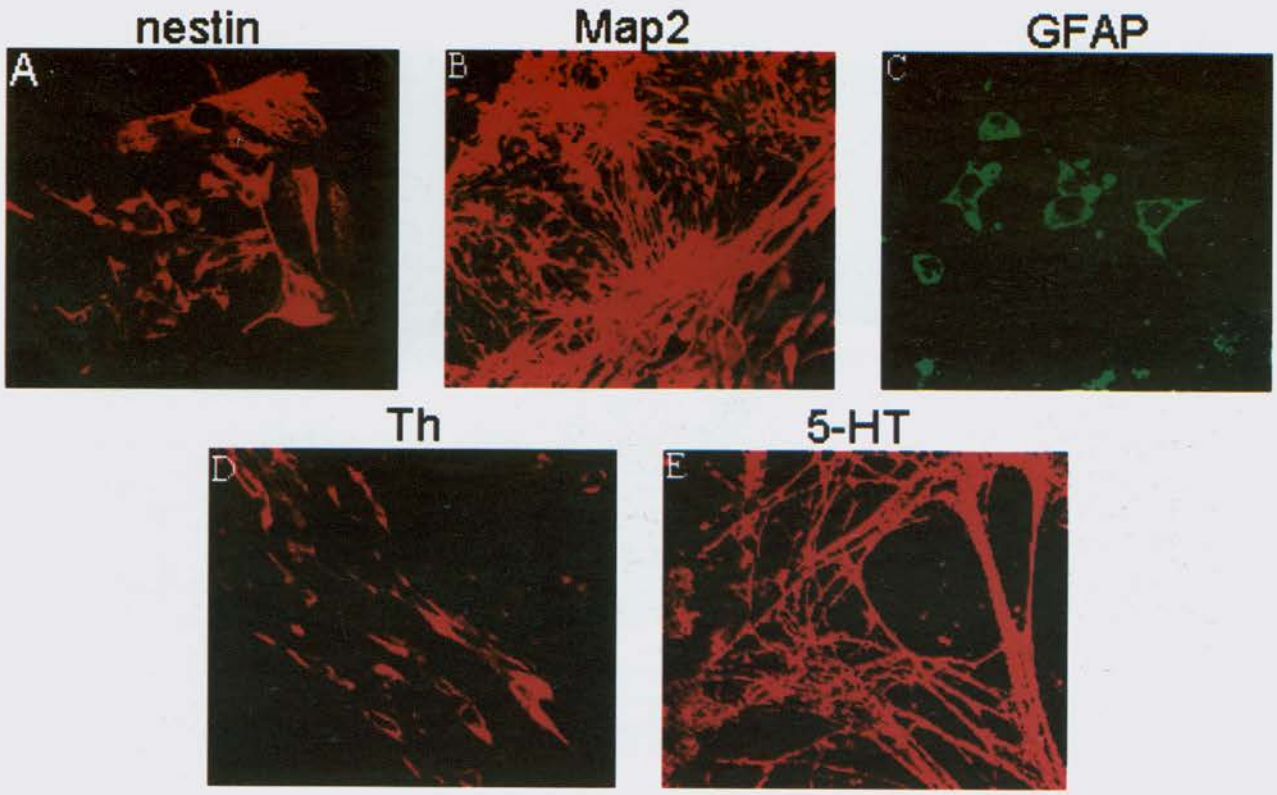

Fig. 4: ES cell differentiation into nestin positive neural progenitors, Map2 positive differentiated neurons, GFAP positive astroglia, Th positive dopaminergic neurons and 5-HT positive serotonergic neurons.

in order to demarcate the cells of interest in the heterogenous population differentiating from the ES cells. Accordingly, we have chosen two different neuronal specific genes expressed at different times during embryonic development i.e. the early onset intermediate filament gene, the nestin which is the marker for mitotically active CNS precursors and the late expressed dopaminergic neuron specific gene, the tyrosine hydroxylase. The nestin transgenic ES cell clones express EGFP at the undifferentiated state itself, however, remained confined to the neural lineage upon differentiation. The immunocytochemical verification showing the overlapping expression (Fig 5C) pattern of EGFP (Fig. 5A) with the endogenous nestin (Fig 5B) proves this fact. While some of the differentiating neurons as evidenced by Map2 immunoreactivity (Fig 5F) retained EGFP expression (Fig. 5D,E), the more mature ones were EGFP negative following the characteristics expression pattern of endogenous nestin. Detailed analysis and characterization of EGFP/ nestin expressing cells would help delineating the early embryonic neural development profile.

The TH transgenic ES cells which remained EGFP negative at the undifferentiated ES cell state express EGFP during

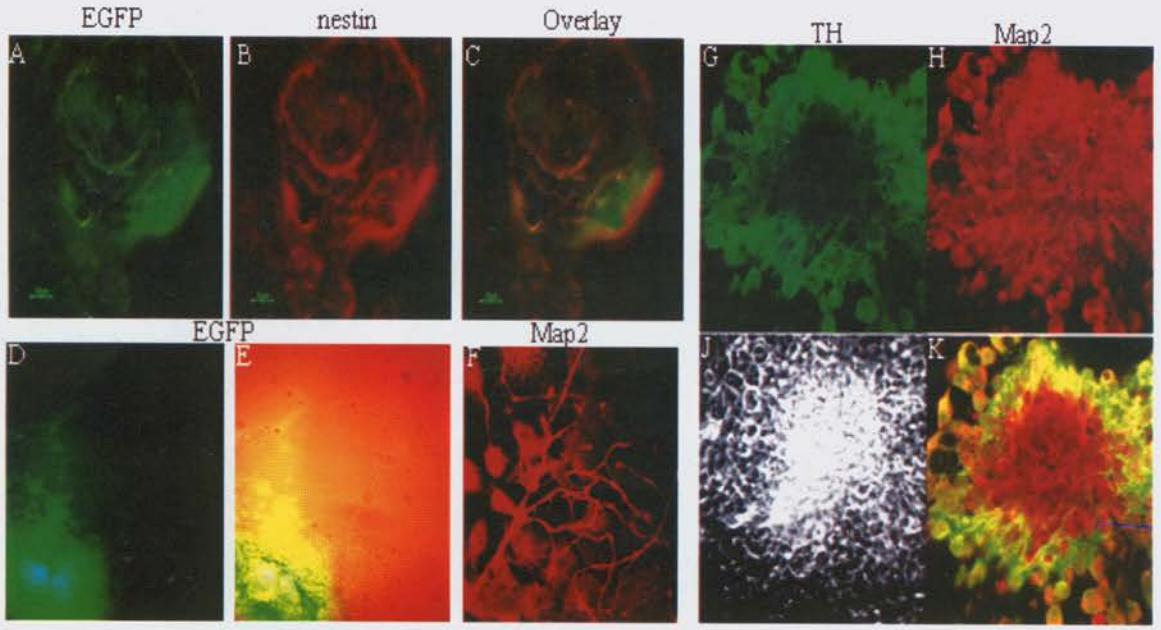

Fig. 5: Nestin and TH Transgenic ES cells differentiation into Neural cells and dopaminergic neurons: A, B showing nestin positive neural progenitors and D, E, the differentiated neurons showing either no or low EGFP expression. F shows Map2 positive differentiated neurons. The overlapping expression pattern of EGFP and nestin (C) indicates the authenticity of nestin transgenic ES cell clones and the specificity in EGFP expression towards neural lineage. G-K: the EGFP expression TH transgenic ES cells shows Th immunoreactivity (G) indicating the EGFP expression in dopaminergic neuron specific manner and all the Th positive neurons show Map2 immunoreactivity (H, K) as well.

differentiation in a dopaminergic neuron specific manner as evidenced by Th immunoractivity (Fig 5G). All the EGFP-Th expressing cells were also Map2 positive (Fig 5H,K) specifying their neuronal phenotype. Efforts are being made to enrich the ES cell derived dopaminergic neurons and detailed characterization of these cells are underway.

Future Directions:

1. In depth mechanistic understanding of the cell fate commitment and specification.

2. Exploration of transdifferentiation potential of lineage commited ES cell-derived stem cells as well as stem cells derived from human umbilical cord blood.

3. Transplantation of ES cell derived neural progenitors/ dopaminergic neurons in xenogenic parkinson rats.

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Cellular and Molecular Mechanisms of Action of IL-3 on Osteoclast Differentiation

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Abstract and background:

Bone remodelling is the predominant metabolic process that maintains bone homeostasis and structural integrity of skeleton. Osteoclasts, the only cells responsible for bone resorption play a crucial role in bone remodeling. Osteoclasts differentiate from haemopoietic precursors of the monocyte/ macrophage lineage that also give rise to macrophages or dendritic cells. Lineage choice and differentiation process is guided by lineage restricted key regulatory molecules and transcription factors. Cytokines produced by activated T cells, as well as by other cell types regulates osteoclastogenesis in physiological and pathological conditions. However, it is not clear how these immune cell-derived cytokines regulates osteoclast differentiation. The recognition of receptor activator of NF-κB ligand (RANKL) that induces osteoclast differentiation enables us to obtain new insights into the control of osteoclast differentiation by cytokines.

IL-3 is a product of $CD4^+$ T cells that act on the immature bone marrow progenitors inducing growth, proliferation and differentiation of these cells. In this study we identified molecular mechanisms for the action of IL-3 in osteoclast differentiation. IL-3 inhibits RANKL and TNF-α-induced osteoclast differentiation and prevents RANKL-induced nuclear translocation of NF-κB by inhibiting the phosphorylation and degradation of IκB. We revealed first time that IL-3 acts directly on early osteoclast precursors isolated from bone marrow cells and irreversibly block RANKL-induced osteoclast differentiation by diverting the cells to macrophage lineage.

Aims:

1. To identify the role of IL-3 on RANKL-induced osteoclast differentiation in stromal cell-free cultures of early osteoclast precursors, and on TNF-α-induced osteoclast differentiation in bone marrow-derived macrophages.

2. To investigate the molecular mechanisms of action of IL-3 on osteoclast differentiation.

Work achieved:

IL-3 inhibits RANKL and TNF-α-induced osteoclast differentiation.

Previously, in co-culture model, IL-3 has been shown to have both stimulatory and inhibitory action on osteoclast formation. We show that IL-3 significantly inhibits RANKL-induced osteoclast differentiation by directly acting on early osteoclast precursors. Osteoclast formation was evaluated by quantification of tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells. IL-3 inhibited osteoclast differentiation in stromal cell-free cultures of osteoclast precursors and also in both stromal and lymphocytes-free cultures of osteoclast precursors (Fig. 1). IL-3 has no inhibitory effect on mature osteoclasts. Indeed, we showed in this study that mature osteoclasts do no express IL-3R. Anti-IL-3 neutralizing antibody abolished the inhibitory effect of IL-3 on osteoclast differentiation, which suggests that IL-3 is the factor responsible for inhibition of osteoclast formation. Recently, TNF-α has been shown to induce differentiation of osteoclast from M-CSF-dependent bone marrow macrophages independent of RANKL. In further investigation we found that IL-3 also inhibits TNF-α-induced osteoclast differentiation in bone marrow-derived macrophages, suggesting that IL-3 can inhibit osteoclast differentiation induced by both RANKL and TNF-α.

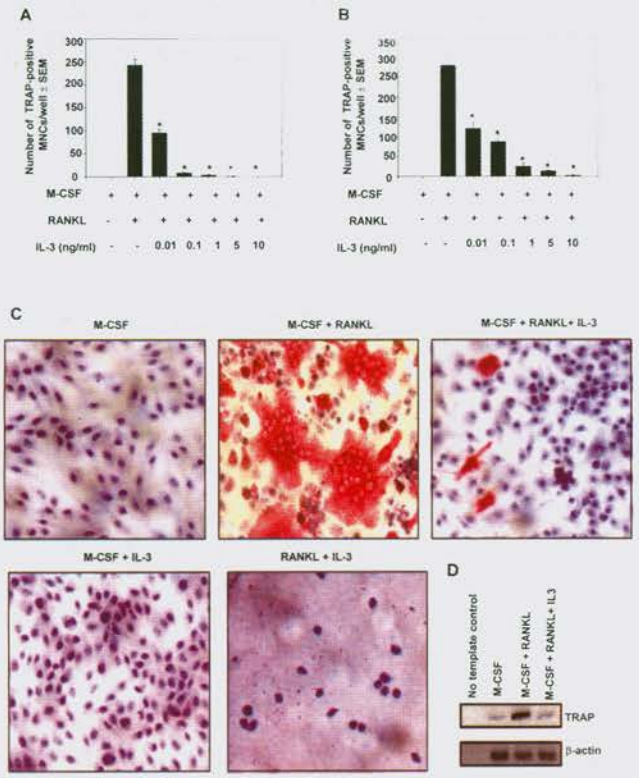


Fig. 1: Effect of IL-3 on osteoclast formation in osteoclast precursors. M-CSF-dependent stromal cell-free osteoclast precursors (A) or Stromal cell and lymphocytes-free osteoclast precursors (B) were incubated in the presence of M-CSF (30 ng/ml) or M-CSF and RANKL (30 ng/ml) in the absence or presence of increasing concentrations of IL-3. After 5 days number of TRAP-positive MNCs was counted. Results are expressed as the mean ± SEM of 8 cultures per variable. *, $p < 0.01$ vs. cultures with M-CSF and RANKL. Similar results were obtained in three independent experiments. C) TRAP staining of osteoclast precursors incubated with M-CSF in the presence or absence of IL-3 (1 ng/ml); M-CSF and RANKL with or without IL-3; and RANKL and IL-3 for 5 days. Photographed at 20x. C) RNA was extracted from osteoclast precursors treated with M-CSF, M-CSF and RANKL ± IL-3 (1 ng/ml) for 5 days and subjected to RT-PCR analysis for TRAP and β-actin genes. Similar results were obtained in two independent experiments.

IL-3 prevents RANKL-induced nuclear translocation of NF-κB by inhibiting the phosphorylation and degradation of IκB.

Osteoclast precursors sequentially express c-Fms and RANK receptors, both required for osteoclastogenesis. IL-3 inhibits osteoclast formation at early stages of differentiation so to investigate its effect on proximal signalling we examined mRNA expression of c-Fms and RANK. Expression of both the receptors was unchanged with IL-3 treatment. These results suggest that the inhibitory effect of IL-3 on osteoclastogenesis is not mediated by blockade in receptor expression.

IL-3 does not affect proximal signalling so we looked for downstream molecules in the cascade. Distal events in RANK signalling include activation of NF-κB complex. As

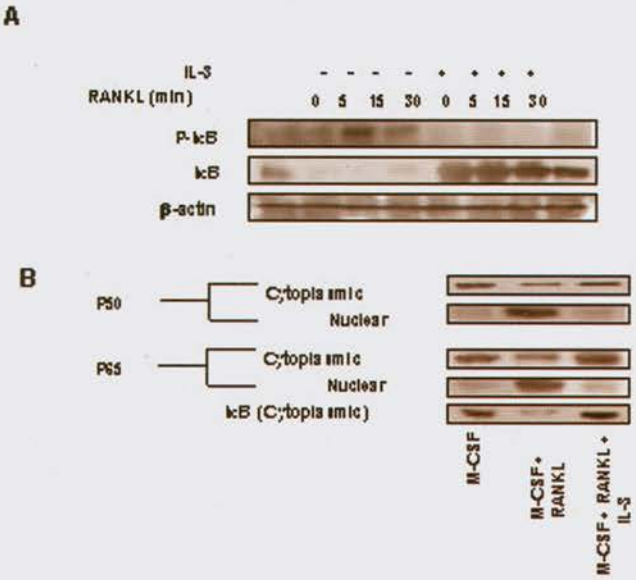


Fig. 2: Effect of IL-3 on RANKL-induced NF-κB signaling. A) Osteoclast precursors were preincubated for 3 days in the presence of M-CSF (30 ng/ml) with or without IL-3 (1 ng/ml) and stimulated with RANKL (30 ng/ml) as indicated. Cells were lysed, fractionated by SDS-PAGE, and analyzed by Western blotting. B) Osteoclast precursors were preincubated for 3 days in the presence of M-CSF with or without IL-3 and stimulated with RANKL for 15 min. Cells were lysed, and cytoplasmic and nuclear fractions were separated. Cytosols were analyzed for IκBα, p50 and p65 NF-κB and nuclear extracts were analyzed for p50 and p65 NF-κB by immunoblots. Results are representative of two independent experiments.

shown in Fig. 2A IL-3 treatment for 3 days inhibits RANKL-induced phosphorylation and degradation of IκB. Next, to examine the effect of IL-3 on RANKL-induced nuclear translocation of NF-κB, osteoclast precursors were incubated for 3 days with or without IL-3 and stimulated with RANKL for 15 min. The data in Fig. 2B shows that RANKL induces nuclear translocation of p50 and p65 subunits and decreases cytoplasmic levels of these two proteins, and also degrade IκB. Consistent with IL-3 inhibition of phosphorylation and degradation of IκB, IL-3 when added prior to RANKL, totally prevents the nuclear translocation p50 and p65 and accumulates these proteins in the cytoplasm and prevent degradation of IκB.

Commitment of mononuclear precursors to mature osteoclasts involves transcription factors such as PU.1 and c-Fos. PU.1, a myeloid cell-specific transcription factor is essential for the development of osteoclasts. The expression of PU.1 was not changed by addition of IL-3 for 5 days indicating that the cells in the presence of IL-3 are of myeloid lineage. However, RANKL induced expression of c-Fos was significantly suppressed by IL-3. These results suggest that IL-3 also inhibits osteoclastogenesis by down regulation of RANKL-induced c-Fos expression.

IL-3 inhibits osteoclast differentiation by diverting the cells to macrophage lineage.

Characteristics of IL-3 treated cells were examined by immunostaining with antibodies against MOMA-2, Mac-1 and F4/80 antigens, which are specific for macrophages. Osteoclast precursors incubated for 5 days with M-CSF alone expressed MOMA-2, Mac-1 and F4/80 antigens, (Fig. 3, upper panel). Expression of these macrophage antigens was not detected on multinucleated osteoclasts (Fig. 3, middle panel). However, strong expression of all these macrophage antigens was detected on cells incubated in

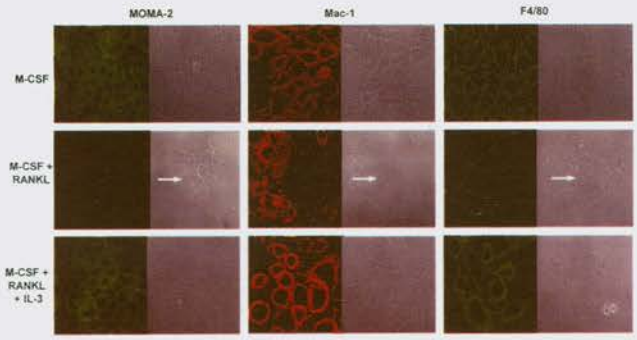


Fig. 3: Effect of IL-3 on expression of macrophage-associated antigens. Osteoclast precursors were cultured in the presence of M-CSF; M-CSF and RANKL with or without IL-3 for 5 days. Cells were washed, fixed and blocked by Fc block. Cells were immunostained with MOMA-2 (green), Mac-1 (red) and F4/80 (green) Abs. Arrows indicates multinuclear osteoclasts.

Table 1: IL-3 irreversibly inhibits osteoclast formation

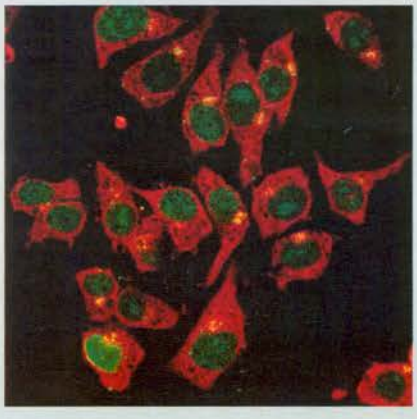
Culture conditions		Number of TRAP-positive MNCs/well ± SEM		
3 days in		day 3	day 5	day 8
M-CSF + RANKL	then 3, 5 and 8 days in M-CSF + RANKL	239.3 ± 19.2	166.6 ± 6.3	47.0 ± 11.6
M-CSF + RANKL + IL-3		4.3 ± 2.4	6.0 ± 2.3	0
M-CSF		203.0 ± 11.2	119.0 ± 8.0	34.6 ± 9.9
M-CSF + IL-3		5.0 ± 1.73	5.6 ± 1.7	5.3 ± 1.8

the presence of IL-3 (Fig. 3, lower panel). Furthermore, the inhibitory effect of IL-3 on osteoclast differentiation was irreversible and the osteoclast precursors preincubated in IL-3 were resistant to RANKL action (Table 1). These results suggest that IL-3 inhibits osteoclast differentiation by diverting the cells to macrophage lineage that are resistant to RANKL action. Thus, our results first time reveal that IL-3 acts directly on early osteoclast precursors and irreversibly block RANKL-induced osteoclast differentiation by diverting the cells to macrophage lineage.

Osteoclast precursors were incubated for 3 days in the presence of M-CSF (30 ng/ml) and RANKL (30 ng/ml) with or without IL-3 (1 ng/ml); and M-CSF with or without IL-3. Cells were washed vigorously to withdraw IL-3 and cells were further cultured for 3, 5 and 8 days with M-CSF and RANKL. Number of TRAP-positive MNCs was scored. Results are expressed as the mean ± SEM of 8 cultures per variable. Similar results were obtained in three independent experiments.

Future work:

IL-3 inhibits RANKL-induced NF-κB activation. However, the means by which IL-3 inhibits the phosphorylation and degradation of IκB, and prevents nuclear translocation of NF-κB are presently unclear. Since IL-3 mainly acts through Janus Kinases (JAKs) and Signal Transducer and Activators of Transcription (STAT) pathway the possible interaction between the two pathways will be delineated. Furthermore, *in vivo* role of IL-3 on bone mass in mice and on ovariectomised induced bone loss in rats will be studied.



Cancer Biology

Manoj Kumar Bhat
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Wild-type tumour suppressor gene in human cancer cells: Its role in chemosensitivity and functional consequences of it's over activation/expression

Rishi Raj Chhipa, Sandeep Singh, Sachin V. Surve, Vijay Kumar M.V. (*Technician*)

Background:

One of the goals of the scientists is to develop novel rational therapeutic strategies based on specific molecular defects in human cancer. In this direction one needs to identify targets that are different between normal cells/cancer cells and the genes that are activated and are responsible for drugs induced cell killing. One such target is "Genomic Care Taker" tumour suppressor p53 gene. Because p53 is most commonly mutated gene in human cancer and the p53 pathway is involved in vast majority of tumours without mutations in p53, this p53 protein becomes an ideal target for studying in roles in cancer therapeutics. Major mechanisms through which p53 function is controlled are regulation at protein levels, control of the localization of the p53 protein and modulation of the activity of p53 particularly its ability to function as a sequence specific transcription factor. p53 is a multifaceted transcription factor regulating multiple cellular processes including cell cycle progression, apoptosis, DNA repair and differentiation. After DNA damage, p53 levels increase and mediate multiple cellular responses i.e., (a) G1 arrest via transcriptional induction of p21, a CDK inhibitor; (b) DNA damage repair via transcriptional induction of GADD45; and (c) induction of apoptosis in some cell types, if the damage is excessive. In addition, p53 status in a cell has been implicated in the outcome of cancer cells treated with various cancer therapeutic namely irradiation and chemotherapy. So far results have been controversial. Studies with mouse cells have provided evidence of drug resistance after p53 inactivation, but the extrapolation of these results to humans is far from straightforward. The requirement of wild-type p53 for apoptosis after genotoxic damage caused by anticancer agents; including irradiation has been demonstrated in tissues of lymphoid origin. However, the influence of p53 on apoptosis in malignant tissues of non-hematological origin is by no means clear. More over most of the studies undertaken have been carried out in systems in which p53 is over-expressed. This has added further confusion to already complex molecule. Therefore, the present study is under taken to investigate the expression pattern of endogenous p53 and other cell growth regulatory genes and to correlate chemotherapy drugs induced effects.

Aims:

In view of the important role played by p53 in cancer cells, we hope to study:

1. The correlation, if any, between p53 status and other cell growth regulatory genes responsible for drug sensitivity of human cancer cells.

2. Effect of p53 over-activation on cells and role if any, on anticancer chemotherapeutic drug sensitivity.

Work Achieved:

- **Effect of anticancer drugs on human cancer cell lines differing in p53 status:**

Human breast cancer cell MCF-7 which contain wild-type p53 gene and MDA-MB-231 cells contain mutated p53 gene together with human oral cancer cells KB and HEP-2, in which p53 function is abrogated by the expression of HPV E6 oncoprotein were treated with varying concentrations DNA damage causing drugs carboplatin, 5-fluouracil and cyclophosphamide. Cytotoxicity assay was carried out. No significant differences in cellular toxicity of MCF-7 and MDA-MB-231 were noticed, whereas KB and HEP-2 seem to be more sensitive to carboplatin and 5-fluorouracil. Cyclophosphamide was toxic to the cells at a very high concentration with the exception being MDA-MB-231 cells. Therefore no correlation could be drawn between drugs mediated cytotoxicity and p53 status. Even though chemotherapeutic drugs induced cell death in all the cells irrespective of p53 status, we observed that there were significant differences in the mechanisms leading to cell death. In wild-type p53 possessing cells, p53 and downstream molecule Bax are involved, whereas in other cells GADD45 and Bcl-2 seems to be involved.

- **Development of cell line exhibiting very high p53 trans-activation activity: Dissecting role of activity and protein:**

MCF-7 T clone was developed from MCF-7 by process of selection for a stable clone being developed for Tetracycline based gene regulators system. MCF-7T cells were compared to parental MCF-7 cells with respect to their ability to activate reporter gene driven by p53 responsive element. MCF-7T cells exhibited significantly higher p53 trans-activation activity by at least 5 folds compared to that exhibited in MCF-7 cells. MCF-7T in-spite of exhibiting very high p53 trans-activation activity did not show significant differences in growth pattern compared to MCF-7 cells. Therefore enhanced p53 trans-activation alone may not be sufficient to induce p53 mediated biological effects under normal growth conditions. Even though MCF-7T cells exhibited high p53 trans-activation activity, the protein levels did not show differences when compared to that of MCF-7 cells. Thus indicating that increased p53 trans-activation activity is not probably due to increased protein expression in MCF-7T cells. Initial experiments with drug sensitivity of MCF-7T cells to chemotherapeutic drugs indicate that MCF-7T

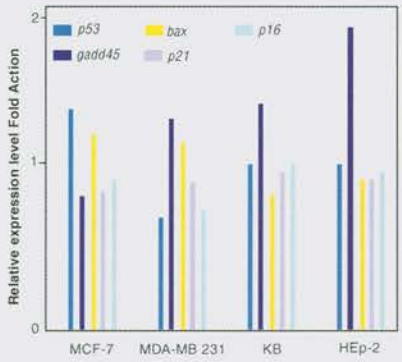
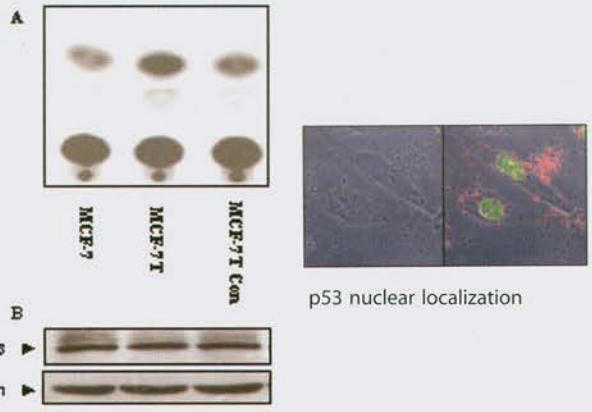


Fig. 1: Induction pattern of cell growth regulatory genes in various human cells treated with cyclophosphamide

cells are more sensitive to DNA damaging chemotherapy drugs mediated cell killing compared to MCF-7 cells. All these results suggest that increased p53 trans-activation may be independent of p53 protein levels. Moreover p53 trans-activation property may play an important role in chemotherapeutic drugs mediated cell killing even though the role played by protein level cannot be totally ruled out at present.



p53 nuclear localization

Fig. 2: (A) p53 trans-activation activity in MCF-7 and MCF-7T cells, transiently transfected with p53 responsive CAT reporter plasmid. (B) p53 protein levels in MCF-7 and MCF-7T cells.

Future Work:

- Study the chemotherapeutic drugs mediated cell killing mechanisms in detail and to investigate gene expression at protein level.
- The cellular factors responsible for increased p53 trans-activation activity in MCF-7T cells will be explored. The in vivo tumor development by MCF-7 and MCF-7T will be studied.
- The drug responsiveness of MCF-7T cells will be studied and compared to that of MCF-7 cells. The role played by enhanced p53 trans-activation will be investigated.

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Identification and Characterization of *M3TR*, A Non-coding RNA From Cloudman Melanoma Cells Inducing Cellular Transformation

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Collaborator: Dr. L.C. Padhy, *TIFR, Mumbai*

Abstract and Background:

The project aims at understanding the mechanism of transformation elicited by the 600 bp gene identified and cloned in our lab from Clone M3 melanoma cells. The gene has been localized as a 600 bp contig on the X chromsome and is expressed as a non-coding RNA. We have generated sufficient experimental evidence to show that the molecule as untranslated RNA itself induces transformation *in vivo* and *in vitro*. Hence, now the present understanding of the biology of Cancer besides focusing on the understanding of the coding genes derived from the proteome, opens up to the role of non-coding RNA's in cellular transformation, as now it is becoming evident that the non-coding RNA's form the primary control architecture that underpins eukaryotic differentiation and development.

Aims:

1. Study the pathway of transformation and the downstream effectors implicated in inducing cellular transformation by an untranslated RNA-M3TR.

2. Gene Expression Profiling in cell-lines exogenously expressing M3TR.

Work achieved:

We have identified a 557 bp novel gene, *M3TR*, from mouse Melanoma cell-line derived from Cloudman Melanoma, and found it to be belonging to the new category of NCR's with the propensity for transformation similar to H-RAS and pSV3 as assayed by colony forming potential invitro. Extensive sequence analyses at the DNA and cDNA level have showed that there are no genes homologous to *M3TR* reported in the available computer databases. However, several EST's have been reported that are homologous to different parts of the M3TR gene. Significantly, the sequence aligned completely with the shotgun sequence on Chromosome X available on the Ensembl Mouse Sequence database . Characteristically, the *M3TR* gene could alone immortalize and transform human diploid cultures on stable ectopic expression, lacked clear long ORF's. and was expressed as a 3.2 Kb. antisense transcript in Melanoma cell-line under study. Despite few reports of NCR's like DD3, PCGEM1 and NCRMS

over-expressed in tumorigenesis, there are no direct studies to demonstrate that their expression alone resulted in tumor development *invivo* or transformation *invitro*. Taken together, this is the first report of a novel NCR gene *M3TR* involved in eliciting cell transformation without need of cooperation from other known oncogenes/tumor suppressor genes, and signifies towards existence of a unique pathway effected by this category of molecules in cancer development and progression.

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Understanding the pathway of tumor progression in Glioblastoma using a novel model system of two developed neuroepithelial cell lines

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Collaborator: Padma Shastry, *NCCS*

Abstract and Background:

We have established a novel model system from human malignant glioma comprising of two human cell lines - Human Neural Glial Cell-line HNGC-1 and HNGC-2 which express neural stem cell-like characters. The HNGC-1 is a non-transformed cell line with differentiation potential and has crossed 50 passages. The HNGC-2 is a clonal cell-line that arose from HNGC-1 by spontaneous transformation and has crossed 250 passages and is maintained in culture since last 3 years. The cell lines HNGC-1 and HNGC-2 together form an excellent model for studying the mechanisms related to transformation and in furthering the knowledge in unraveling the signal transduction pathways in gliomagenesis.

Aims:

1. Study the role of EGFR, c-erb2 and c-erbB3 in tumor progression.

2. Understand the pathway of neural differentiation with respect to the 2 cell-lines under study.

Work Achieved:

Gliomas are the most common of brain tumors and account for more than 40% of CNS neoplasms. The precise study of the molecular mechanisms involved in tumor progression from a low grade astrocytoma to the most malignant Glioblastoma multiforme (GBM) have been hampered due to lack of suitable experimental models. We have established a model of tumor progression comprising of 2 cell-lines HNGC-1 and HNGC-2 derived from the same astrocytoma tumor with a set of features corresponding to low grade glioma (as in HNGC-1) and high grade GBM (as in HNGC-2). The HNGC-1 cell line is a slow growing, contact inhibited, non-tumorigenic, non-invasive cell-line, whereas HNGC-2 is a rapidly proliferating, anchorage independent, highly tumorigenic and invasive cell-line. The proliferation of cell lines is independent of the addition of exogenous growth factors. Interestingly, almost all cells in HNGC-1 and HNGC-2 express the neuronal precursor and progenitor markers vimentin, nestin, MAP-2, NFP160 as well as glial differentiation S100â. The HNGC-1 cell-line also expressed markers of mature neurons like Tuj1 and GFAP, an astrocytic differentiation marker, hence contributing towards a more

morphologically differentiated phenotype with a propensity for neuronal differentiation *in vitro*. Such study would be useful to decipher the events involved in neuronal differentiation. Additionally, over-expression of EGFR, c-erbB2 and loss of fibronectin was observed only in the HNGC-2 cell-line implicating the significance of these pathways in tumor progression. This *in vitro* model system assumes importance in unraveling the cellular and molecular mechanisms in transformation and in gliomagenesis.

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Molecular mechanism of osteopontin (OPN) regulated promatrix metalloproteinase-2 (MMP-2) activation and its role in cell migration and tumor growth (melanoma formation)

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Abstract and Background:

Matrix metalloproteinases (MMPs) degrade extracellular matrix (ECM) components and play important roles in tissue repair, tumor cell invasion and metastasis. MMPs are known to be regulated by ECM-proteins, different cytokines and other factors. However, the molecular mechanisms by which osteopontin (OPN), an ECM-protein regulates activation of pro-MMP-2 and controls cellular invasiveness and tumor growth using murine melanoma (B16F10) cells are not well defined. We have demonstrated that purified, human OPN induces pro-MMP-2 production and activation in these cells. The data revealed that OPN induced MMP-2 activation is mediated by upregulation of MT1-MMP and further demonstrated that OPN induced MT1-MMP expression correlates with translocation of NF-κB into the nucleus. Moreover, OPN-induced NF-κB activation is occurred through phosphorylation and degradation of IκBα by enhancing the activity of IKK in these cells. OPN also enhances cell motility, ECM-invasion and tumor growth. Curcumin, an anti-inflammatory and anticarcinogenic compound suppresses the OPN-induced cell motility, tumor growth and NF-κB-mediated pro-MMP-2 activation. Taken together, OPN stimulates NF-κB activity through phosphorylation and degradation of IκBα by activating IKK that ultimately triggers the activation of pro-MMP-2 and further demonstrates that curcumin potently suppresses OPN-induced cell migration, tumor growth and NF-κB-mediated pro-MMP-2 activation by blocking the IKK/IκBα signaling pathways.

Aims:

1. To examine whether OPN induces pro-MMP-2 activation through IKK dependent NF-κB-mediated pathways in B16F10 cells.

2. To determine whether OPN enhances cell motility, ECM-invasion and tumor growth and whether MMP-2 plays any direct role in these processes.

3. To investigate whether curcumin (diferulolylmethane), an indigenous anti-inflammatory and anticarcinogenic compound down regulates the OPN-induced NF-κB-mediated pro-MMP-2 activation, cell motility and tumor growth.

Work Achieved:

We have demonstrated earlier that purified OPN activates pro-MMP-2 through activation of NF-κB in B16F10 cells. However, the molecular mechanisms by which OPN-induces NF-κB-mediated pro-MMP-2 activation and the effect of curcumin (diferulolylmethane) on these activation processes are not well defined. We have shown that OPN induces phosphorylation and degradation of IκBα by enhancing the activity of IKK in these cells. OPN also stimulated the nuclear accumulation of p65 subunit of NF-κB, NF-κB-DNA-binding and NF-κB transcriptional activity. Curcumin suppressed the OPN-induced IKK activity and NF-κB transactivation by inhibiting the phosphorylation and degradation of IκBα. Curcumin also inhibited the OPN-induced cell motility, tumour growth and pro-MMP-2 activation. These data demonstrate that OPN stimulates NF-κB-mediated pro-MMP-2 activation through phosphorylation and degradation of IκBα by inducing the IKK activity and curcumin suppresses the OPN-induced cell motility, tumour growth and NF-κB-mediated pro-MMP-2 activation by blocking the IKK/IκBα signalling pathways.

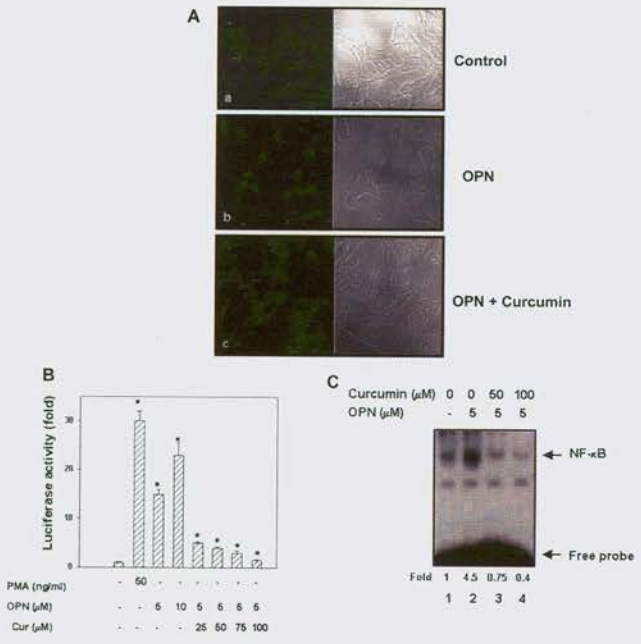

Fig. 1: Curcumin inhibits OPN induced nuclear translocation of NF-κB, p65(A), NF-κB-DNA binding (B) and NF-κB transcriptional activity (C). A: Cells were treated with OPN or pretreated with curcumin prior to treatment with OPN. The cells were immune stained and analyzed under confocal microscopy. In the untreated cells, p65 was detected in the cytoplasm (panel A) but in the OPN-treated cells, p65 translocated from the cytoplasm to the nucleus (panel B). In contrast, upon treatment of cells with curcumin and then with OPN, majority of p65 staining was detected in the cytoplasm (panel C). B: Cells transfected with luciferase reporter construct (pNF-κB-Luc) were either stimulated with PMA (50 ng/ml) or different doses of OPN (0-10 μM) for 6 h or with various doses of curcumin (0-100 μM) for 45 min and then treated with OPN (5 μM) for 6 h. The cells lysates were used to measure the luciferase activity. The values were normalized to renilla luciferase activity. The fold change were calculated. C: The cells were stimulated with or pretreated with curcumin (0-100) μM) for 45 min and then stimulated with OPN. Nuclear extracts were prepared and analyzed by EMSA. OPN treatment (lane 2) induces NF-κB binding compared with the untreated cells (lane 1). Curcumin inhibits OPN-induced NF-κB-DNA binding in a dose dependent manner with 50 μM (lane 3) and 100 μM (lane 4) concentrations.

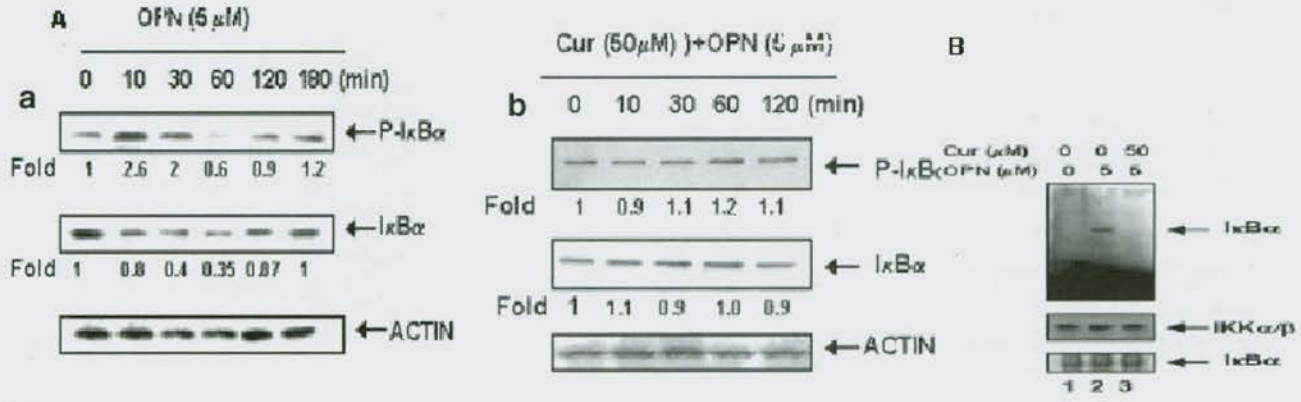

Fig. 2: OPN stimulates IκBα phosphorylation (A) and IKK activity (B) and curcumin blocks the OPN-induced IκBα phosphorylation and IKK activity. A: Cells were treated with 5μM OPN alone for 0-180 min (panel a) or with 50 μM curcumin for 45 min and then with 5 μM OPN for 0-120 min (panel b). The cells were lysed and the lysates were used for Western blot analysis using anti-phospho-IκBα antibody (upper panels a & b). The blots were reported with anti-IκBα (middle panels a & b) or anti-actin (lower panels a & b) antibody. The blots were analysed densitometrically and the values were normalized to actin. The relative values of phospho-IκBα or IκBα in terms of fold changes are indicated. Note that the maximum phosphorylation of IκBα occurs at 10 min in OPN treated cells in panel "a", whereas in panel "b" curcumin blocks the OPN-induced phosphorylation and degradation of IκBα. B: Cells were stimulated with 5 μM OPN for 10 min or with 50 μM curcumin for 45 min followed by 5 μM OPN for 10 min. The cell lysates were immunoprecipatated with anti-IKK α/β antibody and used for kinase assay using recombinant IκBα as substrate (upper panel). The immunoprecipitated samples wre analyzed by Western blot analysis using anti-IKK α/β antibody (middle panel). Equal volumes of samples from kinase assay were analyzed by Western blot analysis using anti-IκBα antibody (lower panel). Lane 1: untreated; lane 2: with 5 μM OPN and lane 3: with 50 μM curcumin and 5 μM OPN.

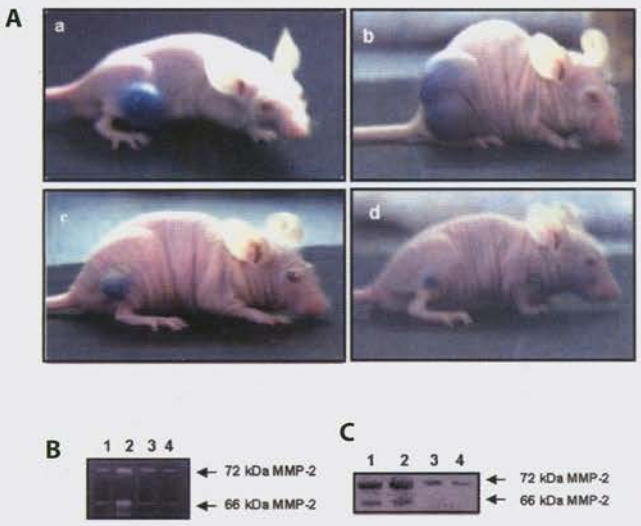

Fig. 3: Cucumin inhibits OPN-induced tumor growth and MMP-2 activation in OPN-induced tumors of nude mice. A: Cells were treated with or without OPN (10 μM) and injected subcutaneously into the flanks of nude mice. In separate experiments, the cells were pretreated with curcumin followed by treatment with OPN and injected into the nude mice. OPN (10 μM) alone or mixture of curcumin (0-100 μM) was injected to same site twice a week. Panel a, cells with PDG; panel b, cells with OPN; panel c, cells with OPN and curcumin (50 μM) and panel d, cells with OPN and curcumin (100 μM). B: detection of MMP-2 expression in the tumors of nude mice by gelatin zymography. The tumor samples from 'A' were lysed in lysis buffer and analyzed by gelation zymography. Equal amount of total proteins were used in each lane. C, The tumor samples from 'A' were lysed. Equal amounts of total proteins were electrophoresed and analyzed by Western blotting using mouse monoclonal anti-MMP-2 antibody. Lane 1: PBS; lane 2: with 10 μM OPN; lane 3: with 10 μM OPN and 50 μM curcumin; and lane 4: with 10 μM OPN and 100 μM curcumin. The arrows indicate the 72- and 66 kDa MMP-2-specific bands.

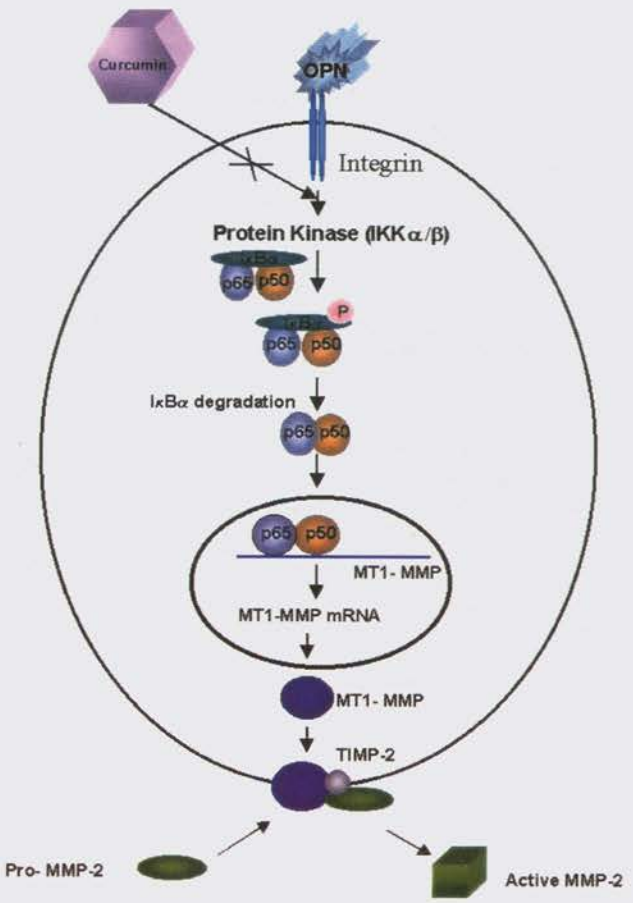

Fig. 4: Mechanism of OPN induced NF-κB mediated pro-MMP-2 activation and action of curcumin on this pathway. Binding of OPN to its integrin receptor on cell surface induces NF-κB signaling pathway by inducing the activity of IKK α/β followed by phosphorylation and breakdown of IκBα. NF-κB then moves from cytoplasm into the nucleus and elevates the levels of MT1-MMP mRNA. Increased MT1-MMP on the cell surface facilitates the activation of pro-MMP-2. Curcumin inhibits this activation process by blocking the signals that lead to IKK/IκBα, activation.

Future Plan:

The signalling pathways by which upstream kinases regulate OPN-induced NF-κB mediated pro-MMP-2 activation in melanoma cells will be delineated.

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Role of osteopontin (OPN) in regulation of cell motility and NF-κB-mediated uPA secretion through PI 3'-kinase/Akt signaling pathways in human breast cancer cells

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Background and Abstract:

We have recently reported that OPN induces NF-κB-mediated promatrix metalloproteinase-2 activation through IκBα/IKK signaling pathways in murine melanoma (B16F10) cells. However, the molecular mechanisms by which upstream kinases regulate the OPN-induced NF-κB activation and urokinase type plasminogen activator (uPA) secretion which are involved in breast cancer cell motility are not clearly understood. Here we report that OPN induces the phosphatidylinositol 3'-kinase (PI 3'-kinase) activity and subsequently stimulates the phosphorylation of Akt in highly invasive MDA-MB-231 and low invasive MCF-7 cells. OPN enhances the interaction between IκBα kinase (IKK) and phosphorylated Akt. OPN also induces NF-κB activation through phosphorylation and degradation of IκBα by inducing the IKK activity in these cells. OPN also enhances uPA secretion, cell motility and chemoinvasion. Pretreatment of cells with PI 3-kinase inhibitors or NF-κB inhibitory peptide (SN50) reduces the OPN-induced uPA secretion, cell motility and invasion. Taken together, we demonstrate that OPN induces cell motility, uPA secretion and NF-κB activity through PI 3'-kinase/Akt/IKK-mediated signaling pathways in human breast cancer cells.

Aims:

1. To investigate whether OPN induces NF-κB activation through PI 3'-kinase/Akt dependent pathways in human breast cancer cells.
2. To examine whether OPN stimulates cell motility, chemoinvasion and uPA secretion through NF-κB-mediated pathways.
3. To delineate whether OPN regulates cell motility and NF-κB-mediated uPA secretion through PI 3'-kinase/Akt/IKK-mediated signaling pathways in breast cancer cells

Work Achieved:

We have shown that OPN induces PI 3'-kinase activity and subsequently phosphorylates Akt in highly invasive MDA-MB-231 and low invasive MCF-7 cells. The OPN-induced Akt phosphorylation was inhibited when cells were

transfected with dominant negative mutant of p85 domain of PI 3-kinase (Δp85) and enhanced when cells transfected with activated form of PI 3-kinase (p110CAAX) indicating that PI 3′-kinase is involved in Akt phosphorylation. OPN enhanced the interaction between IκBα kinase (IKK) and phosphorylated Akt. OPN also induced NF-κB activation through phosphorylation and degradation of IκBα by inducing the IKK activity in breast cancer cells. However, both pharmacological (wortmannin and LY294002) and genetic (Δp85) inhibitors of PI 3′-kinase

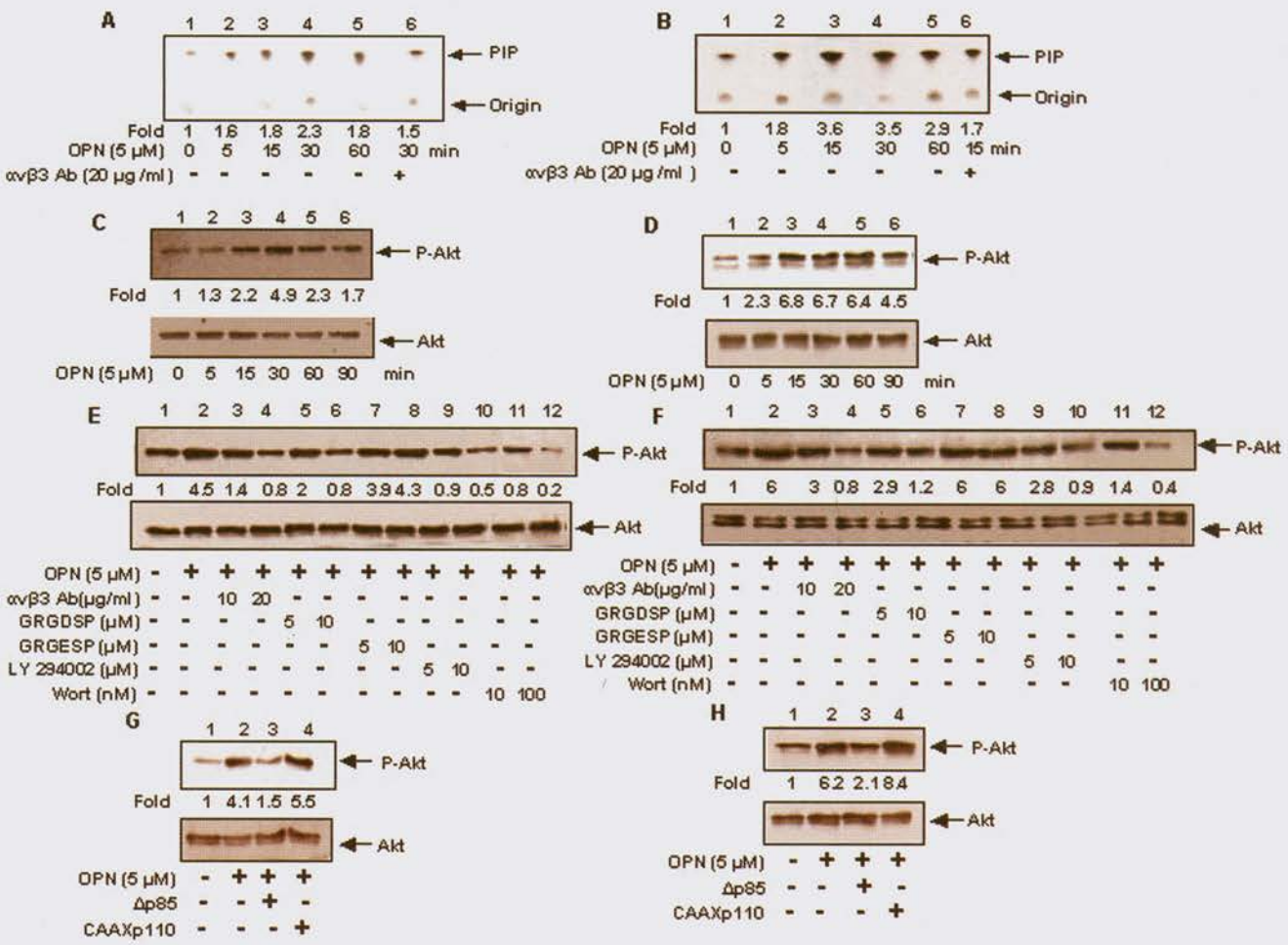

Fig. 1: **OPN induces PI 3′-kinase activity (A, B) and Akt phosphorylation (C-H) in MCF-7 and MDA-MB-231 cells. A and B**, PI 3′-kinase activity. Both MCF-7 (A) and MDA-MB-231(B) cells were treated with 5 μM OPN for 0-60 min or pretreated with -αvβ3 integrin antibody, then treated with OPN. Cell lysates were immunoprecipitated with mouse monoclonal anti-phosphotyrosine antibody, and the immunocomplexes were assayed for their ability to phosphorylated PI to PIP using [γ-^{32}p]ATP. The PIP was resolved by TLC and autoradiographed. **C and D**, MCF-7 (C) and MDA-MB-231 (D) cells were treated with 5 μM of OPN for 0-90 min; lysates containing equal amount of total proteins were analyzed by Western blot using anti-phospho Akt antibody. **E and F**, MCF-7 (E) and MDA-MB-231 (F) cells were individually pretreated with αvβ3 integrin antibody, GRGDSP, GRGESP, LY294002 and wortmannin and then treated with OPN. Cell lysates were analyzed by Western blot using anti-phospho or anti-non phospho Akt antibodies. **G and H**, MCF-7 (G) and MDA-MB-231 (H) cells were transiently transfected with a dominant negative p85 domain of PI 3′-kinase (Δp85) or activated form of p110 domain of PI 3′-kinase (p110CAAX) in presence of LipofectAMINE Plus and then treated with 5 μM OPN. The levels of phospho Akt and Akt in the cell lysates were detected by Western blot analysis.

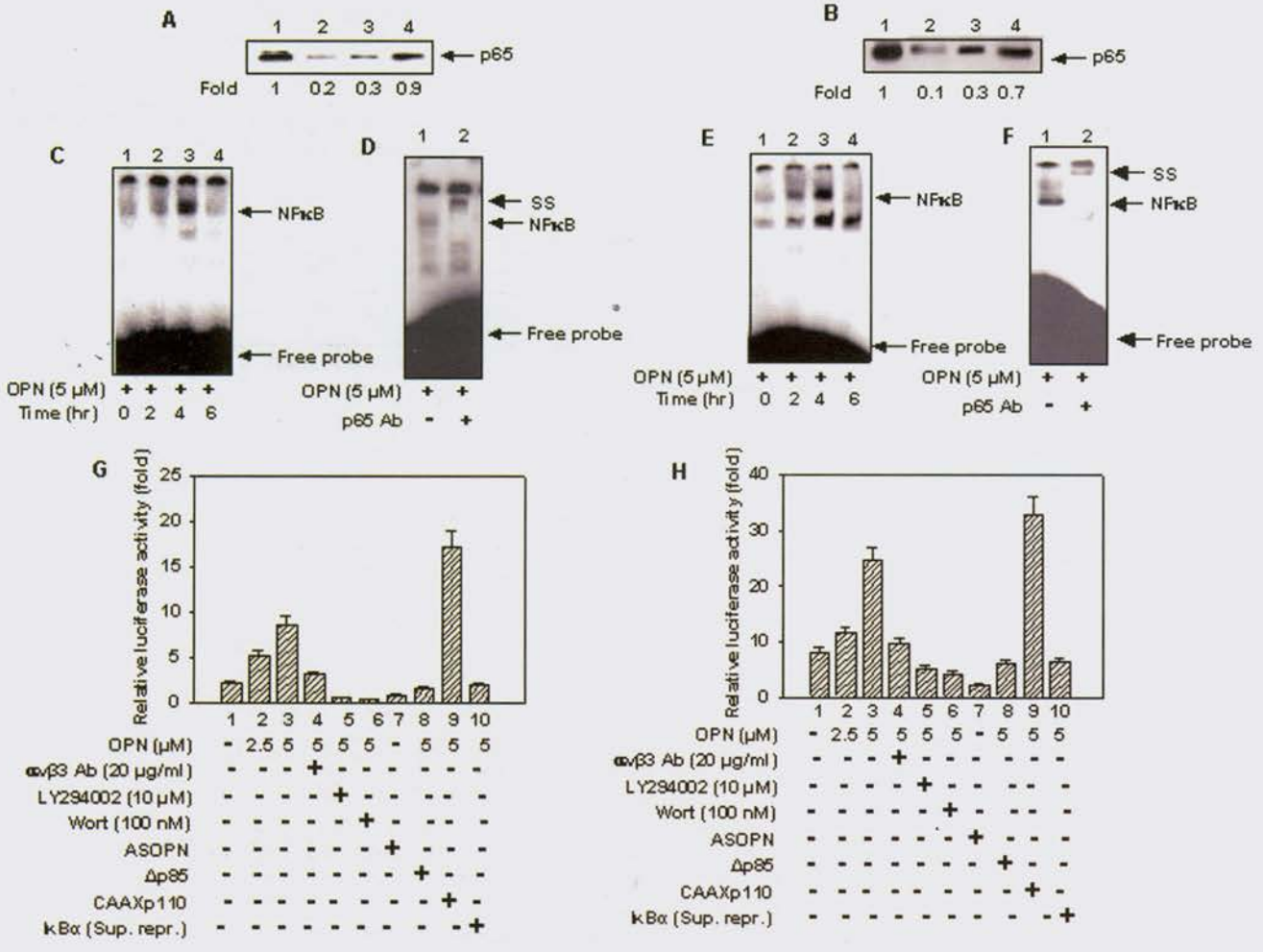

Fig. 2: OPN enhances nuclear accumulation of p65 subunit of NFκB, NFκB-DNA binding and NFκB activation. A and B, nuclear translocation of p65 into nucleus by Western blot analysis. Both MCF-7 (A) and MDA-MB-231 (B) cells were treated with or without OPN (5 μM) for 4 h. The nuclear and cytoplasmic extracts from untreated and treated cells were immunoblotted with rabbit polyclonal anti-p65 antibody. C and E, electrophoretic mobility shift assay. MCF-7 (C) and MDA-MB-231 (E) cells were treated with 5 μM OPN for 0-6 h. The nuclear extracts were prepared and analyzed by EMSA. D and F, supershift assay. The nuclear extracts from OPN treated MCF-7 (D) and MDA-MB-231 (F) cells were incubated with anti-p65 antibody and analyzed by EMSA. G and H, luciferase reporter gene assay. MCF-7 (G) and MDA-MB-231 (H) cells were transiently transfected with luciferase reporter construct (pNFκB-Luc) with LipofectAMINE Plus. Transfected cells were either stimulated with various doses of OPN (0-5 iM) for 6 h or pretreated with anti-αvβ3 integrin antibody or PI 3'-kinase inhibitor and then treated with OPN. In other experiments, cells were individually transfected with Δp85, p110CAAX, super-repressor form of IκBα, or ASOPN in presence of pNFκB-Luc and then treated with OPN. The cell lysates were used to measure the luciferase activity.

inhibited OPN-induced Akt phosphorylation, IKK activity and NF-κB activation through phosphorylation and degradation of IκBα. OPN also enhanced uPA secretion, cell motility and ECM-invasion. Furthermore, cells transfected with Δp85 or super-repressor form of IκBα suppressed the OPN-induced uPA secretion and cell motility whereas cells transfected with p110CAAX enhanced these effects. Pretreatment of cells with PI 3-

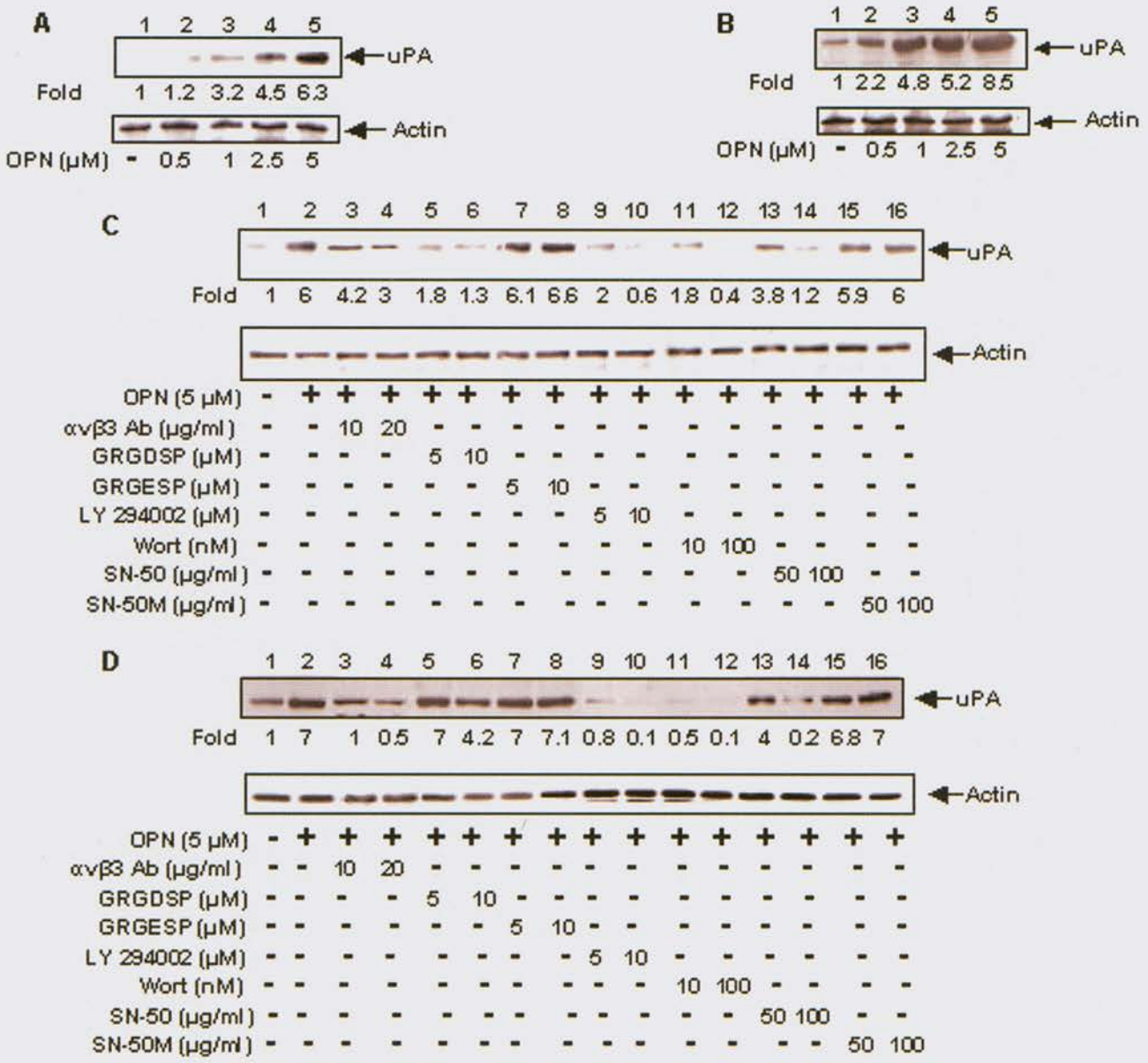

Fig. 3: **Effect of OPN on uPA secretion. A-D,** MCF-7 (A and C) and MDA-MB-231 (B and D) cells were treated with OPN (0-5 μM) for 24 h (A and B). Cells were also pretreated with anti-αvβ3 integrin antibody, GRGDSP or GRGESP peptide, PI 3'-kinase inhibitor NFκB inhibitory peptide, SN-50 or SN-50M and then treated with OPN for additional 24 h (C and D). The level of uPA in the lysates was detected by Western blot analysis using anti-uPA antibody. The same blots were reprobed with anti-actin antibody.

kinase inhibitors or NFκB inhibitory peptide (SN50) reduced the OPN-induced uPA secretion, cell motility and invasion. This is the first report that OPN induces NF-κB activity and uPA secretion by activating PI 3'-kinase/Akt/IKK-mediated signalling pathways and further demonstrates a functional molecular link between OPN induced PI 3'-kinase dependent Akt phosphorylation and NF-κB-mediated uPA secretion, and all of these ultimately control the motility of breast cancer cells.

Future Plan:

The molecular mechanism of OPN-induced NF-κB mediated uPA secretion and cell motility will be further delineated.

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Differential sensitivity of cell cycle phases to apoptosis induced by staurosporine in human neuroblastoma cell – SK-N-MC cells

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Abstract and Background:

One of the important features for an anticancer drug treatment to be effective is that the cancer cells must be sensitive to the effects of the drug, before resistance emerges. Cell cycle-dependent resistance is an emerging concept in combination sequential chemotherapy. Chemotherapeutic drugs induce damage at a number of different loci, and the balance between the pro-apoptotic and survival signals determines the cellular fate. Several tumor cells express proteins such as the members of the Bcl-2 family and the Inhibitor of apoptosis (IAP) family rendering them resistant to chemotherapy. Survivin, a 16.5 kDa protein is a bifunctional protein that suppresses apoptosis as well as regulates cell division. It is reported to be highly expressed in a number of tumor types especially neuroblastoma, colorectal carcinomas, gastric carcinomas and correlates with poor prognosis of the disease. Though survivin is specifically present in the G2/M cells, its role in protecting cell populations in this phase to chemotherapeutic agents has not been reported.

Aims:

1. To investigate the sensitivity of synchronized cell populations in G1 and G2/M phases of cell cycle to staurosporine (STS) induced apoptosis in neuroblastoma cell line.

2. The role of pro and antiapoptotic proteins and IAP in sensitivity or resistance to apoptosis.

Work achieved:

The neuroblastoma cells SK-N-MC were synchronized in G1 and G2/M phases of cell cycle using aphidicolin and nocodozole and the enrichment of specific population was assessed by cell cycle analysis using flowcytometry. As shown in Fig. 1A aphidicolin treated cells showed significantly higher percent of G1 cell population and nocodozole treated cells had higher G2/M population compared to unsynchronized population. On induction of apoptosis by STS, the G1 synchronized cells demonstrated a higher sensitivity ($p<0.01$) compared to G2/M and unsynchronized populations, evaluated by TUNEL (Fig. 1B) and cleavage of PARP (Fig. 1C). The expression of antiapoptotic proteins Bcl-2 and

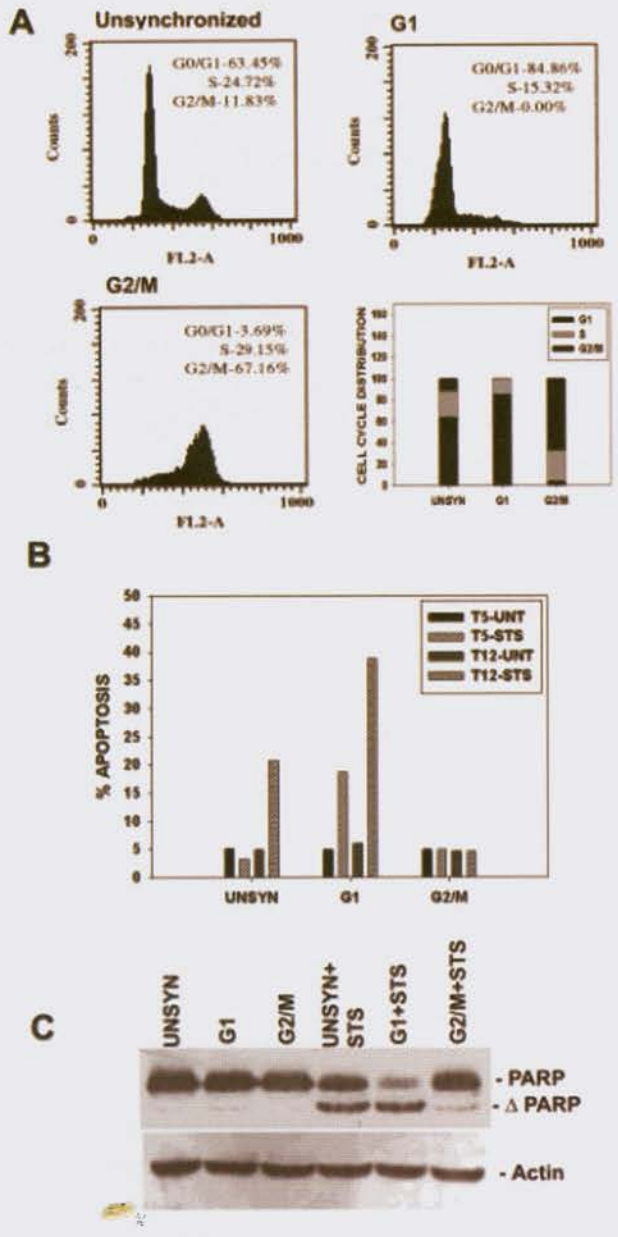

Fig. 1: A. DNA profiles of SK-N-MC cells synchronized in the G1 and G2/M phase with aphidicolin and nocodazole respectively. B. The selective sensitivity of the arrested cell populations to apoptosis induced by STS with 6 hr and 12 hr of treatment assessed by TUNEL assay. C. Cleavage of PARP in unsynchronized and synchronized cells treated with STS for 12 hr by western blotting.

Bcl-xl and survivin was studied western blotting. There was no significant difference in Bcl-2 and Bcl-xl in the control and STS treated cells and during different phases of cell cycle. However a remarkable difference in expression of survivin was observed. As compared to the unsynchronized cells, G1 arrested populations showed a 93.18% decrease in the survivin expressed whereas G2/M arrested cells showed a 52.75% increase in survivin expression that was sustained with STS treatment. Confocal laser scanning microscopy experiments demonstrated different staining patterns of surviving, with low expression and localization in nucleus in unsynchronised cultures and in G1 cells while it was distributed uniformly in G2/M cells and was colocalized with tubulin. (Fig. 2) The expression of cdc2, a protein important for phosphorylation of survivin was highly expressed in G2/M cells in comparison with G1 cells suggesting that survivin in G2/M might be important in protecting G2/M cells against apoptosis induced by STS.

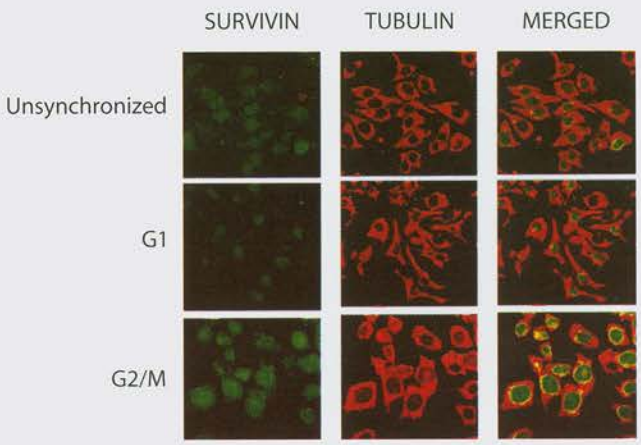

Fig. 2: Localization of antiapoptotic molecule survivin in untreated and STS treated synchronized cell populations by confocal laser-scanning microscopy. Cells were stained for survivin (green) and tubulin (red) and co-localization of survivin and tubulin is seen as yellow fluorescence in the merged images.

Down regulation of survivin in SK-N-MC cells, transfected with antisense survivin oligonucleotide showed the loss of protection of cells to apoptosis with a four-fold increase in sensitivity of cells to STS and cleavage of PARP within 3 h of STS treatment This data highlights the importance of survivin as a molecule that is mandatory for the survival of cells against insult caused by apoptotic insult. The expression of caspase 9, the key initiator caspase that is activated during mitochondrial dependent apoptosis was increased in unsynchronized and in the G1 arrested cells treated with STS. However no caspase 9 activity was detected in the G2/M arrested population with STS treatment. The data demonstrates the differential responses of the cell cycle phase populations and provide evidence for the role of survivin in conferring resistance G2/M cells to STS induced apoptosis.

Future work:

The precise role of survivin in and the mechanisms in conferring resistance to cells in context with caspases cascade will be studied in the STS induced apoptosis in neuroblastoma cells.

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Isolation and characterization of biologically active novel anti-cancer agents from *Momordica Charantia* Linn. fruit and evaluation of its anti-cancer efficacy/potency *in vitro* and *in vivo*

Tanuja Bankar *(Technician)*

Collaborators: Abhay Harsulkar, Vidya Gupta, *N.C.L. Pune.*

Abstract and background:

Potent anti-tumor action of *M. charantia* (commonly called bittergourd) fruit and seed extracts has been documented *in vivo* and on a variety of malignant cells *in vitro*. However, the active principle responsible to induce cell death has not been isolated and the mechanism of how it induces the cell death in a tumour cells is not clearly understood. Present studies are therefore being carried out considering the possibility of discovering a new anti-cancer drug. In this quest, we have isolated and purified a 4-5 kDa protein named BGPI3 from seeds of Momordica charantia Linn. fruit using sepharose 4B affinity column. The current study was conducted to investigate the efficacy of BGPI3 on murine melanoma cells B16F10 both *in vitro* and *in vivo*. Treatment of B16F10 mouse melanoma cells with 12 U of BGPI3 resulted in inhibition of cancer cell proliferation *in vitro* (90%). When B16F10 cells were transferred into C57BL/6 mice developed extensive metastases and all mice succumbed to tumour by day 30. Treatment of the mouse melanoma bearing C57BL/6 mice with BGPI3 at 15 U/injection EOD for 8 injections resulted in significant tumour regression in about 75% of the animals treated; however, tumours recurred in about 50% of these regressors after 30 days' remission. Mice injected with only BGPI3 did not reveal any toxicity. Thus, anti-tumour agent BGPI3 is effective against murine melanoma B16F10 *in vitro* and *in vivo*. This agent may therefore be a potential therapeutic use against melanomas.

Aims:

1. To ascertain the potential anti-cancer activity of crude *Momordica charantia* Linn. (Bittergourd) fruit towards the cancer cell line.

2. To isolate and purify biologically active chemical compounds responsible for their potential anti-cancer activity from seeds of *Momordica charantia* Linn. fruit.

3. To evaluate their biochemical mechanism and potential anti-cancer activity *in vitro* and *in vivo*.

Work achieved:

Potent anti-tumor action of *M. charantia* (commonly called bittergourd) fruit and seed extracts has been documented *in vivo* and on a variety of malignant cells *in vitro*. Our previous results have shown that crude seed extract of bittergourd inhibited proliferation of murine melanoma cells B16F10. Therefore we have isolated and purified a 4-5 kDa protein named BGPI3 from seeds of Momordica charantia Linn. fruit using sepharose 4B affinity column. Treatemnt of B16F10 cells with different concentrations of BGPI3 (0-12 U/ml) resulted in a dose-dependent decrease in the proliferation of B16F10 cells as seen by MTT assay and [^{3}H] thymidine incorporation. Transfer of B16F10 cells into C57BL/6 mice developed extensive metastases and all mice succumbed to tumor by day 30. Treatment of the mouse melanoma bearing C57BL/6 mice with BGPI3 at 15 U/injection EOD for 8 injections resulted in significant tumour regression in about 75% of the animals treated; however, tumours recurred in about 50% of these regressors after 30 days' remission. Mice injected with only BGPI3 did not reveal any toxicity. Thus, anti-tumour agent BGPI3 is effective against murine melanoma B16F10 *in vitro* and *in vivo*. This agent may therefore be a potential therapeutic use against melanomas (Fig. 1).

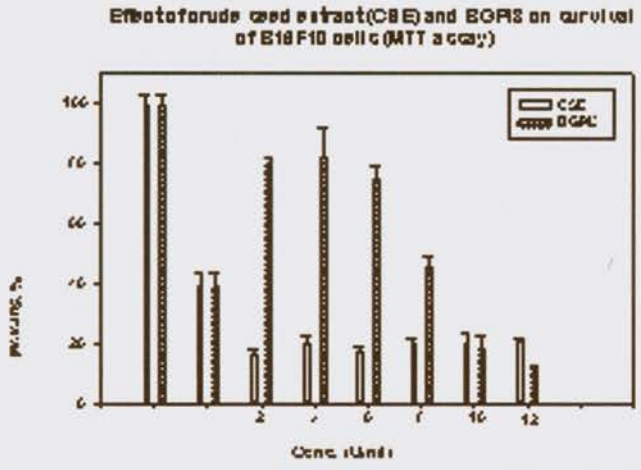

Fig. 1: Dose response relationship of crude seed extract (CSE) and BGPB induced cell death. BIGFI0 cells were treated with CSE and BGPB with indicated concentrations (0-12 Utml). After 24 h MIT assay was performed to evaluate cells viability that was expressed in terms of % survival. Error bars represent the means and standard errors of triplicate determinations of representative of three reproducible experiments.

Future work:

Study the biochemical mechanism of this protein and the signalling pathways.

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Investigation into mitochondrial dysfunstion, oxidative damage and apoptotic cell death stress mechanism as a critical pathway in endothelial and cardiomyocyte death induced by hyperglycemia/ hyperinsulinemia (diabetic cardiomyopathy)

Tanuja Bankar (*Technician*)

Abstract and background:

Diabetes causes various cardiovascular complications, which have become the major cause of morbidity and mortality in the diabetic population. Several studies have shown that hyperglycemia as an independent risk factor directly causes cardiac damage, leading to diabetic cardiomyopathy. Reactive oxygen species (ROS, *e.g.* superoxide, H_2O_2, hydroxyl radicals) have been proposed to participate in the induction of hyperglycemia induced myocardial apoptosis using STZ diabetic mice and rat cardiac myoblast H9c2. However, their source and mechanism of induction are unclear i.e. their source, where they are metabolized, and the relative contributions of different oxidant species to the induction of myocardial apoptosis remains to be studied. The purpose of our study is to investigate the role of mitochondrial ROS in the induction of hyperglycemia-induced myocardial apoptosis, and to clarify which ROS are required for the cell death response (signaling pathways) using STZ diabetic mice and H9c2 rat cardiac myoblast cells. Treatment of H9c2 cells with high glucose (33mM/L) induced apoptotic cell death as revelaed by TUNEL assay and PI staining, showed increased 2',7'-Dichlorofluorescein diacetate (DCF-DA) fluorescence indicative of H_2O_2 generation, increased dihydro-rhodamine 123 (DHR-123) fluorescence indicative of peroxynitrite generation, and increase in rhodamine 123 (Rh-123) fluorescence indicative of disruption of mitochondrial membrane potential. The DCF-DA fluorescence and the change in membrane potential was inhibititable by Catalase and GSH but not by SOD, thus confirming the role of ROS in high glucose-induced myocardial apoptosis. Further studies are being carried out using human endothelial cells and STZ diabetic mice.

Aims:

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

2. To clarify which ROS are required for the cell death response (signalling pathways) including their sources using STZ diabetic mice and H9c2 rat cardiac myoblasts.

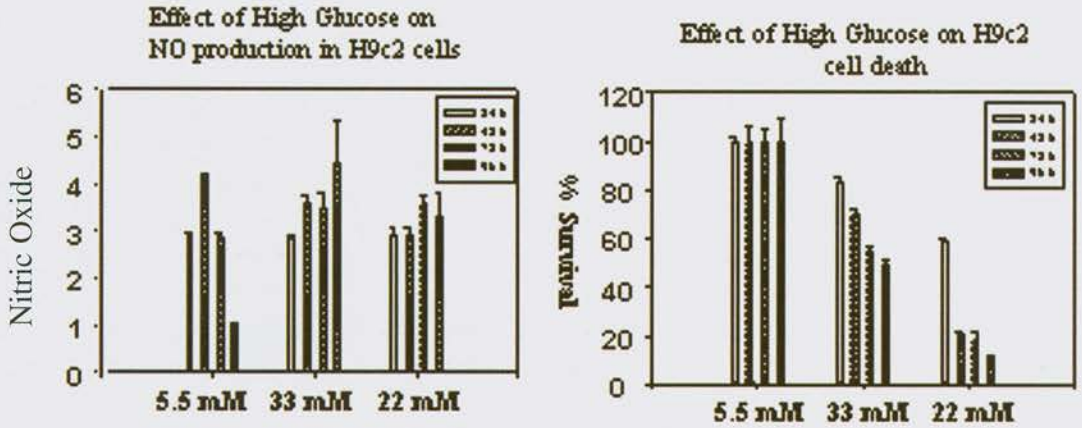


Fig. 1: Dose and time response relationship of glucose-induced cell death. H9c2 cells were treated with glucose with indicated concentrations and time above. After 34 h MIT assay was performed to evaluate cell viability that was expressed in terms of % survuval. Error bars represent the means and standard errors of triplicate determinations of a representative of three reproducible experiments.

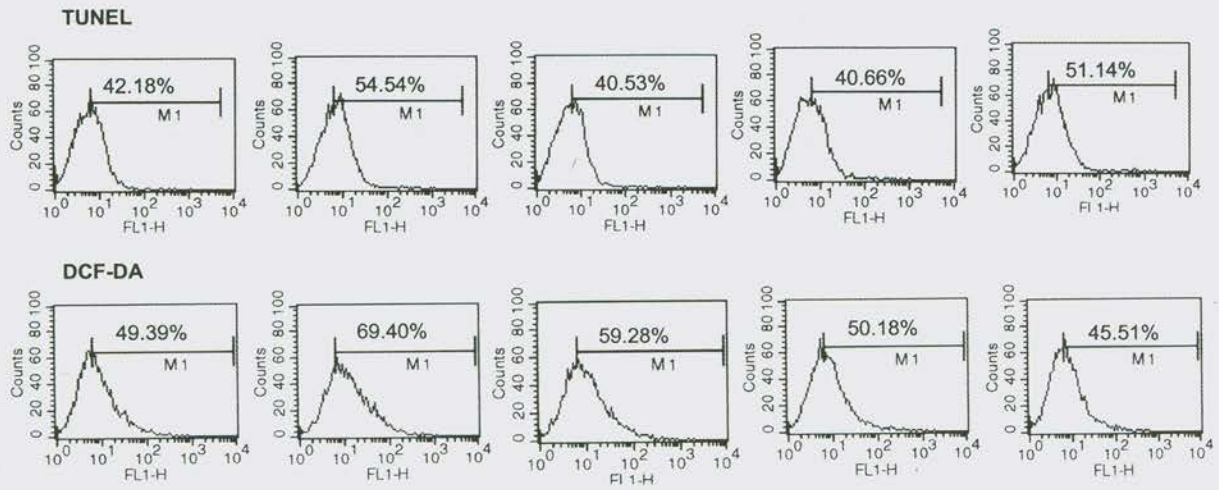


Fig. 2: Glucose-induced changes in H9c2 cells morphology. Shown are phase-contrast views of H9c2 cells cultured for 24 h in control (A), medium containing 33 μM Glucose (B), Glucose +100U SOD (C), Glucose +100U Catalase (D) and Glucose +100 μM GSH (E).

Work achieved:

In vitro studies were carried out using FACS analysis and confocal microscopy. Treatment of H9c2 cells with high glucose (33 mmol/l) for 24-96 h showed decrease in viability as measured by MTT assay and induced a significant increase in the number of TUNEL-positive cells as well as an increase in the number of condensed and fragmentized nuclei in the cells exposed to high levels of glucose as observed by PI fluorescence staining compared with a control (5.5 mmol/l glucose). Exposure of H9c2 cells to high glucose showed increased 2', 7'-Dichlorofluorescein diacetate (DCF-DA) fluorescence indicative of H_2O_2 generation, increased dihydrorhodamine 123 (DHR-123) fluorescence indicative of peroxynitrite generation, and increase in rhodamine 123 (Rh-123) fluorescence indicative of disruption of mitochondrial membrane potential. The DCF-DA fluorescence and the change in membrane potential was inhibititable by Catalase and GSH but not by SOD. All these results confirm the involvement and role of ROS in high glucose-induced myocardial apoptosis and ascertains the role of H_2O_2 as an initiator.

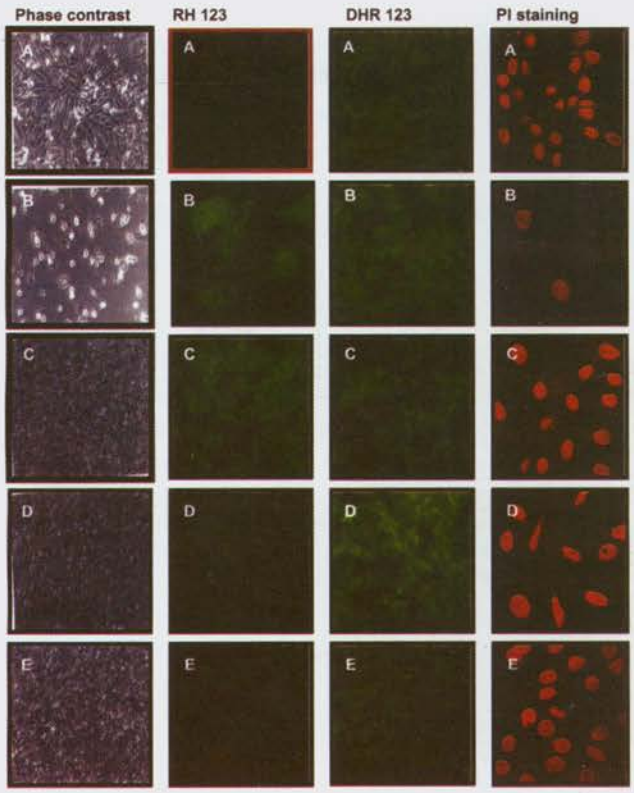
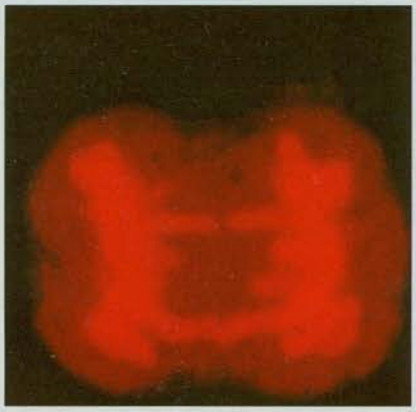


Fig. 3: Glucose-induced changes in H9c2 cell morphology. Shown are views of H9c2 cells cultured for 24 h in control (A), medium containing 33 mM Glucose (B), Glucose+100U SOD (C), Glucose+100U Catalase (D) and Glucose+100μM GSH (E)

Future work:

Further studies will be using human endothelial cells and using STZ diabetic mice.



Signal Transduction

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Caveolae and signal transduction

Kavitakumari, Vasudha Laxman, George Fernandes *(Technician)*

Collaborators: Dr. L.C. Padhy, *TIFR, Mumbai.*

Abstract and background:

Caveolae are 50-100 nm flask shaped vesicular invaginations of the plasma membrane found most abundantly in fibroblasts, endothelial cells, adipocytes and muscle cells. These membrane microdomains are rich in cholesterol, sphingolipids and a 21-24 kD protein, caveolin. Of the three iso-forms known to date the Cav1 and Cav2 are widely distributed while the expression of Cav3 is more restricted to muscle cells.

The main structural features of caveolin are:

a. A 41 amino-acid region that self-associates to form homo-oligomers which are the assembly units for the formation of caveloae.

b. A 20 amino acid long cytosolic, membrane proximal "scaffoding domain" which binds several important signaling molecules in their 'less active' state and sequester them to caveolae.

These molecules include Ha-ras, Src family of tyrosine kinase, eNOS, PKC isoforms, EGF-R, heterotrimeric G proteins etc. Hence the caveolae are also called as "centers for signal transduction."

Caveolins, the primary coat proteins of plasmalemmal caveolae, are implicated in cholesterol trafficking, signal transduction and tumor suppression. Their genes are localized to the suspected tumor suppressor locus, 7q31 and are down regulated in cancers like leukemias, adenocarcinomas, etc,.

Caveolin-1 dysfunction may have important bearings on the genesis of or deciding on the nature of mammary cancers. This is supported by the observations that the loss of caveolin-1 function in homozygous null mouse leads to mammary hyperplasia at a young age and a dominant mutation, P132L is found frequently in a form of aggressive human breast cancers. We therefore propose to examine the role of Caveolin expression in breast cancer cells using both cell line models as well as archived breast carcinoma tissue sections.

Aim:

To study the regulation of Caveolin expression in tumor cells and its role in the tumorigenesis.

Work achieved:

1. Assessment of Caveolin expression in breast tumor sections:

Our earlier data indicated that estrogen receptor activity could negatively regulate the Caveolin 1 expression we examined the Caveolin 1 expression in some known ER positive and ER negative breast tumor tissue sections by immuno fluorescence. The sections were deparafinized and then processed for immuno fluorescence staining using anti Caveolin1 antibody. Caveolin positivity was seen in majority of ER negative sections (12/18 positive; 67% positive) while less number of ER positive cases were Caveolin 1 positive. (5/16 positive; 30% positive). We propose to expand these data using more number of cases to reach conclusive results.

2. Development of MCF 7 cell line with dominant negative ER:

In order to establish the regulation of Caveolin expression by ER activity we have initiated the development of MCF 7 cell line using an expression plasmid having dominant negative ER (N terminal truncation). The vector expresses a fusion protein of DNER with GFP making it easy to visualize the transfectants. MCF 7 cells were transfected with the plasmid vector using Lipofectin(GIBCO BRL) and the cells were transferred to 24 well plates in a medium containing G418 . The clones were monitored for GFP expression using inverted fluorescence microscope. We have isolated several independent GFP positive clones and we will study them further when stable transfection is achieved.

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How does α-hemolysin dephosphorylates via a Protein Tyrosine Phosphatase?

Dr. Nandula S. Rao, Ravi Vijayavargia, Anag Sahasrabuddhe, Singdha Tripathi, Satyabratha Pany, Anil Lotke *(Technician)*

Abstract:

An in depth study of effect of α-HL on EGFr bearing cells is an absolute must as the EGFr serves as a current paradigm of receptor tyrosine kinases and signal transduction initiated by it. Aberrant expression of EGFr has been lined to cancer in humans. In addition, the identity of receptor-attenuating molecules for most receptor tyrosine kinases is still not known. Hence, the information on molecules that associate or work (or supposed to work) in synergy with EGFr are of tremendous importance for various reasons *viz.* (i) to know their role in signal transduction (discovery of even new pathways) and cell proliferation (ii) to serve as starting steps for rational design of new generation molecules. In addition, considering the involvement of *Staphylococcus aureus* in pathogenesis in humans, It is highly desirable to gain an in depth knowledge about the mechanism of action of α-HL on dermal cells at molecular level. Such a deeper knowledge on the function of α-HL on dermal cells will help in the design of new generation molecules for therapeutic purposes.

Aims:

1. Which phosphatase is participating in the dephosphorylation of EGFr?
2. Is that phosphatase membrane or a cytoplasmic phosphatase.
3. If phosphatase activation is positive, how such activation is taking place?

Work achieved:

Our earlier observations have comprehensively suggested that upon α-HL treatment of A431 cells, all signals including down stream targets were completely withdrawn. Hence, we exclusively focused on the identification of the PTPase involved in the process. It is highly relevant to mention here that in the past several authors have attempted to identify endogenous PTPase by all feasible means but could not succeed for the following reasons: (i) extremely low endogenous concentrations of the PTPases in any cell type. (ii) going by the Kcat values calculated, it is believed that only a few thousand molecules may be present in the cell. We have designed our strategy by keeping limitations in mind.

In order to identify the PTPase involved in the process, we attempted immunoprecipitation with anti-EGFr antibody of untreated, TGFα stimulated and α-HL treated cells. Although, several authors have attempted to identify the endogenous PTPase by immunoprecipitation in the past, the attempts were not successful probably due to the low endogenous concentrations coupled with poor transfer efficiencies of high molecular weight glycosylated proteins. In order to overcome these difficulties, we employed dot blot detection by both HRP labelled primary antibodies (to increases the sensitivity level) as well as highly sensitive HRP labelled secondary antibodies. By using this procedure we identified the phosphatase responsible for this irreversible dephosphorylation as - the receptor like protein tyrosine phosphatase σ (Fig. 1).

We clearly see an unambiguous signal with both anti-rPTPσ-N and anti-rPTPσ-C antibodies (specific for intact amino and carboxyl termini respectively). Under identical conditions either the HRP conjugated secondary antibody alone (in the absence of primary antibody) or the primary antibody in the presence of the peptide used for its generation gave no signal at all. In addition, the anti-rPTPσ-N antibody specifically recognized the cloned 45 kDa fragment of rPTPσ which consists of intact amino terminus and the first fibronectin domain of rPTPσ. All these observations authenticate our identification. Interestingly enough, we did not see any signal with anti-PTP1B (known to be present in the endoplasmic reticulum) as well as anti-PTPβ antibodies. To the best of our knowledge, this is the first instance where the endogenous rPTPσ has been shown to be responsible for the dephosphorylation of EGFr and its downstream recipients under a well-defined situation. In addition, the total phosphatase activity observed in the immunoprecipitates obtained with anti-EGFr is at least two fold higher in α-HL treated cells when compared to both untreated and TGFα stimulated cells. We, in fact, anticipated the presence of PTP1B in place of rPTPσ because the former was shown to be strongly associated with the EGFr. Recent data on PTP1B employing confocal microscopy has once again shown that it is predominantly present in the cytoplasmic side of endoplasmic reticulum. Moreover, catalytically inactive mutant of PTP1B (PTP1B$^{D/A}$ which carries a D to A mutation) was found to complex with EGFr only after its internalisation. Our data shows the absence of PTP1B suggesting the absence of EGFr internalisation or the present situation does not warrant the corrective action by PTP1B. It is interesting to note that TGFα stimulated cells exhibited lower PTPase activity which is in excellent agreement with the observation that growth factor stimulation reduces the phosphatase activity due to the production of intracellular H_2O_2 that reversibly inactivates the PTPases *in vivo*. The striking feature of our data is that the dephosphorylation brought out by rPTPσ (induced by α-HL) is irreversible as the TGFα is present throughout the

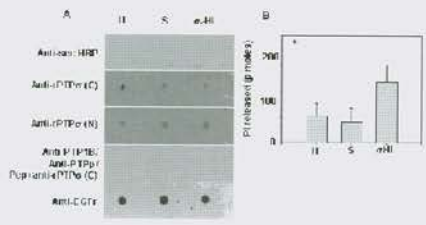

Fig. 1: Detection of PTPases: The IP after mock (**U**), TGFα (**S**) and α-HL (**α-HL**) treatment of A431 was carried out as described. The same blot was stripped and reprobed with indicated antibodies. The anti-EGFr panel confirms the equal loading as determined by densitometric scanning. Note that the antibody concentrations, blocking, probing and exposure to the film and film developing conditions were same in all the cases. The data shown here was one of the five independent experiments. (B) Tyrosine Phosphatase assay of the immunoprecipitates (IP): The tyrosine phosphatase assay was carried out with the IP samples using a non-radioactive tyrosine phosphatase assay system. The phosphate released (p moles) by the samples was calculated from the standard curve. The U, S and α-HL respectively represent the IPs obtained by mock, TGFα and α-HL treatments. The data represents the average of 3 independent immunoprecipitation experiments and student test value $p < 0.05$.

experiment, providing a constant stimulation. In addition, the PTPase targets its substrates even in the presence of potent inhibitors like, Na_3VO_4, H_2O_2 and Iodoacetamide. Together these observations support our interpretations further. Thus, the molecular ensemble formed by α-HL on the cell surface has resulted in the activation of PTPase in an irreversible manner such that it cannot be turned 'off' by any of the means available so far.

Future work:

We wish to devote our efforts to identify all the interactions that occur at molecular level between α-HL and target membrane to elucidate the mechanism of activation of protein tyrosine phosphatase.

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Role of Syk, a protein tyrosine kinase in suppression of cell motility and NF-κB-mediated uPA secretion by inhibiting tyrosine phosphorylation of IκBα in breast cancer cells

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Background and Abstract:

Tumor growth and metastasis are multifaceted processes that mainly involve cell adhesion, proteolytic degradation of extracellular matrix, and cell migration. Syk is a member of a tyrosine kinase family that is expressed mostly in haematopoitic cells. Syk is expressed in the cell lines of epithelial origin, but its function in these cells remains unknown. Here we report that Syk is expressed in MCF-7 cells but not in MDA-MB-231 cells. The overexpression of wild type Syk kinase but not the kinase negative Syk ($SykK^-$) suppresses the cell motility and inhibits the activation of PI 3'-kinase in MDA-MB-231 cells. In contrast, Syk specific antisense S-oligonucleotide (ASSyk) but not the sense S-oligonucleotide (SSyk) when transfected to the MCF-7 cells, the level of PI 3'-kinase activity as well as cell motility were increased. Syk also suppresses the NF-κB transcriptional activation and interaction between IκBα and PI 3'-kinase by inhibiting the tyrosine phosphorylation of IκBα. Syk, PI 3'-kinase inhibitors and NF-κB inhibitory peptide (SN50) inhibits the uPA secretion and cell motility in these cells. These data demonstrate that Syk suppresses the cell motility and inhibits the PI 3'-kinase activity and uPA secretion by blocking the NF-κB activity through tyrosine phosphorylation of IκBα.

Aims:

1. To examine the effect of Syk, a protein tyrosine kinase on PI 3'-kinase dependent activation of NF-κB through tyrosine phosphorylation of IκBα in low (MCF-7) and highly (MDA-MB-231) invasive breast cancer cells.

2. To investigate the role of Syk in cell motility and uPA secretion in these cells.

3. To establish a functional correlation between Syk regulated PI 3'-kinase activity and NF-κB-mediated uPA secretion in breast cancer cells.

Work achieved:

We have shown that Syk is expressed in low invasive breast cancer (MCF-7) cells but not in highly invasive (MDA-MB-231) cells. The overexpression of wild type Syk kinase but not the kinase negative Syk ($SykK^-$) suppressed the cell

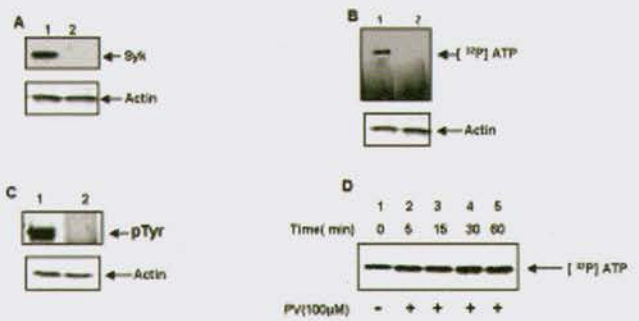


Fig. 1: Expression and autophosphorylation of Syk in breast cancer cells. A, equal amount of total proteins in cells lysates from MCF-7 and MDA-MB-231 cells were resolved by SDS-gel and analyzed by Western blot analysis using rabbit polyclonal anti-Syk antibody. Lane 1: MCF-7 cells and Lane 2: MDA-MB-231 cells. B, equal amount of total proteins in both cells lysates were immunoprecipitated with rabbit polyclonal anti-Syk antibody, and the immunoprecipitates were incubated with 2 μCi of [γ32p] ATP in kinase assay buffer according to the "Experimental Procedures". The sample was resolved by SDS-PAGE and autoradiographed. Lane 1: MCF-7 cells and Lane 2: MDA-MB-231 cells. C, cell lysates containing equal amount of total proteins were immunoprecipitated with anti-Syk antibody, the immunocomplex was resolved by SDS-PAGE and analyzed by Western blot analysis using rabbit anti-phosphotyrosine antibody. Lane 1: MCF-7 cells and Lane 2: MDA-MB-231 cells. The arrow indicates the Syk specific band. As loading controls, all these blots were reprobed with goat polyclonal anti-actin antibody (lower panels A-C). D, effect of pV on autophosphorylation activity of Syk in MCF-7 cells. The cells were treated with 250 mM pV for 0-60 min and the cells lysates containing equal amount of total proteins were immunoprecipitated with anti-Syk antibody. The immunoprecipitates were incubated with 2 μCi of [γ32p] ATP in kinase assay buffer as described above. The sample was resolved by SDS-PAGE and autoradiographed. Lane 1: control; Lane 2: pV with 5 min; Lane 3: pV with 15 min; Lane 4: pV with 30 min and Lane 5: pV with 60 min.

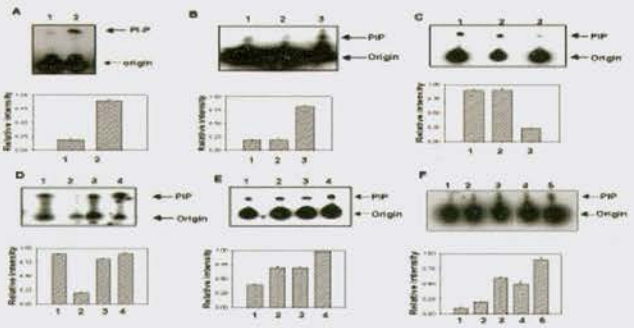


Fig. 2: P13'-kinase activity. A, equal amount of total proteins from the lysates of MCF-7 and MDA-MB-231 cells were immunoprecipitated with mouse monoclonal anti-p85α antibody and the immunocomplexes were assayed for their ability to phosphorylate phosphatidylinositol to phosphatidylinositol phosphate (PIP) using [γ32p] ATP at 300 C for 10 min. The PIP was resolved by Thin Layer Chromatography (TLC) and autoradiographed. Lane 1: MCF-7 cells and Lane 2: MDA-MB-231 cells. B, the serum starved MCF-7 cells were transfected with Syk specific S-oligonucleotides and then P13'-kinase activity was measured as described under "Experimental Procedures". Lane 1: with Lipofect AMINE Plus; Lane 2: with SSyk and Lane 3: with ASSyk. C, MDA-MB-231 cells were transfected with Syk cDNA and used for P13'-kinase assay. Lane 1: with Lipofect AMINE Plus; Lane 2: with SykK- and Lane 3: with wide type Syk. D, MDA-MB-231 cells were transfected wtih wild type Syk cDNA followed by treatment with increasing concentrations of piceatannol (D0-10 mM) at 37°C for 30 min and used for P13'-kinase assay. Lane 1: with Lipofect AMINE Plus alone; Lanes 2-4: cells were transfected with wild type Syk cDNA and then treated with increasing concentrations of piceatannol. Lane 2: without piceatannol; Lane 3: with 5 mM piceatannol and Lane 4: with 10 mM piceatannol. E, MDA-MB-231 cells were treated with 250 μM pV at room temperature for 0-20 min and the cell lysates were used for P13'-kinase assay. Lane 1: untreated cells; Lane 2: with pV for 5 min; Lane 3: with pV for 15 min and Lane 4: with PV for 30 min. F, MCF-7 cells were treated with piceatannol (0-10 mM) for 30 min or with PV (250 mM) for 0-30 min and used for P13'kinase assay. Lane 1: untreated cells; Lane 2: with 5 mM piceatannol; Lane 3: with 10 mM piceatannol; Lane 4: with pV for 15 min and Lane 5: with pV for 30 min. In all the cases, the upper arrows indicate the phosphatidylinositol phosphate (PIP) specific bands. All these bands in panels A-F were quantified by desitometric analysis and are represented in the form of a bar graph. The mean value of triplicate experiments is indicated.

motility and inhibited the activation of PI 3'-kinase in MDA-MB-231 cells. In contrast, Syk specific antisense S-oligonucleotide (ASSyk) but not the sense S-oligonucleotide (SSyk) when transfected to the MCF-7 cells, the level of PI 3'-kinase activity as well as cell motility were increased. The MDA-MB-231 cells transfected with wild type Syk cDNA followed by treatment with piceatannol, a Syk inhibitor, enhanced the cell motility and PI 3'-kinase activity. Pervanadate (pV), a phosphotyrosine phosphatase inhibitor induced the PI 3'-kinase activity and stimulated the interaction between IκBα and p85α domain of PI 3'-kinase through tyrosine phosphorylation of IκBα which ultimately resulted in NF-κB activation. pV had no effect on activation of Syk in these cells. However, Syk suppressed the NF-κB transcriptional activation and

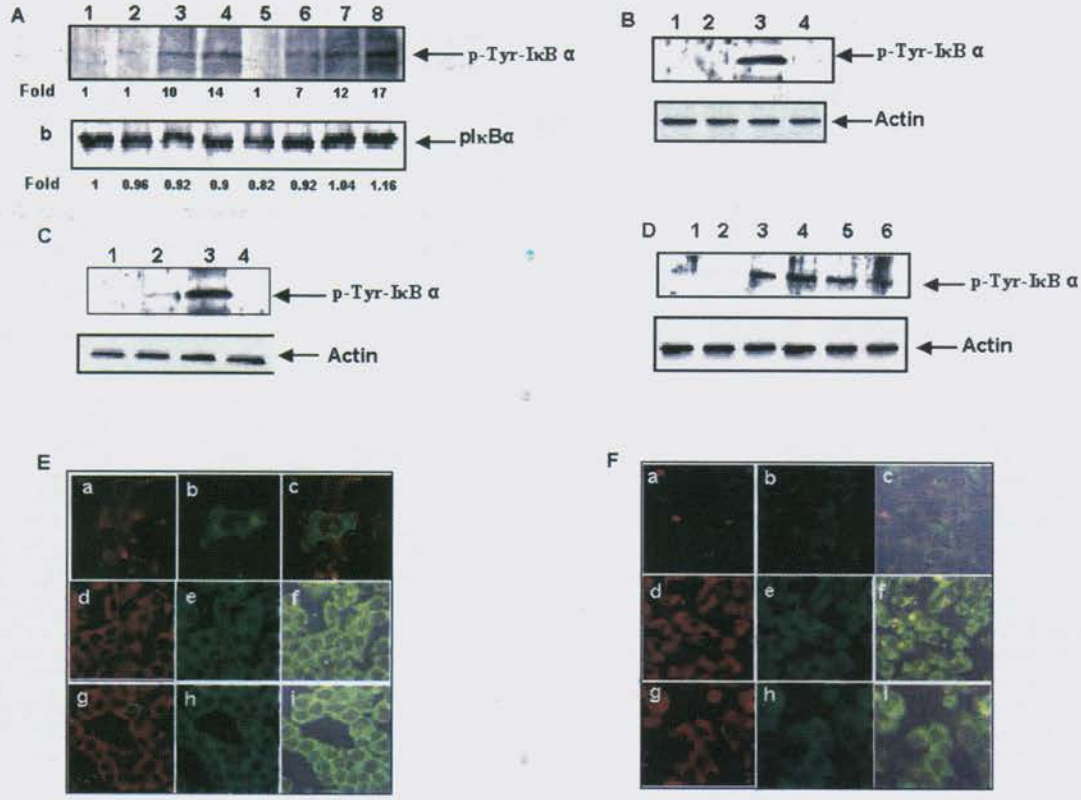
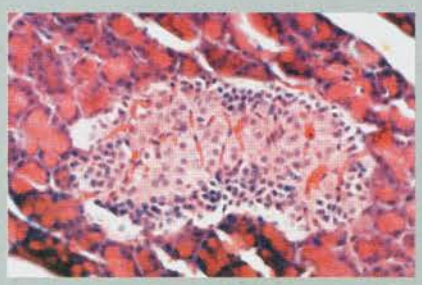


Fig. 3(A-F): Effects of pV on stimulation of interaction between phosphorylated IkBa and p85 domain of PI-3 kinase

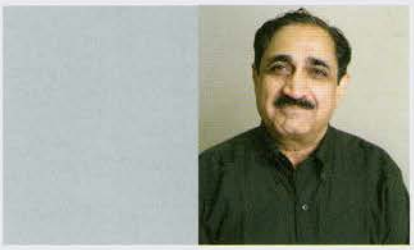
interaction between IκBα and PI 3′-kinase by inhibiting the tyrosine phosphorylation of IκBα. Syk, PI 3′-kinase inhibitors and NF-κB inhibitory peptide (SN50) inhibited the uPA secretion and cell motility in these cells. To our knowledge, this is the first report that Syk suppresses the cell motility and inhibits the PI 3′-kinase activity and uPA secretion by blocking the NF-κB activity through tyrosine phosphorylation of IκBα. These data further demonstrate a functional molecular link between Syk regulated PI 3′-kinase activity and NF-κB mediated uPA secretion, all of these ultimately control the motility of breast cancer cells.

Future work:

The molecular mechanisms of Syk regulated cell motility and PI 3′-kinase dependent NF-κB-mediated uPA secretion in breast cancer cells will be further studied.



Diabetes



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Regeneration of pancreatic β cells and reversal of experimental diabetes

Meenal Banerjee, Sameet Mehta, Ruchi Shukla, Meghana Kanitkar, Ravi Shukla, Malati Umrani *(Technician)*

Collaborators: Sanjeev Galande, P.B. Parab, Subhash Padhye *(University of Pune)*
Visiting fellow: Savita Datar

Background:

The number of functionally intact beta cells in the islet organ is of decisive importance in the development, course and outcome of diabetes mellitus. Elucidation of the regenerative potential in experimentally induced diabetes is of interest as an alternative therapy for diabetes. The pathogenesis of diabetes has been viewed as a balance between the destructive and regenerative forces. The therapeutic goal would be then to down regulate the destructive process and/or up-regulate the regenerative process. The general approach of regenerative biology is to recreate an embryonic environment in a malfunctioning tissue. Hence it is important to define growth factors responsible for proliferation of the islets and differentiation of islet stem cells. Such studies would enhance our understanding of the pathophysiology of the diabetic pancreas and perhaps offer a novel approach to the cure of diabetes. Equally important is the protection of islets from oxidative stress and the cytoprotective mechanisms by which this can be accomplished, both *in vivo* and *in vitro*.

Aims:

1. To find out factors affecting growth of beta cells *in vivo* and *in vitro*.
2. To examine the cytoprotective action of common food supplements.
3. To examine the potential of intra and extra pancreatic tissue for islet neogenesis.
4. To search for alterative sources of insulin and islets for therapeutic purposes.

Work Achieved:

1. **Isolation, identification and characterization of the factors from islet culture supernatant (ICS) responsible for pancreatic regeneration in vivo.**

Last year we reported two molecules from ICS having potential to induce pancreatic regeneration in experimental diabetic mice. These molecules were found to be STI and Fetuin. During current year we have studied the role of STI

and Fetuin alone or in combination for its regenerating potential. Dose Response and time course was studied for STI, Fetuin, alone and in combination. It was observed that STI (80 µg/200 µl) and Fetuin (40 µg/200 µl) alone or in combination (80 µg + 40 µg/200 µl) was able to reduce hyperglycemia, however complete reversal of experimental diabetes was not seen. On the contrary, ICS (w/o STI) injection alone led to reversal of experimental Diabetes in 60% of the cases and stabilized euglycemia for the next one to two months without relapse. A similar experiment was carried out using Rat ICS w/o STI. No reversal was observed, although limited decrease in hyperglycemia was noted suggesting that the reversing factors present in ICS are species specific. Fetuin (600 µg/ml/5 days/mouse) was injected IP and serum samples were analyzed for the amount of insulin present by ELISA. The insulin levels were indicative of insulin resistance, which was subsequently confirmed by IPGTT, HOMA-IR and QUICKI and % beta cell activity indicating insulin resistance without overt diabetes. The data obtained was comparable to IR cases in humans, thus opening plausibility for developing a novel *in vivo* model for IR without diabetes.

Future Work:

Further characterization of the factors responsible for pancreatic regeneration and reversal of diabetes will be undertaken. Efforts will be made to develop a novel animal model for Insulin Resistance.

2. Induction of islet neogenesis in vitro.

Last year we demonstrated the potential of intra-islet precursor cells for islet neogenesis. This was further developed as an in vitro model for screening agents inducing islet neogenesis as well as for depicting in vivo differentiation of islets. Employing this model we have studied the potential of commonly consumed nutrients for their nesidioblastotic activity. Here we show for the first time the potential of trace elements like vanadium to induce islet neogenesis in vitro without supplementing any other growth factor. The presence of new islets was confirmed by DTZ staining.

Studies on intra-islet precursor cells were further extended to include diabetic pancreas. Here we report for the first time that the islets from the pancreas of drug-induced diabetic mice retain the capacity to give rise to neoislets when stimulated appropriately. This has opened up an alternative therapeutic approach for the better management of diabetes. The neoislets obtained from

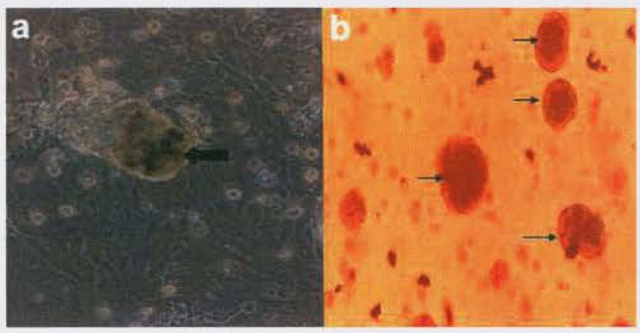


Fig. 1: In response to the growth factor KGF, the intra islet precursor from the epithelial cell sheet starts differentiating into islet like clusters which primarily form an outgrowth-like structure within the cell sheet as shown by the arrow (Figure a). The islet bud turns into a well-defined islet in another 4 days under the influence of KGF. Specificity of the neogenerated islets of varying size were examined by islet specific stain DTZ; neogenerated islets appeared crimson red in colour (islets shown by arrow marks) after DTZ staining.

intra-islet precursor cells of diabetic pancreas were subjected to morphological and physiological characterization. The secretagogue response of the neoislets to L-Arginine depicts their fetal ontogeny. Although the intra-islet precursor cells in a diabetic pancreas are protected from STZ-induced destruction the in vitro exposure of islets to STZ leads to loss of intra-islet precursor cells.

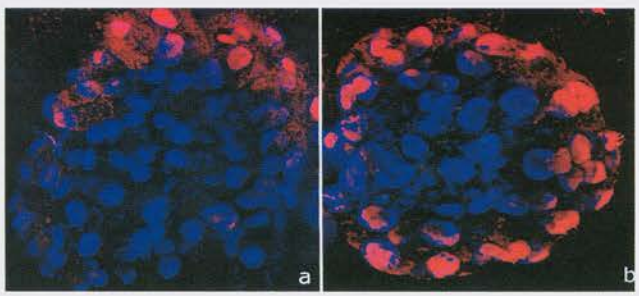


Fig. 2: Confocal images of experimental (a) and control (b) islets depicting population of live and dead cells. Blue color indicates live cells stained with Hoechst 33342, while red color indicates dead cells stained with ethidium bromide.

Future Work:

It is planned to screen commonly consumed nutrients and minerals for their potential to induce islet neogenesis.

3. Screening of food supplements for their potential cytoprotective activity.

Curcumin, an active principle of *Curcuma longa* has been traditionally used in Indian medicine as an antiseptic, anti-inflammatory compound. It is also known to reduce secondary complications in diabetes, especially renal legions and wound healing. We were interested in testing the hypothesis that curcumin may exert a cytoprotective action on islets of langerhans thus ameliorating the diabetic complications. Therefore, we treated the isolated islets with different concentrations of curcumin for varied length of time and then subjected to STZ (diabetogenic compound) challenge. Viability was tested by using Trypan Blue dye exclusion test and confocal laser microscopy using Hoechst and EtBr staining. It was observed that curcumin (10 μM) pretreatment for 24 hours is able to protect the islets from STZ insult (Fig. 1). *In vitro* functionality was tested by stimulating the control and curcumin pretreated islets with two different glucose concentrations and calculating the Stimulation Index. It was observed that curcumin pre treated control and experimental islets secreted more insulin than their curcumin untreated control and diabetic counterparts indicating the islet protective property of curcumin. Encouraged by our *in vitro* data we then extended our studies to examine effect of curcumin treatment on progression /prevention of low dose STZ induced diabetes in C57 /BL6 mice. It was observed that the curcumin-pretreated mice did not become diabetic, while the curcumin untreated mice showed signs of overt diabetes as evidenced by the data from Plasma Glucose estimation, change in body weight, IPGTT, pancreatic and serum insulin levels. These experiments suggest the islet protective role of curcumin against STZ insult.

Future work:

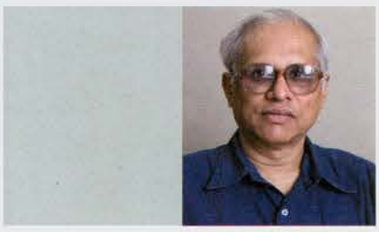
It is planned to test the potential of commonly consumed condiments for their islet protective activity and to investigate the mechanism of cytoprotection. The study would provide valuable information for better control and management of diabetes.

4. Investigation on alternative sources of islets and insulin.

It is claimed that the insulin has been conserved through evolution. Transplantation of the Brockman bodies (found in the teleost fishes) have been shown to reverse experimental diabetes in mice. Moreover the recent finding of novel source of insulin from adipocytes of Common Indian Carp (*katla katla*) having hypoglycemic activity have stimulated the search for alternative sources of insulin. Since Aves and Mammals stem from a common ancestor in the vertebrate evolution we undertook studies on isolation of islets from chick pancreas and to test the chick insulin for its efficacy and activity in mammalian system. Preliminary studies on intraperitoeal injection of chick islets into STZ induced diabetic mice indicated restoration of euglycemia indicating possibility of using chick islets as a source for Xeno- transplantation without immunosuppression. Moreover the chick insulin cross-reacted with human antibody against insulin as revealed by ELISA. Isolated islets of chick release copious amount of insulin *in vitro* compared to mouse islets.

Future Work:

The number of islets to be transplanted, and their site of transplantation will be standardized for its commercial exploitation in mammalian system.



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Immune reactions in type-1 diabetes

Varshiesh Raina, Sunita Koul, Mamata Katdare (*Technician*)

Abstract and Background:

Type-1 diabetes or insulin dependent diabetes is an autoimmune disease that culminates in the destruction of insulin producing beta cells in the islets of pancreas. In humans or in animal models of the type-1 diabetes, intervention of the immunotoxic response can be effected by tolerance or by administration of beta cell specific autoantigens such as insulin and glutamic acid decarboxylase (GAD65) either during the prediabetic stage or at the manifestation of disease. The other way to prevent overt diabetic condition in type-1 diabetes is to increase the biomass of insulin producing beta cells either through islet transplantation or by stimulating new pancreatic islet regeneration.

Aims:

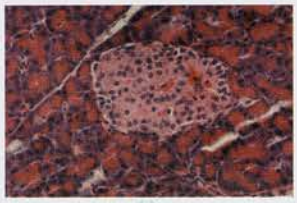
1. In vitro generation of islets from pancreatic ductal epithelial cells.
2. Identification and characterization of autoantigens in rat model using FDNB tagged RIN cells.
3. Development of monoclonal antibodies to FDNB tagged RIN cells.

Work achieved

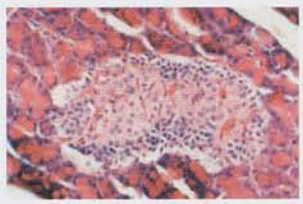
1. In vitro regeneration of islets from pancreatic ductal epithelial cells

A methodology has been developed for regeneration of islets from pancreatic ductal cells. Functional characteristics of these neoislets were assessed by glucose challenge experiments and immunoblots with antiinsulin. These islets when injected into diabetic animals intraperitoneally were found to reduce glucose levels for up to seven days. After seven days again glucose levels showed elevation in blood glucose levels. Emergence of two different populations of islets was noticed in the ductal cultures. Small sized islets respond to L-arginine stimulation depicting their fetal ontogeny whereas large sized islets respond to glucose stimulation depicting their mature nature. Neo islets were cryopreserved in liquid nitrogen for 6 months. These islets were revived after 6 months. The comparison of insulin secretory response of newly generated and freshly isolated islets from normal adult pancreas before and

after cryopreservation revealed similarity to glucose challenge. However 20% reduction in viability was observed in cryopreserved islets.



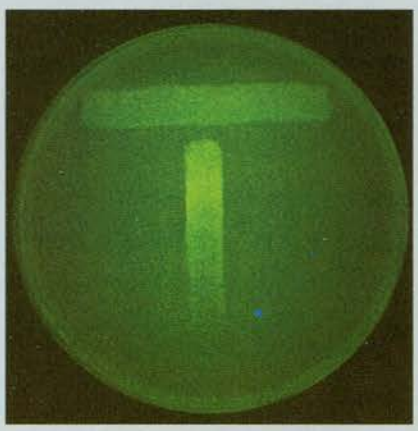
Normal Islet



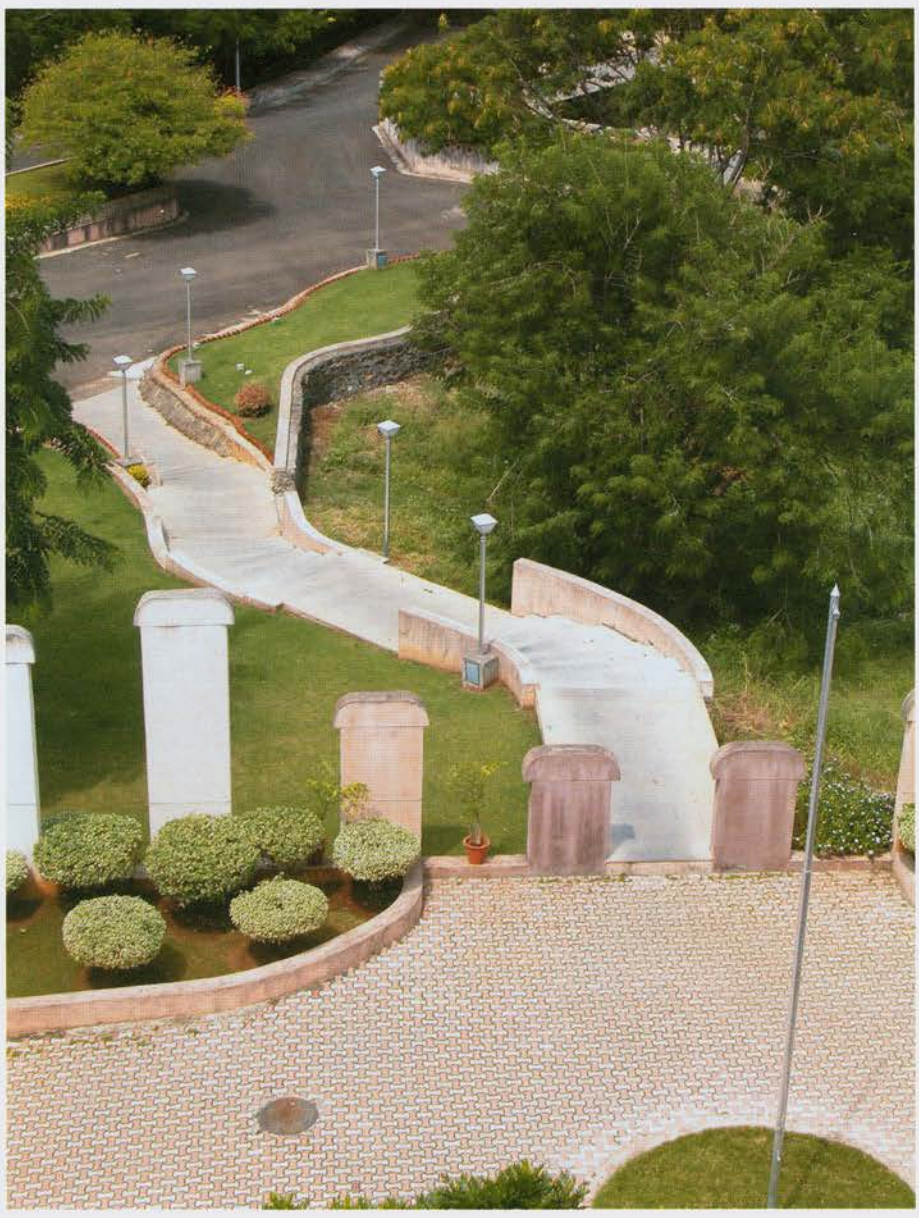
Islet Infiltrated with Mononuclear Cells

2. Characterisation and Identification of autoantigens in rat model.

In the present study we have tagged rat insulinoma cells with 1 Fluoro 2,4 dinitrobenzene (FDNB) and immunized the group of rats with RIN and F-RIN cells. Loss of weights observed in F-RIN immunised animals may be due to insulitis as evident by mononuclear cell infiltration in islets observed in pancreatic sections of F-RIN immunized animals. RIN cell immunized animals did not show either loss of weights or islet cell infiltration in pancreatic sections. RIN and F-RIN immunized rabbit sera recognized different sets of antigens. 26 kDa, 18 kDa and 15 kDa are the antigens only recognized by F-RIN immunized sera. Further studies are in progress.



Insect Molecular Biology



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Cloning and characterization of Hexokinase genes

P. Cyril Jayakumar, P.K. Umasankar, Vijay V. Musande

Collaborator: Yogesh S. Shouche

Background:

Hexokinase is a rate-limiting enzyme in glucose metabolism. It plays important role in energy metabolism in flying insects as it functions very close to Vmax in muscles. We have cloned two complete coding sequences for Hexokinase isoenzymes from *Drosophila melanogaster.* The differential expression of the two cloned enzyme indicated that the nature of 5'upstream non-coding regions may have different cis-acting elements necessary for their expression. This prompted us to clone and characterize the promoter elements. The promoter of DM1 was found to be a TATA less and has typical Inr element and DPE element. This made us to search for other upstream elements involved in enhancing the transcription of housekeeping gene like hexokinase.

Aim:

To analyze the cloned 5' upstream sequences of Hexokinase isoenzymes for testing its ability for transcription initiation.

Work achieved:

1. Analysis of DM1 promoter showed presence of TCATT and TCAAT sequences. Analysis of several housekeeping genes from drosophila showed presence of TCATT and TCAAT sequences at the vicinity of reported Inr and DPE elements of these genes. This implicated the possible role of these sequences in expression of housekeeping genes in fruit-fly. A consensus TCAWT sequence was derived for the analysis.

2. The ability of TCAWT sequence to specifically interact with DNA binding proteins was evaluated by oligonucleotide competition assay. The competition assay indicated that this sequence can specifically interact with DNA binding proteins and at position W, only A and T are functional. Replacement with G or C in the oligonucleotide can not compete out the DNA binding. Mutant promoter sequences have been constructed by site directed mutagenesis and are being tested for their transcriptional activity in vivo.

3. Nearly 90% coding sequence for hexokinase gene is cloned from parasite *Leishmania major.* Some 60 base pairs from 3' end needs to be cloned and

the work to obtain complete coding sequence are being done. Expression pattern and genomic localization of the locus has been done by using pulse field gel electrophoresis.

4. Using total RNA and degenerate oligonucleotides, RACE was performed to clone a partial coding sequence (nearly 70%) for hexokinase gene from *Aedes albopictus*, another dipteran than fruit-fly. 5'-RACE has been performed to obtain the remaining coding sequence. Preliminary genomic analysis indicates that unlike fruit-fly, mosquito has only one gene sequence, coding for all the hexokinase isoenzymes.

Future work:

Experiments will be done to study the synthesis of hexokinase isoenzymes in mosquito and targeting of leishmanial hexokinase to glycosomes.

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Molecular taxonomy and diversity studies using rRNA gene sequence and other tools

N. Badri Narayanan, Kamlesh Jangid, Gurdeep Rastogi, K.M. Dayananda, Mahesh Dharne, Kaushal K. Sharma, Hitendra Munot

Collaborator: M.S. Patole

Abstract and background:

Macromolecules have been described as documents of evolutionary history and they have been used to explore the phylogenetic diversity and evolutionary relatedness of organisms. Ribosomal RNAs are essential components of all living cells that are functionally and evolutionarily conserved. This has made them a valuable tool in molecular taxonomy, phylogeny and diversity studies. However, it suffers from certain shortcomings like differences in the copy number and limited resolving power. In the past few years many additional gene sequences are being increasingly used for diversity and evolutionary studies.

We have been using ribosomal RNA and other markers for the diversity studies including those on "uncultivable" microbes. Knowledge of this will provide information about metabolic diversity of these microbes further enabling their exploration for biotechnological purposes.

Aims:

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

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

Work achieved:

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) Mosquito Midgut Flora:

A total of 152 clones from the library of PCR amplified 16S rRNA genes were analyzed. The 16S rRNA gene library comprised of 47% unidentified and the

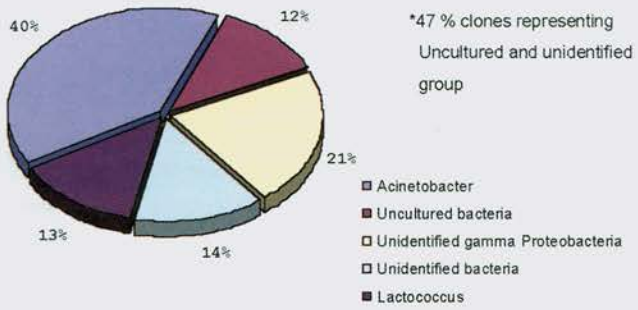


Fig. 1: Distribution of the clones of 16S rRNA gene library from the midgut of Culex quinquefasciatus. A total of 152 clones were sequenced and the analysis was done using 500 bp sequence length at the BLASTn site at the NCBI serever.

uncultured bacterium, 40% *Acinetobacter* spp., and 13% *Lactococcus* spp (Fig. 1). Majority of the cultured isolates and the 16S rRNA gene library clones belonged to the gamma proteobacteria class. Most of the bacteria have earlier been reported to inhabit the midgut of different mosquito species. This study indicates that different mosquito species harbor common representatives of the microbiota that may be the potential candidates for genetic manipulation to control the disease transmission capabilities of the host.

b) Soil from Antarctica:

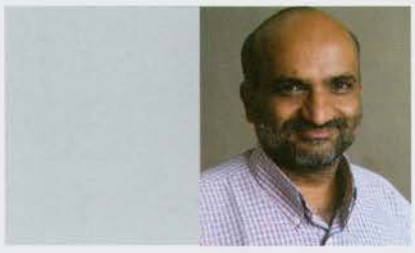
DNA was extracted from this soil sample and PCR was done using universal primers which can amplify both eubacterial (28A) and archeal (28B) 16S rRNA. Also PCR was done using universal primers specific to Cyanobacteria.

Two libraries were prepared for above PCR products and screened for positive clones. Sixty positive clones randomly picked from library 28A and 90 clones from library 28b were partially sequenced.

Similarly three soil samples from different parts of the lake bed were processed for DNA isolation and PCR was done to amplify above mentioned 16S rRNA genes using universal primers. Above mentioned method will be applied for these samples to study uncultured diversity of microbes.

c) Microbial Diversity of Anaerobic Digesters:

To assess the diversity of microorganism in anaerobic sludge from room temperature and 20°C anaerobic digester libraries of PCR amplified 16S rRNA, Archeal 16S rRNA, hsp70 and gene cassette PCR were prepared and the clones are being analyzed.



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Genome diversity of *H. pylori* strains from patients of Indian origin

Mahesh Dharne, Hitendra Kumar Munot

Collaborators: CDFD, Hyderabad; Deccan Medical College, Hyderabad; AIIMS, New Delhi; SGPGI, Lucknow

Molecular Taxonomy & Genetic Diversity

A number of molecular markers are used to delineate taxonomic affiliations and estimation of diversity of various organisms. We have used these markers to understand taxonomy of some important organisms and also to assess diversity. The range of organisms studied ranges from viruses to mammals and some important examples are given here.

a) *Aeromonas* Taxonomy:

In order to resolve the prevailing confusion in the taxonomy of genus *Aeromonas*, molecular markers as 23S rRNA, *gyrB*, and *rpoD* genes were used (Fig. 2). In addition, the validation of the phylogenetic inference using cophenetic correlation coefficient (CCC) and the correlation between gene sequence similarities and DNA hybridization values was also done. Further taxonomic analysis of the genus was done using Amplified Fragment Length Polymorphism (AFLP). Amongst all the fingerprinting techniques, AFLP analysis was found to be the most discriminating genomic methods to distinguish among *Aeromonas* hybridization groups.

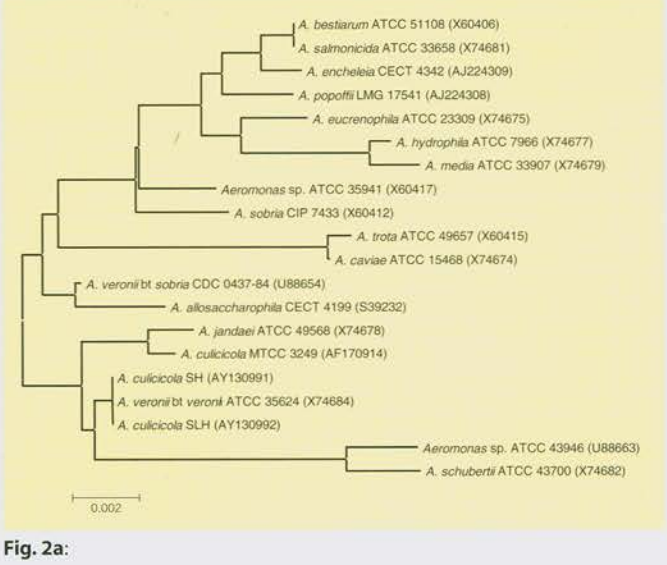


Fig. 2a:

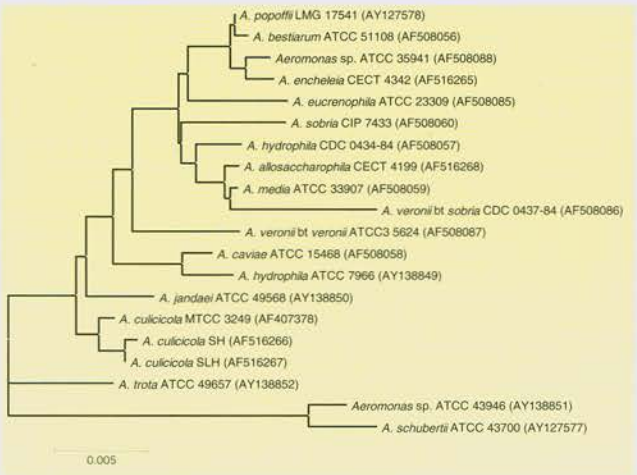


Fig. 2b:

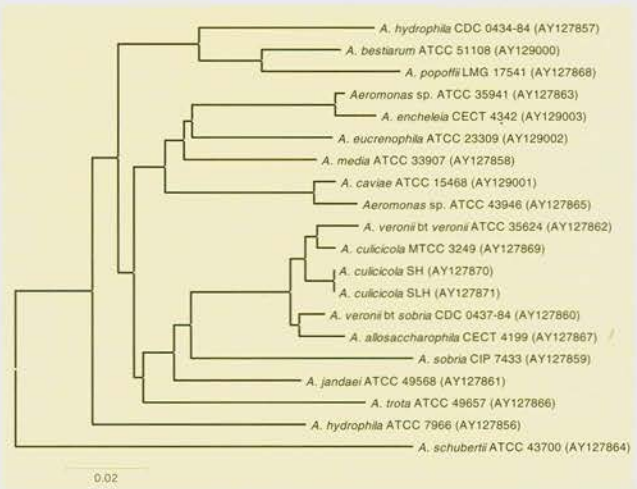


Fig. 2c:

Fig. 2: Phylogenetic relationship of Aeromonas culicicola MTCC 3249^T with reference strains of the genus Aeromonas based on: a) 16S rRNA; b) 23S rRNA and c) rpoD gene sequences. A midpoint rooted phylogenetic tree based on the gene sequences (1371 bp for 16S rRNA; 2762 bp for 23S rRNA and 825 bp for rpoD) was constructed from Kimura 2 distances and the neighbor-joining algorithm. The numbers near the nodes indicate percentage of 500 bootstrap replicates. The scale bar indicates genetic distance. Numbers in bracket indicate Gene bank accession number.

Aeromonas Quorum sensing:

Earlier we have isolated a new member of genus *Aeromonas* from mosquito mid gut and named it *Aeromonas culicicola*. Our studies showed that its number increases more than thousand fold after mosquito take blood meal. We undertook a study on the quorum sensing ability of *A. culicicola* that would help in understanding the regulatory mechanisms involved in the sudden increase in its cell density after blood meal of the mosquito. The production of a quorum-sensing signal was detected using *gfp* based bioassay (Fig. 3). PCR amplification of the QS regulon from *A. hydrophila* was done. The PCR product will be used as a probe for *A. culicicola* QS gene identification.

b) *Methanobrevibacter* Taxonomy:

The phylogeny of the genus *Methanobrevibacter* was established almost 25 years ago on the basis of the similarities of the 16S rRNA oligonucleotide catalogs. We tried to reorganize the 16S rRNA gene sequences of this genus and revise the taxonomic affiliation of the isolates and clones representing the genus *Methanobrevibacter*. The phylogenetic analysis of the genus revealed five

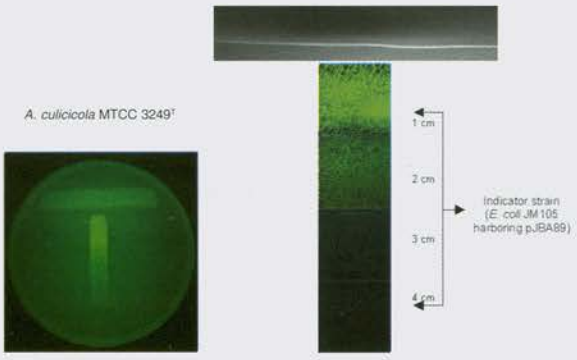


Fig. 3: Detection of production of Quorum sensing signal by Aeromonas culicicola MTCC 3249^T using gfp based bioassay.

groups namely; Ruminantium, Smithii, Curvatus, Arboriphilicus and the Termite gut symbionts. The CCC was found to be 0.913 indicating the high degree of goodness of fit of the tree topology. A significant relationship was found between the 16S rRNA sequence similarity (*S*) and the extent of DNA hybridization (*D*) for the genus with the correlation coefficient for log*D* and log*S* being 0.75, implying that it is possible to predict *D* from *S* with a known precision for the genus. In addition, we deduced group specific signature positions that have remained conserved in evolution of the genus.

c) Genome diversity of *H. pylori* isolates from Indian patients (DBT funded project):

A large number of putative *H. pylori* isolates were received from Center for DNA Fingerprinting and Diagnostics and analyzed for various markers. It was discovered that majority of the isolates were in fact, belonging to (probably) new species of genus *Ochrobactrum*. Gastric biopsies were obtained from patients and attempts were made to isolate *Helicobacter pylori* from these. Out of 135 biopsies received, 33 were positive for *H. pylori* when tested by PCR with specific primer and from these cultures could be made from 9. These isolates are being further characterized.

d) Genetic Diversity of *Bacillus anthracis* isolates from India (LSRB funded project):

DNA of twenty *B. anthracis* isolates were prepared at DRDO, Gwalior and brought to NCCS. PCR was done by primers specific for 16S rRNA gene, Gyrase B gene, 16S-23S rRNA intergenic spacer region [ISR], Protective antigen gene and *vrrA* gene. We sequenced Gyrase B gene of 7 isolates and protective antigen gene of 3 isolates.

e) Phylogeny of Turtle beetles:

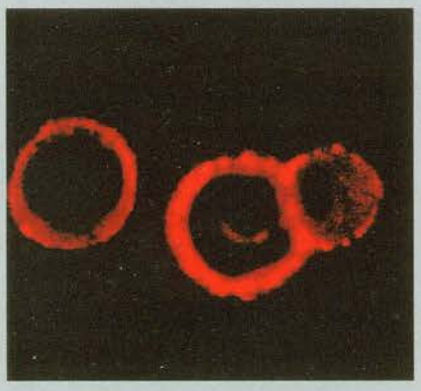
The protocol for DNA isolation from leaf feeding turtle beetles was standardized and PCR was done for ***mt12S, mt16S, Cox I, Cox II*** and ***ND4*** regions.

f) Molecular systematics of Amphibians, Fresh Water Fishes, Uropeltid snakes and Bats:

Molecular systematics of the above specimens involved PCR amplification and sequencing of mt12S, mt16S, CytochromeB, Cytochrome Oxidase I and NADH-dehydrogenase subunit 4. For Uropeltids, Nuclear gene Cmos is under analysis. For taxonomical analysis a total of 70 specimens of amphibians, 20 Snake specimens, 96 Fish specimen were used.

Future work:

The libraries of PCR amplified 16S rRNA and hsp genes from Antarctica and anerobic digester will be analyzed further. Quorum sensing molecules from the *Aermomonas culicicola* will be characterized and attempts will be made to clone and characterize this regulon. Work on *Bacillus anthracis* and *Helicobacter pylori* will be further extended to include more isolates and additional markers.



Infection & Immunity

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Studies on promoters of genes of merozoite surface proteins of *Plasmodium falciparum*

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Collaborator: P. Khandekar, *University of Pune*

Abstract and Background:

Malaria, caused by infection with *Plasmodium* sp., is a resurging disease that infects 300-500 million individuals and results in 1-3 million deaths annually. The majority of these are the result of infection with *P.falciparum*. Due to lack of an efficacious vaccine and the growing resistance to antimalarial drugs, the need to investigate the biology of this parasite is high priority. Little is known about sequences that regulate gene expression in this parasite, even though the complete sequence of the *P.falciparum* genome has been determined.

The development of new control tools to roll back malaria is being, unfortunately, hampered due to the complexity of the parasite's life cycle which involves a vertebrate and a non-vertebrate host, and to our limited knowledge of the basic parasite biology.

A tightly regulated gene expression pattern controls the differentiation of the parasite from one stage to another during its life cycle. At the cellular level, obvious changes in morphology are accompanied by distinct pattern of mRNA expression and protein synthesis. In fact, the mechanisms uderlying the regulation of gene expression in *P.falciparum* are poorly understood. The promoter sequences of for only three of the approximately 5,000-6,000 *P.falciparum* genes have been elucidated. Merozoite surface proteins of *P.falciparum* are known to play an important role in the RBC invasion. The studies of elucidation of promoters of MSP genes are important in understanding the gene expression.

Aims:

To investigate the promoter sequences of merozoite surface protein genes of 3D7 and k1 isolates of *P. falciparum*.

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B cell mediated activation of CD8⁺ T cells

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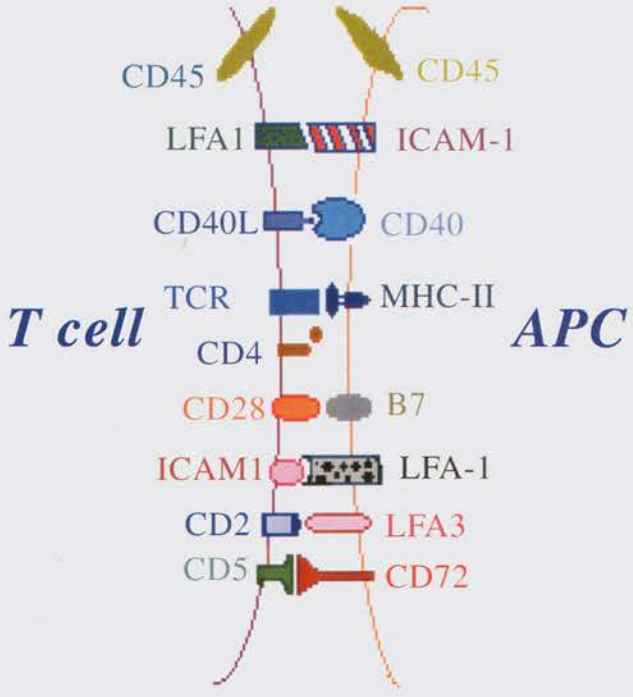
Collaborator: Anil Kumar, *School of Biotechnology, Devi Ahilya Vishwavidyalaya, Indore*

Abstract and background:

B cells recognize antigen through their surface Ig-receptors and present it in the context of MHC-II molecules to CD4⁺ T cells. Recent evidences indicate that B cells also present exogenous antigens in the context of MHC-I to CD8⁺ T cells and thus, may play an important role in the modulation of CTL responses (Mode-1). However, in this regard conflicting reports are available. One group of studies suggest that the interaction between B cells and CD8⁺ T cells leads to the activation of the T cells while others propose that it induces T cell tolerance. For discerning this dichotomy, we used B cells that were either activated with LPS or anti-Ig + anti-CD40 antibody, which mimic the T-independent and T-dependent mode of B cell activation respectively, to provide accessory signals to resting CD8⁺ T cells. Our results show that in comparison to anti-Ig + anti-CD40 antibody activated B cells (CD40-B), the LPS activated B cells (LPS-B) failed to induce significant levels of proliferation, cytokine secretion and cytotoxic ability of CD8⁺ T cells. This hyporesponsiveness of CD8⁺ T cells activated with LPS-B was significantly rescued by anti-TGF-β1 antibody. Moreover it was found



Model 1: A schematic showing distribution of receptor and co-receptor molecules on the cell surface and possible interactions between CD8⁺ T cells and APC's.

that such hyporesponsive CD8⁺ T cells activated with LPS-B had entered a state of anergy. Further, LPS-B expresses significantly higher level of TGF-β1 on the surface, which caused the observed hyporesponsiveness of CD8⁺ T cells. Therefore, this study for the first time provides a novel mechanism of B cell surface TGF-β1 mediated hyporesponsiveness leading to anergy of CD8⁺ T cells.

Aims:

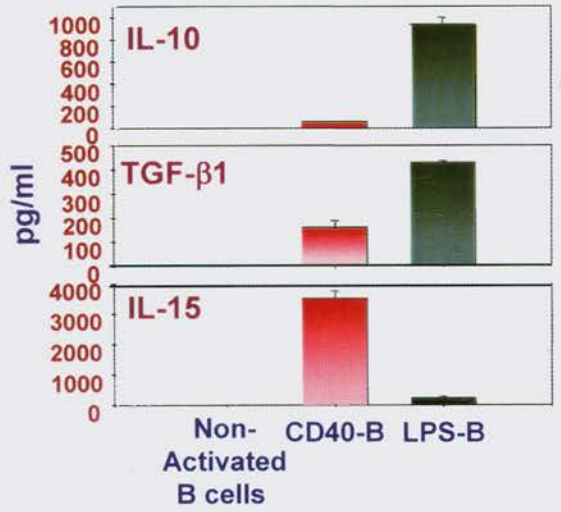
1. To investigate the interaction of activated B cells with CD8⁺ T cells.
2. To investigate the effect of LPS and anti-CD40 mediated activation of B cells.
3. To study the involvement of surface TGFβ in LPS mediated anergy.

Work achieved:

Cytokines synthesized by CD8⁺ T cells activated by LPS stimulated B cells

We investigated the molecular mechanism of CD8⁺ T cell activating capabilities of LPS-B (LPS activated B cells) as compared to CD40-B (anti-CD40 activated B cells). To investigate the mechanism in detail, we analyzed the profile of cytokines secreted by CD8⁺ T cells activated in the presence of B cells. We examined for cytokines such as IL-2, IFN-γ, TNF-α, IL-6, IL-10, IL-13 and TGF-β1 released in the culture supernatant, by sandwich ELISA. The results showed that IL-2, IFN-γ, TNF-α, IL-6 and IL-13 were detected at a significantly higher level in culture supernatants of CD8⁺ T cells activated with CD40-B, as compared to CD8⁺ T cells activated with LPS-B (Fig. 1). In contrast, IL-10 and TGF-β1, which are T cell inhibitory cytokines, were found to be much higher in the culture supernatant of CD8⁺ T cells and LPS-B as compared to the culture supernatant of CD8⁺ T cells and CD40-B. Apart from this, we also performed intracellular cytokine staining for IFN-γ, TNF-α, IL-2 and IL-

A



B

Cytokines	Culture Conditions			
	CD8⁺T cells+ antiCD3+ LPSB (pg/ml)	CD8⁺T cells+ antiCD3+ CD40B (pg/ml)	Irradiated LPSB alone (pg/ml)	Irradiated CD40B alone (pg/ml)
IL-2	155	490	ND	ND
IFNγ	1650	3425	ND	ND
TNFα	192.5	1135	ND	65
IL-6	205	717.5	ND	ND
IL-13	187	631	ND	ND
IL-10	570	142.5	100	ND
TGFβ1	780	265	190	45

ND = Not Detected

Fig. 1: Purified CD8⁺ T cells ($1x10^5$) were cultured with anti-CD3 Ab and irradiated LPS-B or CD40-B ($5x10^4$) in a 96 well plate. Supernatants were harvested at 24 h for IL-2 & IFN-γ, 48 h for IL-6, IL-4, IL-15, TNF-α, IL-10 & IL-13 and 72 h for TGF-β1, after stimulation.

6. Results of this experiment also suggested that LPS-B are much poor inducer of cytokine synthesis from $CD8^+$ T cell as compared to those activated with CD40-B, as observed by the percent of T cells positive for cytokine staining. Taken together, all these results prove that even though LPS-B expresses sufficiently high surface costimulatory molecules, when used as accessory cells, their ability to induce $CD8^+$ T cells responses were severely compromised as compared to the ability of CD40-B. This was evident in terms of not only proliferation and maturation of CTLs, but also in terms of the synthesis of cytokines by the T cells.

TGF-β1 but not IL-10 is responsible for hyporesiveness of LPS-B

We next asked a question whether or not neutralizing the activity of TGF-β1 or IL-10 would restore the hyporesponsiveness of $CD8^+$ T cells activated in the presence of LPS-B as compared to $CD8^+$ T cells activated with CD40-B. We used anti-TGF-β1 and anti-IL-10 Abs to neutralize their activities in $CD8^+$ T cell and activated B cell cultures. The results show that anti-TGF-β1 indeed, significantly restored the proliferation of $CD8^+$ T cells activated with LPS-B, as compared to its isotype control normal mouse IgG1 (**Fig. 2A**). On the other hand, anti-IL-10 had no effect on the proliferation of $CD8^+$ T cells as compared to its rat IgG2b isotype control (Fig. 2A).

To further prove the inhibitory role of TGF-β1, we also performed experiments where CD40-B activated $CD8^+$ T cells were cultured in the presence of recombinant TGF-β1, IL-10 and a combination of TGF-β1 and IL-10. For this, we chose to chemically fix CD40-B with paraformaldehyde The results from such experiments show that while recombinant TGF-β1 completely inhibited the proliferation of $CD8^+$ T cells in a dose dependent manner, IL-10 had no effect. Further, in contrast to earlier observation with $CD4^+$ T cells, no synergism between TGF-β1 and IL-10 could be

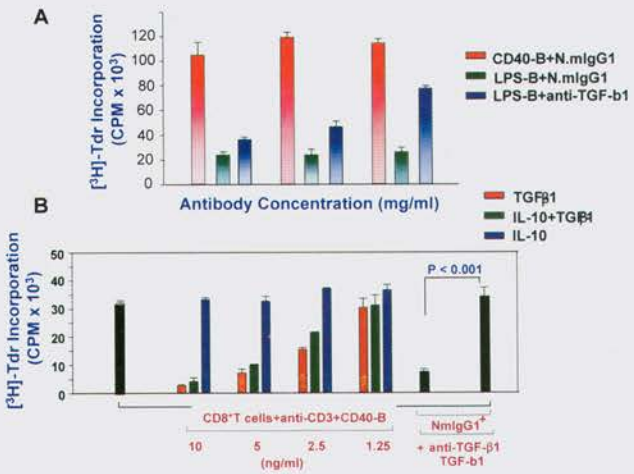


Fig. 2A: Purified $CD8^+$ T cells ($1x10^5$) were activated with anti-CD3 Ab (1 mg/ml) and irradiated LPS-B or CD40-B ($5x10^4$) in the presence of anti-TGF-β1 (1D11) Ab or its isotype control mouse IgG1 Ab or in the presence of anti-IL-10 Ab or its isotype control rat IgG2b. Cells were cultured for 72 h with [3H]-thymidine incorporation (1 mCi/well) for the last 12 h of the culture. **B**. Purified $CD8^+$ T cells ($1x10^5$) were activated with paraformaldehyde (0.4%) fixed CD40-B ($5x10^4$) and anti-CD3 (1 mg/ml) in the presence of varying concentrations of recombinant TGF-β1 or IL-10 or a combination of TGF-?β1 plus a fixed concentration of IL-10 (10 ng/ml). Cells were cultured for 72 h with [3H]-thymidine incorporation (1 mCi/well) for the last 12 h of the culture.

observed. Moreover, addition of high affinity mouse anti-TGF-β1 Ab was found to completely rescue the $CD8^+$ T cell proliferation from the inhibitory effects of rTGF-β1 (Fig. 2B). These results link TGF-β1 to the hyporesponsiveness of $CD8^+$ T cells.

LPS-B expresses higher levels of surface TGF-β1 than CD40-B

The fact that anti-TGF-β1 significantly restored the proliferation of $CD8^+$ T cells activated with LPS-B to the level of those activated with CD40-B, while the secreted cytokines from such cultures do not play a role in causing the hyporesponsiveness of $CD8^+$ T cells, suggests that TGF-

β1 is expressed on the surface of LPS-B. To examine for such a possibility, LPS-B and CD40-B were stained with chicken anti-TGF-β1 and goat anti-LAP (TGF-β1) antibodies that recognize the active form and the latent form of TGF-β1, respectively. We observed that LPS-B and CD40-B both expressed active TGF-β1 on the surface (Fig. 3). To ensure the specificity of the surface staining obtained for TGF-β1, a competition experiment was carried out in the presence of recombinant TGF-β1. The results showed that the staining of TGF-β1 on the surface of LPS-B was diminished by 8-10 times when stained with anti-TGF-β1 in the presence of recombinant TGF-β1, suggesting that anti-TGF-β1 binding on the surface of LPS-B is specific to TGF-β1. Comparing the level of expression of TGF-β1 on these B cells, it was found that LPS-B expresses 10 fold higher levels of TGF-β1 on the surface as compared to CD40-B. The presence of TGF-β1 on the surface of LPS-B was confirmed by confocal microscopy (**Figure 3).**

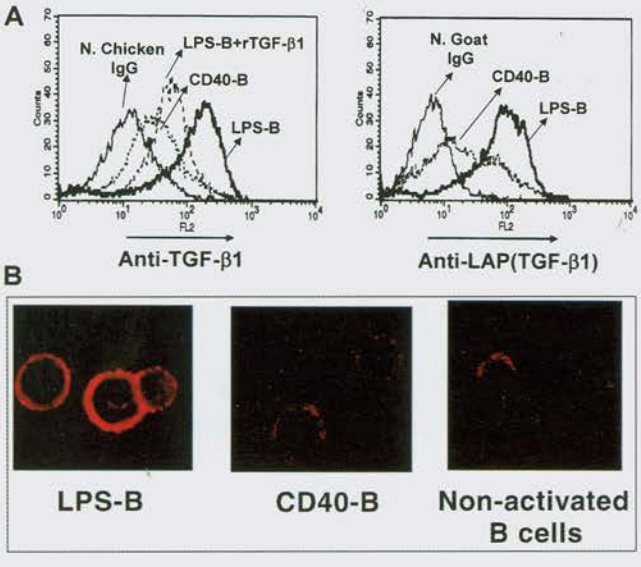


Fig. 3: The enriched B cells were activated with LPS or anti-Ig+anti-CD40. Non-activated B cells or activated B cells were stained with anti-TGF-β1 antibodies and then viewed under confocal microscope. The images of confocal microscopy (63X magnification) showing the surface localization of TGF-β1 on LPS-B (left), CD40-B (middle) and non-activated B cells (right).

Collectively these results prove the presence of TGF-β1 on the surface.

Our results show that LPS-B is less efficient in activating $CD8^+$ T cells as compared to CD40-B. Further, the $CD8^+$ T cells receiving signals from LPS-B enter into state of anergy, which can be rescued by exogenous IL-2. The observed hyporesponsiveness of $CD8^+$ T cell could be attributed to higher expression of TGF-β1 on the surface of LPS-B. Therefore, the type of activation the B cell originally receives, either T-independent or T-dependent, seems to play an important role in deciding the fate of $CD8^+$ T cell response. These results thus illuminate the immune evasion strategies adopted by both gram -negative bacteria and retroviruses that specifically target TLR-4 signaling in B cells.

Future work:

One of the key issues unresolved is the signal required for the initiation of generation of memory $CD8^+$ responses. Our lab currently takes the lead for focusing on this issue.

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Molecular and Cellular Basis of HIV Pathogenesis

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Collaborators: Bhaskar Saha, G.C. Mishra, Anil Chatterji *(National Institute of Oceanography)*, M.K. Gurjar *(National Chemical Laboratory)*, S.P. Joshi *(National Chemical Laboratory)*, K.N. Ganesh *(National Chemical Laboratory)*

Abstract and 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 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:

1. Role of viral regulatory proteins Tat and Nef in HIV pathogenesis, and differential gene expression studies in HIV-1 infected cells.

2. Immune response to HIV infection towards generation of DNA vaccine.

3. Identification of anti-HIV activity in plant extracts and marine animals.

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 is not yet clear. The current study has been undertaken to understand the role of Nef in viral transcription and replication, in presence of the transactivating protein, Tat. Using co-immunoprecipitation and pull down analysis of HIV-1 infected cells, we have shown earlier that HIV-1 Nef protein physically interacts with Tat (Fig. 1) both *in vitro* and *in vivo*. We have now been able to show co-localization of Nef and Tat proteins in transfected 293T cells (Fig. 2). In our endeavour to identify Nef interacting host cell proteins, we have screened a

human cDNA library using yeast two hybrid system, which has resulted in identification of several clones. These clones are currently being characterized.

Despite significant advancement in our understanding of the pathogenesis of AIDS, the mechanisms by which HIV-1 infection induces CD4+ T cell depletion is not clearly understood although apoptosis has been shown to be one major mechanism. The objective of the present work is to identify differentially expressed molecules in cells undergoing apoptotic cell death as compared to non-apoptotic cells in the HIV infected T- cell population and to elucidate the interaction of those molecules in the signaling cascade leading to cell death. Using a reporter T cell line, CEM-GFP and NL4.3 virus, we have been able to purify the apoptotic cells from the non-apoptotic cells and differential gene expression studies have been performed by differential display technique and microarray analysis. Several differentially regulated genes are being characterized.

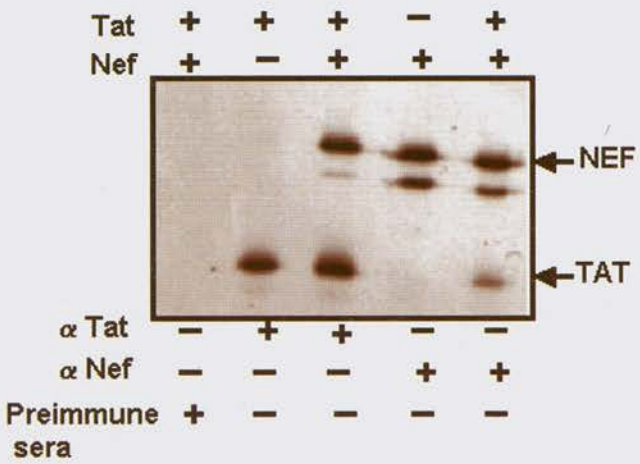

Fig. 1: Interaction of HIV-1 Tat and Nef proteins in vitro. [^{35}S]-Met labeled Tat and Nef proteins were synthesized using in vitro coupled transcription and translation. The two proteins were incubated with each other followed by immunoprecipitation with Tat or Nef antisera. The immunoprecipitated complexes were analyzed on SDS-PAGE followed by autoradiography.

Fig. 2: Localization of HIV-1 Tat and Nef in co-transfected 293T cells. 293T cells were grown on coverslips and transfected with GFP-Tat and RFP-Nef expression vector using Calcium phosphate precipitation. The transfected cells were visualized after 24 hours by confocal microscopy.

Immune response to HIV infection towards generation of DNA vaccine.

HIV-1 is a uniquely difficult target to develop immunological intervention against it. The high rate of replication, mutation and recombination of HIV enable the virus to evolve rapidly in the host and so outsmart immune response evoked by natural immunity or a vaccine. In order to identify candidate molecules for generating protective immune response, we have constructed bicistronic mammalian expression vectors expressing either HIV-1 subtype C (prevalent in India) gp120 and Tat or gp120 and Nef using internal ribosomal entry site in between the two genes. We have confirmed protein expression from the vectors. Furthermore, DNA immunization studies in mice have indicated the generation of both humoral and cellular immune response.

Identification of anti-HIV activity in plant extracts and marine animals.

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 initiated screening of anti-HIV activity in marine bivalves of Indian coastline and also in plants and trees of medicinal importance in western region. Our initial study reveals the presence of anti-HIV activity in some marine bivalves, which are being fractionated to identify the active components.

Future work:

The future work involves identification of the domains in Nef or Tat proteins, which are important in the interaction and also elucidation of the signal transduction pathway. Furthermore, as the role of Nef in HIV induced pathogenesis is yet to be clearly understood, we have initiated studies to identify Nef interacting host cell factors. Characterizations of some of the Nef interacting clones are in progress. Identification of differentially expressed genes and their relevance to HIV induced cell death will be continued. Finally, marine bivalve extract showing anti-HIV activity is being fractionated to identify the active molecule/s.

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Role of T cells and non-T cells in the resistance or susceptibility to Leishmania infection

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Trainees: Nitya Jain, Amit Ashtekar

Collaborator: Debashis Mitra

Abstract and background:

Neutrophils and macrophages are known to play a crucial role in phagocytic clearance of *Leishmania*, a protozoan parasite that induces immuno-suppression in a susceptible host. We have proposed that neutrophils, known to be terminally differentiated and transcriptionally inactive, do actively acquire antigens, transcribe and express the genes for major histocompatibility complex (MHC) class I and class II molecules, co-stimulatory molecules and several functionally diverse cytokines and chemokines that induce T-cell migration and differentiation. On the other hand, macrophages infected with *Leishmania* have impaired CD40 signaling resulting in disease-promoting T cell response. A novel pharmacological intervention that rectifies the signaling serves as a unique anti-leishmanial chemotherapeutic strategy.

Aims:

1. How does *Leishmania* survive in a susceptible host?

2. Since Leishmania resides within macrophages, does it impair anti-leishmanial functions of macrophage?

3. Neutrophils are the first cells to migrate to the site of infection. Do they have any other roles in *Leishmania* control than just phagocytosis? If they do, how do they work at the interface of macrophage and T cells?

Work achieved:

1. We have shown that CD40, a costimulatory molecule on macrophages, signals through p38MAP kinase to induce inducible nitric oxide synthetase 2 (iNOS2) that catalyzes nitric oxide formation. Nitric oxide is an important reactive oxygen species that kills the parasite. It is observed that during *Leishmania* infection, CD40 signaling through p38MAP kinase is impaired resulting in unrestricted parasite growth. Rectification of the signaling results in parasite killing and host-protective T cell response.

2. Polymorphonuclear leukocytes (PMNs), comprising about two-thirds of peripheral blood leukocytes, are absent from uninfected, uninjured,

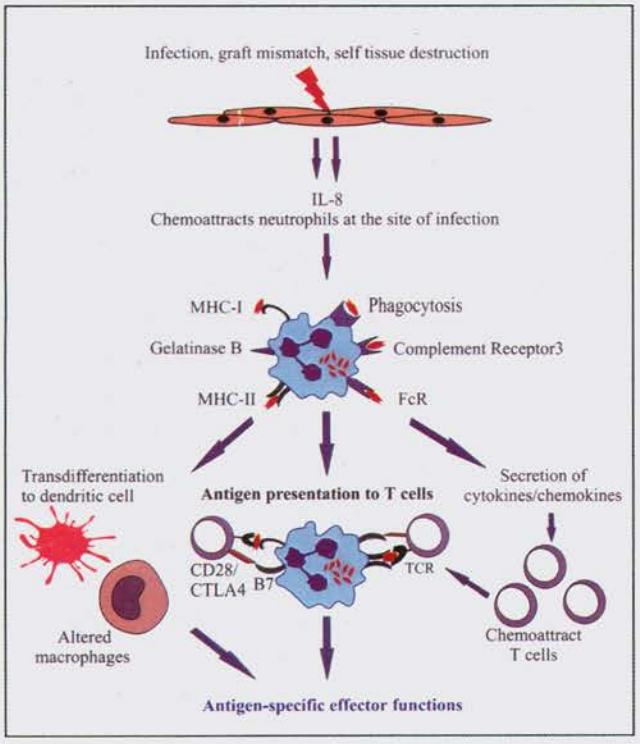


Fig. 1: A unified view of the modus operandi of neutrophils as antigen-presenting cells (APCs) and their role in the pathogenesis of different diseases. The infection or injury to tissues as in mismatched grafts or autoimmune diseases results in chemokine release, chiefly interleukin-8 (IL-8) that activates and chemoattracts neutrophils to the site. The activated neutrophils acquire antigen by phagocytosis, by F_c receptor (FcR) or by other means, release matrix metalloproteinases (Gelatinase B) that dissolute the matrix facilitating migration of other cells including T-cells, and generate peptides that bind to major histocompatibility complex (MHC) molecules. The antigen-MHC complex is recognized by T-cell receptor (TCR) generating the first activation signal in T-cells. B7 molecules on neutrophils bind to CD28 or Cytotoxic T Lymphocyte Associated antigen4 (CTLA-4) on T-cells to deliver the second signal or costimulatory signal to T-cells. Delivery of the first signal without the second signal results in antigen-specific hyporesponsiveness of T-cells. Also, the trans-differentiation of neutrophils to dendritic cells, interaction with macrophages and release of cytokines and chemokines modulate the size and quality of APC pool at the tissue site. The effector T-cells respond to direct antigen presentation by neutrophils, and also by other APCs, to execute various effector mechanisms.

'healthy' tissues. PMNs, proposed to be terminally differentiated and transcriptionally inactive, are the first cells to migrate to the site of infection to perform phagocytosis and release cytotoxic compounds. Recent data reveals that PMNs actively acquire antigens, transcribe and express the genes for major histocompatibility complex (MHC) class I and class II molecules, co-stimulatory molecules and several functionally diverse cytokines and chemokines that induce T-cell migration and differentiation. Thus, PMNs act not only as phagocytic scavengers and non-specific effectors of the innate immune system but also as significant inducers of T-dependent immune responses by processing and presenting antigens to T-cells in infectious and autoimmune diseases.

Future work:

1. We will further investigate the mechanism of Leishmania-induced impairment of CD40 signaling. If a novel mechanism is observed, a therapeutic strategy for *Leishmania* elimination will be examined.

2. We will further investigate the non-phagocytic role of neutrophils in controlling *Leishmania* infection.

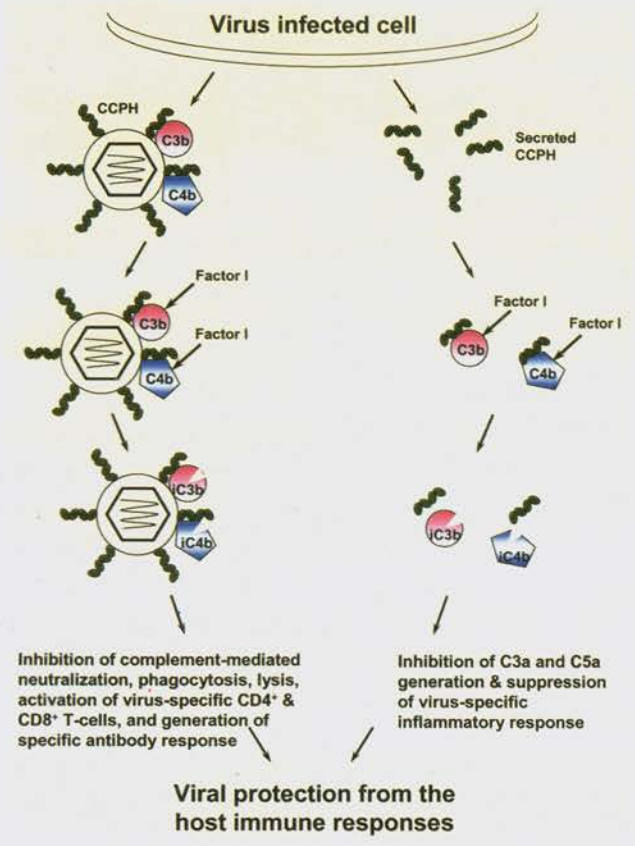


Fig. 1: Working model of CCPH-mediated protection of viruses from the host immune responses. CCPH, either expressed on the viral surface, or secreted in the medium, bind to C3b and C4b and support their proteolytic inactivation by factor I. Regulation of complement on the viral surface would lead to inhibition of complement-mediated viral neutralization, phagocytosis, lysis, activation of virus-specific $CD4^+$ and $CD8^+$ T-cells, and generation of specific antibody response. In addition, inhibition of fluid phase complement activation at the site of infection by soluble CCPH would reduce the specific inflammatory response against the virus. Together this would lead to protection of virus from the host immune responses.

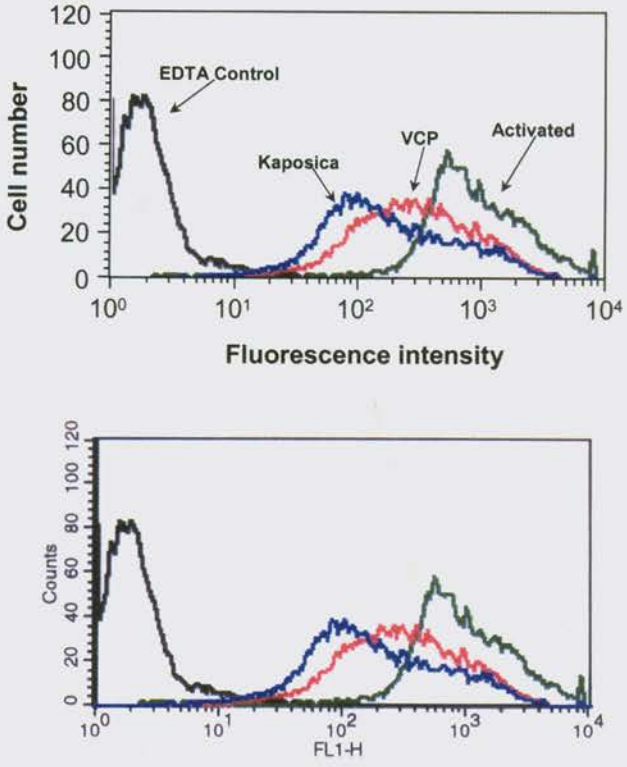


Fig. 2: Inhibition of C3b deposition on erythrocytes during complement activation by kaposica and VCP. Like VCP, kaposica also inhibits complement activation at the C3 level.

VCP inactivates complement by inactivating C3b and C4b, the subunits of C3 convertases. Since KSHV is also known to encode for a complement control protein-like molecule (ORF 4) with significant (44-55%) sequence similarity to human complement control proteins, we expressed this ORF to assign a function to this protein. Like VCP, the ORF 4 protein of KSHV was also expressed using the *Pichia* expression system. The purified ORF 4 protein migrated as a single band of 56,000 Da on SDS-PAGE; its identity was confirmed by automated Edman degradation. The expressed protein inhibited both the classical as well as the alternative pathway of complement activation, blocked the cell surface deposition of C3b (**Fig. 2**) and served as cofactor for factor I-mediated inactivation. Of complement proteins C3b and C4b (**Fig. 3**), indicating thereby that it acts at the level of C3 convertases. Interestingly, this protein also showed binding to heparin. Among the human complement regulators, factor H and

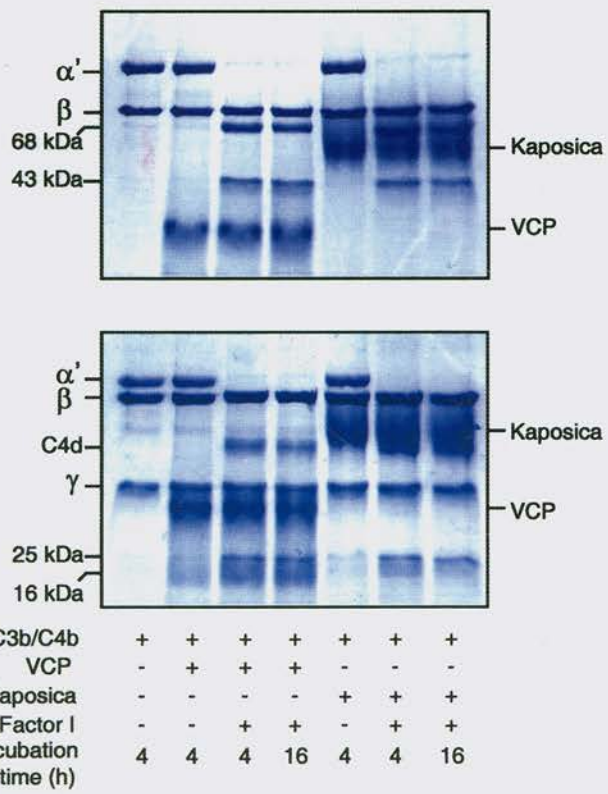


Fig. 3: Analysis of factor I cofactor activity of kaposica and VCP for complement proteins C3b (upper panel) and C4b (lower panel). Like VCP, kaposica also supports the factor I-mediated inactivation (cleavage of α' chain) of C3b and C4b into their inactive forms.

C4-binding protein bind to heparin, and interaction of factor H with heparin is an important step in the regulation of activation of the alternative pathway. Whether the interaction of the ORF 4 protein with heparin is fortuitous or has any physiological relevance requires further study. Based on its activities, the protein was named as kaposica (the Kaposi's sarcoma-associated herpesvirus inhibitor of complement activation).

Earlier mutation analyses of charged residues of host complement proteins have highlighted the importance of ionic interactions in host CCP-C3b/C4b interactions. Our data obtained using surface plasmon resonance technology indicates that ionic contacts are also important in VCP-C3b/C4b as well as kaposica-C3b/C4b interactions.

Identification and characterization of functionally important determinants of viral homologs of complement control proteins

Identification of vital structural determinants of viral complement control proteins that are important in interacting with host complement proteins would not only provide an essential insight into its biology, but would also act as potential targets for the development of pharmacological ligands to neutralize viruses. Our laboratory is using two different approaches to identify the functionally important determinants of viral complement control proteins. 1) The first strategy involves generation of deletion as well as chimeric mutants wherein individual domains will be either deleted or swapped by structurally similar proteins. 2) The second approach involves use of monoclonal antibodies.

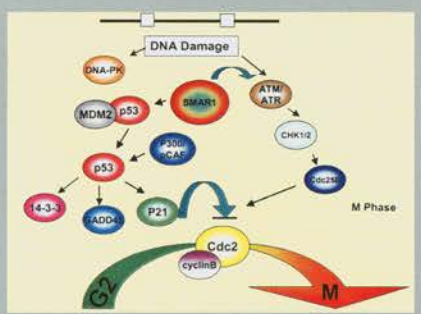
VCP is entirely composed of four tandemly repeating domains termed complement control protein (CCP) domains. We have generated five deletion mutants of VCP wherein one or more CCP domains of VCP have been deleted. These mutants are cloned in *Pichia* and the clones expressing these mutants have been obtained. A large-scale expression, purification and characterization of these mutants is underway. In addition to VCP, we have also initiated the construction of kaposica deletion mutants.

In our efforts to generate monoclonal antibodies against VCP, we had generated over 40 hybridoma clones. We have now sub-cloned nineteen of them. These monoclonal antibodies are characterized with respect to their binding to VCP and inhibition of functional activities of VCP. Seven

out of nineteen mAbs inhibited the factor I cofactor activity of VCP for C3b and C4b and their equilibrium dissociation constants varied from 2 nM to 22 μM. Deletion mutants of VCP that are generated in the laboratory will be useful in identification of CCP domains to which these antibodies bind. These mAb will also be useful for characterization of in vivo function of VCP. In particular, mAbs with good affinities and slow off rates will be good candidates for in vivo studies.

Future work:

1. Expression and functional characterization of CCPH of HVS.
2. Functional characterization of deletion mutants of VCP and generation of deletion mutants of kaposica.
3. Characterization of in vivo role of VCP using skin lesion model.



Chromatin Architecture and Gene Regulation

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A novel tumor suppressor protein SMAR1 that regulates cell cycle through activation of p53

Ruchika Kaul, Asavari Kulkarni, Shravanti Rampalli, Archana Jalota, Kamini Singh
Devraj Mogare *(Technician)*, Gazalla Sheikh *(Trainee)*

Collaborators: Debashis Mitra, Sanjeev Galande and Bhaskar Saha *(NCCS)*

Abstract and background:

My laboratory is interested in understanding how the chromatin structure changes during the transformation of a normal cell to a cancerous cell. In this regard, we have seen that the MARs (Matrix Associated Region), small AT rich sequences, and MAR binding proteins modulate chromatin architecture regulating T cell development and HIV integration. Many of these MAR binding proteins have been shown to be unregulated in various cancers. We have identified a new gene SMAR1 that is conserved both in mouse and human. In human SMAR1 is located in chromosome 16q24 and the loss of heterozygosity of this region causes colon, prostrate and breast cancers. We observed that the expression of SMAR1 is low or none in various human cancers. We now partly understand the mode of its action through p53 (tumor suppressor protein). It directly binds to p53 and modulates it posttranslationally. Our studies with SMAR1 transgenic mice show that overexpression of SMAR1 results in enlargement of various organs and also perturb T cell development in thymus and lymph nodes. Currently, we have extended work towards the understanding the regulation of SMAR1 expression on upon various stresses especially in diabetic conditions. Along with these results, our recent finding shows that MAR sequences also play a critical role in the processive transcription from HIV-LTR promoter and allows HIV late genes expression at a distance. These MAR sequences are cloned into viral vectors that can be used in future for gene therapy.

Aims:

1. To delineate the signaling by SMAR1 that mediates activation of tumor suppressor protein p53.

2. How the repressor protein SMAR1 works at the TCRβ locus in the presence of Eβ enhancer through recruitment of CDP/Cux, another MAR binding protein.

3. To elucidate the possible mechanisms of MAR mediated processive elongation of transcription from HIV-LTR promoter.

4. To find out molecular mechanisms of extracellular HIV1 Tat mediated activation of T cells resulting IFNγ secretion and overexpression of transcription factor T-bet.

Work achieved:

1. SMAR1 interacts and activates p53 causing cell cycle arrest

The tumor suppressor p53 is a multifunctional protein mainly responsible for maintaining the genomic integrity. The p53 induces its tumor suppressor activity by either causing cell cycle arrest (G1/S or G2/M) or by inducing the cells to undergo apoptosis. We reported that SMAR1 as one such p53 interacting protein and the interaction occur in the absence of either UV damage or other external stimuli (**Fig. 1A**). Overexpression of SMAR1 in mouse melanoma cells (B16F1) and their subsequent injection in C57BL/6 mice delays tumor growth. SMAR1 activates p53-mediated reporter gene expression in mouse melanoma cells, breast cancer cells (MCF-7) and p53 null cells (K562), the activation being more pronounced with $SMAR1^{S}$than $SMAR1^{L}$ (longer form. These data together suggest that among MAR-binding proteins, SMAR1 is the only known protein that delays tumor progression via direct activation and interaction with tumor suppressor p53.

To further analyze the function of SMAR1 in vivo, transgenic mice were generated and V(D)J recombination was studied in detail. SMAR1 was found to downregulate some of the important Vβs such as Vβ8.1 and 8.2, Vβ8.3, Vβ5.1 and 5.2. SMAR1 transgenic mice were also found to exhibit a defect in the V(D)J recombination, particularly those that were found to be downregulated. Thus, SMAR1 regulates V(D)J recombination at the TCRβ locus. We hypothesize that SMAR1 mediated regulation of V(D)J recombination might be controlled through chromatin modulation and proper assembly with the Eβ enhancer. In other studies we have seen that SMAR1 works in combination with another MAR binding protein CDP/Cux. Together they bind to MARβ, a MAR sequence present next to the Eβ enhancer. By confocal analysis we show that these two proteins associate together (**Fig. 1B**).

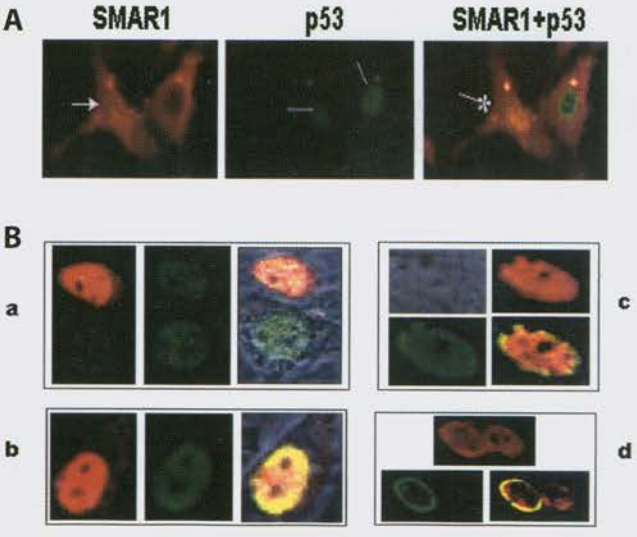

Fig. 1A: Colocalization of SMAR1 and p53 within the nucleus. A part of the picture that was published on the cover page of 20th February, 2003 issue of International Journal of Cancer (Kaul *et al.*, 2003).
B: Colocalization of SMAR1 with the MAR binding protein Cux. Red and green colors showing the presence of SMAR1 and Cux respectively. Yellow colour corresponds to the merged feature showing colocalization of these two proteins in the nucleus and also at the perinuclear region.

2. SMAR1 phosphorylates p53 and upregulates cdc2 to control cell cycle

Various stresses and DNA damaging agents trigger transcriptional activity of p53, a global regulatory switch that controls cell proliferation and apoptosis. These genotoxic insults cause post-translational modification of p53 through its phosphorylation and acetylation. SMAR1 directly binds, phosphorylates and stabilizes p53 within the nucleus. By truncations and deletions, we identified a novel nuclear localization signal (NLS). Interestingly, a short Arginine-Serine rich (RS) motif present next to NLS strongly phosphorylates the p53 at Serine-15 residue. We also show that the same domain downregulates MAPK and upregulates p21, mdm2 and phospho-cdc2. The upregulation of p21 triggers accumulation of phospho-

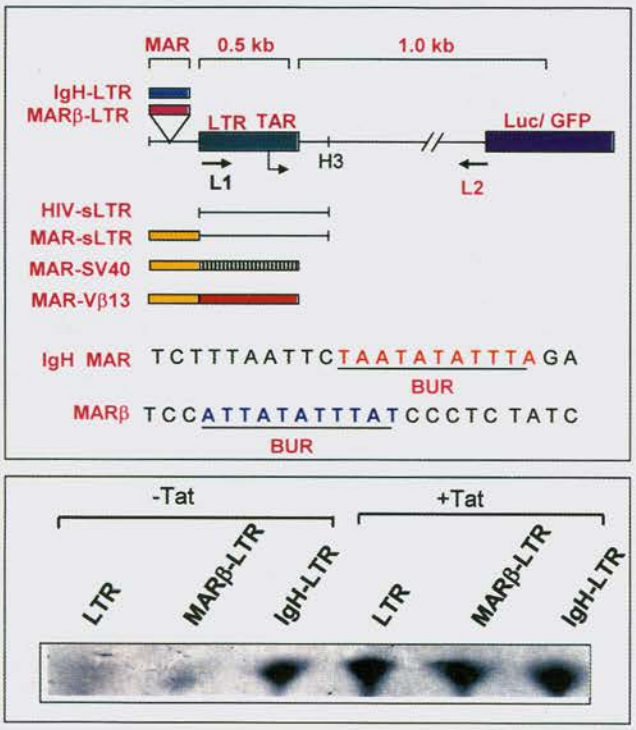

Fig. 2: SMAR1, a novel regulator of cell cycle. It interacts and activates p53, p21 and mdm2. It also upregulates phospho-Cdc2. We hypothsize that SMAR1 is another new member in the signal transduction cascade.

cdc2 causing cell cycle arrest. Thus, SMAR1 is a new sensor protein that directly allows functional modulation of p53 in the absence of any external insults and arrest cells (Fig. 2). In future we will further unravel the stresses that control SMAR1 and cause suppression of cancer.

3. Tat independent HIV-1 LTR transcription at a distance

Chromosome dynamics and the sites of integration in the host genome are critical determinants of HIV transcription and replication. Depending on the chromosomal location of provirus integration within the genome, HIV1-LTR mediated transcription may vary from 0-70 folds. cis elements such as Topo-II cleavage sites, Alu repeats and MARs are thought to be targets for retroviral integration. We have shown that a novel MAR sequence from TCRβ locus (MARβ) and IgH MAR mediate transcriptional augmentation when placed upstream of HIV1-LTR promoter. The effect of transcriptional augmentation is seen both in transient and stable transfection indicating its effect even upon integration in the genome. MAR mediated transcriptional elevation is independent of Tat, and occurs synergistically in the presence of Tat (**Fig. 3**). Further we show that MAR mediated transcriptional elevation is specific to HIV1-LTR promoter. In a transient transfection assay using overexpressed IκB, inhibitor of NFκB, we show that MAR induced processive transcription is NFκB dependent, signifying the role of local enhancers within LTR promoter. Furthermore, by RNase protection experiments using proximal and distal probes, we showed that MAR mediated transcriptional up regulation is more prominent at the distal rather than proximal, thus indicating potential role of MARs in promoting elongation.

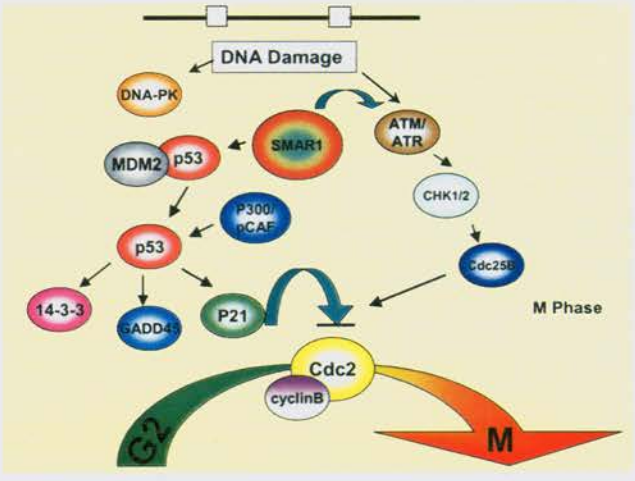

Fig. 3. The MAR upstream of the HIV-1 LTR promoter shows strong upregulation of processive transcription. Upper panel showing the constructs used to show the effect of various MARs upstream of LTR. Lower panel shows transcription at a distance as seen RNase protection assays. (Rampalli et al., Nucleic Acids Research, 2003).

Future work:

1. The specific domain of SMAR1 that activates p53 will further be analyzed and will be overexpressed for tumor suppression in mice

2. We have observed organomegaly in the SMAR1 transgenic mice indicating that SMAR1 is also involved in the development of organs. These mice will further be analyzed and immuno-histochemistry will be performed to show expression of various related signaling molecules.

3. We find that SMAR1 is upregulated upon various stress and external stimuli. p53 phosphorylation upon SMAR1 upregulation will be studied in detail. Regarding the p53 promoter function we.

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

Pavan Kumar P., Arnab Nayak, Prabhat Kumar Purbey, Nitin Sonawane (*Technician*)

Collaborator: Debashis Mitra

Abstract and background:

Special AT-rich sequence binding protein 1 (SATB1) participates in the maintenance of chromatin architecture in a cell-type specific manner by organizing it into domains via periodic anchoring of base-unpairing regions (BURs) to the nuclear matrix. In thymocyte nuclei, SATB1 has a cage-like 'network' distribution circumscribing heterochromatin and selectively tethers BURs onto its network resulting in coordinated regulation of distant genes. In regard to mouse *IL-2Rα* locus, SATB1 recruits histone deacetylase (HDAC1) from the nucleosome remodelling and histone deacetylase (NURD) complex to its binding site and mediates the deacetylation of histones over long distance within the locus. SATB1 therefore regulates large chromatin domains by acting as a 'landing platform' for several chromatin-remodelling enzymes in T cells. SATB1 possesses a MAR-binding domain (MD) and homeodomain (HD) that are both essential for recognition of the core-unwinding element within BURs. SATB1 exists as a homodimer, and that dimerization is essential for its DNA binding activity. Interestingly, the dimerization domain of SATB1 is homologous with many other PDZ domains. As a PDZ- and homeo domain-containing protein SATB1 may provide a framework that mediates assembly of specific protein complexes onto a discrete set of BURs. We set out to search for proteins that interact with SATB1 via its PDZ signalling domain and to investigate the outcomes of such interaction on the function of SATB1. We report that HIV-1 transactivator (Tat) interacts with SATB1 via its PDZ domain *in vivo* and causes derepression of SATB1 regulated genes via displacement of SATB1-bound HDAC1 corepressor.

Aims:

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 domain.

Work achieved:

We prepared constructs for yeast two-hybrid analysis towards the search for SATB1's interacting partners. We used both near full length SATB1 (amino acids

53-763) as well as the PDZ domain (amino acids 90-204) as baits for two-hybrid screening. After screening of the human T cell leukemia library in pACT2.1 vector, we identified four candidate gene products as interaction partners of SATB1. We are in the process of characterizing these at a molecular level.

To test whether the negative regulation by SATB1 is manifested via recruitment of the HDAC1 corepressor, we explored the possibility of their direct interaction. HEK 293 cells were transfected with a mammalian expression construct FLAG-HDAC1 and the cell-free lysate was then incubated with in vitro translated ^{35}S-labeled SATB1. Anti-FLAG antibody was used to immunoprecipitate HDAC1 and its associated protein(s). Analysis of the immunoprecipitate revealed that HDAC1 forms a complex

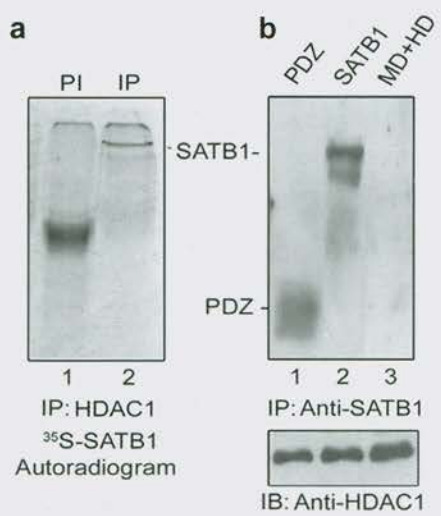


Fig. 1: SATB1 directly interacts with the HDAC1 corepressor via its PDZ domain. a, *In vitro* translated ^{35}S-labeled SATB1 was mixed and incubated with cell lysate of HEK 293 cells. Co-immunoprecipitation was carried out using normal rabbit serum (lane 1) and anti-HDAC1 (lane 2). SATB1 was visualized by autoradiography (lane 1). **b,** Interacting domain of SATB1 with HDAC1 was analyzed by FLAG pull down assay followed by immunoblot using anti-SATB1. Anti-FLAG pulled down the PDZ domain (lane 1) and full-length SATB1 (lane 2) but not the MD+HD domain (lane 3). Lower panel represents immunoblot with anti-HDAC1 to serve as loading control.

with SATB1 (Fig. 1a, lane 2), consistent with previous studies showing complex formation between mouse SATB1 and HDAC1 *in vitro*. We further delineated the region of SATB1 that interacts with HDAC1. The recombinant PDZ or MD and HD domains of SATB1 were separately incubated with cell-free extract from HEK 293 cells transfected with the construct expressing FLAG-HDAC1. Coimmunoprecipitation analysis using anti-FLAG antibody showed that HDAC1 is associated with the PDZ domain (Fig. 1b, lane 1) and full length SATB1 (Fig. 1b, lane 2), but not with its DNA-binding domain (Fig. 1b, lane 3). Thus, human SATB1 may also act as a transcriptional repressor by recruiting HDAC1 corepressor to its specific genomic targets.

One hallmark of HIV-1 infection is the dysregulation of cytokine gene expression in CD4$^+$ T cells. All transcription factors implicated cytokine induction are ubiquitously expressed and no T-lineage-specific factor has yet been identified that regulates their expression. Since SATB1 is a T lineage-specific global suppressor, we explored its involvement in the regulation of cytokine gene regulation. We hypothesized that for HIV-1 Tat to be able to upregulate the expression of multiple cytokines and other T cell-specific genes and activate T cells, the repression mediated by SATB1 should be alleviated.

To investigate how Tat might overcome negative regulation by SATB1, we tested whether SATB1 specifically associates with HIV-1 Tat. Cell-free lysate from Jurkat (Fig. 2a, Lanes 1-4 and 6-9) or HEK 293 (Fig. 2a, lanes 11-14) cells was passed over glutathione-S-transferase (GST)-Tat bound Sepharose matrix. The bound protein was eluted in stepwise manner using buffers containing 1 M NaCl or reduced glutathione. The eluted protein was analyzed by SDS-PAGE followed by immunoblot using anti-SATB1 (Fig. 2a, lanes 1-5 and upper panel of lanes 11-14) or anti-GST (Fig. 2a, lanes 6-10) antibody. Their co-elution (Fig. 2a, lanes 3 and 13, upper panel) suggested association between

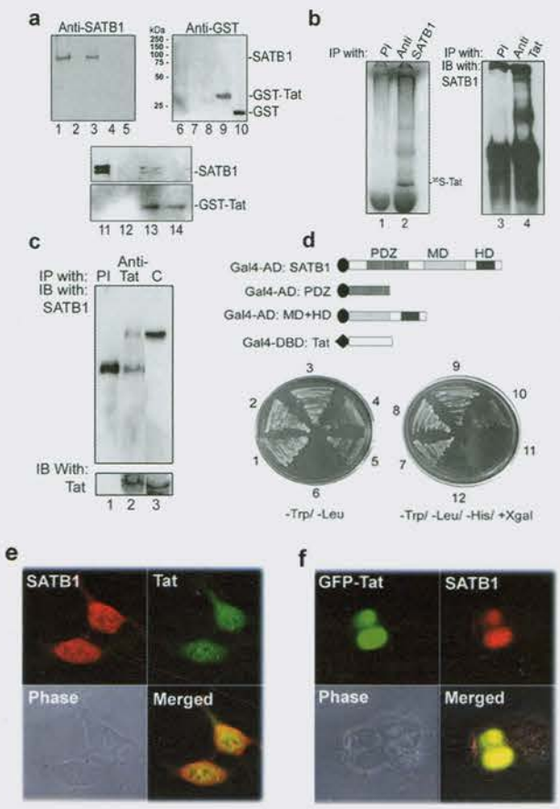


Fig. 2: **HIV-1 Tat physical interacts with SATB1 *in vitro* and *in vivo*. a**, SATB1 is eluted in GST-Tat affinity chromatography. Nuclear extracts from Jurkat and 293 cells were passed separately on GST-Tat and GST affinity columns prepared by incubating the respective recombinant proteins with glutathione Sepharose. Bound proteins were eluted from the GST-tat affinity column with phosphate buffer containing 1 M NaCl (lanes 3 and 13) or with reduced glutathione (lanes 9 and 14). Proteins from GST-bound Sepharose were eluted with reduced glutathione only (lanes 5 and 10). Column input (lanes 1, 6 and 11), flow through (lanes 2, 7, and 12). **b**, *In vitro* translated ^{35}S-labeled Tat and cold SATB1 were mixed and immunoprecipitated with normal rabbit serum (lane 1 and 3), anti-SATB1 (lane 2), and anti-Tat (lane 4). The immunoprecipitated proteins were detected by autoradiography (left panel) or immunoblot analysis using anti-SATB1 (right panel). PI, normal rabbit serum, IP, immunoprecipitation, IB, immunoblot. **c**, SATB1 immunoprecipitated using anti-Tat antibody in extract from NL4.3 infected CEM-GFP cells (lane 2) was detected by immunoblot analysis using anti-SATB1. Lane 3 (C, control) shows proteins in cell extract used for coimmunoprecipitation. Lower panel represents an immunoblot with anti-Tat. **d**, Yeast two-hybrid analysis. Various constructs used for yeast two hybrid analyses are schematically represented on top of the two plates depicting growth of cotransformants. Numbers 1-6 represents the –Trp and –Leu selection medium and 7-12 represents the –Trp, -Leu, -His and X-gal selection medium. One and 2, 7 and 8 represents the co transformation of Gal4-AD:SATB1 and Gal4-AD:PDZ with Gal4-DBD:Tat. Three and 9 represent p53 and T-antigen as a positive control, 4 and 10 represent the p53 and lamin C as a negative control, 5 and 11 represent the Gal4-AD:MD+HD and Gal4-DBD:Tat cotransformation and 6 and 12 represent the mock transformed cells as control. AD, activation domain, DBD, DNA-binding domain, MD, MAR-binding domain, HD, homeodomain. **e** and **f**, Immunofluorescence analysis of SATB1 and Tat expression in HEK 293 and SKBR3 cells respectively. Tat was expressed in both these cell lines by transient transfection using pCDNA3.1-Tat (in HEK 293) or pEGFPN1-Tat (in SK-BR-3). The interaction of this exogenously introduced Tat with endogenous and transiently transfected SATB1 in HEK 293 and SK-BR-3 cells respectively was monitored by indirect immunofluorescence analysis using laser scanning confocal microscope (Zeiss LSM 510).

SATB1 with Tat. In HEK 293 cell-free lysates we often observed two closely migrating bands when stained with anti-SATB1 (Fig. 2a, lane 11, upper panel). Control experiments using GST-bound glutathione Sepharose beads did not yield anti-SATB1 cross-reactive species (Fig. 2a, lane 5). Only GST eluted from this column after elution with buffer containing glutathione (Fig. 2a, lane 10). We then tested the possibility of physical interaction between these two proteins by incubating *in vitro* translated Tat with Jurkat cell-free lysate. By co-immunoprecipitation using polyclonal antibodies against each protein, we observed that SATB1 and Tat formed a complex *in vitro* (Fig. 2b, lanes 2 and 4). The interaction of these two proteins was further confirmed by coimmunoprecipitation using cell-free lysates from NL4.3 infected CEM-GFP cells. Anti-tat antibodies immunoprecipitated SATB1 in these lysates indicating that SATB1 directly interacts with Tat *in vivo* (Fig. 2c, lane 2). Their *in vivo* functional interaction was further demonstrated by yeast two hybrid assays. In a manner that is identical to coimmunoprecipitation of these with HDAC1 (Fig. 1b), we found that both the full length (Fig. 3d, 2 and 8) as well as PDZ domain (Fig. 2d, 3 and 9) of SATB1 interacted with Tat. The MD and HD domains failed to interact with Tat (Fig. 2d, 4 and 10). The strength of this interaction was quantified by β-galactosidase (β-gal) assay. Interaction of full length SATB1 with Tat yielded a value of 41.2 units while that of PDZ domain and Tat yielded 26.7 units compared to the undetectable β-gal activity of MD and HD and Tat cotransformed yeast cells. Thus, these results suggest that functional interaction between HIV-1 Tat and SATB1 occurs via its PDZ-like domain. We next tested whether SATB1 and Tat associate with each other *in vivo* by indirect immunofluorescence using antibodies against each protein. When Tat was expressed in HEK 293 cells by transient transfection it was found to colocalize with endogenous SATB1 by indirect immunofluorescence analysis (Fig. 2e). Interestingly, when SATB1 was expressed exogenously along with GFP-Tat in the human breast cancer cell line SK-BR-3 that does not express SATB1 endogenously, a similar nuclear colocalization pattern was observed (Fig. 2f). In both cell types, the colocalization was evident in more than 90% of Tat expressing cells.

Future work:

1. Map the acetylation site(s) in SATB1 and monitor the effect of acetylation on the MAR-binding activity of SATB1 in vitro and in vivo.

2. To investigate the functional significance of the PDZ-mediated interactions in T cells.

Support Units

Experimental Animal Facility

Dr. B. Ramanamurthy, Dr. K. N. Kohale

The Team: Mr. Md. Sheikh, Mr. A. Inamdar, Mr. P.T. Shelke, Ms. Vaishali Bajare, Mr. Anand Bidlan

Visiting Fellow/Trainee: Dhairyasheel Patil, Sonia Menenzes

The Experimental Animal Facility is an infrastructural service department of the Institute. It is a barrier-maintained facility for the breeding, maintenance and supply of high quality and standardized laboratory animals viz. inbred 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

Strain: BALB/cJ
C57BL/6J
DBA/2J
SWISS
BALB/c*
Nude Mice
Gene knock-out mice

RATS

Strain: 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 three different inbred lines viz. BALB/c, C57BL/6, and DBA/2 is structured in a two-tier format, i.e. the Foundation colonies (FC) and the Production colonies (PC). Strict full-sib pairing only propagates the animals in the FC. The three different strains of mice namely BALB/c, C57BL/6 and DBA/2 are currently at F17, F15 and F13 respectively. These colonies are the nuclear colonies for the long-term propagation of an inbred strain.

A single male mouse with spontaneous congenital cataract and micropthalmia was detected in a production colony of BALB/c strain of mice. Breeding studies undertaken suggested that the defect is inheritable and that this mutation is caused by a gene defect inherited in an autosomal recessive manner. The morphological characterization revealed that the defect is manifested at the 14th postnatal day when the eyes are open. The other phenotypic features of this congenital defect include clear cornea, central lens opacity and intact lens capsule. Slit lamp examination studies also confirmed the central lenticular opacity with clear zone at periphery and the disturbance of the typical iris pattern together, with the maldevelopment of the pupillary muscles. The rupture of lens and the protrusion of lens materials through the lens capsule into the posterior space as reported in other mutants are not noticed in this case. Histology of the mutant lenses during embryonic stage revealed loss of longitudinal fibrillar structure, failure of the elongation of primary fibres, abnormal morphology of the cells, vacuolation and degeneration of the lens fibre cells. Efforts have been initiated to study the various lens proteins like alpha, beta and gamma crystallins. Attempts shall also be made to identify the genes responsible for this defect with the help of molecular biological tools.

The mutant colony is currently at F21 level of inbreeding.

The facility has imported a total of 73 mice comprising of 15 different gene knock-out mice/mutant mice. Attempts made to establish and breed these mice for supply and use in the ongoing R&D projects have been mostly

successful and efforts are ongoing to fully establish these strains at NCCS. Some of these mice have also 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. and the procurement of animals.

As a part of the health-monitoring program around 33 samples were subjected for hematological screening and around 13 samples for clinical biochemistry.

Histopathology: Over 200 stained and unstained slides have been processed and prepared for hispathological examination.

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.

1-4-2002 to 31-03-2003

Sr. No.	Strains/Species	Animals Procured	Animals Bred	Animals Supplied
1.	**RATS**			
	Wistar	—	86	139
	Lewis	—	88	5
2.	**MICE**			
	BALB/c	393	4306	3202
	C57bl/6	300	1546	1120
	SWISS	95	909	528
	DBA/2	—	—	8
	Nude (nu/nu)	146	—	131
	BALB/c*	—	309	166
	Mutant Mice	73		260
3.	**MASTOMYS COUCHA**	—	28	—
4.	**RABBIT**(NZW)	5	7	4

* **BALB/c with cataract mutation.**

• Fluorescence Activated Cell Sorter (FACS)

During the period approximately 4000 samples were acquired and analysed. The samples were of surface staining, DNA staining, analysis of GFP fluorescence, calcium uptake. We have also analyzed samples from other institutes like NARI, Hyderabad University etc.

• The Confocal Laser Scanning Microscope (CLSM)

The confocal laser scanning microscope (CLSM) is a state of the art laser scanning microscope (Zeiss LSM 510) having four lasers converting the UV, 488 nm and 590 nm ranges. Presence of four lasers enhanced the choice of

various fluorophores during real time observation. This year our facility has aquired more than 5000 images from NCCS users and approximately 260 images for users from various other institutes.

• The Automated DNA Sequence Analyser

We have been using an ABI 310 Single capillary based machine for cycle sequencing. Since its installation in April 2001, more than five thousand samples have been processed for sequencing. The machine is being used at its maximum capacity with virtually no downtime. This also includes samples from other organizations like National Chemical Laboratory, Pune University, Agharkar Research Institute, Serum Institute, Central Institute of Fisheries, Mumbai, National Environmental Research Institute, Nagpur etc.

• Transgenic Facility

Last year NCCS had procured the entire set up for micro-manipulation and microinjection. This year, this set up is complete with the addition of a new Pipette pulling machine. We have also optimized procedures for superovulation and embryo collection. Within next few months we will be able to successfully inject embryos and transfer them into pseudopregnant females to generate transgenic mice. The laboratory is equipped for performing routine procedures in molecular biology and the screening protocols are also being developed simultaneously.

• Library

The NCCS Library has collection in the frontier areas of biotechnology having relevance to NCCS research activities. The Library holds approximately four thousand bound journals, sixteen hundred books, and subscribes to seventy-five scientific journals and thirty other periodicals.

In the development of its collections, the Library's priority is to support NCCS research activities. The Library collection is expanded in consultation with NCCS scientists. The Library's print collections are growing by approximately 700 volumes per year. During the period of 2002-2003, the Library has added seventyeight books and 675 volumes of journals to its collection. In order to provide faster access to research information, the Library also subscribes for limited full text material online.

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

• Computer Centre

Computer section of NCCS has been involved in following main activities:

Expansion of LAN & INTERNET Bandwidth Up gradation:
We have further expanded institute wide LAN by installing and configuring network cards in Library, store and Purchase and I&M Division computers. To meet the increased user demand for Internet/E-mail access, we have upgraded INTERNET leased line bandwidth from 64 Kbps to 256 Kbps this year.

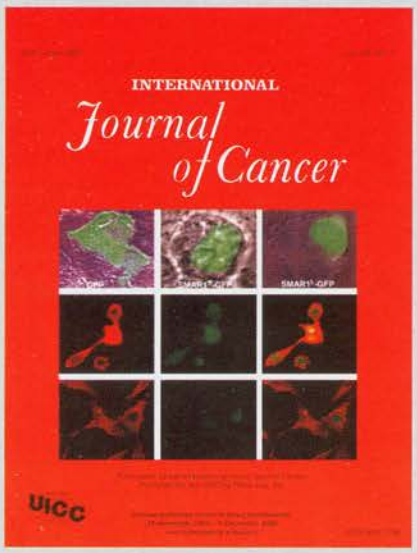
Server Up gradation:
We have enhanced server capacity by installing additional 256MB RAM and 9.1GB SCSI hard disk to the main Internet/E-mail server. We also upgraded Corporate antivirus server RAM to 128 MB.

Computer Facilities provided:

To increase the quality of scientific presentation of NCCS staff and students attending national and international conferences/seminars, computer section is helping for DTP work, CD writing, scanning images and transparency printing on color LaserJet printer.

Computer System Management, Maintenance and Up gradation:

Computer section is providing technical support for More than 80 computers and 45 printers, Anti-Virus package install & upgrade, Installation and configuration of new computers, CDROM/RAM/Network card installation in old computers, Operating System, Software's and Drivers, Server operating system up gradation and maintenance, Website up gradation.



Publications

Awards and Honours

Funds Received

Seminars Attended

Committees of NCCS

Publications

1. Madhura R. Vipra and Jayant M. Chiplonkar. 2002. **Vital stain to study cell invasion in modified Boyden chamber assay.** BioTechniques, 33: 1200-1204.
2. S. Kazemi, D. Wenzel, E. Kolossov, N. Lenka, A. Raible, P. Sasse, J. Hescheler, K. Addicks, B.K. Fleischmann, W. Bloch. 2002. **Differential Role of bFGF and VEGF for Vasculogenesis.** Cell Physiol Biochem. 12:55-62.
3. Khapli SM, Mangashetti LS, Yogesha SD, Wani MR. 2003. **IL-3 Acts Directly on Osteoclast Precursors and Irreversibly Inhibits Receptor Activator of NF-kappaB Ligand-Induced Osteoclast Differentiation by Diverting the Cells to Macrophage Lineage.** J. Immunol. 171:142-145.
4. Parekh VV, Prasad DV, Banerjee PP, Joshi BN, Kumar A, Mishra GC. 2003. **B cells activated by lipopolysaccharide, but not by anti-Ig and anti-CD40 antibody, induce anergy in CD8(+) T Cells: Role of TGF-beta1.** J Immunol. 170: 5897-5911.
5. Sharma, V., Sangha, N., Kaur, S., Krishnasastry, M.V. 2003. **Modulation of EGF receptor autophosphorylation by α-hemolysin of Staphylococcus aureus via protein tyrosine phosphatase.** FEBS lett. 535, 71-76.
6. Mahabeleshwar GH, Kundu GC. 2003. **Syk, a protein tyrosine kinase, suppresses the cell motility and NF-kappa B mediated secretion of urokinase type plasminogen activator by inhibiting the phosphatidylinositol 3'-kinase activity in breast cancer cells.** J. Biol. Chem. 278: 6209-6221.
7. Philip S and Kundu GC. 2003. **Osteopontin induces NF-kappa B mediated promatrix metalloproteinase-2 activation through IKK/IκBα signaling pathways and curcumin (diferulolylmethane) down regulates these pathways.** J. Biol. Chem. 278: 14487-14497.
8. Takada Y, Mukhopadhyay A, Kundu GC, Mahabeleshwar GH, Singh S, Aggarwal BB. 2003. **Hydrogen peroxide activates NF-κB through tyrosine phosphorylation of IκBα and serine phosphorylation of p65: Evidence for the involvement of IêBá kinase and Syk protein tyrosine kinase.** J. Biol. Chem. 278: 24233-24241.
9. Das R, Mahabeleshwar GH, Kundu GC. 2003. **Osteopontin stimulates cell motility and NF-κB-mediated secretion of urokinase type plasminogen activator through phosphatidylinositol 3-kinase/Akt signaling pathways in breast cancer cells.** J. Biol. Chem. 278(31):28593-28606.
10. Ajith Mathew J, Jayashree S. Ladha, Manoj Mojamdar, Debashis Mitra. 2003. **Human Immunodeficiency Virus-1 Nef protein interacts with Tat and enhances HIVgene expression.** FEBS Lett., 548: 37-42.
11. Jayashree C. Jagtap, Anmol Chandele A .Chopde, Padma Shastry. 2003. **Sodium pyruvate protects against H2O2 mediated apoptosis in human neuroblastoma cells.** J. Chem. Neuroanatomy ,In press.
12. R R Bhonde. 2003. **Human Pancreatic beta cells have potential to regenerate in vivo.** Diab Res and Clin Pract, 61(1):78-79.
13. Sadhana Joshi, Shobha Rao, Bhonde R. R. 2003. **Chronic malnutrition affects birth weight and increases susceptibility to Syndrome X.** J Nutrition. In press.
14. Shweta Shah, Rameshkumar K, S Bodhankar, R Bhonde, Dharmendra Goswami. 2003. **Hypoglycemic activity of CSP in alloxan induced diabetic mice and rats.** J. Ethnopharm, In press.
15. Panchnadikar A, Bhonde R. 2003. **Can stress provide protection to pancreatic beta-cells and prevent diabetes?** Med Hypotheses. 2003 Mar; 60(3): 356-9.
16. Risbud MV, Dabhade R, Gangal S, Bhonde RR. 2002. **Radio-frequency plasma treatment improves the growth and attachment of endothelial cells on poly (methyl methacrylate) substrates: implications in tissue engineering.** J Biomater Sci Polym Ed. 13:1067-1080.
17. Gudrun UM, Metzner C, Bhonde RR, Malsch G, Schiermeyer B, Vetter H. 2002 **Simultaneous isolation of endothelial and smooth muscle cells from human umbilical artery or vein and their growth response to low-density lipoproteins.** In Vitro Cell Dev Biol Anim. 38:265-272.
18. Joshi B, Chatterji A, Bhonde R. 2002 **Long-term in vitro generation of amoebocytes from the indian horseshoe crab tachypleus gigas (muller).** In Vitro Cell Dev Biol Anim. 38: 255-257.
19. Risbud MV, Bhonde RR. 2002 **Models of pancreatic regeneration in diabetes.** Diabetes Res Clin Pract. 58: 155-165.
20. George S, Nair PD, Risbud MV, Bhonde RR. 2002 **Nonporous polyurethane membranes as islet immunoisolation matrices — biocompatibility studies.** J Biomater Appl. 16: 327-340.
21. K. Venuprasad, S Chattopadhyay, Bhaskar Saha. 2002. **CD28 signaling in neutrophil induces T cell chemotactic factor(s) modulating T cell response.** Hum. Immunol. 46: 38-43.
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23. Amit R Ashtekar, Bhaskar Saha. 2003. **Poly's plea: membership to the club of APCs.** Trends in Immunol. 24(9):485-490.
24. Mullick J, Kadam A, Sahu A. 2003. **Herpes and pox viral complement control proteins: 'the mask of self'.** Trends in Immunol. 24(9):500-507.
25. Mullick, J., Bernet, J., Singh, A.K., Lambris, J.D., Sahu, A. 2003. **Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) open reading frame 4 protein (kaposica) is a functional homolog of complement control proteins.** J. Virol. 77: 3878-3881.
26. Soulika, A.M., Morikis, D., Sarrias, M.R., Roy, M., Spruce, L.A., Sahu, A. Lambris J.D. 2003. **Studies of structure-activity relations of complement inhibitor compstatin.** J. Immunol. 170:1881-1890.
27. Sahu A., Morikis D., Lambris J.D. 2003. **Compstatin, a peptide inhibitor of complement, exhibit species-specific binding to complement component C3.** Mol. Immunol. 39: 557-566.
28. Kaul, R., Mukherjee, S., Ahmed, F., Bhat, M. K., Chhipa, R., Galande, S. Chattopadhyay, S. 2003. **Direct interaction and activation of p53**

by SMAR1 causes cell cycle arrest at G2/M phase and delays tumor growth in mice. Int. J. Cancer, 103: 606-615.

29. Rampalli, S., Kulkarni, A., Kumar, P., Mogare D., Galande, S., Mitra, D Chattopadhyay, S. 2003. **Stimulation of Tat independent transcriptional processivity from the HIV-1 LTR promoter by Matrix Attachment Regions.** Nucleic Acids Research, 31: 3248-3256.
30. Sarkar, A. Chattopadhyay S., Kaul R., Pal J. K. 2002. **Lead exposure and heat shock inhibit cell proliferation in human HeLa and K562 cells by inducing expression and activity of the heme-regulated eIF-2αk Kinase.** J. Biochem. Mol. Biol. Bioph, 6: 391-396.
31. D. T. Mourya, M. D. Gokhale, Vyankatesh Pidiyar, P. V. Barde, Milind Patole, A. C. Mishra, Yogesh Shouche. 2002. **Mid gut bacterial flora of Culex quinquefasciatus affects the susceptibility of mosquitoes to Japanese Encephalitis virus.** Acta Virologica 46: 257–260.
32. Pidiyar, V.J., Patole, M.S., Yogesh S. Shouche. 2003. **Identification of Wolbachia sequences in Indian populations of Culex and Aedes.** Current Science 84: 1136-1139.
33. Pidiyar, V.J., Jangid, K., Patole, M.S., Yogesh S. Shouche. 2003. **Analysis of 16S-23S intergenic spacer regions and rrn operon copy number of Aeromonas culicicola MTCC 3249T.** DNA Sequence 14: 183-194.
34. Pidiyar, V.J., Jangid, K., Dayanand, K.M., Patole, M. S., Jose M. Gonzales, Kaznowski, A., Yogesh S. Shouche. 2003. **Phylogenetic affiliation of Aeromonas culicicola MTCC 3249T based on gyrB gene sequence and PCR–Amplicon Sequence Analysis of cytolytic enterotoxin gene.** Syst. Appl. Microbiol. 26: 197-202.
35. DT Mourya, DK Singh, P Yadav, MD Gokhale, PV Barde, PC Jayakumar, JP Thakare, AC Mishra, YS Shouche. 2003 **Role of Ascogregarina culicis (Apicomplexa: Lecudinidae) parasite in the maintenance and vertical transmission of Chikungunya virus in the vector mosquito Aedes aegypti.** J. Eukaryot. Microbiol, In press.
36. Meenal Banerjee, Ramesh Bhonde. 2003. **Islet generation form intra islet precursor cells of diabetic pancreas: in vitro studies depicting in vivo differentiation.** J. Pancreas, 4(4):137-45, Online journal.
37. Ashwini Hinge, Sandhya Sitaswad. 2003 **Decreased antioxidant enzymes and membrane essential polyunsaturated fatty acids in schizohrenic and bipolar mood disorder patients.** Psychiatry Res, In press.
38. Mahabeleshwar GH, Kundu GC. 2003. **Tyrosine Kinase, A56 lck regulates all mortility & nuclear factors kappa B-mediated secretion uPA through tyrosine ph osphorylation of IKKα following hypoxia/ reoxygenation.** J. Biol. Chem, In Press.
39. Anmol Chandele, Vandana Prasad, Jayashree C. Jagtap, Ravi Shukla, Padma Shastry. 2003. **Upregulation of survivin in G2/M cells and inhibition of caspase 9 activity enhances resistance of SK-N-MC cells to staurosporine-induced apoptosis.** Neoplasia, In press.
40. Lalita M. Sasnoor, Vaijayanti P. Kale & Lalita S. Limaye. 2003. **Supplementation of Conventional Freezing Medium with a Combination of Catalase and Trehalose Results in Better Protection of Surface Molecules and Functionality of Hematopoietic Cells.** J. Hematoth. Stem Cell Res, In press.
41. Anjali Shiras, Arti R Bhosale, Varsha S. Shepal, Ravi Shukla, V.S. Baburao, Prabhakara K, and Padma Shastry. 2003. **A unique model system for tumor progression in GBM comprising two developed human neuro-epithelial cell lines with differential transforming potential and co-expressing neuronal and glial markers.** Neoplasia (In Press).
42. Deepak B. Salunke, Braja G. Hazra, Vadana S. Pore, Manoj Kumar Bhat, Pallavi B. Nahar, Mukund V. Deshpande, 2003. **New Steroidal Dimers with Antifungal and Antiproliferative Activity** J. Med. Chem. (In Press).

Bulletins

1. Meenal Banerjee and Ramesh Bhonde 2003. Islet generation form intra islet precursor cells of diabetic pancreas: in vitro studies depicting in vivo differentiation. *J. Pancreas* (In press).
2. D T Mourya, V Pidiyar, M Patole, M D Gokhale and Y Shouche. Effect of Midgut Bacterial Flora of *Aedes aegypti* on the Susceptibility of Mosquitoes to Dengue Viruses. *Dengue Bulletin* (2002) 26: 190-194.

Patents filed

1. US Patent filed.
   Durbaka V. R. Prasad, Pradeep Parab and G C Mishra.
   Patent file # FP01583
2. Process for the cure and control of diabetes mellitus using natural products from perna viridis.
   R R Bhonde & Anil Chatterji
   Provisional Patent # 1005/MUM/2003
3. In vitro culture of ameobocytes from Tachypleus gigas in Leibovitz culture medium.
   R R Bhonde & Anil Chatterji.
   Patent # 20030186432
4. Osteoporosis
   Patent # 412/NF/2003
5. Anti-Malaria
   Patent # 409/NF/2003
6. Indian green mussel (Perna viridis) as a source of anti-HIV activity
   Debashis Mitra, Anil Chatterji
   Patent # 20020168416

Dr. Gyan Chandra Mishra, Director, NCCS receiving "Padmashree" award from Honourable President of India, April 2003

Honours/Awards/Memberships:

JM Chiplonkar
- Life Member, Indian Society of Cell Biology.

MR Wani
- Life Member, Indian Society of Cell Biology (2002 onwards)

Arvind Sahu
- Member of the International Complement Society (1999-onwards)

Gopal C Kundu
- Member of the Indian Society of Cell Biology (life member)
- Member of Molecular Immunology Forum (2002)
- Received Travel Award from Indian National Science Academy (INSA) for attending Third International Conference on Osteopontin and Related Proteins (3rd ICORP).

Debashis Mitra
- Member, Molecular Immunology Forum, 2002
- Visiting Student Fellowship to Jayashree S. Ladha Albert Einstein College of Medicine, Bronx, NY, USA September 2002 – March 2003.

Bhaskar Saha
- Member of Indian Immunology Society.
- FIMSA Young Investigator Award.

Pradeep B Parab
- Wellcome Travel Award offered to visit Diabetes & Metabolism Dept. SouthMead Hospital at Bristol, U.K. Sept.-Oct. 2003.

Gyan Chandra Mishra
- 'Padmashree' conferred to Dr. Gyan Chandra Mishra by the honourable President of India, 3 April, 2003.
- Ranbaxy Research award for excellence in Medical Science-Medical Research.

Extramural funding received by NCCS Scientists:

Yogesh S Shouche

1. The *Helicobacter pylori* Genome Programme: Genome Sequencing, Functional Analysis and Comparative Genomics of the strains obtained from Indian patients.
   In collaboration with Centre for DNA Fingerprinting and Diagnostics, Hyderabad, All India Institute of Medical Sciences, New Delhi, Sanjay Gandhi Post Graduate Institute of Medical Sciences Luknow and Deccan College Medical Sciences, Hyderabad.
   Duration 2001-2004
   Funding Agency: Department of Biotechnology

2. Studies on Genetic Relatedness of *Bacillus anthracis* strains of Indian origin.
   In collaboration with Defense Research and Development Establishment, Gwalior
   Duration: 2002-2005
   Funding Agency: Life Sciences Research Board.

3. Looking for evidence of Life in outer space: Studies on meteor crators.
   In collaboration with Agharkar Research Institute, Pune.
   Duration: 2003-2006
   Funding Agency: Indian Space Research Organization

Sanjeev Galande

Bridging signal transduction and chromatin architecture: Role of PDZ domain in the function of SATB1
Duration: 2003-2006
Funding Agency: Department of Biotechnology

Gopal C Kundu

1. Received funding from Department of Science and Technology, Govt. of India on "Role of Osteopontin on Matrix Metaloproteinase-2 expression, Cell migration and ECM-invasion in melanoma cells".

2. Received funding from Department of Biotechnology, Govt. of India on "Role of novel factor in suppression of breast cancer cells migration and metastasis".

3. Received funding from The British Council through the Higher Education Link Program on "Development of Breast Cancer Specific Drugs".

Debashis Mitra

1. DBT project, BT/PRO/897/AAQ/03/099/00.
   Identification and characterization of anti-HIV compounds in Indian marine bivalves.

2. ICMR project, 4/3-2/2000, NM/BMS/TRM.
   New Natural products as HIV-1 reverse transcriptase inhibitor from the genus *Calophyllum.*

3. CSIR project, 31/NIO/124/2001-RPBD
   Isolation of active compound in the extract of mussels to develop drugs.

Nibedita Lenka

Research Grant from Department of Biotechnology, Government of India (2003).

Arvind Sahu

Wellcome Trust Overseas Senior Fellow (2001-2006)

Seminars/Invited talks given by NCCS Scientists at other places

Yogesh Shouche

- Invited talk "DNA: The molecular clock" at World Science Festival-2003 & International Dialogue to celebrate golden jubilee of DNA Double Helix discovery.
- Invited Talk on "Gene, Genomes and Genomics, Life after 50 years after Discovery of DNA double Helix" at Nagpur University as a part of Science Day Celebrations on February 28, 2003.
- Talk on "Biological Weapons" at Pune in March 2003 under the auspices of Marathi Vigyan Parishad, Pune.

Lalitha S. Limaye

- Invited Talk on "Applications of FACS in Haematology research" at Indian toxicology research Center, Lucknow.

Debashis Mitra

- Invited Lecture "Molecular Biology of HIV Pathogenesis", 11th July, 2002 at School of Life Sciences, Debi Ahilya Vishwavidyalaya, Indore.
- Invited Lecture in UGC Refresher Course in Genetics "HIV Genome Function and regulation", 27th Nov, 2002 at Department of Botany, University of Pune, Pune.
- Invited Lecture on National Science Day Celebrations "Fifty years of Double Helix: current understanding of retroviral gene expression", 28th Feb, 2003 at National Institute of Virology, Pune.

Ramesh R. Bhonde

- Invited to give a talk at Maratha Chamber of Commerce Industries and Agriculture, Pune on "Emerging Technologies in Biotechnology for Small Scale Industries" April 23, 2002.
- Invited to deliver a lecture at Agharkar Research Institute for Exploratory students on Animal Tissue Culture, April 26, 2002.
- Radio talk in Marathi at Pune Radio Station on "New Direction in Diabetes Research" May 14, 2002 in Vidyan Parichay.
- Radio Talk in Hindi on All India Radio Delhi "Madhumeh Anusandhan Kae Nayae Aayam" August 5, 2002.
- Invited to deliver a lecture at Dayanand college, Solapur on 'The Role of Animal Tissue Culture in Biotechnology' August 10,2002
- Invited to give a lecture at Fergusson College, Department of Chemistry on "Overview of Biotechnology Today" August 16, 2002

- Invited to deliver Dr. S N Ghosh Memorial Oration at NIV, Pune November14, 2002 (World Diabetes Day) "Pancreatic Regeneration and Reversal of Experimental Diabetes"
- Invited to attend a WHO /ICMR workshop on "Strategies for prevention and control of Diabetes mellitus" held at New Delhi during January 10-12,2003
- Invited to deliver a talk on " In vitro models as an alternative to animal experiments" at The International Seminar organized by CPCS at New Delhi during February 17-20,2003
- Invited to give a talk at Pandharpur College of Arts Science and Commerce on "Tissue Banking and Engineering" February 24-25,2003

Bhaskar Saha

- Immunology Study Group, West Bengal. Bose Institute. November 2002. CD40 signaling in *Leishmania*-infected macrophages.
- Roving Seminars in Immunology for Teachers. School of Health Science. Pune. December 2002. Host-parasite interaction.
- University of Hull, UK: April 2003. Impairment of CD40 signaling as an immune evasion strategy of *L major*.
- London School of Hygiene and Tropical Medicine, UK. April 2003. CD40 signaling is impaired in *Leishmania*-infected macrophages.

Arvind Sahu

- Structure-function studies on viral homologs of complement control proteins : May 2002 : Department of Infectious Diseases, University of Pennsylvania, Philadelphia, PA 190104, USA.

Samit Chattopadhyay

- Delivered popular lecture as invited speaker at NCCS on the occasion of "50th years of DNA double helix". The topic was "Chromatin modulation and its implication in disease". (February 15, 2003)

Seminars given at NCCS by visiting Scientists:

Dr. Tim Paget
University of Hull, Hull, UK.
Applications of Bacteriophage Antibody Display Technology to Clinical Microbiology,
26th November, 2002

Prof. Umesh S. Deshmukh
University of Virginia School of Medicine
Autoantibody Diversification in Systemic Lupus Erythematosus,
19th December, 2002

Dr. Ashok Kulkarni
The National Institutes of Health, Maryland, USA
Functional Genomics: A Gold Standard for In-vivo Gene Function Analysis
16th December, 2002

Dr. Rahul Bakshi
Johns Hopkins University, School of Medicine, USA.
Trypanosoma brucei topo I : a twisted tail,
11th February, 2003

Dr. Nagendra Hegde
Oregon Health & Science University, Portland, Oregon.
Immuno evasion: arm-twisting the MHC to fit a viral lifestyle
13th February, 2003

Dr. Hari S. Sharma
Institute of Pharmacology, Erasmus University, Netherlands
DNA Micro-array
21st February, 2003

Prof. Aaron Lewis
Nanonics Imaging System
16th July, 2003

Prof. Srinivas Pentyala
School of Medicine, State University of New York, USA.
Structure and function and control of phospholipase C-delta 1
17th July, 2003

Conferences/workshops attended:

Jayant M Chiplonkar

XXVI All India Cell Biology Conference and Symposium, 12-14 December 2002, ACTREC, Navi Mumbai.
Poster presentations: Epithelial mesenchymal transition in cervical carcinoma cells.
Authors: Madhura R. Vipra and Jayant M. Chiplonkar

Lalita S Limaye

XXVI All India Cell Biology Conference and Symposium, 12-14 December 2002, ACTREC, Navi Mumbai.
Poster presentations: Use of membrane stabilizers and bio-antioxidants in the conventional freezing medium to improve the quality of the frozen haematopoietic graft. Lalita M. Sasnoor, Vaijayanti P. Kale and Lalita S. Limaye

Mohan R Wani

XXVI All India Cell Biology Conference and Symposium, 12-14 December 2002, ACTREC, Navi Mumbai.

Anjali Shiras

Participated in Symposium on
"Current Trends in Biology" held at CCMB Hyderabad in October-2003.
Participated in Workshop on "Hands-on Training" on DNA Microarrays held at NBRC, Manesar, Haryana in June-2003

Gopal C Kundu

1. Osteopontin regulates activation of promatrix metalloproteinase-2 through nuclear factor-κB-mediated induction of membrane type 1 matrix metalloproteinase in B16F10 cells. Third International Conference on Osteopontin and Related Proteins, May 10-12, 2002, San Antonio, Texas, USA (Invited talk).
2. Osteopontin induces nuclear factor-κB-mediated promatrix metalloproteinase-2 activation through IκBα/IKK signaling pathways. XXVI All India Cell Biology Conference and Symposium, December 12-14, 2002, ACTREC, Navi Mumbai.
3. Matrix metalloproteinase-2: mechanism and regulation of NF-κB-mediated activation and its role in cell motility and ECM-invasion. 6th International Symposium on Biochemical Roles of Eukaryotic Cell Surface Macromoleculaes, January 16-18, 2003, Indian Institute of Chemical Biology (IICB), Kolkata (Invited talk).
4. Matrix metalloproteinase-2: mechanism and regulation of NF-κB-mediated activation. XIth Annual Meeting of Molecular Immunology Forum, January 31-February 2, 2003, Centre for Cellular & Molecular Biology (CCMB) and Shantha Biotechnics Pvt. Ltd., Hyderabad (Invited talk).

Ramesh R Bhonde

1. All India Cell Biology Conference Dec 11-13, 2002 ACTREC Mumbai. (Oral Presentation) "Encashment of intra-islet precursor cells from diabetic mouse pancreas for islet generation – A new dimension in diabetes control" Meenal Banerjee and R. R. Bhonde.
2. National Symposium on Development and Reproduction March 7-8, 2003 Dharwad (Poster Presentation) "Analysis of factors from ICS inducing pancreatic regeneration in vivo" Sameet Mehta, Sanjeev Galande and R. R. Bhonde.

YS Shouche

World Science Festival-2003 & International Dialogue to celebrate golden jubilee of DNA Double Helix discovery, at New Delhi February 2003.

D Mitra

Studies on anti-HIV activity of *Calophyllum inophyllum* Linn and *Calophyllum apetulam* wild. S. P. Joshi, S. R. Kulkarni, D. Ravi and D. Mitra. 2nd World Congress on Biotechnological Developments of Herbal Medicine. 20-22nd Feb, 2003, Lucknow, India.

Bhaskar Saha

FIMSA Conference on Molecular mechanism of infection and immunity. 2002. Bangkok. K Venuprasad and Bhaskar Saha. Neutrophil macrophage interaction controls Leishmania infection.

Arvind Sahu

1. Arvind Sahu, "Structure-function analysis of viral homologs of complement control proteins".
   Name: First International Senior Research Fellows Meeting
   Duration: 29th April – 1st May 2002
   Venue: Royal College of Physicians, 11 St Andrews Place, Regent's Park, London NW 1 4LE.
2. Arvind Sahu, "Viral complement control proteins: 'the mask of self' for viruses".
   Name: First Indian Senior Fellow Meeting
   Duration: 3rd April – 4th April 2003
   Venue: ICGEB, New Delhi.

3. Jayati Mullick, "Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) open reading frame 4 protein is a functional homolog of complement control proteins"
   Name: First Indian Senior Fellow Meeting
   Duration: 3rd April – 4th April 2003
   Venue: ICGEB, New Delhi.
4. Akhilesh K. Singh, Jayati Mullick, and Arvind Sahu, "Molecular cloning and expression of Herpesvirus saimiri complement control protein homolog"
   Name: First Indian Senior Fellow Meeting
   Duration: 3rd April – 4th April 2003
   Venue: ICGEB, New Delhi
5. Archana Kadam and Arvind Sahu, "Identification of peptide inhibitors using phage-displayed random peptide libraries for factor B: An approach for specific inhibition of alternative pathway activation".
   Name: First Indian Senior Fellow Meeting
   Duration: 3rd April – 4th April 2003
   Venue: ICGEB, New Delhi

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