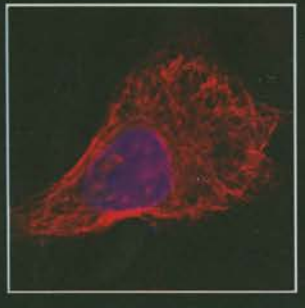
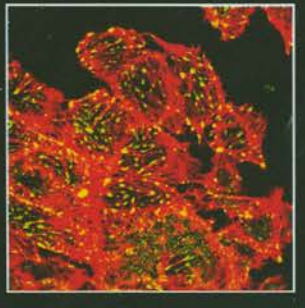
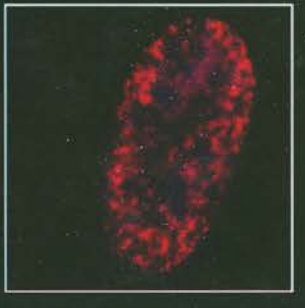
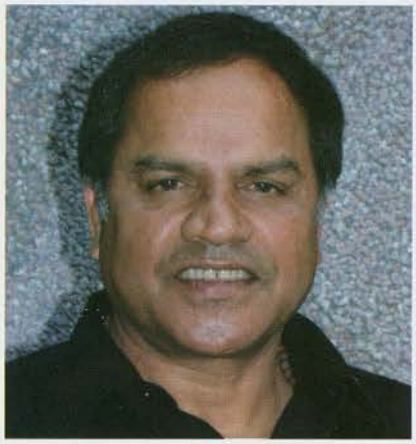
# Annual Report 2001–2002


nccs

NATIONAL CENTRE FOR CELL SCIENCE

## Contents



## From Director's Desk

It is my pleasure to present the Annual Report of National Centre for Cell Science (NCCS) for the year 2001-2002. In our continued mission to research and development in various areas such as Stem Cell Biology, Cancer Biology, Cell Biology, Immunology, Diabetes and HIV Biology and Teaching & Training. We have achieved significant progress in all our endeavours. We also serve as National Cell Repository and this year we have supplied 617 cell lines to about 110 scientific institutions and have initiated a programme on establishment and characterization of new cell lines.

In cell biology, cell migration has been shown to play a critical role in tissue formation, angiogenesis, tumorigenesis and wound healing. Our studies revealed that the osteopontin induced activation of matrix metalloproteinase-2, a protein that degrades extracellular matrix components, occurs through nuclear factor κB mediated pathway. In addition, we further characterized a bone derived chemoattractant protein involved in cancer cell migration. In our effort to understand wound healing using SiHa cell model system, the cell shape analysis revealed a distinct change in the cell morphology in response to the growth factor stimulation. Osteoclasts are multinucleated cells that are involved in bone resorption. We have shown that interleukin-3 inhibits the osteoclast formation mediated by receptor activator of NF-κB ligand (RANKL) as well as other proinflamatory cytokines.

Our interests in cancer biology have been focused on the tumour formation, discovery of genes involved in tumorigenesis, role of p53, signal transduction pathways and new factors responsible for tumour cell migration. Though two dimensional cell cultures are useful in acquiring insight into tumour biology, we have initiated studies on three-dimensional cultures, which would be a better model system for understanding tumorigenesis. Earlier we have identified a 600 bp gene from mouse melanoma clone M3. This has now been mapped to a 3.4 kb transcript located in the X-chromosome.

The molecular basis of the function of tumour suppressor p53 is still not fully understood. Our experiments with antisense p53 exhibited a differential inhibition of endogenous p53 in different cancer cell types. Our studies with anti-cancer drugs revealed that the cells expressing wild type p53 were more sensitive to DNA damaging and protein synthesis inhibitors and the mechanism appears to be different.

Oxidative stress stimulates a variety of receptors by inhibiting protein tyrosine phosphatases. Interestingly, α-haemolysin, a membrane binding protein is able to induce signal attenuation even in the presence of adverse agents such as hydrogen peroxide. In another study, role of antioxidants such as sodium pyruvate, β-carotene, rutin, quercetin in suppressing hydrogen peroxide induced apoptosis was studied. Our experiments suggest that sodium pyruvate could be exploited for prevention of neurodegeneration. In this context, bitter gourd extract displayed a strong growth inhibitory of breast cancer cells through membrane disruption and necrosis. We have also initiated studies on the role of caveolin and Syk in tumorigenesis and signal transduction.

I am happy to mention here that NCCS has further strengthened the stem cell biology programme, in an effort to understand and develop a knowledge base regarding the differentiation of embryonic stem cells into various lineages. It should be recalled that NCCS has mastered the cryopreservation and revival of stem cells, which led to a successful transplantation of autologous marrow cells in a neuroblastoma child. Our studies on erythropoietin treated bone derived mononuclear cells have shown secretion of transforming growth factor β1 that led to activation of multiple signal transduction pathways. In order to further strengthen our base, current research is being focused on differentiation of pluripotent ES cells into wide variety of cell types. As a first step, we have initiated studies on the role of various neuronal specific genes expressed at different times during embryonic development. In addition, in relation to cardiomyocyte differentiation from ES cells, we have observed spontaneous beating areas, as early as day 2 and there is enough evidence of the formation of pace making centres.

NCCS has well-established scientific programmes to address diabetes, which is today's major concern. Our interests include the identification of new factors affecting the growth of pancreatic β cells and islet neogenesis. Analysis of a previously identified fraction responsible for pancreatic regeneration has revealed that one of the components has high homology with soybean trypsin inhibitor. We have also developed a methodology for regeneration of islets from pancreatic ductal cells.

After diabetes, malaria is a serious public health concern. During the course of *Plasmodium falciparum* infection, there is an impairment of immune response and the mechanism involved is not clear. Our studies suggest that immunosuppression induced by hemozoin, an important catabolite released into blood, is due to altered cytokine production. In addition, understanding the mid-gut flora of mosquito and identification of new bacteria is important to unravel the host-parasite relationship. In this regard we have identified a new bacterial species, *Aeromonas culicicola* and developed a new PCR based technique for the identification of *Ochlorobactrum* species. In another study on insects, we have identified the transcription start site for *Drosophila melanogaster* hexokinase promoter.

The incidence of HIV infection has reached alarmingly high levels worldwide and India is not an exception. We have been able to demonstrate that Nef and Tat, the two most important regulatory proteins necessary for HIV pathogenesis, interact physically and positively regulate viral gene expression. Our initial efforts on screening of anti-HIV activity from plant and marine extracts show positive results. Further we show that AT rich matrix attachment region (MAR) plays an important role in the HIV-1 promoter mediated processive transcription at a distance.

NCCS has a strong immunology programme which is devoted to understand the basics of immune response towards the pathogenesis of mycobacterium, leishmania, viruses and malaria. We have identified and thoroughly characterized two co-stimulatory molecules from macrophage and B cells, which are lysosomal associated membrane protein-1 and gp96, that elicit Th1 and Th2 like responses respectively. In another study, we observed that human neutrophils, a part of afferent limb, plays an important role in immune response against leishmania and this precedes T-cell response. Studies are also focused on how viruses evade the complement attack. In an attempt to understand the mechanism at the molecular level, we have cloned the complement control protein homologues of vaccinia, herpes virus saimiri and Kaposi's sarcoma associated herpes virus and observed complement inhibitory activities in these proteins.

The chromatin architecture plays an important role in the regulation of gene expression. The anti tumorigenic MAR-binding protein SMAR1 has been shown to directly interact with p53 and arrest cell cycle at G2/M phase. SATB1, the hallmark BUR-binding protein, has been shown to exist in two forms that seem to be generated by two different mechanisms.

This year NCCS has 40 publications in national and international journals with an average impact factor of 3.007. Our average publication impact factor per scientist is 4.005. This year the scientists at NCCS continued to attract peer reviewed extramural funding and trained 12 young graduates. Currently, there are 71 doctoral research fellows and 4 research associates engaged in the ongoing research. In addition, our library and documentation facility has procured 670 journals and 90 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 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). This year NCCS has procured 14 new cell lines, which are available for distribution upon request. At present, the total number of culture strain is 1141, of which about 314 are available for distribution to users on registration. Approximately 510 researchers from 275 institutes have registered with NCCS for the same. During the year 2001-2002, the cell repository of NCCS has supplied 617 cell cultures comprising of 148 different cell types to 110 research organizations in the country. The repository has initiated programmes to develop, immortalize and characterize cell lines from different tissue/tumour types. We have also planned to generate lineage specific cell lines from mouse and human embryos and preserve them for future use.

## 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. The following are the courses conducted during the year 2001-2002:

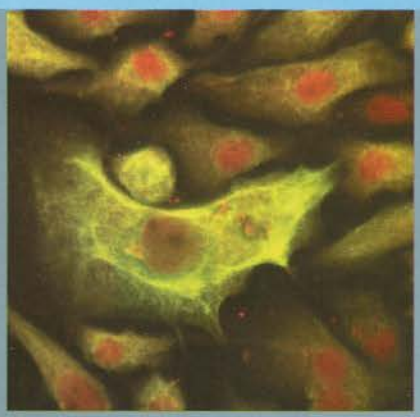
⇒ **Principles of Animal Tissue Culture**

⇒ **Basic Techniques in Animal Tissue Culture**

The total number of people trained in the above programmes during this year is 12. In addition, the centre has also attracted about 20 summer trainees from various universities all over India. The projects were appreciated by scientists of other institutes and many of the summer trainees showed interest to pursue future Ph.D. programme at NCCS on those projects.

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 local collages.

During this year 19 new Junior Research Fellows joined at NCCS for Ph.D. programme. The total strength of research fellows reached to 71. In addition four 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

Although a considerable amount of research and epidemiological data is available in India regarding breast cancer, the same is not the case for ovarian cancer, which is believed to have a similar etiology. Ovarian cancer ranks as the fourth leading cause of cancer-related deaths in women and represents the most fatal gynaecological malignancy. The poor prognosis in ovarian cancer in a majority of cases has been attributed to an inability to identify definitive premalignant lesions as well as lack of diagnostic tools for early detection.

Primary ovarian tumour cultures and cell lines can be used advantageously in numerous studies including the assessment of molecular events associated with ovarian tumour progression and mapping of new suppressor genes or oncogenes. Permanent cell lines have a number of advantages over primary cell cultures or fresh clinical material, viz. the purity and reproducibility of research material, elimination of necrotic growth as well as the ability to generate sufficient quantities of DNA, RNA and protein extracts for numerous studies. 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. The ovarian cell cultures, which will be established, would also find several applications including pharmacological testing of new anti-cancer drugs. The resulting cell lines/ models shall be available not only for any further work at the NCCS, but will also be made available to other researchers who wish to use the specific cell system for their research.

### Aims

1. To enrich cell repository at NCCS by establishing cell lines from ovarian epithelia including normal epithelium (often referred to as OSE – ovarian surface epithelium) and benign and malignant epithelial tumours. In addition, establishment of lymphoblastoid cell lines from the same individuals will also be attempted. These would serve as normal genotypic controls in monitoring the changes due to *in vitro* culture and/or transition of normal cells to a tumorigenic phenotype.

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

**Work achieved**

Several samples have been procured and processed towards achieving the above objectives. Primary cultures have been successfully established from normal ovarian epithelia and stroma, tumour epithelia (Fig. 1), and stroma from primary sites as well as from secondary sites such as omentum and ascitic fluid. Several of the cultures established have overcome the first crisis period (M1) that sets in around the eighth passage; some of them have been maintained over more than 20 passages. Whenever possible, aliquots of each sample and passage are being cryopreserved. Immortalization using SV40 large T antigen is currently being standardised in order to establish cell lines with extended life span. EBV transformation of B lymphocytes from peripheral blood has been established (Fig. 2). Such lymphoblastoid cultures are of special significance in the establishment of paired cell lines that prove to be useful in the genetic analysis of specific tumours.

The phenomenon of epithelial to mesenchymal transition (EMT) has been observed in both normal and tumour epithelia. Normally associated with wound healing and metastasis, it involves a transition of the epithelial phenotype into a motile mesenchymal one. Using confocal microscopy, the corresponding change in cytoskeletal protein expression from keratin to vimentin has been tracked (Fig. 3). EMT has been largely held responsible for the hitherto inability of researchers to establish cell cultures/lines that retain characteristics of the normal OSE *in vivo*. It would be interesting to further study the mechanistic differences occurring in the two cell types derived from normal and transformed epithelia.

The primary stromal cultures established have yielded a pliable cell culture system of diploid fibroblasts. Most cultures have, so far, shown a capability of around 40-60 population doublings before senescence sets in (Fig. 4). In adult human tissues, such a culture system is rivalled only by that of skin fibroblasts. This system thus developed is being further tested for several applications such as use as feeder layers for fastidious cells, investigating stromal-epithelial interactions, etc.

**Future plan**

Immortalization/life-span extension and detailed phenotypic and functional characterization of the cell cultures thus established will be undertaken. This would provide valuable defined research resources useful for the understanding of the underlying mechanisms in ovarian cancer such as EMT and stromal-epithelial interactions in progression of the cancer.

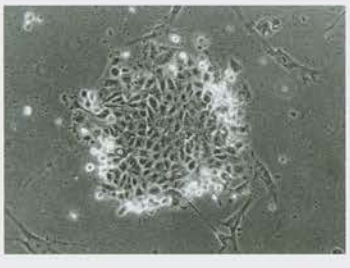


Fig. 1: Epithelial colony developing from an ovarian adenocarcinoma (100X)

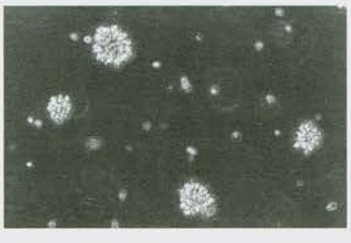


Fig. 2: Lymphoblastoid cell line – ON3LBL (100X)

Fig. 3: Vimentin expression seen after epithelial Mesenchymal transition (EMT) in normal ovarian epithelial cells

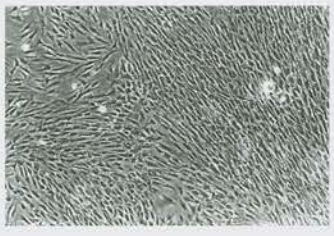
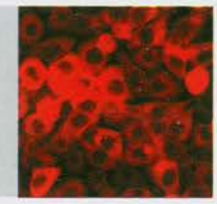


Fig. 4: Ovarian stroma derived culture – ON6 (100X)



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

Epithelial cells form a tight continuous layer of cells covering a body surface or lining a body cavity. However, cohesiveness of epithelial cells gets modulated at times and they are induced to dissociate, disperse and migrate. Epithelial-mesenchymal transition (EMT) provides one of the most striking examples of cellular transformations leading to cell motility, and characterized by loss of epithelial and acquisition of mesenchymal features and plays a central role in many physiological processes such as embryogenesis, angiogenesis, wound healing and tumour invasion and metastasis. Cells loose their cohesive nature and are converted into individual motile fibroblastic cells, and could result from interaction of more than one regulator. Many factors are thought to be involved in the acquisition of cell motility. Growth factors, primarily known as regulators of cell proliferation can also stimulate cell motility in vitro. Growth factors are also essential for tissue repair and morphogenesis, role in the expansion, invasion and metastasis of tumour cells. A number of growth factors like scatter factor, epidermal growth factor (EGF), transforming growth factor (TGF$\beta$) have been shown to induce epithelial mesenchymal transition both *in vivo* and *in vitro*. Some types of tumour 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 the possible 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

We have shown the serum-dependent effects of EGF on proliferation and invasion of human cervical carcinoma cell lines SiHa.

Our earlier work has reported the enhanced migration and invasion by SiHa cells when treated with EGF. A strong synergistic effect with serum was noted. The cells typically underwent epithelial mesenchymal transition like changes characterised by up-regulation mesenchymal markers like vimentin and fibroblast surface protein and down regulation

of epithelial makers like cytokeratins, epithelial specific antigen. Cell shape analysis revealed a distinct change in the cell morphology in response to the growth factor as analyzed by the image analysis system. The cells showed enhanced motility and invasion capacity in response to the growth factors during in vitro wound healing assay, invasion assay, and colony growth in EHS-ECM gel. The cells were extensively characterised by immunocytochemistry for the different markers associated with cell-cell adhesion and migration like uvomorulin, beta catenin, desmosomal proteins, desmoplakins, actin, vinculin, talin, β1 integrin, etc. The results seen by immunocytochemistry were confirmed by western blotting.

We also analysed the cell cycle progression of cells undergoing epithelial mesenchymal transition like changes and migration by blocking SiHa cells at different points during ell cycle. Cells synchronized at serum restriction point (R) in G1, when induced to migrate delayed their progression thorough the G1 phase compared to the non migrating cells present in the same population indicating that in cells not committed to the cell cycle, migration gets the priority. However in cells committed to the cell cycle the progress of migrating cells through the replicative phase of cycle was unaffected when cells were induced to migrate at G1/S. The progress through G2 however was slower again for the migrating cells but the migration was completely halted when cells reached mitosis and mitosis was completed normally. Thus we can say that cancer cells if come across a mitogenic signal during G0 or G1 undertake migration and showed a delayed entry into the cell cycle. However, once committed to cell division the cells continue their progress through the cell cycle, migrating simultaneously except at the time of mitosis when the migration halts. The data indicated that carcinoma cells continued their migration irrespective of the stage they were in the cell cycle except Mitosis.

Further characterization to assess the possible role of matrix metalloproteinases in invasion, we analyzed the conditioned medium by gelatin zymography. It showed presence of MMP-2 which showed a significant increase in pro form as cells were induced to migrate. Both pro- and active MMP-2 were detected by western blotting of the migrating cells. Similarly, MMP-2 could be detected by immunocytochemical fluorescence of SiHa cells. Thus, we have gathered enough evidence to demonstrate the epithelial mesenchymal transition associated changes in a cervical

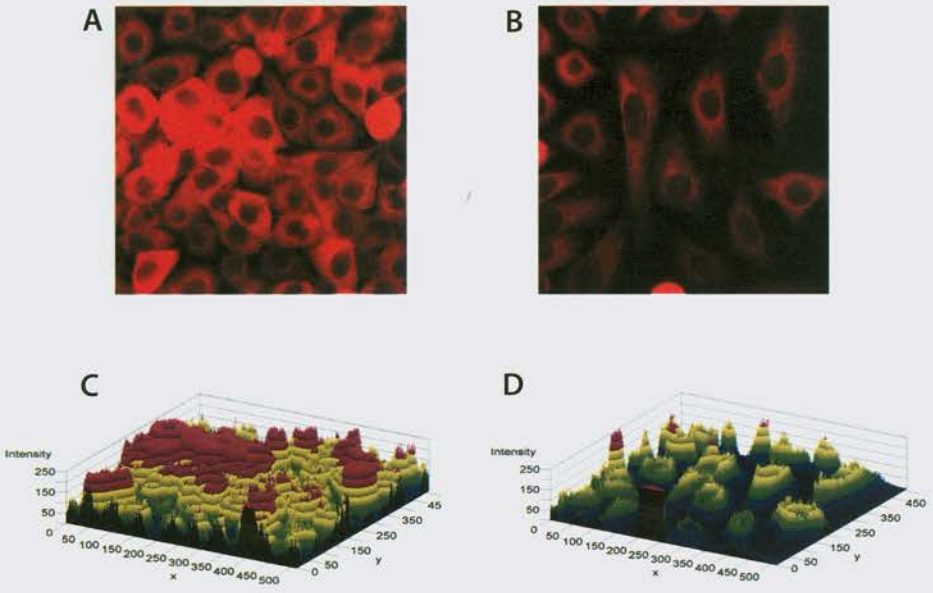


Fig. 1: Down regulation of cytokeratin 18 in the cells induced to migrate in the wound healing assay, as analysed by immunofluorescence and 2.5 D reconstruction of the fluorescence intensities by laser confocal microscope. (A) Cells in the intact distal monolayer (non-migrating) 24 Hours post wound and (C) their fluorescence intensity distribution. (B) Cells migrating into the wound 24 Hours post wound and (D) their fluorescence intensity distribution.

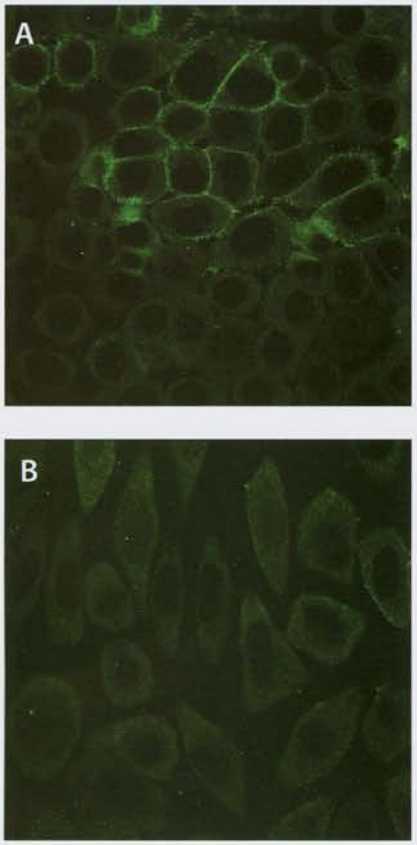


Fig. 2: Altered expression of a member of zonula adherens (beta catenin) in the cells induced to migrate as analysed by immunofluorescence. (A) Non-migrating cells in the intact distal monolayer, 24 Hours post wound. (B) Cells migrating into the wound, 24 Hours post wound.

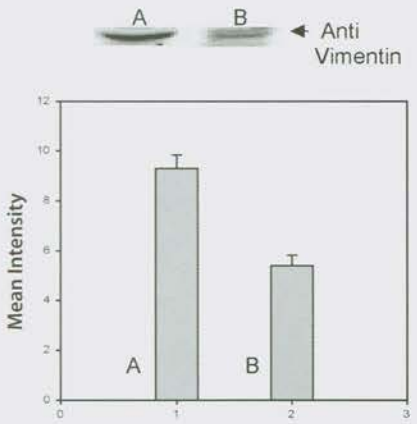


Fig. 3: Up regulation of the mesenchymal marker vimentin in the cells induced to migrate as analysed by western blotting and densitometry. (A) Cells migrating into the wound, 24 Hours post wound. (B) Non migrating cells in the intact distal monolayer, 24 Hours post wound.

carcinoma cell line SiHa. These findings are all the more important as cervical carcinoma is the second most prevalent cancer found in women all over the world.

**Future plan**

We would now assess the role of EGF receptor tyrosine kinases and how and when the signalling pathways trigger either mitogenic or motogenic response, as higher concentrations of EGF have been shown to induce mitogenic response in these cells.

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## Role of stromal cell-mediated signalling cascade in the development of stem cells

Development of haematopoietic stem cells (HSC) into fully differentiated blood cells is a multi step process. This functional maturation takes place in the bone marrow microenvironment composed of many types of stromal cells and the extra-cellular matrix (ECM) molecules secreted by them. In a steady state condition the HSC are believed to be present in a quiescent state. Recent reports, however, indicate that HSC do undergo a continuous, though very slow, turn over. Since body needs a constant supply of differentiated cells, it is necessary that a pool of HSC be maintained throughout the life. The self-renewal and commitment to differentiation have to be balanced to achieve this. Thus, a finely tuned and tightly controlled process is involved in the generation of blood cells from HSC.

Several experiments have underscored the role of stromal cells in the development of haematopoiesis. Our data strongly indicate that the signals generated by stromal cells indeed affect the fate of the stem cells exposed to them. We are, therefore, exploring the role of stromal cell mediated signalling cascade in the development of stem cells.

### Aim

To understand the nature of stromal cell mediated signalling pathways playing deterministic role in the stem cell fate.

### Work achieved

Our earlier experiments have shown that treatment of bone marrow derived stromal cells with transforming growth factor (TGF$\beta$1) makes them more competent to support the growth of stem cells. We, therefore, carried out several experiments to footprint the signalling pathways involved in the process. We have used pharmacological modulators of various pathways and colony formation or $CD34^+$ cell proliferation as the read out systems.

The treatment of stromal cells with inhibitors of nitric oxide (NO) signalling such as cPTIO prior to TGF$\beta$1 treatment inhibited the proliferation of $CD34^+$ stem cells co-cultured with them while the treatment with stimulators like SNN or SNAP stimulated the $CD34^+$ cells. Our results indicate that activation of nitric oxide signalling pathway could be one of the important down stream events in the TGF$\beta$1 mediated signal transduction in the context of haematopoietic support.

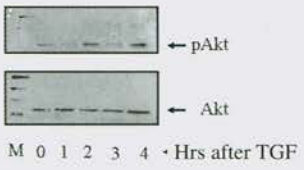


Fig. 1: Treatment of adult bone marrow derived fibroblasts leads to phosphorylation of Akt-Ser.

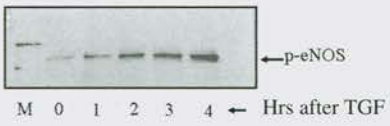


Fig. 2: Treatment of foetal bone marrow derived fibroblasts with TGF b1 leads to both increased expression and phosphorylation of eNOS.

Using western blot analysis we found that treatment of stromal cells with TGFβ1 leads to activation of Akt pathway as indicated by the presence of phosphorylated Akt in TGF b1 treated cells when probed with phospho-Akt-specific antibody (Fig. 1). We also found that the treatment of stromal cells with PI3K inhibitor LY 294002 prior to TGFβ1 treatment leads to quiescence of $CD34^+$ cells exposed to them. Additionally, we detected the phosphorylation of ERK+, SAPK/JNK as well as p38 MAPK in the stromal cells as a result of TGFβ1 treatment. This activation was however transient, declining 1-2 hours after treatment as against sustained activation of Akt pathway.

We also found by western blot analysis that TGFβ1 treatment leads to the phosphorylation of eNOS, indicating its activation (Fig. 2).

It will be interesting to examine whether these various pathways interact with each other in a temporal manner in stem call fate determination or each pathway plays a distinct role in the process.

**Future plan**

1. Investigation of the role of eNOS mediated signalling pathway in haematopoiesis.

2. Examination of the cross-talk amongst various signalling pathways in the stromal cells and the net effect of the cross-talk on the stem cell fate.

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## Studies on Caveolin expression in tumour cells and its role in tumorigenesis

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 kDa protein, caveolin. Of the three isoforms 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 include a 41 amino-acid region that self-associates to form homo-oligomers which are the assembly units for the formation of caveloae and a 20 amino acid long cytosolic, membrane proximal "scaffolding domain" which binds several important signalling 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 "centres for signal transduction". Caveolins, the primary coat proteins of plasmalemmal caveolae, are implicated in cholesterol trafficking, signal transduction and tumour suppression. Their genes are localized to the suspected tumour suppressor locus, 7q31 and are down regulated in cancers like leukaemias, adenocarcinomas, etc.

### Aim

The aim of the project is to study the regulation of Caveolin expression in tumour cells and its role in tumorigenesis.

### Work achieved

MCF-7 is a mammary adenocarcinoma cell line which we found to be positive for Caveolins 1, 2 and 3, at the mRNA level (Fig. 2 upper panel). MCF-7 is an estrogen receptor positive ($ER^+$) cell line and estrogen is known to down regulate caveolin expression. We examined this effect using MCF-7 transfected with antisense ER (Courtesy of Dr. Indraneel Mitra, TMH, Mumbai). We also studied the effect of Progesterone and Estrogen inhibitors on Cav-1 expression.

Immunofluorescence and Western blotting analysis showed that Caveolin-1 expression is up regulated in quiescent cells, as evidenced by effect of serum starvation (Fig. 1 and Fig. 2, middle panel). The CDK inhibitor, olomoucine, increased the protein levels of Caveolin-1, which is in accordance with the above observation (Fig. 2 lower panel).


Fig. 1: Effect of Serum Starvation on Caveolin-1 Expression: 24hrs growing MCF 7 cells serum starved for the indicated times, fixed and immunostained with anti Caveolin 1 polyclonal antibody.

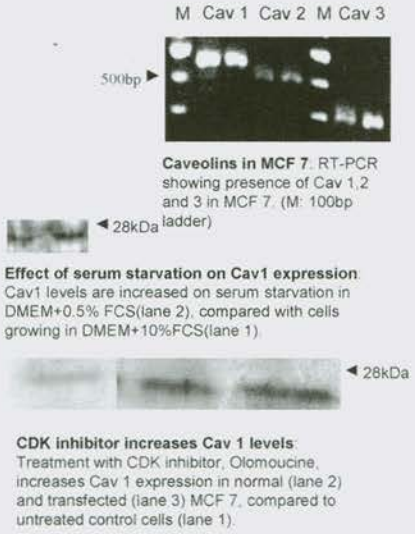
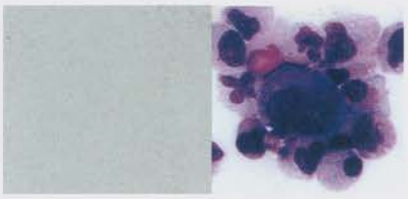



Fig. 2: Effect of serum starvation and cell cycle regulators on caveolin-1 expression in MCF-7 cells.

Treatment of MCF 7 with Progesterone and Estrogen inhibitors (Tamoxifen and 4-chloro phenyl acetate) also showed an increase in the levels of Caveolin-1 when compared with untreated control cells MCF-7 cells transfected with anti sense ER constructs also had elevated levels of Caveolin-1.

## Future plan

We will continue these studies on the regulation of Caveolin-1 expression in the tumour cells to understand the role of Caveolins in the tumour progression.



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## Studies on cryopreservation of haematopoietic cells

Cryopreservation of haematopoietic cells is important for both clinical as well as research purposes. We have standardized the protocols of cryopreservation of haematopoietic cells. This technology was transferred to Armed Foreces Medical College jointly by NCCS, Cancer Research Institute and National Institute of Virology. A programmable freezer was purchased by NCCS and installed at AFMC. Haematopoietic stem cell banking work is being initiated there. In the research aspect our aim has been to get improved recovery post freezing. Freezing is a multiple stress phenomenon and causes thermal, osmotic, mechanical and chemical stress to the cells. We used physiologically acceptable additives to the conventional freezing medium and tested the efficacy of freezing by in vitro and in vivo assays. The samples used were mouse bone marrow and human haematopoietic cells like cord blood and foetal liver. The parameters studied before and after cryopreservation were CFU-S assay, long term cultures and engraftment assays. The results suggest that a combination of Catalase and Trehalose in the conventional freezing medium is beneficial to the cells.

### Aims

1. Setting up long term cultures from both MNCs and $CD34^+$ cells of fresh and frozen cord blood and foetal liver and weekly estimation of cells by CFU assay.

2. Studying short term engraftment of fresh and frozen cells by CFU-S assay.

### Work achieved

1. AFMC has frozen 30 cord blood samples so far using this technology. AFMC has successfully cryopreserved and transplanted autologous marrow to a child suffering from Neuroblastoma. The samples are presently stored at -80°C as they have not yet got their liquid nitrogen plant sanctioned. We are helping them out in standardization of CFU assays.

2. Long term cultures were set up by recharging fresh and frozen cells on irradiated M210B4. A total of 23 CB and 11 foetal liver samples could be successfully grown in long term cultures for a period of 10 to 12 weeks both before and after freezing. Two each of these cultures were set up from isolated $CD34^+$ cells. Representative result of CFU obtained at the termination of the experiment is shown in Fig. 1.

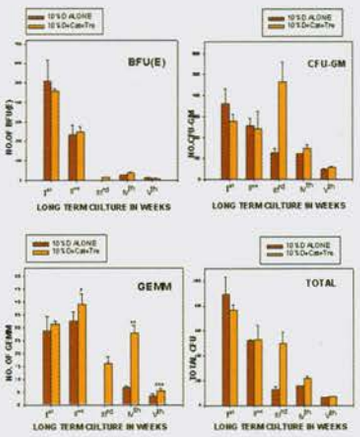



Fig. 1: Differential and total colonies formed per week in LTBMC by frozen foetal liver haematopoietic cells.

**Table 1**: Mouse Bone Marrow CFU(S) assay Pre and Post Cryopreservation.

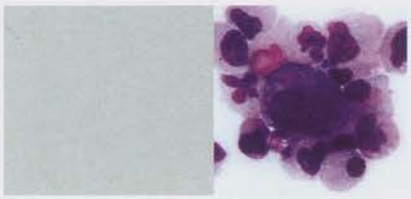
| Types of Cells Infused | Number of Animals Infused | Average CFU(S)* |
|---|---|---|
| Fresh Bone Marrow | 5 | 14.8±3.70 |
| Frozen (10% DMSO alone) | 5 | 15.4±6.06 |
| Frozen (10% DMSO+Cat+Tre) | 5 | 19.8±2.68 |
| Frozen (10% DMSO+Cat+Taur) | 5 | 16.6±3.71 |
| Frozen (10% DMSO+α+Taur | 5 | 15.2±4.08 |
| Frozen (10% DMSO+α+Tre) | 5 | 17.25±2.75 |

* Values obtained after deducting endogenous colonies formed on spleen of irradiated and PBS infused mice

3. Short term engraftment of fresh and frozen mouse bone marrow was assessed by CFU-S assay. Results indicate that cells frozen with additives are better protected (Table 1).

**Future plan**

In vitro homing experiments will be performed by labelling fresh and frozen cells with $Cr^{51}$ and then studying their adhesion pattern.



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## Cryopreservation of Bone marrow with membrane stabilizers and antioxidants as additives in the conventional freezing medium

This project is funded by DRDO Life Science Research Board for a period of 3 years from April 2000-March 2003. In this project the aim is to optimize freezing protocols by use of membrane stabilizers and bioantioxidants in the conventional freezing medium and to study the behaviour of fresh and frozen cells. The parameters studied are status of growth factor receptors, adhesion molecules, homing molecules, growth factor responsiveness and engraftment potential. It is also proposed to make attempts to reduce or replace the DMSO with sugars and other cryoprotectants. The data obtained so far indicates that a combination of antioxidants and membrane stabilizers restores the expression of adhesion molecules, growth factor receptors to some extent and improves the growth factor responsiveness. Experiments on reduction of DMSO are in progress.

### Aims

To study the behaviour of fresh and frozen cells with special reference to;

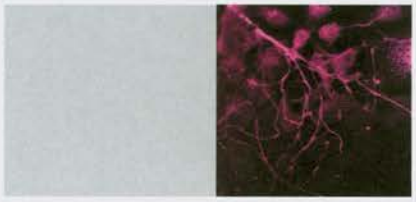
1. Growth factor responsiveness and growth factor receptor status
2. Adhesion molecule and homing molecule status
3. *In vivo/in vitro* engraftment

### Work achieved

Cytokine receptor status of frozen $CD34^+$ isolated cells from cord blood and foetal liver MNCs is very well conserved with additives in the freezing mixture. However the beneficial effect of additives in conserving the IL-3R and SCF-R receptors of frozen TF-1 cell line is marginal. Growth factor responsiveness of frozen cord blood mononuclear cells is restored to some extent when membrane stabilizers and antioxidants are present in the conventional freezing medium, as indicated by CFU assays. Long term culture initiating cells of both cord blood and foetal liver are better protected due to additives in the cryopreservation medium. Additives in the freezing medium improve adhesion molecules especially l-selectin expression of frozen human haematopoietic cells. It is possible to store cord blood and foetal liver haematopoietic cells at -80°C and additives improve the CFU ability of such stored cells. In our experiments a combination of 5% DMSO and vitrifying concentrations of sugars has so far not proven to be effective in freezing TF-1 cell line.

### Future plan

In vivo experiments in mice to study engraftment potential of frozen marrow using Ly5.1 as donors and Ly5.2 as recipients.



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

Stem cells are unique cell population capable of self-renewal, differentiation and regeneration. In vertebrates, the stem cells could be categorized into various types based on their source of derivation, (i) the embryonic stem cells (ES Cells) residing in the developing embryo, and (ii) the foetal and adult stem cells residing in distinct subsets in foetus and adult respectively. Fascinating though, the discrete population of ES cells residing in the inner cell mass (ICM) of blastocyst bears the complete repertoire of the complex organizational blueprint of an organism. The embryonic stem cell research is quite well known to the scientific community since the establishment of the murine ES cell line about twenty years ago. Subsequently, attempts have been made to obtain ES cells from other rodents and primate species. In fact, a major breakthrough has been accomplished in this field at the end of 1998 with the advent of human ES and EG cells reported respectively by two independent groups. The promising potential of ES cell system has led to the discovery of (i) various cell signalling cascades and (ii) understanding their role during development by generating several transgenic and knockout mice. Although initial interest was centred on using these cells as vehicles for germ line transmission and analysis of specific traits, the recent focus has been extended to explore the ES cell system in understanding the early embryonic developmental processes. In this regard, our interest lies in the exploration of molecular basis of cell commitment and differentiation into various lineages using both murine and human ES cell system. 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, foetal and adult stem cells. This in future would help in cell replacement therapies in case of various degenerative diseases.

### Aims

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:** One of the most critical parameters in ES cells research is to maintain the cells in undifferentiated state. Failure to do so results in the loss of pluripotency. Accordingly, parallel to the widely used cytokine LIF (Leukaemia inhibitory factor) we are also investigating the influence of the marine mussel extracts (ME) on the retention of undifferentiated status. Using various concentrations of ME we observed ME to induce cytotoxicity compared to LIF, hence resulting in a concentration dependent increase in cell death. In contrast, the texture of ES cells was more compact compared to LIF treated ones and seemed undifferentiated in presence of ME (Fig. 1A-B). Further experiments would be carried out to obtain conclusive understandings in this context.

**Neural Differentiation:** ES cells are pluripotent and hence give rise to wide array of cell types upon differentiation. In order to establish the ES cell derived neuronal progeny and demarcate the cells of interest in the heterogeneous cell mass we have taken advantage of live reporter based cell trap system. 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.

*Nestin*: Previous studies on the nestin gene using transgenic mouse demonstrated the second intron to have the necessary enhancer motifs for driving the reporter gene expression in a neuronal specific manner. The human nestin intron II (1852 bp) fused to a heterologous 160 bp thymidine kinase (tk) basal promoter was subcloned into the pEGFP vector taking advantage of the fact that, the live EGFP expression does not interfere with the *in vivo* gene expression. Using this vector construct (hnestin-EGFP, Fig. 2A) the murine ES cells (D3) were transfected by electroporation and we have successfully obtained a number of stable neomycin resistant ES cell clones. Upon analysis these clones were observed to be EGFP positive at the undifferentiated state (Fig. 3A, B) supporting the earlier findings (Lenka et al., 2002). The neural differentiation (Fig 3C, D) was carried out by cell aggregation and retinoic acid induction. Detailed characterization of these cells is underway by both, immunocytochemistry and Fluorescent Activated Cell Sorting (FACS) analyses.

*Tyrosine hydroxylase (TH)*: We have taken 9 Kb of murine TH promoter region (Fig. 2B) to drive the EGFP expression in murine ES cells derived differentiated neuronal cells. Since, TH expression is confined to dopaminergic neurons of both CNS and PNS and the relative percentage of these neurons with respect to the entire neoronal population is quite insignificant in *in vitro* culture system, we would like to establish the culture conditions for efficient induction of the dopaminergic neurons from transgenic ES cell derived clones. We have been successful in obtaining several neomycin resistant clones and currently we are characterising these clones following similar approaches as has been described for nestin clones. TH is the rate limiting enzyme of catecholamine neurotransmitter biosynthesis. The degeneration of dopaminergic neurons is the root cause for the neurodegenerative disorder, the Parkinson's disease. Thus, the establishment of efficient dopaminergic neuronal differentiation from ES cells would help us to extend this investigation further in transplanting the cells and explore their potential in cell replacement therapy.

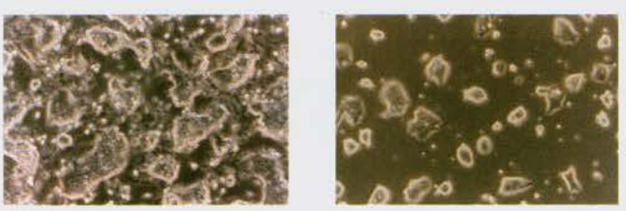


Fig. 1: Influence of factors on maintenance of undifferentiated state in ES cells: Colonies exhibit more compact morphology with Mussel Extract (B) compared to LIF (A).

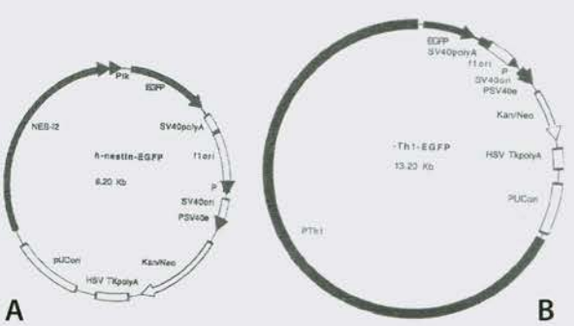



Fig. 2: The h-nestin-EGFP (A) and Th1-EGFP (B) vector constructs used for obtaining stable transfectants in ES cells.

**Cardiomyocyte differentiation:** The murine ES cells were maintained on fibroblast feeders and differentiated into cardiomyocytes by cell aggregation and plating. Spontaneous beating areas were seen on day 2 of plating of the embryoid bodies (EB). By day 5 a number of such areas were observed covering a vast area (Fig. 4) implying excitation spread through gap junctions. The beating pattern in various areas was either simultaneous or consecutive indicating the probable presence of different pace making centres. Futher investigation is underway to specify these cells by immunocytochemistry. Subsequent studies would be undertaken to isolate the cells and analyse the gene profile that would shed light on understanding the events leading to cardiac development during embryogenesis.

Fig 4: Cardiomyocyte differentiation from murine ES cells day 5 post-plating; the demarcated areas (black) showing different beating clusters within a single EB and the arrowhead indicating the cardiomyocytes.

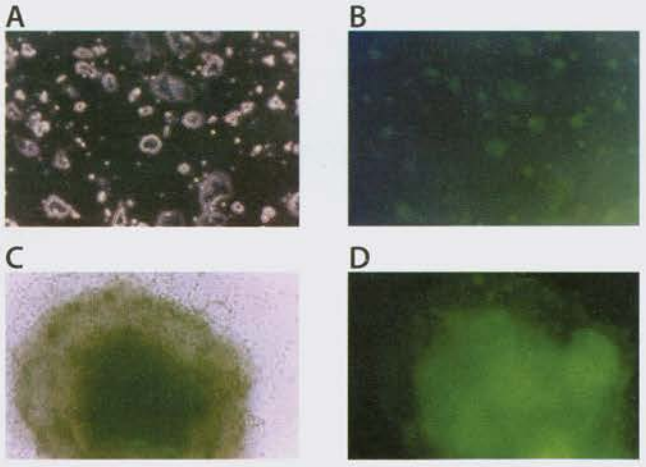



Fig. 3: EGFP expression in hNESEGFP transfected ES cell clones. A, B: Undifferentiated ES cells during propagation (A) show EGFP expression (B). C, D: Embryoid bodies exhibiting both EGFP positive (neural lineage) and EGFP negative (other lineages) cells during differentiation day 6 post plating. (A, C: transmission light; B, D: Fluorescent light).

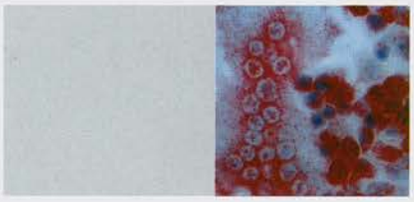
**Future plan**

1. Differentiate the ES cells into lineages other than neural and cardiac and carry out comparative in depth mechanistic understanding of cell fate commitment and specification.

2. Establishment of stem cell lines using surplus IVF human embryos and maintaining those in an undifferentiated state in long term cultures.

3. Differentiation of human ES cell lines into various lineages and their characterization and developing strategy for the enrichment and purification of lineage specific stem cells.

4. Exploration of transdifferentiation potential of lineage committed ES cell-derived stem cells as well as stem cells derived from human umbilical cord blood and other adult tissues/organs.



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## Regulation of osteoclast differentiation and activation by interleukin-4

Osteoclast, the cells that resorb bone play a crucial role in bone remodelling. Osteoclasts differentiate from haemopoietic precursors of the monocyte/macrophage lineage. To promote osteoclastogenesis two molecules, M-CSF and receptor activator of NF-κB ligand (RANKL) are essential and sufficient. M-CSF is required for the proliferation and survival of osteoclast precursors, while the TNF family member RANKL commits precursors towards formation of bone resorbing osteoclasts. RANKL induces osteoclast differentiation through binding to its receptor RANK on osteoclast progenitors and exerts its biologic effect via activation of NF-κB transcription factor. Mice deficient in both p50 and p52 subunits of NF-κB are osteopetrotic because of the failure in osteoclast differentiation. The similar phenotype was observed in c-Fos knockout mice, which can be rescued by Fra-1 as well as c-Fos over expression. The c-Fos is a component of the dimeric transcription factor AP-1. These results indicate that both NF-κB and AP-1 activation are necessary for osteoclast development. Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL that inhibits RANKL-induced osteoclast formation. TNFα, like RANKL also potently activates osteoclasts through a direct action independent of and strongly synergistic with RANKL. Increased osteoclasts activity is seen in osteoporosis, rheumatoid arthritis, bone metastasis and other skeletal diseases of clinical importance.

Osteoclast formation and bone resorption is regulated by many hormones, growth factors and immune cell-derived cytokines. The lack of clear mechanisms for many bone-active factors is largely attributable to the complexity of co-culture model previously used for osteoclastogenesis and bone resorption. The use of soluble RANKL in stromal cell free culture model will reveal the cellular and molecular mechanisms of action of these osteotropic factors.

Interleukin-4 (IL-4) is a pleiotropic immune cytokine secreted by the activated TH2 lymphocytes, mast cells and eosinophils. IL-4 influences immunologic and haemopoietic processes and also modulates macrophage function. Previous studies using co-culture model have shown that IL-4 inhibits osteoclast differentiation, however, the mechanism of action of IL-4 in osteoclastogenesis and bone resorption is poorly understood. In this study we report the role of IL-4 in RANKL and TNFα-induced osteoclastogenesis and bone resorption.

### Aims

1. To study the role of IL-4 in RANKL and TNFa-induced osteoclastogenesis in stroma free cultures of haemopoietic precursors.

2. To study the effect of IL-4 on mature and isolated osteoclast, and bone resorption.

3. To investigate further the molecular mechanism(s) of IL-4 action on RANKL and TNFa signalling pathways.

4. To investigate the role of IL-4 on expression of OPG and RANKL genes in osteoblasts.

## Work achieved

In our earlier studies we have shown that IL-4 has a direct action on osteoclast precursors and exerts a potent dose-dependent inhibitory effect on *in vitro* osteoclast formation and bone resorption in mice. Our data also showed that anti-IL-4 antibody completely neutralized the inhibitory effect of IL-4 on osteoclast formation. In further investigation, we examined the effect of IL-4 on osteoclast formation in the presence of cytokines such as TGFβ, prostaglandin $E_2$ and IL-1α. Recently, we have shown that these cytokines alone do not induce the osteoclast formation but they enhance the RANKL-induced osteoclast formation. These cytokines are known to mediate the cascade of molecular pathways resulting in the production of matrix-degrading enzymes. We observed that IL-4 inhibited the osteoclast formation induced by these cytokines (Fig. 1). These results suggest that IL-4 also inhibits osteoclast formation in the presence of co-factors.

TNFα is crucial to the pathogenesis of bone and joint destruction in rheumatoid arthritis and chronic inflammatory postmenopausal osteolysis. TNFα induces osteoclast formation by RANKL-independent mechanism. So we examined and compared the effect of IL-4 and OPG on TNFα-induced osteoclast formation. We revealed that IL-4 but not OPG inhibited the TNFα-induced osteoclast formation. Our data shows that IL-4 inhibits osteoclast formation induced by both RANKL and TNFα. Further investigation of effect of IL-4 on RANKL and TNF-α signalling pathways and mature and isolated osteoclast is in progress.

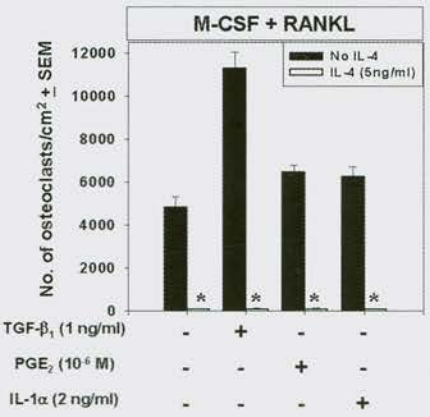


Fig. 1: Effect of IL-4 on RANKL-induced osteoclastogenesis in the presence of proinflamatory cytokines Non-adherent, M-CSF-dependent bone marrow cells were incubated in the presence of M-CSF, RANKL and proinflamatory cytokines including TGF-$\beta_1$, $PGE_2$, and IL-1α. IL-4 significantly inhibited osteoclastogenesis induced by these cytokines, n=8 cultures per variable. Experiment was repeated with similar results. *$p<0.01$.

## Future plan

OPG and RANKL mRNA expressed by osteoblastic/stromal cells are regulated by osteotropic factors such as $1\alpha25(OH)_2D_3$, PTH, IL-11 and $PGE_2$. Role of IL-4 on regulation of these molecules is not known. In this proposed project we also plan to investigate the role of IL-4 on OPG and RANKL gene expression in osteoblasts and bone marrow stromal cells.

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## To investigate the mechanism of action of IL-3 on osteoclastogenesis

Activated T cells regulate osteoclast formation by some unknown mechanisms. T cells support osteoclast formation by RANKL-dependent and RANKL-independent mechanisms. Cytokines produced by activated T cells, as well as by other cell types regulates osteoclastogenesis. However, it is not clear how these cytokines regulates osteoclast formation and bone resorption.

Interleukin-3 (IL-3), secreted by activated T lymphocytes stimulates the proliferation, differentiation and survival of pluripotent haemopoietic stem cells. IL-3 is a broadly acting haemopoietic-regulatory protein with activities on a number of lineages including macrophages, neutrophils, eosinophils, and megakaryocytes. Although osteoclasts differentiate from haemopoietic stem cells, the role of IL-3 in osteoclast differentiation is not clear. In this study we report the role of IL-3 in RANKL-induced osteoclast formation.

### Aims

1. To investigate the role of IL-3 in RANKL-induced osteoclast formation in haemopoietic precursors of monocyte/macrophage lineage.

2. To understand the molecular mechanism of action of IL-3 on osteoclast differentiation.

### Work achieved

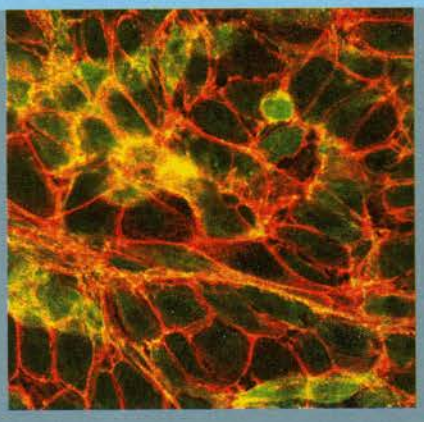
IL-3 has been shown to have both stimulatory and inhibitory action on osteoclast formation in whole bone marrow cells. So in the present study we first examined the effect of IL-3 on RANKL-induced osteoclast differentiation from mouse whole bone marrow cells. IL-3 dose-dependently inhibited the osteoclast formation in these cultures. IL-3 concentration of 0.1 ng/ml was sufficient for the significant inhibition of osteoclast formation. The unfractionated bone marrow cultures used in this study contains mixture of haemopoietic cells including stromal cells and osteoblasts. These cells produce substantial amounts of growth factors that either stimulate or inhibit osteoclast formation. Therefore, to determine the target cell for the action of IL-3 we examined the effect of IL-3 in stroma free population of bone marrow cells. We found that IL-3 dose-dependently inhibited the osteoclast formation and there was complete inhibition of tartrate-resistant acid phosphatase (TRAP) activity in these cultures. IL-3 inhibited RANKL-induced mRNA expression of TRAP and CTR genes. These results show the direct inhibitory effect of IL-3 on osteoclast precursors. We observed that complete inhibition of osteoclast formation occurs within 48 h of IL-3 treatment.

We next examined the effect of IL-3 on mRNA expression of macrophage specific genes c-fms, F4/80 and PU.1. By RT-PCR expression of these genes was observed in cultures treated with IL-3 for 4 days. These results show that IL-3 induce macrophage differentiation in osteoclast precursors of monocyte/macrophage lineage. The expression of RANK was also observed in cultures treated with IL-3 indicating no effect on receptor expression.

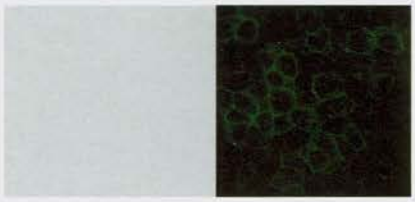
To further investigate the mechanism of action of IL-3, we examined whether IL-3 impacts RANKL activation of NF-κB. We found that IL-3 inhibited IκB degradation and NF-κB nuclear translocation. To examine if the effect of IL-3 is reversible, we incubated the cells with IL-3 for 3 days and withdrawn for 48 h. We found no osteoclast formation after withdrawal of IL-3. Similarly the osteoclast precursors pre-treated with M-CSF and IL-3 were resistant to RANKL action. Thus our results indicate that IL-3 acts directly on osteoclast precursors and inhibits RANKL-induced osteoclastogenesis through inhibition of NF-κB DNA binding activity.

**Future plan**

We are further investigating the mechanism(s) of action of IL-3 on bone resorption.



# Cancer Biology



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## Wild-type tumour suppressor gene in human cancer cells: Its role in chemosensitivity and functional consequences of its over expression

Our goal 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 and cancer cell. One such target is "Genomic Care Taker" tumour suppressor p53 gene. Because p53 is the 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 its roles in cancer therapeutics. 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 by: (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. 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-haematological origin is by no means clear. In order to contribute to the recent ongoing research in the area of cancer therapeutics, we are exploring the effect of wild-type p53 expression (sense/antisense) in human tumour cell lines and studying the drug sensitivity in a set of isogenic cell lines in which the p53 gene is either over expressed or disrupted. Because the only difference among these cell lines is the absence or presence of activator, the interpretation of results will be particularly straightforward and less complicated.

### Aims

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

1. The correlation, if any, between p53 status and drug sensitivity of human cancer cell lines.

2. Role of p53 over expression or deletion on sensitivity of cells to anticancer therapeutic drugs.

3. Explore the reasons of variability if any in these cell lines.

## Work Achieved

### 1. Effect of anticancer drugs on human breast cancer. Their p53 status and mechanism of action.

The MCF-7 and MDA-MB-231 cells were treated with varying concentrations of drugs and cytotoxicity assay was carried out. The $IC_{50}$ values for MCF-7 cells were significantly lower than that of MDA-MB-231 cells with the exception being an anti-metabolite drug. The cells possessing wild type p53 are more sensitive to DNA damaging and protein synthesis inhibitor anticancer drugs when compared to cells with mutant p53. However no such correlation could be drawn with the cells treated with anti-metabolite drug. DNA damaging drug-mediated killing of MCF-7 cells involves activation of endogenous wild type p53 and subsequently leading to increase in p21 activation, resulting in cell cycle arrest/cell death. In the cells with mutant p53, the mechanism of cell death is under investigation. However our preliminary data indicates that GADD45 is not involved in cell death induced by DNA damaging anticancer drug.

### 2. Development of stable cell lines over expressing wild-type p53 or antisense p53.

MCF-7 cells stably expressing tetracycline binding protein (Tet) was generated (MCF-7 Tet-On). In presence of activator tetracycline, Tet is transcriptional activated. The MCF-7 Tet-On cells were transiently transfected with Tet regulated expression plasmid coding for sense and antisense p53. The cell lysates assayed for p53 reporter assay indicate that the endogenous p53 transactivation activity was not inhibited by over expression of antisense p53. However, cotransfection of p53 expression plasmids (both sense and antisense) resulted in inhibition of p53 transactivation activity in a dose dependent manner (Fig. 1). In contrast to MCF-7 Tet-On clones, in SK-Hep-1 Tet-On stable clones, transient expression of antisense p53 significantly inhibited transactivation activity of both endogenous as well as exogenously added p53 expression plasmid (Fig. 2 and 3).

## Future plan

1. Develop stable clones of human breast, liver and oral cancer cells capable of over expressing wild-type p53.
2. The drug responsiveness of these isogenic stable clones will be studied with or without over expression of p53.
3. Determination of activity of cellular regulators like p53, p21, and GADD45 by reporter assays.

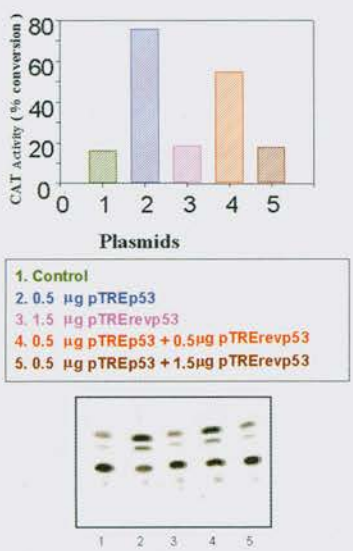



Fig. 1: Inhibition of p53 Transcriptional Activity by antisense p53 in MCF-7 Tet-On Stable Clone

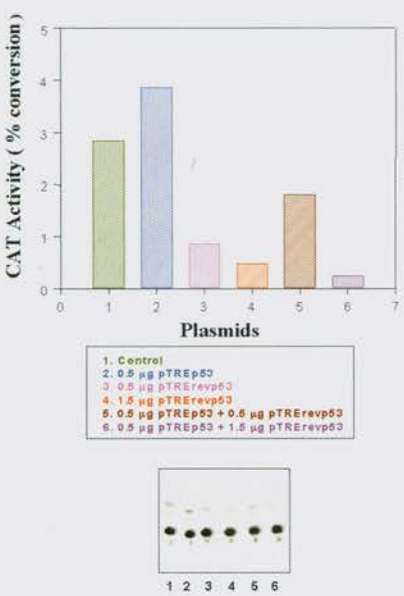



Fig. 2: Inhibition of p53 Transcriptional Activity by antisense p53 in SK-Hep-1 Tet-on Stable Clone

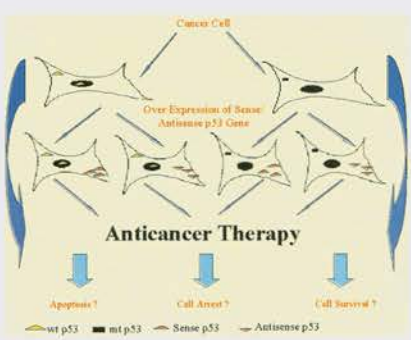
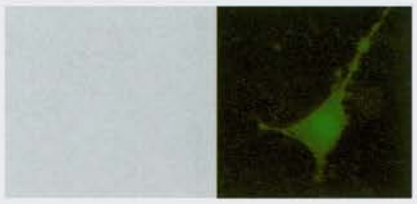



Fig. 3: A putative model for cancer therapy



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## Identification and Characterization of oncogenes implicated in melanoma

Melanoma is a highly metastatic and invasive cancer which occurs due to uncontrolled proliferation of melanocytes. The genetic and molecular mechanisms that lead to progression of melanoma are poorly understood. Mutations of p16 (CDKN2), p53, ras, neurofibromatosis type I gene (NF-1), bcl2 and the retinoblastoma gene have been described, but none are common, implicating for heterogeneous mechanisms of carcinogenesis. Both familial inheritance of potential tumour suppressor genes, e.g. p16, and differences in DNA-repair capacity contribute to the individual risk for melanoma. The genetic events, particularly aberration of cell cycle control and transcriptional control mechanisms are also implicated in the pathogenesis of melanoma. The project involves study of transforming genes implicated in melanoma using an expression cDNA library constructed from Clone M3, a mouse melanoma cell line.

### Aims

1. Characterization of the 600 bp transcript from clone M3 cells.

2. Expression cloning of the 3 different ORFs generated from the 600 bp transcript into GFP vectors, sequencing of the individual clones, transfection analyses of the clones and tumorigenicity studies with the cloned ORFs for understanding the mechanisms of transformation induced by the gene.

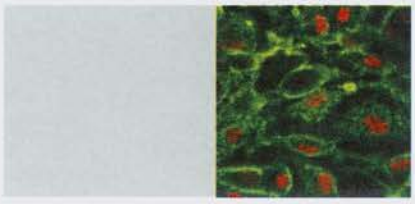
### Work achieved

We isolated, cloned and sequenced the 600 bp gene with tumorigenic potential from mouse melanoma cells clone M3. Sequence analyses of the gene revealed that the molecule lacked long ORFs and belonged to a novel family of proteins termed as *riboregulators*. The 600 bp sequence was cloned in both sense and reverse orientations and the directionality of cloning was ascertained by PCR and sequencing. Using transfection, stable cell-lines were generated with insert in both orientations and assayed for functionality and growth potential. Only cells with 600 bp insert in sense orientation were rapidly proliferating and tumorigenic in nude mice. Expression studies by RT-PCR and Northern hybridization demonstrated the specific expression of the transcript. Northern Expression analyses using the 600 bp riboprobe demonstrated the expression of a specific 3.4 Kb transcript in clone M3 cells. The entire 3.4 Kb sequence was retrieved and the gene was found to be located on the X-chromosome. The 600 bp gene in both sense and antisense orientations and the different reading frames were cloned in pEGFP-N1 and assayed for studies about their localization and transforming potential.

Experimental work has demonstrated that the transforming activity resided only with the 600 bp transcript and not with the individually cloned ORFs demonstrating the importance of untranslated RNAs in growth control. The present study provides evidence for the involvement of untranslated RNAs in oncogenesis and provides further support for the role of noncoding RNAs as riboregulators.

**Future plan**

A detailed study on the cell transformation pathway evoked by the 600 bp transcript in mouse and human cell lines ectopically expressing the gene.



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## Study of molecular markers in two dimensional and three dimensional cultures of mouse melanoma cell lines

Cell cultures of transformed cell lines grown as monolayers represent two dimensional (2-D) cultures that are extensively useful in experiments to acquire insight into tumour biology. Though these culture systems are useful for studying molecular regulatory mechanisms involved in tumorigenesis they are not true representative of *in vivo* conditions in which cells exist in a 3-D micro-environment. The MCS are 3-D spherical aggregates of intermediate complexity between monolayer cultures and tumours, devoid of requirement for adhesion to artificial substrate. As most cancers grow as solid 3-D tumours *in vivo* MCS serve as ideal models for studies involving tumour cell proliferation and micrometastasis. Additionally, heterogeneity is one of the important factors that influences tumour growth which is absent in monolayers but is present to a considerable extent in multi-cellular aggregates which closely mimic situations in primary tumours. Much of the studies dealing with cell proliferation and transformation have been undertaken using cell lines grown as conventional monolayers. Several studies have indicated that anchorage independent survival and proliferation of tumour cells are dependent on cell-cell adhesion which is mimicked when tumour cells form MCS.

### Aims

1. Find out role of cell adhesion related molecules, cyclin dependent kinase in tumorigenesis.

2. To study the role of $\alpha$-and $\gamma$-Catenin in cellular adherence and morphogenesis.

### Work achieved

We have compared the expression of molecular markers with reference to their growth as conventional adherent monolayers (2-D) and anchorage independent cultures (3-D) using two mouse melanoma cell lines- B16F10 and clone M3. The two cell-lines differed in their ability to form spheroids with respect to their aggregation potential, with B16F10 forming large clusters compared to clone M3 (Fig. 1). A panel of molecular markers comprising cell adhesion molecules, cyclin dependent kinase inhibitors and members of the cadherin-catenin complex were analyzed by flow cytometry in 2-D and 3-D cultures. There was a distinct difference in the patterns of expression of CD44(S) and variant isoforms in spheroids compared to cells grown as monolayers in both cell lines (Fig. 2). Also, there was an increase in cells positive for CDK inhibitor p27 in 3-D cultures from B16F10 cell line. The expression of $\alpha$-and $\gamma$-Catenin was down regulated in spheroids. As these molecules are implicated in regulation of cell proliferation, alterations in expression

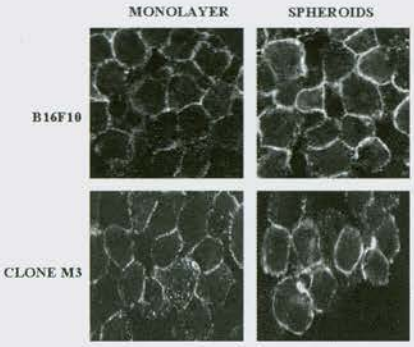


Fig. 1: Qualitative analyses of CD44(S) expression with CFLSM

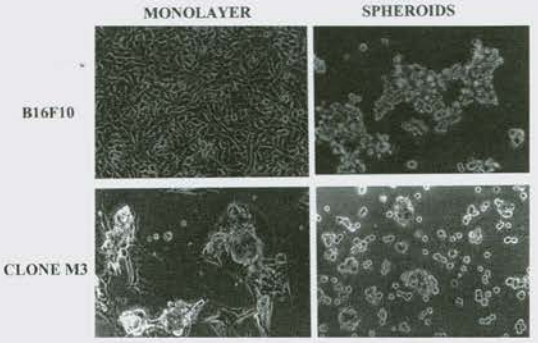


Fig. 2: Phase-contrast photomicrographs of monolayer and Spheroids from 24h cultures

of these molecules in 3-D cultures compared to their 2-D counterparts suggests the importance of spheroids as experimental model for tumorigenesis.

**Future plan**

Further studies are planned to analyze and decipher the changes in gene expression in the two experimental models using DNA microarrays.

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## Matrix metalloproteinase-2: mechanism of activation and its role in cellular invasiveness and tumour growth

Matrix metalloproteinases (MMPs) are a family of enzymes that degrade extracellular matrix (ECM) components and play important roles in tissue repair, tumour invasion and metastasis. MMPs are regulated by ECM-proteins, different cytokines and other factors. However, the molecular mechanisms by which osteopontin (OPN), an ECM-protein regulates activation of MMP-2 and controls cellular invasiveness and tumour growth are not well defined. We have shown that purified OPN induces pro-MMP-2 production and activation in B16F10 cells. The data also indicated that OPN induced MMP-2 activation is mediated by upregulation of membrane type 1-MMP (MT1-MMP) and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2). Moreover, the OPN induced MT1-MMP expression correlates with translocation of p65 (NF-κB) into the nucleus. OPN also enhances cell motility and ECM invasion by interacting with $\alpha v\beta 3$ integrin in these cells. Interestingly, when the OPN treated cells were injected into nude mice, the mice developed larger tumours and the MMP-2 levels in the tumours were significantly higher than in controls. These data demonstrate that OPN stimulates MMP-2 activation and MMP-2 plays significant roles in regulating cellular motility, ECM-invasion and tumour growth.

### Aims

1. To examine whether purified human OPN induces pro-MMP-2 activation and to investigate whether activation of pro-MMP-2 occurs through NF-kB mediated induction of MT1-MMP in B16F10 cells.

2. To determine whether OPN enhances the cellular migration and ECM-invasion and whether it occurs by interacting with integrin receptor. To delineate the direct role of MMP-2 in cellular migration and invasion by transfecting the cells with antisense S-oligonucleotides.

3. To detect the role of OPN in tumour growth and to check the levels of MMP-2 in the tumour of nude mice. To explore the molecular mechanism by which OPN modulates the MMP-2 activation and to demonstrate the functional correlation between OPN induced MMP-2 activation and OPN-enhanced cellular migration, ECM-invasion and tumour growth.

### Work Achieved

We have purified OPN from human milk and shown that OPN induces pro-MMP-2 production and activation in these cells (Fig. 1A and B). Our data revealed that OPN

induced MMP-2 activation is mediated by upregulation of membrane type 1-matrix metalloproteinase (MT1-MMP) and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) (Fig. 2A and C). Moreover, we have demonstrated that OPN induced MT1-MMP expression correlates with translocation of p65 (nuclear factor κB (NF-κB)) into the nucleus (Fig. 2B). However, when the super-repressor form of IκBα (inhibitor of NF-κB) was transfected into cells followed by treatment with OPN, no induction of MT1-MMP expression was observed, indicating that OPN activates pro MMP-2 via NF-κB mediated pathway (Fig. 2C). OPN also enhanced cell motility and ECM invasion by interacting with αvβ3 integrin, but these effects were reduced dramatically when MMP-2 expression was suppressed by MMP-2 specific antisense S-oligonucleotide (ASMMP-2) but not with sense S-oligonucleotide (SMMP-2). Interestingly, when the OPN treated cells were injected into nude mice, the mice developed larger tumours, and the MMP-2 levels in the tumours were significantly higher than in controls. Both, tumour size and MMP-2 expression were reduced drastically when juxta tumoral injection of MMP-2 antibody or ASMMP-2 was performed. Taken together, these data clearly indicated that MMP-2 plays critical role in regulation of cellular motility, ECM-invasion and tumour growth and further demonstrates that OPN-induced MMP-2 activation occurs through NFκB mediated pathway.

## Future plan

The molecular mechanism and signalling pathways by which OPN regulates NF-κB mediated MMP-2 activation will be delineated.

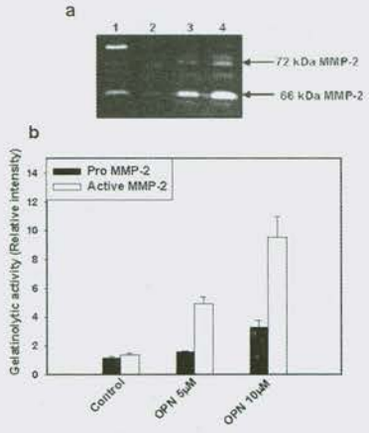


Fig. 1 A: Detection of MMP-2 expression in B16F10 cells by zymography: Panel a: The cells were treated in absence or presence of OPN (0-10.0 åM), conditioned media were collected and MMP-2 activity was analyzed by gelatin zymography. Lane 1: Pure MMP-2 as standard; Lane 2: untreated cells; Lanes 3: with 5 åM OPN; lane 4: with 10 åM OPN. The arrows indicate both 72kDa and 66 kDa MMP-2 specific bands. Panel b: The levels of MMP-2 (pro and active) were quantified by densitometric analysis.

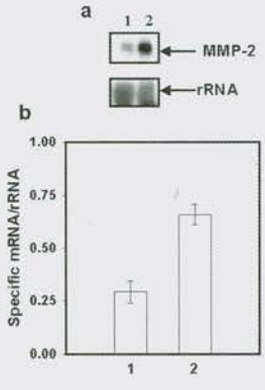


Fig. 1B: Expression of MMP-2 mRNA by Northern blot analysis. Panel a: Lanes 1 and 2 show the faint and intense MMP-2 specific mRNA bands in the untreated and OPN treated cells respectively (upper panel a). The rRNA bands are shown as control (lower panel a). Panel b: RNA bands were quantified by densitometric analysis and the ratio of specific mRNA and rRNA is represented in the form of bar graphs.

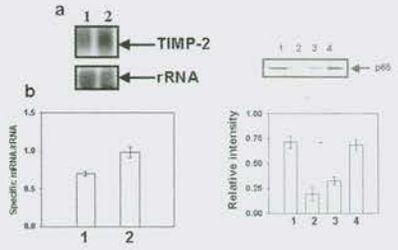


Fig. 2A: Expression of TIMP-2 mRNA by Northern blot analysis. TIMP-2 specific mRNA bands are shown in lane 1 (untreated cells) and lane 2 (OPN-treated cells). Lower panel a, rRNA bands are shown as a control. RNA bands were quantified by densitometric analysis and the ratio of specific mRNA to rRNA is represented in the form of bar graphs (panel b). Fig. 2B, Panel a: Effect of OPN on cellular localization of NF-kB p65. In the untreated cells, majority of the p65 is detected in the cytoplasm but not in the nucleus (panel a, lanes 1&2 respectively). In contrast, upon OPN treatment, p65 is translocated from cytoplasm to the nucleus (panel a, lanes 3 & 4 respectively). The relative intensity of p65 in the cytoplasm and nucleus was analyzed and is represented in the form of bar graph (lower panel a). Panel b: Inhibition of OPN induced MT1-MMP expression by NF-ᵏB super repressor IkBᵅ. The cells were transfected with super repressor form of IkB-a in pCMV4 and then treated in absence or presence of OPN. The cell lysates were used for the detection of MT1-MMP by Western blot analysis (upper panel b). Lane 1: untreated, non-transfected cells; lane 2: OPN-treated, non-transfected cells; lane 3: untreated, transfected cells and lane 4: OPN-treated, transfected cells. Note that there is an increase of MT1-MMP expression in OPN-treated cells (lane 2) as compared to control (lane 1) whereas in transfected cells, no differences of expression of MT1-MMP are observed in both untreated (lane 3) and OPN-treated (lane 4) cells. The bands were analyzed by densitometry and represented in form of bar graph (lower panel b).

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## Identification and characterization of novel, bone derived chemoattractant factor that regulates breast cancer cell migration and bone metastasis

The mechanism of breast cancer metastasis to bone is still not clearly understood. The process by which breast cancer cell invade, migrate and proliferate to bone is remained unclear. We have identified and purified a novel bone derived chemoattractant factor (BDCF) that induces the human breast adenocarcinoma (MDA-MB-231) cell migration in a dose dependent manner. We have radiolabeled the BDCF protein and performed the radioreceptor assay using BDCF as ligand. The data indicated that radiolabeled BDCF binds with its putative receptor with high affinity and specificity in breast adenocarcinoma cells. Our data demonstrate that BDCF induces the breast adenocarcinoma cell migration by interacting with its putative receptor.

### Aims

1. To purify and characterize the factor from human bone extract and bone derived osteosarcoma cells that induces the breast cancer cell migration.

2. To generate the antibody against the factor, identify and characterize the putative receptor of the factor.

3. To study the molecular mechanism of cell motility and invasiveness and to examine the bone metastasis in breast cancer model in nude mice by the factor.

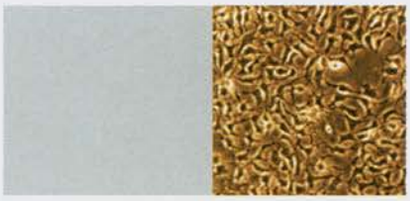
### Work achieved

The BDCF was partially purified from human foetus bone extract by FPLC, Mono-Q column. The different fractions were tested for cellular migration of MDA-MB-231 cells. The protein containing active fraction was rechromatographed by FPLC, Mono-Q column. The final chromatography on FPLC showed a single and symmetrical peak. The purity of protein was analyzed by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie blue which showed a single, sharp band with a molecular mass of 55 kDa. This purified protein showed the induction of MDA-MB-231 cell migration in a dose dependent manner. The antibody against BDCF was raised in rabbits and characterized by Western blot analysis. Pre-incubation of BDCF with its antibody reduces the BDCF induced migration. The N-terminal of BDCF was sequenced and the sequencing data indicated that this protein has 67% sequence identity with human serum albumin (HSA). Moreover, we have radiolabeled the BDCF and performed the radio receptor assay by using BDCF as ligand. The data showed that radiolabeled BDCF binds with a putative receptor and this binding is displaced by unlabeled BDCF but not by HSA in MDA-MB-

231 cells indicating that the putative receptor is BDCF specific. Taken together, these data indicate that BDCF induces the breast adenocarcinoma cell migration by interacting with its putative receptor and further demonstrate that BDCF-induced breast adenocarcinoma cell migration may play potential role in skeletal metastasis.

**Future plan**

Further characterization of the factor and identification and characterization of the receptor are in progress.



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## Effect of antioxidants and sodium pyruvate protects against $H_2O_2$ mediated apoptosis in human neuroblastoma cell line: SK-N-MC

Nerve cell death by apoptosis is the central feature of the human neurodegenerative diseases. Apoptosis can be triggered by a variety of inducers that include reactive oxygen species (ROS) like superoxide anions and hydrogen peroxide. It has been demonstrated that oxidative stress plays an important role as a mediator of apoptosis Hydrogen peroxide; a by-product of oxidative stress has been implicated to trigger apoptosis in various cell types leading to major neurodegenerative diseases – Alzheimer's disease (AD), Parkinson's disease (PD) and amylotrophic lateral sclerosis (ALS). Cells have numerous protective mechanisms that maintain the concentrations of ROS within a range compatible for survival. The brain can efficiently metabolize superoxides but may have difficulties in eliminating the hydrogen peroxide produced. It has been hypothesized that some trophic factors may upregulate neuronal antioxidants.

We investigated the protective effect of antioxidants – β carotene, rutin and quercetin and a free radical scavenger -sodium pyruvate against hydrogen peroxide ($H_2O_2$) induced apoptosis in human neuroblastoma cell line: SK-N-MC. Cells on exposure to $H_2O_2$ (0.025 mM) exhibited apoptosis within 24 h, demonstrating a high caspase 3 activity by 3 h followed by cleavage of PARP that was maximum at 24 h. A breakdown in the mitochondrial membrane potential was also observed 3 h onwards. In our experimental system, antioxidants like β-carotene, rutin and quercetin (concentrations ranging from 0.01 to 1 μM) did not protect cells against $H_2O_2$-mediated apoptosis. Sodium pyruvate (1 mM) protected the cells against apoptosis induced by $H_2O_2$ by inhibiting caspase 3 activity, cleavage of PARP and breakdown of mitochondrial membrane potential. Our data also suggested that the protective effect of sodium pyruvate was by neutralization of the action of $H_2O_2$ and therefore inhibits apoptosis. The potential of pyruvate as a neuroprotectant can therefore be exploited for prevention rather than for treatment.

### Aims

1. To study the apoptotic pathways in neuroblastoma cell line SK-N-MC.

2. To examine the protective effect of antioxidants β carotene, rutin and quercetin and a free radical scavenger -sodium pyruvate against hydrogen peroxide ($H_2O_2$) induced apoptosis in SK-N-MC.

### Work achieved

We observed a time and dose dependent increase in the apoptotic population on treatment of SK-N-MC cells with $H_2O_2$. SK-N-MC cells treated with 0.025 mM $H_2O_2$ showed

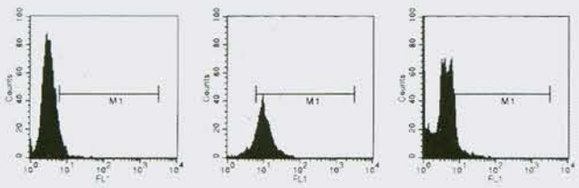


Fig. 1: Inhibition of apoptosis by sodium pyruvate by TUNEL assay. (a-c) Representative histograms of different groups. (a) Control SK-N-MC cells under normal conditions. (b) Cells treated with $H_2O_2$ (0.025 mM). (c) Cells treated with $H_2O_2$ (0.025 mM) and sodium pyruvate (1 mM). Apoptosis was measured by TUNEL assay. The extent of percentage of FITC positivity corresponded to percent apoptosis. Sodium pyruvate (1 mM) significantly inhibited apoptosis stimulated by $H_2O_2$.

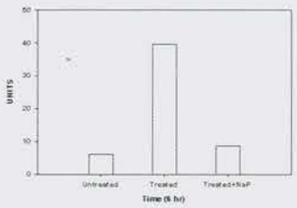
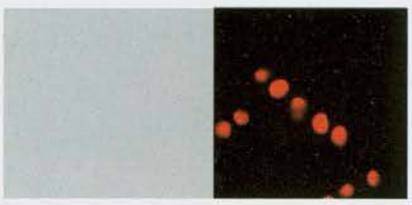


Fig. 2: Inhibition of caspase 3 activity in $H_2O_2$ treated cells by sodium pyruvate. The cells were treated for 6 hr with 0.025 mM $H_2O_2$ alone and in combination with 1mM sodium pyruvate. Cell lysates were prepared and assayed by caspase 3 colorimetric assay kit. The high activity of caspase 3 observed in $H_2O_2$ treated cells was inhibited by sodium pyruvate. The data is representative of 2 independent experiments.

37.40% and 78.20% apoptosis respectively. Reactive oxygen species are known to be quenched by antioxidants; we therefore investigated the effect of antioxidants such as β-carotene, rutin and quercetin (0.01 to 1 μM) and a free radical scavenger sodium pyruvate (0.125 to 1.0 mM) on apoptosis induced by exposure of $H_2O_2$ for 24 h by MTT assay. Beta-carotene, rutin or quercetin did not protect the cells form undergoing apoptosis. In contrast, significant protection (96% viability) was observed with 1 mM sodium pyruvate against $H_2O_2$ induced apoptosis. To confirm whether $H_2O_2$ induced cell death was due to apoptosis, cells were stained with propidium iodide and rate of apoptosis was studied. The hypodiploid population was considered apoptotic and DNA content (DNA fragmentation) was measured by flow cytometry. Reactive oxygen species are known to be quenched by antioxidants; we therefore investigated the effect of antioxidants such as β-carotene, rutin and quercetin (0.01 to 1 μM) and a free radical scavenger sodium pyruvate (0.125 to 1.0 mM) on apoptosis induced by exposure of $H_2O_2$ for 24 h. This was assessed by determining the cell viability by MTT assay. No effect of β-carotene, rutin or quercetin was observed. In contrast, significant protection (96% viability) was observed with 1 mM sodium pyruvate against $H_2O_2$-induced apoptosis. The result was further confirmed by TUNEL assay. Within 9 h of $H_2O_2$ treatment, 59.34% cells were FITC positive which decreased to 15.4% when cells were treated with $H_2O_2$ in combination with sodium pyruvate (1 mM), confirming the protective effect of sodium pyruvate (Fig. 1). Further experiments were conducted to investigate the mechanism of protection by sodium pyruvate. We next examined the status of caspase 3, a key protease in the execution of the apoptotic machinery. The $H_2O_2$ treated SK-N-MC cells showed higher caspase 3 activity (39.49 units) as compared to the control cells (6.07 units). Treatment with sodium pyruvate induced protection in $H_2O_2$ treated cells and showed a decrease in the caspase 3 activity to 8.73 units (Fig. 2). This data suggests the involvement of caspase 3-dependent pathway in $H_2O_2$-induced apoptosis in SK-N-MC cells. The cleavage of PARP on treatment with $H_2O_2$ alone and in combination with sodium pyruvate was studied by Western blotting. The cells treated with $H_2O_2$ alone showed a distinct band of cleaved PARP of 89 kDa. The untreated control cells and the cells treated with sodium pyruvate did not show the presence of the cleaved fragment (Fig. 3). These results confirmed that $H_2O_2$ induces apoptosis in SK-N-MC cells exhibiting typical signs of the apoptotic cascade and sodium pyruvate counteracts this effect. In conclusion, sodium pyruvate may be useful as an efficient, readily


Fig. 3: Inhibition of PARP cleavage by sodium pyruvate. SK-N-MC cells were treated with 0.025 mM $H_2O_2$ alone and in combination with 1 mM sodium pyruvate. The cells were harvested at 3, 6 and 24 h and PARP cleavage was determined by immunoblot analysis. Lane: 1 untreated control; 2, 4, 6 $H_2O_2$ with sodium pyruvate; 3, 5, 7 $H_2O_2$ alone, for 3 h, 6 h and 24 h respectively.

diffusible and specific scavenger of hydrogen peroxide in future studies to determine the mechanism/s of action of ROS and in development of treatment strategies for degenerative diseases and secondly the potential of pyruvate as a neuroprotectant can be exploited for prevention rather than for treatment in neurodegeneration. Future work is planned to investigate the role of the anti-apoptotic and pro-apoptotic molecules in the protection of pyruvate against apoptosis in neuronal 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*

Epidemiological studies suggest that high dietary intake of fruit and vegetables protects against tumorigenesis in many tissues, including the breasts but little is known about the mechanisms by which these types of food serve as cancer chemopreventive agents in humans. Among vegetables with anticarcinogenic properties commonly consumed, members of the Cucurbitaceae family, particularly bitter gourd (*Momordica Charantia* Linn.), has been shown to possess immune-enhancement ability, hypoglycaemic, anti-viral, anti-diabetic and anti-tumour activities. Potent anti-tumour action of *M. charantia* fruit extracts has been documented *in vivo* and on a variety of malignant cells *in vitro*.

The pharmacological safety is demonstrated by its consumption for centuries at levels of approximately up to 70-80 g/day by people in certain countries including India and also been shown by previous *in vivo* and *in vitro* studies using human lymphocytes. However, the mechanism of how it induces the cell death in a tumour cells is not clearly understood and the active principle responsible to induce cell death has not been isolated. Present studies are therefore being carried out considering the possibility of discovering a new anti-cancer drug.

### Aims

1. To ascertain the potential anti-cancer activity of crude bitter gourd fruit juice (BFJ) towards the breast cancer cell line MCF-7.

2. To isolate and purify biologically active chemical compounds responsible for their potential anti-cancer activity from the complex mixtures using biochemical fractionation and purification procedures.

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

### Work achieved

In this study, a preliminary investigation was done by studying the regulation of proliferation and cell death in human breast cancer cell lines, MCF-7, SK-BR-3 and MDA-MB-231. BFJ displayed strong growth inhibitory effects against all the human breast cancer cells, with estimated IC-50 values of only 2.5% with minimal toxicity to the normal epithelial cells MRC-5 (Fig. 1a and 1b).

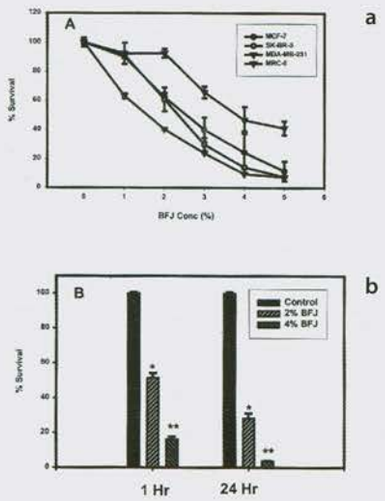



Fig. 1: a: Dose-response relationship of BFJ-induced cell death. b: Time kinetics of BFJ-induced cell death

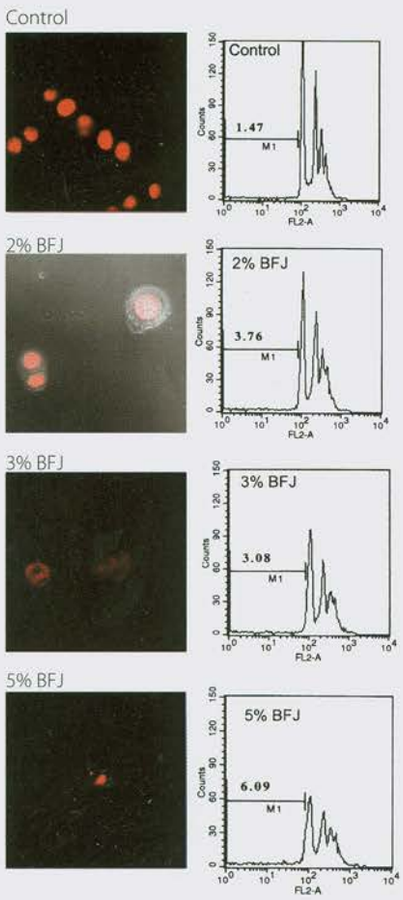



Fig. 2. Morphological changes and death occurring due to BJF at various concentrations

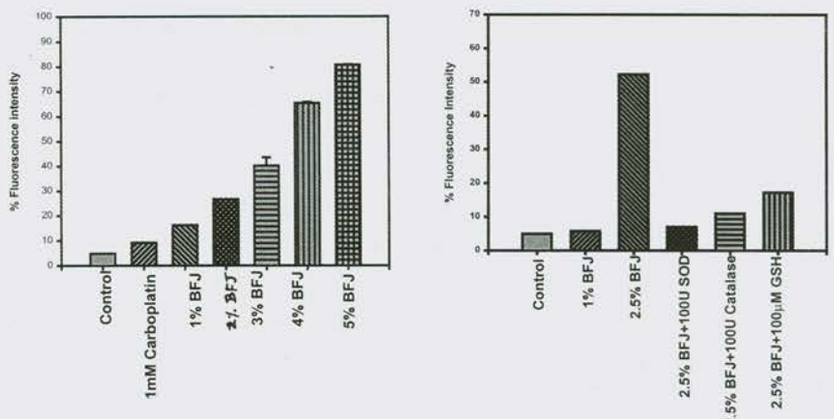



Fig. 3: BFJ-induced generation of Reactive Oxygen Intermediates (ROIs)

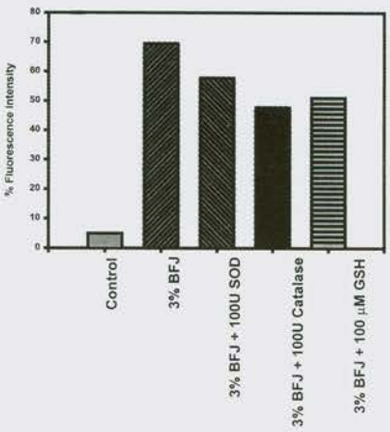



Fig. 4: BFJ-induced expression of Fas

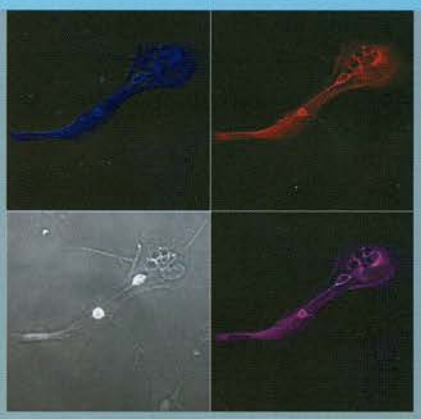
It is generally accepted that the primary cytotoxic mechanism of cisplatin and other DNA-binding antineoplastic drugs is the induction of DNA damage and subsequent induction of apoptosis and/or necrosis depending on the availability of energy and the metabolic condition of the cell. We did not find cell populations undergoing apoptosis as evidenced by flow cytometry studies. However, Confocal laser microscopic studies clearly revealed a necrotic rather than apoptotic cell death includes disruption of cellular membrane and cell lysis with only few cells also displaying a morphological appearance of apoptosis (Fig. 2). The necrotic cell death was further confirmed with the Trypan blue staining. Cell cycle arrest at the $G_0/G_1$ compartment was also observed in the BFJ treated cells.

Reactive oxygen intermediates (ROIs) have emerged as an important mediator of apoptosis and necrosis. Several previous studies also indicate that the anti-tumour immune response and anticancer agents are known to induce this type of cell death by way of generating free radicals. In the present studies, flow cytometry analysis showed a marked dose-dependent induction in the levels of ROIs (Fig. 3) and the death receptor protein Fas (Fig. 4) but apoptotic cell death was not observed in MCF-7 cells treated with BFJ for 24 h. This induction in ROI but not in Fas was inhibitable by the addition of the antioxidants SOD, Catalase and reduced glutathione (GSH).

Present studies thus support the anti-tumour effect of BFJ and provides sufficient documentation to define its role and action for its potential and promising use in treating cancer.

**Future plan**

Isolation and purification of the bioactive compound and evaluation of their biochemical mechanism and potential anti-cancer activity *in vitro* and *in vivo*.



# Signal Transduction

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**ANIL LOTKE**, Technician

## How does α-haemolysin establishes the synergistic function with a Protein Tyrosine Phosphatase?

In our laboratory, we discovered the attenuation of phosphorylation signal of EGFr in the presence of α-haemolysin (α-HL) of *Staphylococcus aureus*. Contrary to the current thinking that pore forming toxins kill target cells by membrane disruption, our studies suggested an involvement of a protein tyrosine phosphatase (PTPase) for the attributed attenuation. However, neither the pathway that led to the activation of PTPase nor the consequences of the presence of α-HL on the cell surface are not understood at molecular level. An in-depth study on the effect of α-HL on EGFr bearing cells is an absolute must because the EGFr serves as the current paradigm for receptor tyrosine kinases (RTKs) and for signal transduction events initiated at it. In addition, the identity of receptor-attenuating molecules for most receptor tyrosine kinases including the EGFr is still not known. The information regarding such molecules that associate or supposed to work in synergy with EGFr or other RTKs are of tremendous importance for two reasons *viz.* (i) To know their role in signal transduction and cell proliferation (ii) the information on function can serve as stepping stone for rational design of new generation molecules that function from outside of a cell. Hence, we wished to find out the molecules involved in the process of dephosphorylation mediated by the α-HL and the associated mechanism.

### Aims

1. What happens after the inhibition of autophosphorylation of EGFr?

2. After the removal of phosphorylation signal of EGFr, are the downstream signals getting affected? If so which ones are affected and what is the time frame at which these occur?

### Work achieved

**Downstream signals are completely withdrawn:** In order to study whether inhibition of RTK activity of EGFr by α-HL has any effect on the downstream signalling events, the Phospholipase Cγ (PLCγ) was selected for this study because this is one of first few molecules that receives signal from EGFr. Binding of EGF or TGFα to EGFr stimulates rapid tyrosine phosphorylation of PLCγ. The tyrosine phosphorylation of PLCγ is maximum after 30 second incubation with 50 nM EGF at 37 °C. At active stimulation stage, antibodies specific to PLCγ are able to co-immunoprecipitate the EGFr and *vice versa*. Approximately 1% of EGFr molecules upon activation are associated with PLCγ. The protein tyrosine kinase inhibitor Tyrphostin (RG 50864), which blocks EGFr dependent cell proliferation, blocked both the EGFr induced tyrosine phosphorylation of PLCγ and its association with the EGFr.

It is clear in Fig. 1 that the addition of α-HL (100nM) has been shown to result in an elevated EGFr phosphorylation after 5 min, which is completely reversed by 30 min. In the upper panel which is probed with anti-phosphotyrosine antibody, the protein that co-immunoprecipitates with the EGFr (170 kDa) is the PLCγ (145 kDa). The immunoprecipitation by PLCγ (instead of EGFr) after a similar treatment results in an identical phosphorylation pattern indicating the strong association between the EGFr and the PLCγ after growth factor stimulation. However, the association between these two cell surface receptors is lost upon α-HL treatment as the EGFr and PLCγ no longer associate with each other (Panel A, lane 3 or lane 5). This indicates that the α-HL treatment inhibits or withdraws the communication between the EGFr and PLCγ as a result, nether of the cell surface receptors carry the phosphorylation signal. This is even more glaring when we used the fusion protein α-HLTGFα, constructed by us earlier for this purpose, which shows that there was an intact communication between the EGFr and PLCγ at 5 min time point as expected, but the same signal is withdrawn upon longer incubation. The amount of PLCγ immunoprecipitated is same in all the lanes, as seen by anti-PLCγ antibody (Panel B).

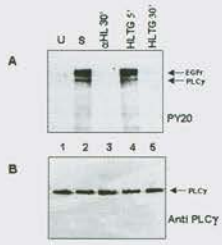



Fig. 1: Phosphorylation status of EGFr.

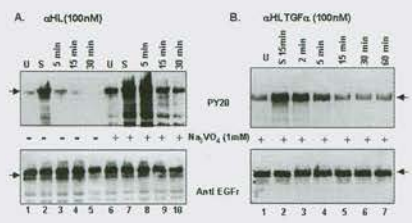



Fig. 2: The effect of α-HL on EGFr phosphorylation in presence and absence of $Na_3VO_4$ (1 μM) was studied.

The αHLTGFα, thus, seems to bring about rapid dephosphorylation of PLCγ as well as the EGFr. These results show that α-HL inhibits or withdraws the phosphorylation signals of all downstream molecules without entering the cell. This observation suggests the presence of powerful mechanism, accessible from outside of the cell that can undo or tilt the balance of phosphorylation-dephosphorylation towards the later.

### Involvement of a protein tyrosine phosphatase

**Reversible inhibitors:** In order to confirm whether αHL was mediating its effect through a PTPase, phosphorylation assays were carried out in presence of $Na_3VO_4$. In absence of $Na_3VO_4$, αHL brings about a complete inhibition of EGFr phosphorylation within 5 min (Fig 2, Panel A).

In presence of $Na_3VO_4$, a 15 minute treatment causes a complete inhibition of EGFr phosphorylation Fig 2, Panel A, lane 9). Note that the $Na_3VO_4$ raises the basal level phosphorylation by inhibiting the reverse reaction (lane 6 compared to lane 1). With α-HLTGFα (100nM), the phosphorylation level returns to basal level in 15 min. This indicates that the $Na_3VO_4$, a well-studied inhibitor of PTPases, only slows down the effect of αHL and αHLTGFα but it is unable to block the action of α-HL completely.

**Irreversible inhibitors:** Since $Na_3VO_4$ inhibition is reversible, the experiments were also done in presence of Iodoacetamide (IAA) which reacts irreversibly with the active site cysteine present in the phosphatases. α-HL is able to inhibit phosphorylation of EGFr to basal level even in presence of IAA, in a manner similar to that seen in non IAA treated cells (Fig 3, Panel A). In IAA treated cells

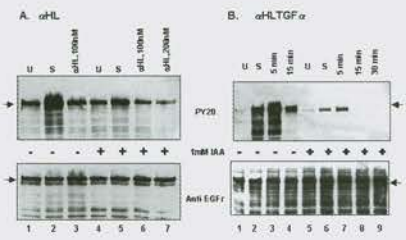



Fig. 3: Cells were treated with IAA (1 mM) for 30 min at room temperature following which αHL was added for 30 min at 37°C. The cells were then incubated with 70 nM TGFα for 10 min. The IAA was present during the entire duration of the experiment.

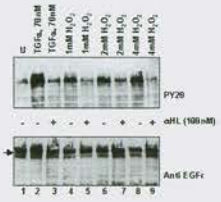



Fig 4: The A431 cells were treated with or without 100 nM of α-HL following which varying concentrations (1-4 mM) of $H_2O_2$ were added. α-HL prevents the elevation in phosphorylation levels of EGFr brought about by $H_2O_2$ (lanes 5, 7, 9 as compared to non-α-HL treated cells lane 4, 6, 8). It should be noted that TGFα was not used for stimulating the cells.

a decrease in autophosphorylation levels of EGFr in response to TGFα is seen, which is consistent with earlier reports, (lane 5) as compared to non-IAA treated cells (lane 2) (Fig 3, Panel A). In the presence of IAA, the increase in phosphorylation signal seen at 5 min (Lane 7) is completely lost by 15 min (lane 8) indicating the reversal of EGFr phosphorylation even in the presence of irreversible inhibitors. The blots were probed with EGFr antibody to indicate that equal levels of EGFr are present in all the lanes.

**Blockage of ligand independent phosphorylation:** Activation of EGFr by its ligand results in transient generation of $H_2O_2$ (which inactivates the PTPases) to allow the safe passage of phosphorylation signal in the natural pathway. In the absence of ligand, it tilts the equilibrium of phosphorylation-dephosphorylation towards the former. The effect of α-HL on $H_2O_2$ induced accumulation of phosphorylation signal on EGFr is studied to understand the phenomenon of signal withdrawal.

It is clear from fig 4 that varying concentrations of $H_2O_2$ causes an increase in EGFr phosphorylation. Cells treated with both TGFα and $H_2O_2$ show marginally higher phosphorylation levels as compared to cells stimulated with TGFα alone and maximum stimulation occurs within 2 min. The phosphorylation signal remains at elevated levels for about 15 min. The phosphorylation level of EGFr obtained by the hydrogen peroxide is almost equal to that caused by stimulation with TGFα.

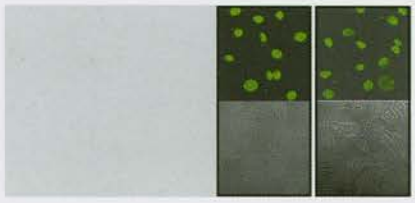
It is important to note that the cells in this experiment were saturated with $H_2O_2$. Under such saturating levels of hydrogen peroxide all the PTPases are expected to remain in inactive state (due to oxidation of active site). However, the αHL is still able to bring the dephosphorylation of EGFr in a dramatic way.

In summary, all our experiments unequivocally illustrate the ability of α-HL to influence the equilibrium of phosphorylation-dephosphorylation reactions that occur at the cytoplasmic side of a cell, while it is outside. We demonstrated, for the first time, that it is possible to withdraw all phosphorylation signals without entering the cell. As a result, it paved the way for the creation new generation molecules which need not enter the cell to arrest uncontrolled proliferation.

**Future plan**

1. Identification of the PTPase performing the dephosphorylation.

2. Mechanism of activation of the PTPase.



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**GANAPATI H.M**

## SYK, a protein tyrosine kinase regulates the cell motility and urokinase type plasminogen activator secretion through activation of phosphatidylinositol 3-kinase in breast cancer cells

Tumour invasion and metastases are multifaceted processes those of which are involved in cellular adhesion, proteolytic degradation of extracellular matrix and cellular migration. Syk is a protein tyrosine kinase that is mostly expressed in haematopoietic cells. It has been reported earlier that Syk is expressed in the breast epithelial cells, but its function in these cells remains unknown. The mechanisms by which Syk, a protein tyrosine kinase regulates the cell motility and activation of phosphatidylinositol 3-kinase (PI 3-kinase) through secretion of Urokinase Plasminogen Activator (uPA) in breast cancer cells is not well defined. Here we report that the expression of Syk is absent in high invasive breast cancer (MDA-MB-231) cells but it is present in low invasive breast cancer (MCF-7) cells. The overexpression of wild type Syk kinase but not the kinase negative Syk (SykK$^-$) suppresses the cellular migration and reduces the activations of PI 3-kinase in MDA-MB-231 cells. However, the activation of PI 3-kinase is much higher in non-transfected MDA-MB-231 cells but it is lower in MCF-7 cells. The secretion of uPA is also higher in non-transfected MDA-MB-231 cells as compared to MCF-7 cells. These results suggest that Syk plays critical role in regulation of cell motility and activation of PI 3-kinase through secretion of uPA in breast cancer cells.

### Aims

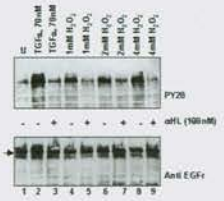
1. To check the expression of Syk in low and high invasive breast cancer cells. To determine how Syk controls the cell motility and tumour growth using low and high invasive breast cancer cells,

2. To examine the effect of Syk on regulation of PI 3'-kinase activation and uPA secretion in breast cancer cells,

3. To establish a functional correlation between Syk mediated cellular motility and Syk regulated PI 3'- kinase dependent uPA secretion in breast cancer cells.

### Work achieved

Our recent data showed that the expression of Syk is absent in high invasive breast cancer (MDA-MB-231) cells but it is present in low invasive breast cancer (MCF-7) cells (Fig. 1). The overexpressed wild type Syk kinase but not the kinase negative Syk (SykK$^-$) suppresses the cellular migration (Fig. 2B) and reduces the activations of PI 3'-kinase in MDA-MB-231 cells. However, the level of PI 3'-kinase activation is much higher in non-transfected MDA-MB-231 cells and lower in MCF-7 cells. Syk specific antisense

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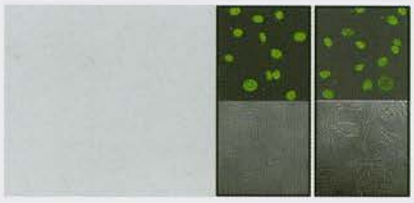

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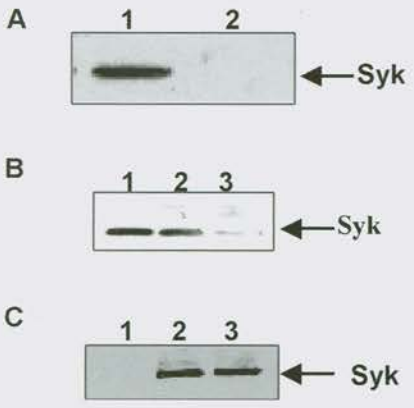



Fig. 1: Expression of Syk in MCF-7 and MDA-MB-231 cells.
Panel A: The equal amount of total proteins from the cell lysates were resolved by SDS-gel and detected by Western blot analysis using anti Syk antibody. Lane 1: MCF-7 cells and lane 2: MDA-MB-231cells.
Panel B: The MCF-7 cells were transiently transfected with LipofectAMINE Plus alone (lane 1) or with Syk specific sense S-oligonucleotide, SSyk (lane 2) or with Syk specific antisense S-oligonucleotide, ASSyk (lane 3). The cell lysates containing equal amounts of total proteins were resolved by SDS-gel and detected by Western blot analysis using anti Syk antibody.
Panel C: The MDA-MB-231 cells were transiently transfected with LipofectAMINE plus alone (lane 1), with wild type Syk cDNA (lane 2) or with kinase negative Syk cDNA (lane 3) in pcDNA3.1. The total proteins in cell lysates were separated by SDS-gel and detected by Western blot analysis using anti Syk antibody as described above. The arrow indicates the Syk specific band.

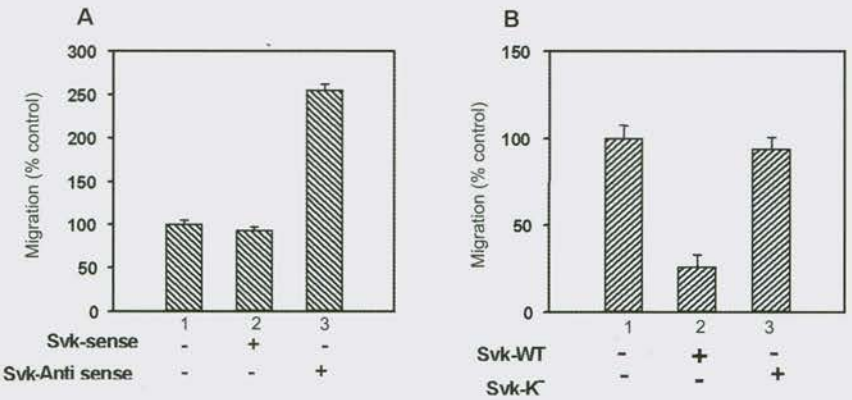
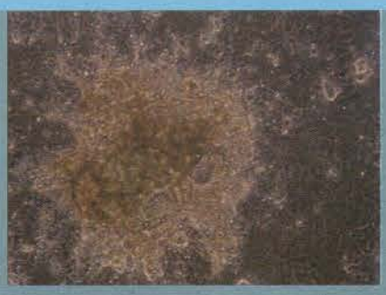



Fig. 2A: Effect of transfection of MCF-7 cells with Syk specific S-oligonucleotides on cell migration. The cells were transfected with LipofectAMINE Plus alone or with SSyk or with ASSyk as described above. The cells transfected with ASSyk showed dramatic increase of cell migration compared with cells transfected with LipofectAMINE plus alone or with SSyk. The results are expressed as the means ± S.E. of three determinations. B. Effect of transfection of MDA-MB-231 cells with wild type and kinase negative Syk cDNA on cell migration. The cells were transfected with LipofectAMINE plus alone or with wild type Syk cDNA or kinase negative Syk cDNA. The transfected cells were used for cell migration assay as described above. The cells transfected with wild type Syk cDNA showed drastic reduction of cell migration as compared to cells transfected with LipofectAMINE plus or kinase negative Syk cDNA. The results are expressed as the means ± S.E. of three determinations.

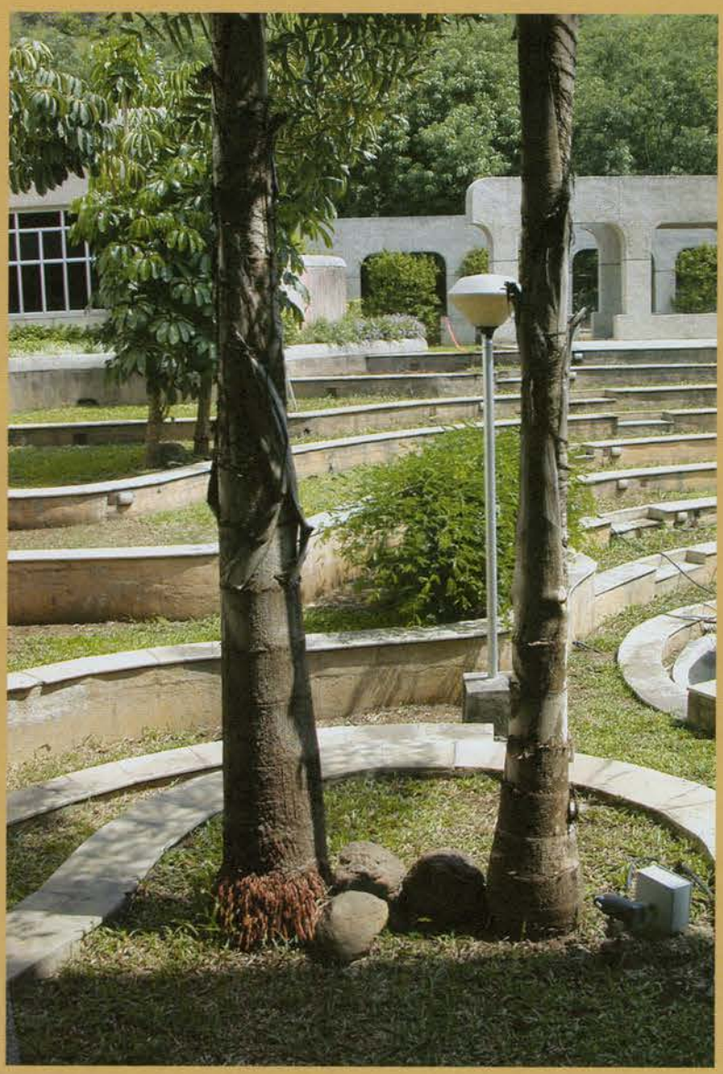
S-oligonucleotide but not the sense S-oligonucleotide when transfected to the MCF-7 cells, the level of PI 3'-kinase activity were increased and the cellular migration was also enhanced (Fig. 2A). The secretion of uPA was also higher in non-transfected MDA-MB-231 cells as compared to MCF-7 cells. These data indicated that Syk regulates the PI 3'-kinase activation, uPA secretion and cellular motility in breast cancer cells.

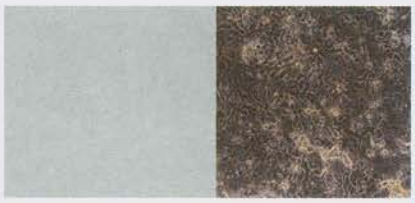
**Future plan**

The molecular mechanisms by which Syk, a protein Tyrosine kinase suppresses the cell motility and regulates the PI 3'-kinase activation through uPA secretion will be studied.



## Diabetes





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**SANJEEV GALANDE**, NCCS, **P.B. PARAB**, NCCS

## Regeneration of Pancreatic β cells and reversal of experimental diabetes

The number of functionally intact β 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 mellitus would be of interest as an alternate therapy for diabetes. The pathogenesis of diabetes has been viewed as a balance between the destructive and regenerative process. The therapeutic goal then would be to down-regulate the destructive processes and/or up-regulate the regenerative processes. The general approach of regenerative biology is to re-create an embryonic environment in a malfunctioning tissue. Hence it is important to define growth factors responsible for proliferation of the islets and islet stem cells. Such studies would enhance our understanding of the pathophysiology of the diabetic pancreas and perhaps offer novel approaches to the cure of diabetes.

### Aims

1. To find out factors affecting growth of β cells *in vitro* and *in vivo*.

2. To ascertain the role of exocrine pancreas/liver in promoting β cell proliferation.

3. To examine the potential of intra- and extra- pancreatic tissues for islet neogenesis.

### Work achieved

1. **Isolation and identification of the molecules from islet culture supernatant (ICS) responsible for pancreatic regeneration *in vivo*.** ICS was prepared in roller bottle in serum free medium (STI) from 20 pancreata obtained from Balb/c mice and subjected to fractionation (on ion exchange columns) and protein purification. The protein bound only to the cation exchange column (HiTrap Q, Amersham Pharmacia). All fractions were checked on 10% SDS-PAGE (Fig. 1). The gel showed two prominent bands, one around 66 kDa, and the other around 16 kDa. Previous studies revealed that the regenerating factor was a 16 kDa protein; the present studies supported the same. The 66 kDa band showed a migration which was very close to that of Serum Albumin. To confirm the identity of the protein the western blot of the proteins was done, employing the polyclonal antibodies developed earlier against Fraction 4 D. The antibodies detected Fraction 4 as STI. The present studies confirm the earlier amino terminal sequencing of fraction 4 which exhibited more than 90% homology with STI. Since fraction 4 was found out to be STI; we injected STI to the STZ induced diabetic mice to ascertain its role in pancreatic regeneration. We found that injections of STI for consecutive 15 days to experimental

diabetic mice led to decrease in hyperglycaemia indicating regenerating of pancreas. The present investigation showed for the first time the role of STI as a nesidioblastotic agent.

**Future work**

It is planned to study the role of STI *in vivo* and *in vitro* in islet neogenesis.

2. **Induction of islet neogenesis *in vitro***: In order to study the potential of intra-islet precursor cells in islet neogenesis, we isolated islets from adult Balb/c mice from either sex and cultured them on various ECM coated plates, such as Fibronectin, Collagen, Matrigel, in a serum free medium supplemented with Nicotinamide, ITS (Insulin, Transferrin and Selenium cocktail) and KGF (Keratinocyte Growth Factor). It was observed that islets attached to matrigel coated plates and spread to from colonies of epithelial cells. After 8-10 days of culture the epithelial cell colonies exhibited islet like clusters protruding out from the monolayer (Fig. 2). Subsequently the islet-like clusters started floating the medium, which stained positive with Dithizone (DTZ), confirming their identity as islets. The immunofluorescence studies employing FITC-tagged anti-Insulin antibodies revealed presence of insulin in the newly formed islets. The present studies thus revealed potential of intra-islet precursor cells to give rise to new islets *in vitro*, when stimulated appropriately.

**Future studies**

It is planned to screen commonly consumed nutrients for their potential to induce islet neogenesis *in vitro*.

3. **Islet neogenesis from extra-pancreatic tissues.** The aim of present study was to assess the potential of pancreatic acinar cells and duodenal epithelial cells to give rise to islets *in vitro*, as a result of transdifferentiation. During the course of study the pancreatic acinar cells and duodenal cells from rat were cultured in Ham-F12K medium supplemented with 10% Foetal Calf Serum (FCS). Attempts were made to culture acinar cells on mouse embryonic fibroblast feeder layer. It was observed that acinar attach to the feeder layer and could be maintained for a week. However they were found to be non-viable after 15 days. Hence, studies were undertaken to culture acinar cells in DMEM + HAMF12 (1:1) medium supplemented with 0.1% STI and Wortmannin (PI3K inhibitor). This led to maintenance of viable acinar cells for up to 1 month. Rat duodenal cultures exhibited presence of islets confirmed by DTZ staining. This indicated duodenum as a site of extra-pancreatic islet-like tissue. Several areas of the duodenal tissues including Payers' patches were stained positive for DTZ staining indicating their potential to give rise to extra-pancreatic islets. However, mouse duodenal cell culture did not reveal presence of islet like cells.

**Future plan**

It is planned to study the potential of foetal and adult duodenal cells and established cell lines from intestine to give rise to extrapancreatic islets.

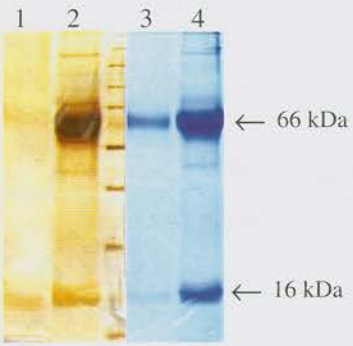



Fig. 1: SDS-PAGE analysis of cation exchange column fractions. Lanes 1 & 2 silver stain; 3 & 4 Coomassie blue stain.

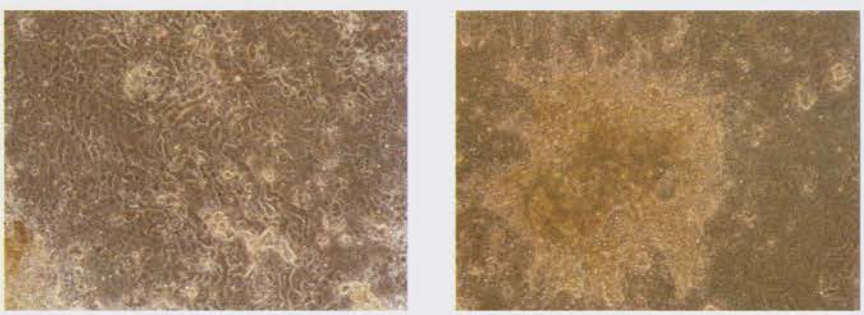
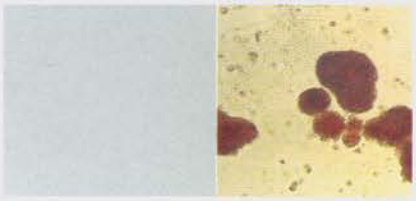


Fig. 2: Figure 2A, Monolayer of islet precursor cells, B–Budding islets from the precursor cells



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**MAMATA KATDARE**, Technician

## Immune reactions in type-1 diabetes

Insulin dependent diabetes mellitus is an autoimmune disease characterized by progressive T cell mediated beta cell destruction. In humans or in animal models of type-1 diabetes, modulation of this response can be effected by tolerance or by immunization by administration of beta cell specific auto-antigens 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 β cells either through islet transplantation or by stimulating new pancreatic islet regeneration.

### Aims

1. In-vitro regeneration of islets from pancreatic ductal epithelial cells.
2. Identification and characterization of auto-antigens.
3. Development of ELISA for estimation of blood insulin.

### Work achieved

A. **In-vitro regeneration of islets from pancreatic ductal epithelial cells**: A methodology has been developed for regeneration of islets from pancreatic ductal cells. Newly generated islets were studied for their functional characteristics such as presence of mRNA transcripts of insulin, glucagons and pdx-1 gene (Fig. 1). Regenerated islets when challenged with glucose found to secrete insulin in dose dependent manner. Regenerated islets were analysed on SDS-PAGE and transferred on nitrocellulose membrane. Immunoblots with anti-insulin antibody show a single characteristic band at 6 kDa. Pancreatic ductal cultures were further expanded and islets were cryopreserved. Diathiazone staining of regenerated islets in the culture (Fig. 2)

B. **Mechanism of action of Streptozotocin (STZ) on pancreatic β cells.** Streptozotocin is a natural toxin and destroys pancreatic beta cells when injected in-vivo. We have developed MAb against streptozotocin. Anti-STZ antibody found to protect mouse islets in presence of STZ. Immunohistochemical studies are in progress to determine site of action of STZ on β cells.

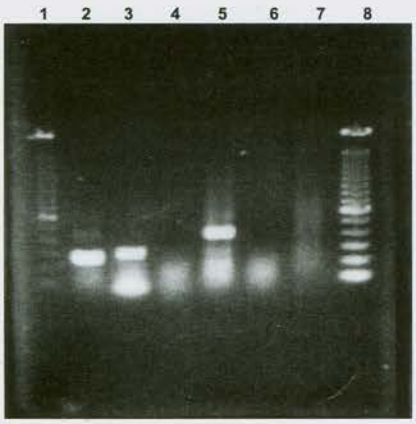



Fig. 1: RT-PCR with different PCR primers corresponding to either Actin (lanes 2), Insulin (lane 3), PP (lane 4), Glucagon (lane 5). Lanes 1 and 8 are DNA ladder whereas lane 6 and 7 are for negative controls.

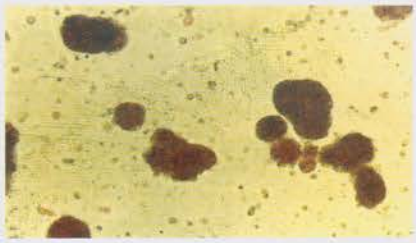


Fig. 2: Dithizone staining of emerging islets

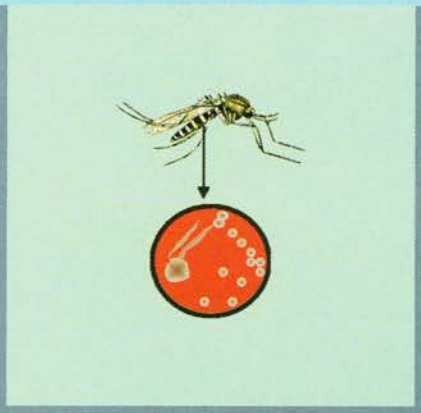
**Future plan**

1. In vitro regenerated islets have been cryopreserved at -70°C. Their revival will be assessed after 6 months.

2. These regenerated islets will be injected into diabetic animals to see reversal of diabetes.

3. Sections of pancreas will be immunostained using anti-STZ antibody to see localized action of STZ with β cells.

4. 1-Fluoro 2, 4 dinitrobenzene (FDNB) treated MIN immunized rabbit sera recognized 2 major protein bands from FDNB treated MIN cells extract. Identification and characterisation of these protein bands will be the next step.



# Insect Molecular Biology

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## Cloning and characterization of Hexokinase genes

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 their promoter elements.

### Aim

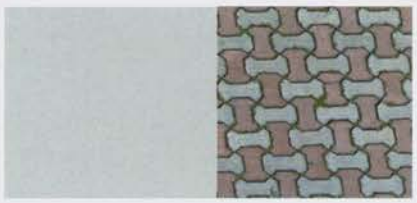
To analyze the cloned 5' upstream sequences of Hexokinase isoenzymes for testing its ability for transcription initiation.

### Work achieved

Two cloned promoter sequences for DM1 and DM2 isoenzymes in pGL3 basic vector were transfected in drosophila Schneider cell line D.Mel.2 along with pRL-Actin5C as an internal control. The dual luciferase assay performed indicated that DM1 promoter was actively used for transcription while DM2 promoter could not drive transcription. The 1600 bp DM1 promoter was deleted to yield 8 different constructs having different regions of promoter. These constructs were sequenced and characterized using luciferase reporter system. The minimum sequence essential for transcription was found to be 214 bp region in which transcription start site was located. Cotransfection experiments conducted with *Spodoptera* and *Aedes* cell line, suggested that Hexokinase promoters were not functional in these cells. Electrophoretic mobility shift analysis using 214 bp DM1 promoter element and nuclear extract obtained from cell line demonstrated sequence-specific interaction of DNA with DM1 promoter elements. Heterologous Southern hybridization using DM1 and DM2 sequences and Western blotting using anti-DM1 antibody suggested that the enzyme is conserved in insects at DNA and protein level. In addition to this Hexokinase cloned from *Leishmania major* is being investigated to obtain complete coding sequence and its expression.

### Future plan

Biotechnological applications of *Drosophila* isoenzymes and promoters will be delineated by doing expression studies *in vitro*.



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## Cloning and characterization of genes for heat shock proteins from Aeromonas

Aeromonas is ubiquitous bacterial genus widely found in different ecological conditions. Many species of Aeromonas are involved in clinical infections in fish, animals and humans. As the bacterium survives in different environmental conditions and most of them are adverse in nature, the key question asked is how the bacteria survive and do heat shock proteins play important role in survival and pathogenicity of Aeromonas.

### Aim

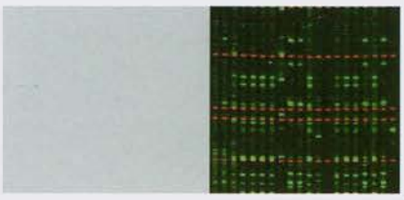
To clone and characterize genes for heat shock protein and chaperone from Aeromonas.

### Work achieved

Degenerate primers were designed for amplification of complete operon of Hsp10 and 60 and Hsp 70. Using these primers complete genes for Hsp10, 60 and 70 have been amplified from *Aeromonas culicicola*. The genes have been completely sequenced and analyzed. The sequences show high degree of homology with known heat shock protein genes from bacteria. Out of these, gene for Hsp60 protein is cloned in pET vector for expression of protein in large quantity.

### Future plan

Experiments will be done to compare the expression at RNA and protein level of various heat shock proteins under different environmental conditions.



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## Molecular taxonomy and diversity studies using rRNA sequence analyses

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. The molecule being a mosaic of conserved and variable regions, varying degree of specificity could be obtained in the design of primers for PCR and hybridization. Thus group, kingdom or strain specific primers could be designed and this has very important application in diversity studies. Due to their inherent limitations, culture based methods allow us to study a very miniscule proportion of microbes. PCR amplification of rRNA genes using "universal primers" and their sequencing makes it possible to study the uncultured majority. Knowledge of this will provide information about metabolic diversity of these microbes further enabling their exploration for biotechnological purposes. In our studies, we have selected few sites like Antarctic soil, anaerobic digester and insect mid guts.

### Aims

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

### Work achieved

A 16S ribosomal DNA (rDNA) clone library was constructed from soil obtained below a dead seal, Cape Evans McMurdo, Antarctica. A total of 499 clones were PCR screened using vector specific primers. 97 different clones were found to be positive for 1.7 kb insert. Direct PCR product sequencing (partial) was done for all the 97 clones. All the 97 sequences were analyzed for their closest similar 16S rRNA sequence from the database "nr" using blastn software. A majority of the clones showed homology to the *Cytophaga–Flavobacterium-Bacteroides* Group. Representative sequences also belonged to the classes of proteobacteria, *Psychrobacter, Aequorivita, Nitrosomonas, Marinobacter, Moraxella, Deniococcus, Gelidibacter, and* thermophilic bacteria. Our observations suggest that an enormous bacterial diversity occurs within the Antarctic soil.

From another Antarctic soil a library was prepared using primers that would amplify both prokaryotic as well as Archaeal 16S rRNA genes. The library contained a total of 1250 clones, which are being analyzed at present. From the same sample a library of PCR amplified Cyanobacterial rRNA genes was prepared and that too is being analyzed.

In addition to Antarctic soils, anaerobic methane producing digester and polluted river sediments are also being studied using same approach. For both, around one

thousand clones have been obtained and these are being screened.

Work on the mosquito mid gut flora was continued. We isolated a strain of *Aeromonas* from the mid gut of wild *Culex quinquefasciatus* and named it *Aeromonas culicicola* There is lot of confusion in the genus *Aeromonas* due to different hybridization groups and lack of congruence between these and 16 S rRNA sequence data. In an attempt to resolve some of the discrepancies, our isolate was further characterized by gyrase B gene sequencing and analysis of Aerolysin gene. We determined the *gyr*B sequence of the type strains of all the 17 hybridization groups of *Aeromonas* and analyzed their phylogenetic structure. The comparison between the phylogenetic trees drawn with 16S rRNA and *gyr*B (Fig. 1) was also done. Our strain, *A. culicicola* MTCC 3249$^T$, grouped with *A. veronii* in the *gyr*B based analysis whereas in the 16S rRNA based tree, it grouped with *A. jandaei*. The number of nucleotide differences in 16S rRNA sequence was very less as compared to the *gyr*B sequence data. Most of the observed nucleotide differences in the *gyr*B gene were synonymous. The Cophenetic Correlation Coefficient (CCC) for *gyr*B sequences was 0.78 indicating this gene to be a better molecular chronometer compared to the 16S rRNA for delineation of *Aeromonas* species.

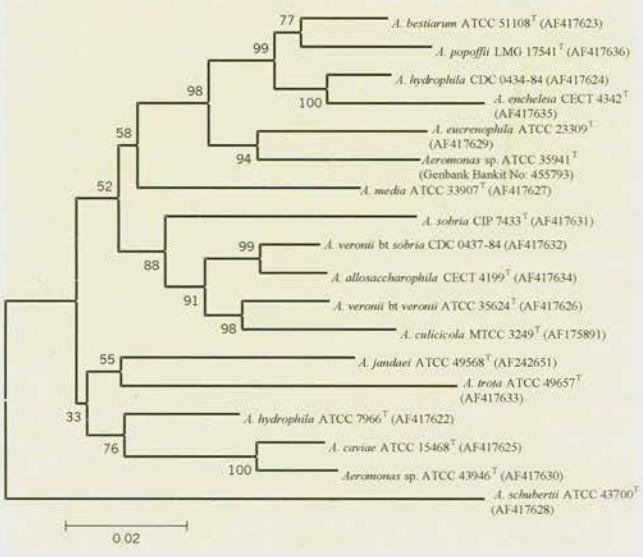



Fig. 1: Phylogenetic tree based on *gyr*B gene sequences. *gyr*B gene sequence based phylogenetic relationship of *Aeromonas culicicola* MTCC 3249$^T$ with reference strains of the genus *Aeromonas*. A midpoint rooted phylogenetic tree based on *gyr*B gene sequences (1,115 bp) was constructed from Kimura 2 distances and the neighbour-joining algorithm. The numbers near the nodes indicate percentage of 500 bootstrap replicates. The scale bar indicates genetic distance. Numbers in bracket indicate Genbank accession number.

Fig. 2: PCR amplification of the 232 bp product indicative of virulence factor presence in *Aeromonas culicicola* MTCC 3249$^T$. Lane 1, Molecular weight marker (100 bp ladder); Lane 3, PCR product (232 bp) from *Aeromonas culicicola* MTCC 3249$^T$.

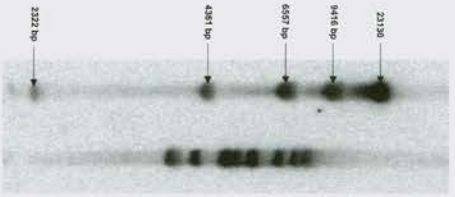


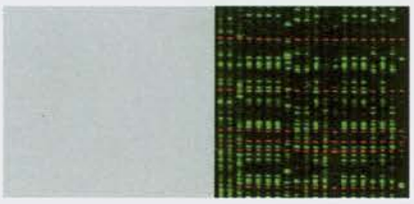

Fig. 3: Southern hybridization of *A. culicicola* MTCC 3249$^T$ using a 16S rRNA PCR product as a probe. Lane 1, Molecular Weight Marker; Lane 2, *A. culicicola* genomic DNA digested with *Sal*I and *Kpn*I.

Polymerase Chain Reaction Amplicon Sequence Analysis (PCR–ASA) of the cytolytic enterotoxin gene showed that the isolate is affiliated to type I (Fig. 2). We were also able to isolate two more strains of this species and named them SH (Small Haemolytic) and SLH (Small Late Haemolytic) and these too were completely characterized. The 16S-23S intergenic spacer and 23S rRNA gene sequence were also determined for this isolate. Ten different ISR, indicative of ten *rrn* operons, were found in the strain that were grouped in three major types. Of the three types, ISR I was non-coding while ISR II and III coded for tRNA$^{Glu\ (UUC)}$. The tRNA$^{Glu\ (UUC)}$ sequence was identical to that of *E. coli*. There were ten *rrn* operons present as confirmed by Southern hybridization analysis (Fig. 3). The upstream and downstream regions of 23S rRNA gene in the strain showed high sequence similarity with *A. hydrophila* and *E. coli* indicating their importance in processing of rRNA molecules.

## Future plan

1. Analysis of libraries generated from Antarctic soils, anaerobic methanogenic digester and river sediment will be completed. Attempts will also be made to isolate genes involved in important metabolic pathways from uncultured bacteria.

2. Gene regulation and molecular biology of Aeromonas *culicicola* will be studied to understand molecular basis of its interaction with mosquito host.



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

The gastric pathogen *H. pylori* constitutes a major cause of peptic ulcer and is an early risk factor for gastric carcinoma. The infection and peptic ulcers are very common in India with two million persons at risk from gastric inflammation, ulcers and carcinoma. There have been very little efforts for genetic characterization of these strains. Due to different ethnic composition, food habits, differential susceptibility to infectious disease and differential immune response make it highly possible that a separately evolved genotype might be colonizing Indian population.

In this project, we are attempting to get *H. pylori* isolates from Indian patients and try to characterize them genetically using known markers.

Genome sequencing of *H. pylori* strains from ethnic Indian population for comparative studies on potential genomic landmarks associated with pathogen survival, gastric ulcer and gastric carcinoma, differential pathogenesis, immune activation, molecular mimicry and essential metabolic cascades. A total of 51 isolates from gastric biopsies from patients with gastritis were obtained from CDFD, Hyderabad. These were Urease positive, resistant to Vancomycin, Trimethoprim, PolymixinB, Bacitracin, Novobiocin, Actidione, Colistin and cephazolin. They were negative of *cag* and *vac* alleles specific for *H. pylori*. At NCCS, we analyzed them by 16S rRNA sequencing and out of 51 isolates, 40 were found to be *Ochrobactrum* sp. Rest were *Achromobacter xyloxida*, *Stenotrophomonas*, *Brevibacterium*, *Enterococcus faecalis*, *Staphylococcus*, *E. coli*, *Ralstonia*, unidentified Eubacteria and unidentified and uncultured bacteria. Chromosomal FAFLP markers in the size range of 50-100 bp were analyzed for these isolates (Fig. 1) and tree (Fig. 2) was generated from this data using Genotyper 2.5 software. Most of the isolates clustered with an intra-strain distance of less than 10%.

*Ochrobactrum sp* was also detected in two biopsies. For one isolate entire 1.5 kb 16S rRNA gene was sequenced. Fig 3 shows the phylogenetic tree derived from this sequence, which indicates this isolate to have a distinct lineage from know species of genus *Ochrobactrum*. We have devised a PCR-RFLP based identification method for detection and identification of this isolate.

Recently we have obtained gastric biopsies isolated from the patients suffering from Duodenal and gastric ulcers from hospitals in Pune (Armed Forces Medical College, Ruby Hall Clinic, Dinanath Mangeshkhar Hospital) and are being processed for the isolation of *Helicobacter pylori* . These biopsies were urease positive and also gave PCR with primers specific for *cag* and 16S rRNA genes of Helicobacter *pylori*. The authenticity of PCR products was confirmed by sequencing. Interestingly, one of the biopsies showed a shorter PCR product for *cag* gene indicative of presence of IS element.

**Automated genotyping and mapping of FAFLP markers**

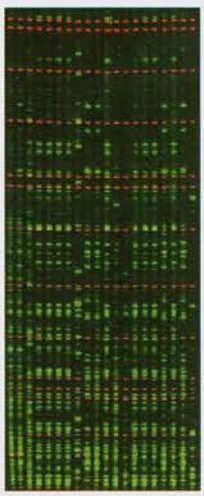


Fig. 1: Two-dimensional gel images were transformed with Genescan-TM 3.1 software (ABI). Gel image indicating fingerprints of the *Ochrobactrum* isolates. The primer pairs [MseI+0/EcoRI+0-FAM (Blue bands) and MseI+0/EcoRI+T-JOE (Green bands)] were used in a multiplex FAFLP reaction. Red coloured bands indicate the internal lane standard, Genescan Rox 500.

**Genetic relationships among Ochrobactrum isolates**

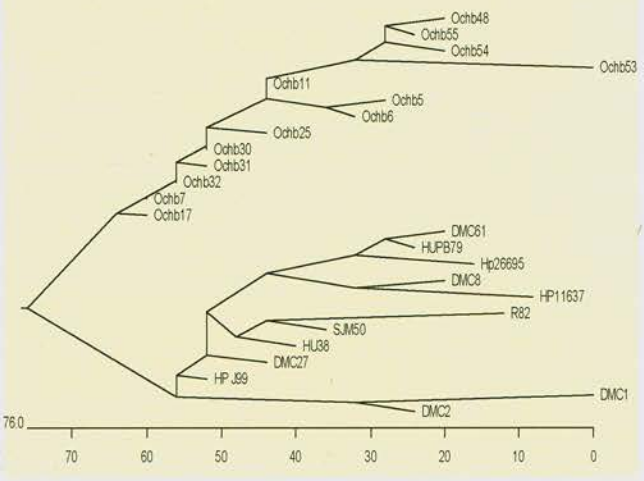



Fig. 2: Neighbour joining tree developed from the binary data obtained by genotypic comparisons made by the Genotyper™ macro. Scale in the bottom shows percent heterogeneity among the isolates based on sharing of FAFLP fragments. Ochrobactrum spp. (strain code Ochb) were compared with epidemiologically related H. pylori (strain code DMC) isolates and standard H. pylori strains from across the world such as the two sequenced strains (HP26695 and HP J99), one murine strain from Australia (HP11637) and patient isolates from Japan (HU38), South Africa (R82), Peru (SJM50) and Spain (HUPB79.).

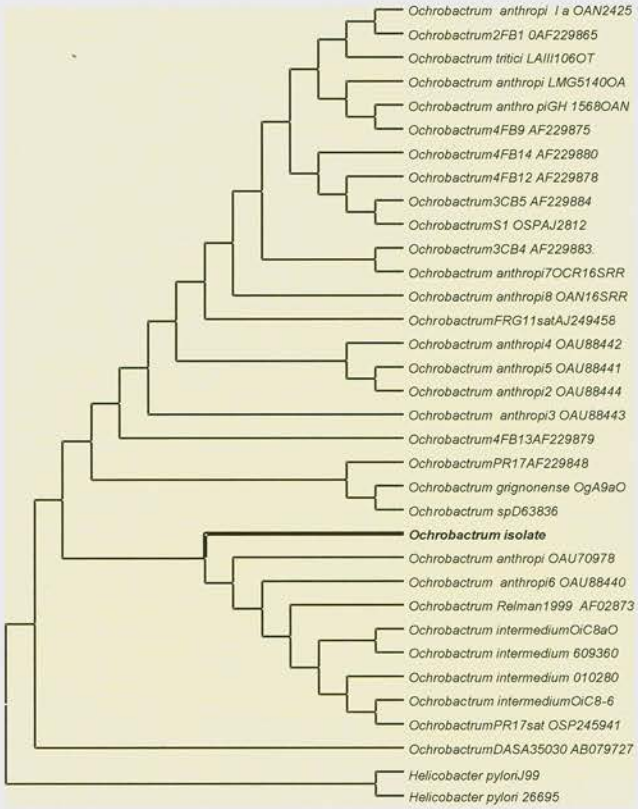
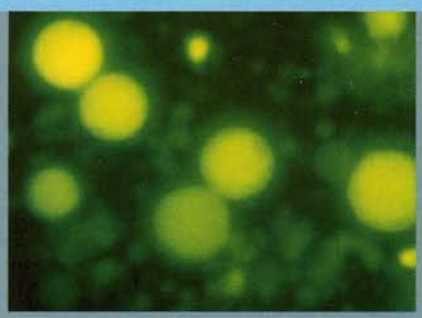
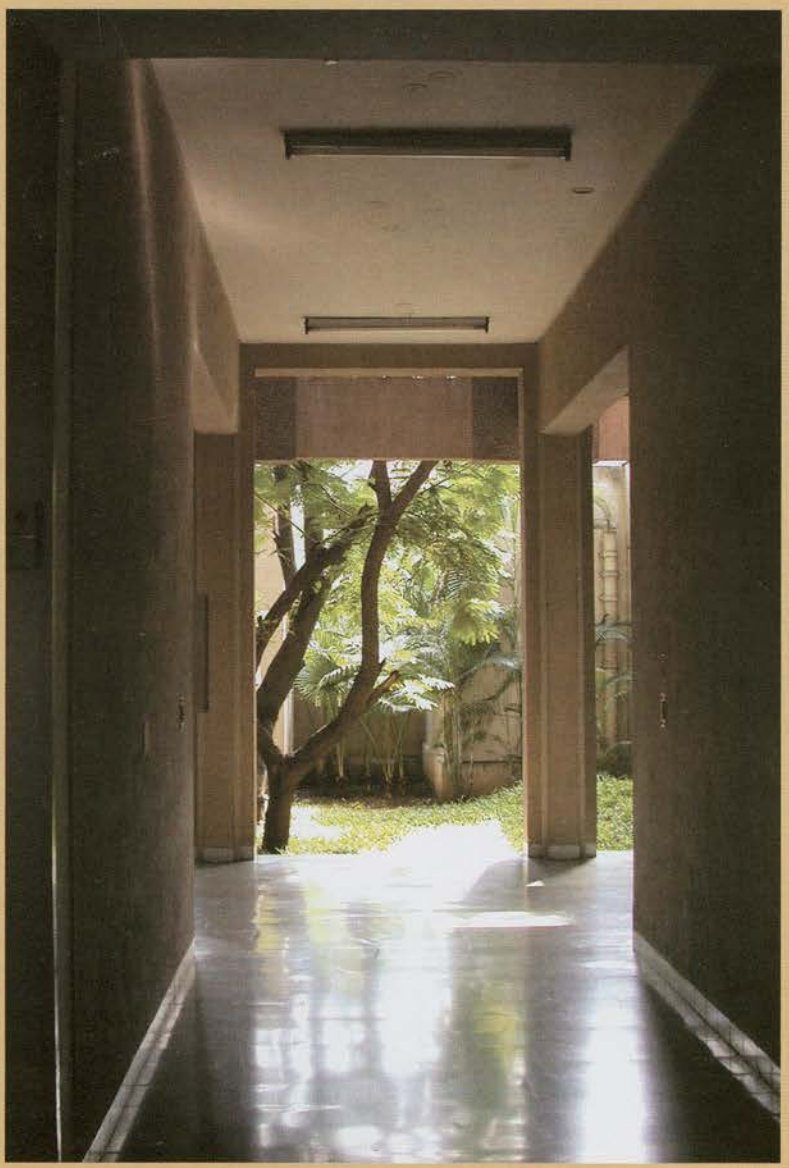



Fig. 3: 16S rRNA phylogeny prepared by using Helicobacter pylori isolates (J99, 26695) as an out group.



# Infection & Immunity



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## Role of hemozoin in immune response

The course of Plasmodium *falciparum* malaria disease is chararacterized by complex interactions of parasite and its products with host immune system. It has been reported that there is a suppression or impairment of immune responses in malaria patients. So far there is no report on malaria parasite-induced immunosuppression. The malaria pigment hemozoin is an important catabolite which is formed during the intraerythrocytic development of the parasites. The pigment is released into blood along with merozoites, when mature schizont infected RBC ruptures. The pigment is accumulated in the phagocytic cells of different organs of malaria patients. We have isolated hemozoin from *P. falciparum* cultures and investigated its role in the immune responses.

### Aims

1. Effect of malaria pigment on mitogen stimulated normal human peripheral blood lymphocytes proliferation.

2. Hemozoin-induced cytokine response and its role in the modulation of immune response.

### Work achieved

Malaria pigment inhibits mitogen (PHA) stimulated normal human peripheral blood lymphocyte proliferation in a dose dependant manner. Except hemozoin, none of the other components such as parasite soluble extract, parasite culture supernatant, normal RBC extract or haemin chloride were able to inhibit mitogen-stimulated cell proliferation. The inhibition of proliferation is mediated only when monocytes/macrophages fed/phagocytosed (Fig. 1) with the pigment only. The immunosuppression is accompanied with drastic reduction of IL-2, IFN$\gamma$ and high level production of TNF$\alpha$, IL-1$\beta$ and IL-10 cytokines. Exogenous supplementation of recombinant IL-2 (human) or neutralization of TNF$\alpha$ does not abrogate hemozoin-induced inhibition of activated lymphocyte proliferation. There is no apoptosis of lymphocytes in the hemozoin induced suppression of cell proliferation and there is no role of nitric oxide (NO) in this phenomenon. Hemozoin also inhibits the concanavalin A (Con A)-stimulated PBL proliferation.

The malaria pigment hemozoin inhibits normal human peripheral blood lymphocyte proliferation activated with $\alpha$-CD3/$\alpha$-CD28, ionomycin+PMA and purified protein derivative (PPD). These results indicate that monocyte/macrophages fed with the malarial pigment inhibit stimulated peripheral blood lymphocyte proliferation.

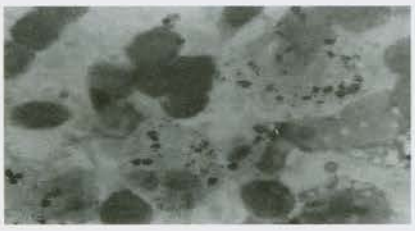
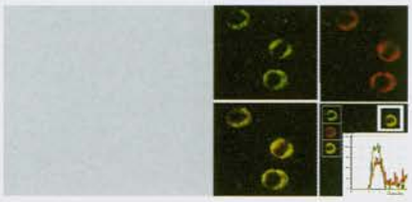


Fig. 1: Monocyte/macrophages phagocytosed with *Plasmodium falciparum* pigment – hemozoin



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## Development of Th1 and Th2 Specific Costimulatory Molecules

According to the present dogma of T cell activation, it is the "Strength of Signal", which denotes the pathway of T helper cell differentiation. To depict, apart from the intensity of interaction between the antigen presenting cells and the T cells, it is the pre-existing cytokine milieu which also plays a pivotal role in skewing the T helper governed immune response to give raise either Th1 or Th2 phenotype. Further, researchers of the present decade have witnessed compelling evidences to accept that, generation of Th2 response from naïve Th cells requires a higher 'strength of signal' in comparison to the 'strength' required for initiating a Th1 biased immunity. Since, in nature, the first signal only comes to the T cells in context of MHC-TCR interaction and it is only the second/ costimulatory signal, which may vary among the APCs, we therefore, hypothesized the role of costimulatory molecules in Th differentiation.

### Aim

Our aim therefore, was to isolate novel costimulatory molecules from activated professional antigen presenting cells like macrophages and B cells and to test their ability for T helper cell differentiation.

### Work achieved

The work under the present title had recently been completed. In brief, previous workers of our lab had identified 4 new costimulatory molecules. Three of them, isolated from LPS activated B cells was found to be Th2 specific and a molecular mass of 150 kDa (M150) isolated from thioglycollate exudate macrophages were found to initiate a potent Th1 response. In the forging account, while characterizing these molecules, we found that M150 was a differential glycosylated form of LAMP-1 (Lysosomal Associated Membrane Protein-1) expressed only in macrophages (1) while one of the molecules isolated from B cell membrane turned out to be gp96. Both these molecules were the product of housekeeping genes and thus our research opened a new era in T cell research, where on one hand differential glycosylation added a new charismatic role to LAMP-1 and on the other; the danger signal (LPS) provoked the gp96 to be a costimulatory molecule (2).

It may be the matter of particular importance that voluminous reports are available on the activation of Th cells by B cells; however their role in $CD8^+$ T cell activation is yet to be elucidated. One school of thought explains how B cells can tolerize or suppress the $CD8^+$ T response while the other depicts an important role of B cells in memory $CD8^+$ T activation. To find a suitable answer to this dilemma we decided to study the role of B

cells in a $CD8^+$ T cell assay. For this we chose B cells, which were either activated by LPS or by anti-Ig and anti-CD40 antibodies. It is known that B cell activation may occur in response to two kinds of antigens viz. T-dependent and T independent antigens. B cell activation in response to T-dependent antigens occurs when B cells capture antigens via surface Ig and process it for presentation with MHC-II to $CD4^+$ T cell resulting in an antigen specific T-B cognate interaction. Moreover, interaction of CD40L on T cells with CD40 on the B cells is an essential prerequisite for the optimum activation of B cells. Therefore, for T-dependent B cell activation, we used anti-mouse Ig as a model antigen in the presence of anti-CD40 activating antibodies to mimic $CD4^+$ T cell help. On the other hand, the best studied T-independent antigen i.e. Lipopolysaccharide (LPS) had been used to activate the second group of B cells. LPS is a product of gram-negative bacteria, which is known to bring polyclonal B cell activation, proliferation and production of cytokines like IL-1, IL-6, IL-8, and TNF-$\alpha$. LPS is also known to stimulate B cells to enhance their antigen presenting capacity, which could be accompanied by secretion of large amounts of LPS neutralizing antibodies. Thus, in the present study, we utilized these two types of B cells i.e. anti-Ig plus anti-CD40 activated B cells and LPS activated B cells as APCs to verify their role in $CD8^+$ T cell activation. For the assay, we used $CD8^+$ T cells from primed mice and cultured them in the presence of activated B cells *in vitro*. Here we show that $CD8^+$ T cells responses in terms of proliferation (Fig. 1), cytokine secretion (Figure 2) and cytotoxic effector function are severely depressed when those were

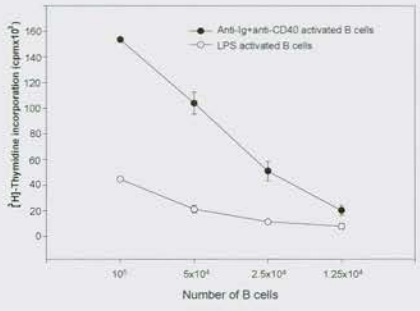


Fig. 1: Anti-Ig+anti-CD40 activated B cells induce higher proliferation of $CD8^+$ T cells compared to LPS activated B cells. Purified $10^5$ $CD8^+$ T cells were activated in the presence anti-CD3e (1mg/ml) and varying numbers of irradiated anti-Ig+anti-CD40 activated or LPS activated B cells. Cells were cultured for 72 hours and T cell proliferation was assessed by [$^3$H]-Thymidine incorporation for the last 12 hours. Irradiated B cells alone did not proliferate as the counts of the highest cell number used ($10^5$) were 3452 ± 1098 and 2353 ± 875 for LPS activated and anti-Ig+anti-CD40 activated B cells, respectively. The results shown are the mean ± SD of triplicate wells and represent five individual experiments.

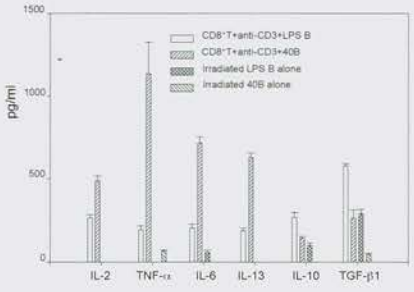


Fig. 2: Cytokine analysis by ELISA. Purified $10^5$ $CD8^+$ T cells were activated with anti-CD3e and $5x10^4$ irradiated LPS activated B cells or anti-Ig+anti-CD40 activated B cells per well of a 96 well plate. Supernatant were harvested at 24 hours for IL-2, 48 hours for IL-6, TNF-a, IL-10 & IL-13 and 72 hours for TGF-b1. Supernatants of T cells alone and T cells with anti-CD3e did not show detectable cytokine levels and IL-15 & IL-4 were not detected in these culture supernatants. For TGF-b1 estimation, cells were cultured in the medium containing 2% foetal bovine serum with Insulin-Transferrin-Selenium-A. Culture supernatants were treated with hydrochloric acid to a pH 2.5 for activation of pro-TGF-b to active TGF-b1 and estimated by ELISA.

activated in the presence of LPS activated B cells as compared to anti-Ig + anti-CD40 activated B cells. The results show that the supernatant of $CD8^+$ T cells activated with LPS activated B cells as APCs, secreted higher levels of TGF-$\beta$1 and IL-10, as compared to the supernatant of $CD8^+$ T cells activated with anti-Ig + anti-CD40 activated B cells. The results of our preliminary experiments suggest that suppressed $CD8^+$ T cell responses by LPS activated B cells was due to much higher synthesis and expression of Transforming growth factor-$\beta$1 + (TGF-$\beta$1) + by them in comparison to anti-Ig + anti-CD40 activated B cells. Therefore, it is possible that these T cell inhibitory cytokines secreted by the LPS B suppress $CD8^+$ T cell responses in our cultures.

Thus our data depicting the role of TGF-$\beta$1 in down regulation of CTL response not only provide the very first line of information about mechanism of CTL activation and regulation at molecular level but also suggest the possible use of anti-TGF-$\beta$1 antibody as an effective barrier against the invasion of gram negative bacteria.

**Future plan**

1. Delineate the Mechanism of signal transduction by M150.

2. Studies and the distribution, in time and space, of costimulatory molecules and their role during the differentiation of APCs from pluripotent stem cell.

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## Molecular and Cellular Basis of HIV Pathogenesis

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 the virus. 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. We are also working towards understanding the immune response to HIV in order to design novel vaccination strategies. Finally, we are also engaged in identification of anti- HIV activity in plant and marine bivalve extracts.

### 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

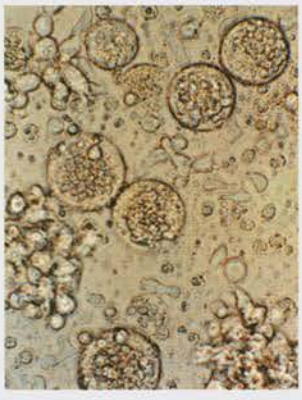
**Role of viral regulatory proteins Tat and Nef in HIV pathogenesis, and differential gene expression studies in HIV-1 infected cells.**

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, and also to determine if these two proteins interact and thereby influence HIV replication and pathogenesis. Using co-immunoprecipitation and pull down analysis of HIV-1 infected cells, we have been able to confirm that HIV-1 Nef protein physically interacts with Tat, the premier transactivating protein of HIV-1. Our observations further indicate that this interaction results in positive regulation of Tat induced HIV-1 LTR mediated gene expression.

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 living cells in the HIV-infected T cell population and to elucidate the interaction of those molecules in the signalling cascade leading to cell death. We have used a reporter T cell line, CEM-GFP to distinguish HIV infected cells from uninfected cells (Fig. 1), and our data clearly indicates that both infected and uninfected cells die by apoptosis. We have been able to purify the apoptotic cells from the non-apoptotic cells and differential gene expression studies are currently in progress using differential display technique (Fig. 2) and microarray analysis.

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

HIV-1 is a uniquely difficult target to develop immunological intervention against it. In this regard, one of the main hindrances has been the lack of understanding as to what constitutes protective immunity to the virus. 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. The present study, therefore, aims at defining the most critical components involved in the anti HIV immune response, which results in elimination of the virus, using DNA immunization protocol. In order to identify candidate molecules for generating protective immune response, we have constructed bicistronic mammalian expression vectors expressing either HIV-1 subtype C gp120 and Tat or gp120 and Nef using



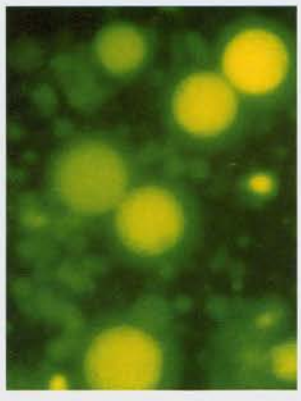


Fig. 1: CEM-GFP reporter human T cell line infected with HIV-1 NL4.3 showing green fluorescent protein expression in infected cells and syncitia formation (400X).

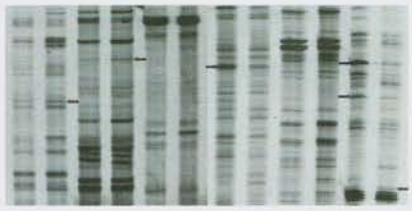


Fig. 2: Autoradiogram showing differential expression of transcripts using RNA from HIV-1 infected and uninfected cells.

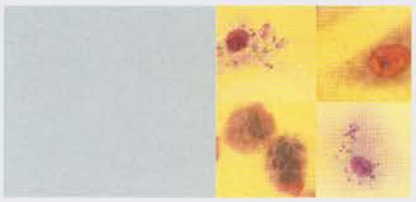
internal ribosomal entry site in between the two genes. We have started DNA immunization studies in mice using these vectors and has been able to observe humoural response. Cellular response studies are in progress. Furthermore, we are also trying to use virosomes as a vehicle for DNA immunization in order to develop a better delivery system for DNA vaccine.

**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 anti-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 preliminary data indicate presence of anti-HIV activity in some marine samples.

**Future plan**

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. Identification of differentially expressed genes and their relevance to HIV induced cell death will be continued. We are also trying to identify novel and efficient delivery systems for DNA immunization as a part of the DNA vaccine work. Finally, crude samples showing anti-HIV activity will be 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

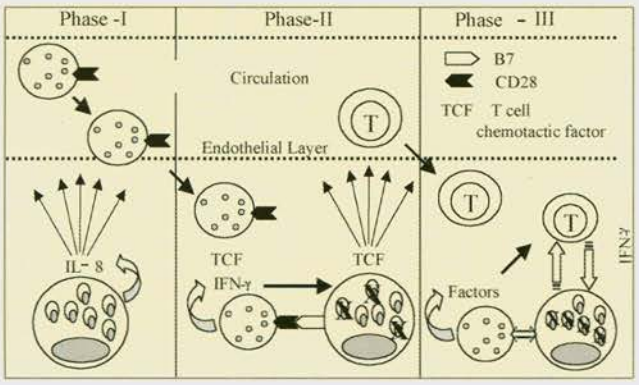
We showed previously that human peripheral blood neutrophils express CD28 and interact with macrophage B7 to generate CD28 signalling through PI-3 kinase. Here, we demonstrate that cross-linking of CD28 on neutrophils results in the release of IFN-γ, which restricts amastigote growth and modulate $CD4^+$ T cells cytokine secretion. CD28 cross-linking also induces a T cell chemotactic factor (TCF) that induces chemotactic migration of $CD4^+$ T cells. Based on our previous and the current set of data, we propose an operational model explaining how neutrophils are involved in *Leishmania* infection and how the reported effect of neutrophils on the control of infection is mediated by alteration of T cell function.

### Work achieved

It is proposed that the host's reaction in response to *Leishmania* infection initiated at this point is mediated by a monoclonal T cell repertoire of $V\beta4^+V\alpha8^+$ T cells, which produce IL-4 to set the bias towards Th2 cells in the susceptible host. In contrast, earlier proposition was that the initial T cell activation results in the production of a wide array of cytokines, which control parasite growth by regulating the macrophage activation or deactivation or by setting a Th subset bias. Moreover, we and others did not observe any alteration in the Vβ repertoire of T cells in *L. major* and *L. donovani* infections under many different conditions. Therefore, involvement of T cells in the initiation of the anti-leishmanial immune response is not well defined raising a possibility of involvement of non-T cells in the initiation of anti-leishmanial immune response.

The culture supernatant of CD28-crosslinked neutrophils inhibited intracellular *L. major* growth in the infected macrophages in IFN-γ+dependent manner.++ The CD28-activated neutrophils also secreted a factor(s), which chemoattracted $CD4^+$ T cells. Because IFN-γ was present in the CD28-activated neutrophil supernatant, we tested whether or not IFN-γ induced the $CD4^+$ T cell chemotaxis. We did not observe any significant chemotaxis of $CD4^+$ T cells in response to IFN-γ. Since the cytokine microenvironment is proposed to effect the Th subset polarization, we investigated whether or not the CD28-activated neutrophil supernatant had any effect on $CD4^+$ T cells cytokine secretion profile. The $CD4^+$ T cells pre-incubated with the CD28-activated neutrophil culture supernatant secreted more IFN-γ but? less IL-4 upon subsequent activation. IFN-γ neutralization in the supernatant inhibited the augmented T cell IFN-γ secretion suggesting that IFN-γ secreted by neutrophils plays an important role in setting a Th subset bias. Based on our observations, we propose the following model (Model-1) defining the role of neutrophils in anti-leishmanial immune response.

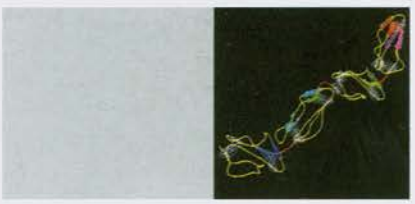
**Model 1:**




According to the model, the inflammatory reaction against *Leishmania* infection is initiated with the release of chemokines such as IL-8 by *Leishmania*-infected macrophages, followed by IL-8-dependent neutrophil recruitment to the site of infection (Phase-I). CD28 on neutrophils engages CD80/CD86 on macrophages to generate CD28 signal inducing IFN-γ secretion (Phase-II). In addition to restriction of *Leishmania* growth, neutrophil secreted IFN-γ alters the cytokine microenvironment at the site of infection, affecting the IL-4 and IFN-γ secreting T cells differentiation as a consequence (Phase-III). Thus, although the current hypothesis for initiation of the disease leishmaniasis by setting a Th subset bias relies heavily on T cell secreted cytokines, our observations define for the first time a significant role of neutrophils in the afferent limb of the anti-leishmanial immune response, especially that which precedes T cell response.

**Future plan**

Since both neutrophils and macrophages belong to the innate immune system, they will be compared for their reactivity to common antigens like LPS, anti-LFA-1, etc. in terms of cytokine production and other functions.



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## Structure-function analysis of viral homologs of complement control proteins

The complement system is a potent innate immune mechanism consisting of cascades of proteins, which are designed to fight against and annul intrusion of all the foreign pathogens. Although viruses are smaller in size and have relatively simple structure, they are not immune to complement attack. Thus, activation of the complement system can lead to neutralization of cell-free viruses, phagocytosis of C3b-coated viral particles, lysis of virus-infected cells, and generation of inflammatory and specific immune responses. However, to combat host responses and succeed as pathogens, viruses not only have developed/adopted mechanisms to control complement, but have also turned these interactions to their own advantage. Vaccinia virus (V V), herpes virus saimiri (HVS), and Kaposi's sarcoma associated herpes virus (HHV-8) in particular, are known to encode for complement control protein homologs (CCPHs). Our working hypothesis is that these viruses encode CCPHs to protect themselves against host's complement attack and are essential for their survival in vivo (Fig. 1). Currently our laboratory is interested in unravelling the molecular mechanisms underlying the interaction between host's complement proteins and CCPHs of V V, HVS and HHV-8. It is our conviction that this study would provide valuable insight into the essential aspects of viral pathogenesis.

### Aims

1. Expression and characterization of the biological properties and mechanism of complement inactivation of viral homologs of complement control proteins.

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

### Work achieved

**Expression and characterization of the biological properties and mechanism of complement inactivation of viral homologs of complement control proteins**

Previously it has been reported that CCPHs of V V and HVS inhibit complement, however, the detailed mechanisms by which these viral CCPHs regulate complement are not known. In case of CCPH of HHV-8, data is still awaited to assign the function to this protein.

We have expressed CCPH of vaccinia virus (VCP) using *Pichia* expression system. While designing the construct we did not include His tag, which is normally added during expression to aid purification. We expressed VCP in its native form since addition of His

could put a stretch of charge on the protein (if pKa of His is altered upon binding) which may influence its activity. Standardization of expression resulted in secretion of rVCP at the level of 50-150 μg/ml. The expressed protein was functionally active as judged by its binding to complement proteins C3b and C4b and inhibition of complement-mediated erythrocyte lysis. Functional analysis revealed that VCP acts as a cofactor and supports factor I mediated inactivation of both C3b as well as C4b. In order to understand molecular mechanisms involved in the interaction of VCP with its ligands C3b and C4b we employed a surface plasmon resonance technology. Analysis of binding data revealed that binding of VCP to C3b and C4b does not follow a simple 1:1 Langmuir binding model and involves more than one

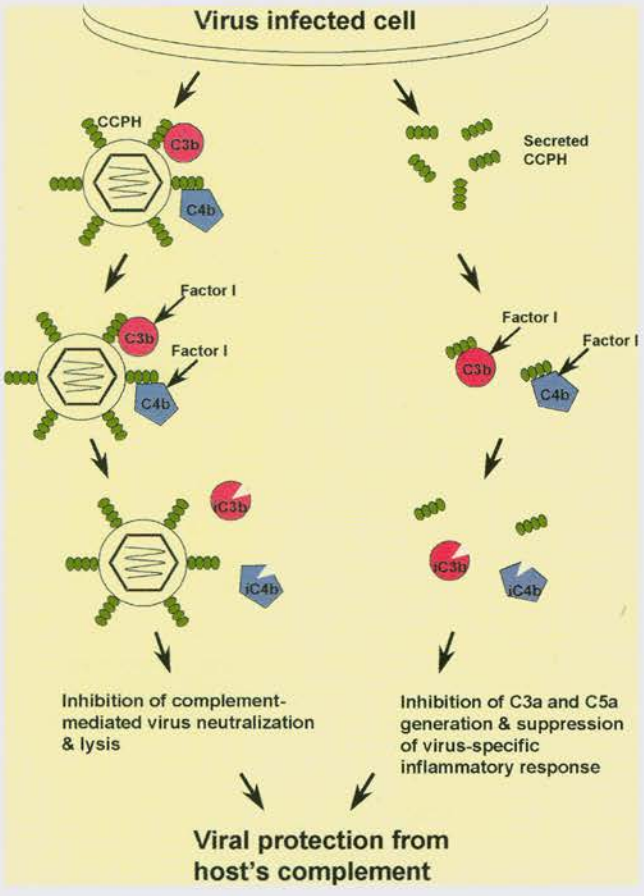



Fig. 1: Working model of CCPH-mediated protection of viruses from the host complement system. CCPH, either expressed on the viral surface, or secreted in the medium, binds to C3b and C4b and supports their proteolytic inactivation by factor I. Regulation of complement on the viral surface would lead to inhibition of complement-mediated viral neutralization and lysis, while inhibition of fluid phase complement activation at the site of infection would reduce the specific inflammatory response against the virus. Together this would lead to protection of virus from the host's complement system.

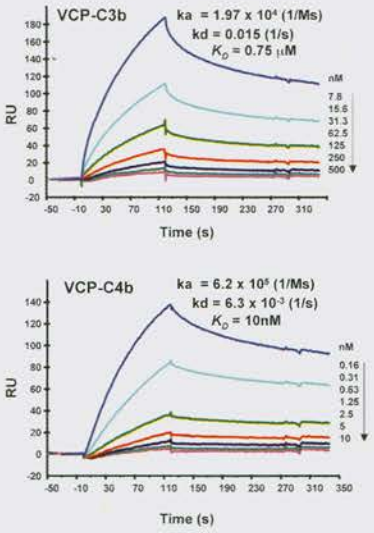



Fig. 2: Analysis of binding of VCP to human C3b and C4b by surface plasmon resonance.

component (Fig. 2). Interestingly its affinity for C3b is approximately 73-fold less than that for C4b. This was primarily due to decrease in on-rate of C3b. It is pertinent to point out here that although the virus encodes a small protein, it is capable of regulating both C3b as well as C4b. The only human complement regulator that has these activities is CR1, which is a 200 kDa protein. Thus, small size of VCP (30 kDa) reflects the limited size of the virus genome, but this does not limit its activities.

We have also cloned CCPHs of HVS and HHV-8 in the pPICZ α expression vector and have validated its identity by sequencing. Experiments are underway to integrate these homologs in the *Pichia* genome for expression.

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

Identification of vital structural determinants would not only allow us to better understand the structural features of viral CCPHs that are important in its biology but would also identify a significant control point in the molecule that would direct the development of pharmacological ligand molecules to neutralize these viruses.

To characterize the functional determinants of CCPHs we plan to generate deletion as well as chimeric mutants where individual short consensus repeats (SCRs) of viral homologs will be deleted

and swapped by SCRs of human complement proteins, respectively. This approach is feasible since previous structural data have shown that individual SCRs are capable of folding into a bead like structure. To this end, we have generated deletion mutants of VCP. We have obtained the required PCR products of VCP and have cloned them in the pPICZ α expression vector (Fig. 3). The structure of all the mutants have been validated by DNA sequencing. Integration of these mutants into *Pichia* genome for expression is underway.

Use of monoclonal antibodies (mAbs) to block the function of a protein is an important approach utilized for the identification of functionally important determinants. The advantage of this approach over use of deletion and chimeric mutants is that functional determinants are identified in the context of a native protein. Previously we had generated over 40 hybridoma clones. We have sub-cloned, purified, and characterized four of them (Table 1). Functional analysis of these mAbs revealed that mAb NCCS 67.1 inhibits the factor I cofactor activity of VCP for C3b, but not for C4b indicating thereby that physically distinct domains of VCP are involved in the interaction with C3b and C4b. Deletion mutants of VCP that are being generated in the laboratory will be helpful in identification of the domain to which mAb 67.1 binds. This mAb will also be useful for characterization of the in vivo function of VCP. We are in a process of subcloning more mAbs with the hopes of generating a panel of mAbs that would block individual functions of VCP.

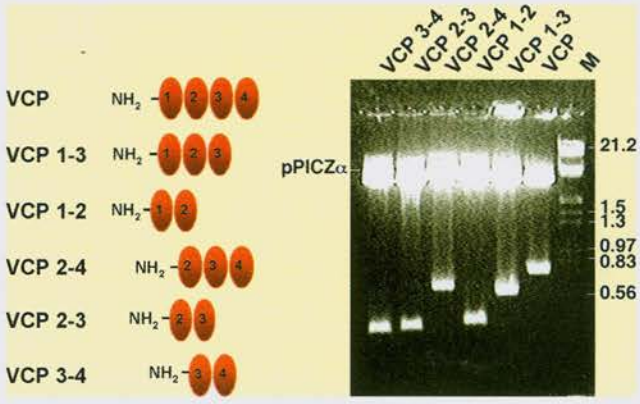


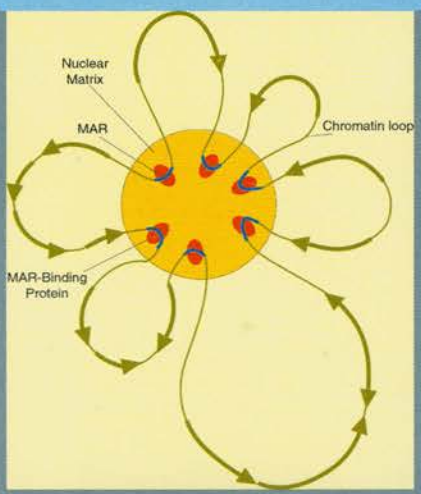

Fig. 3: Deletion mutants of VCP.

## Future plan

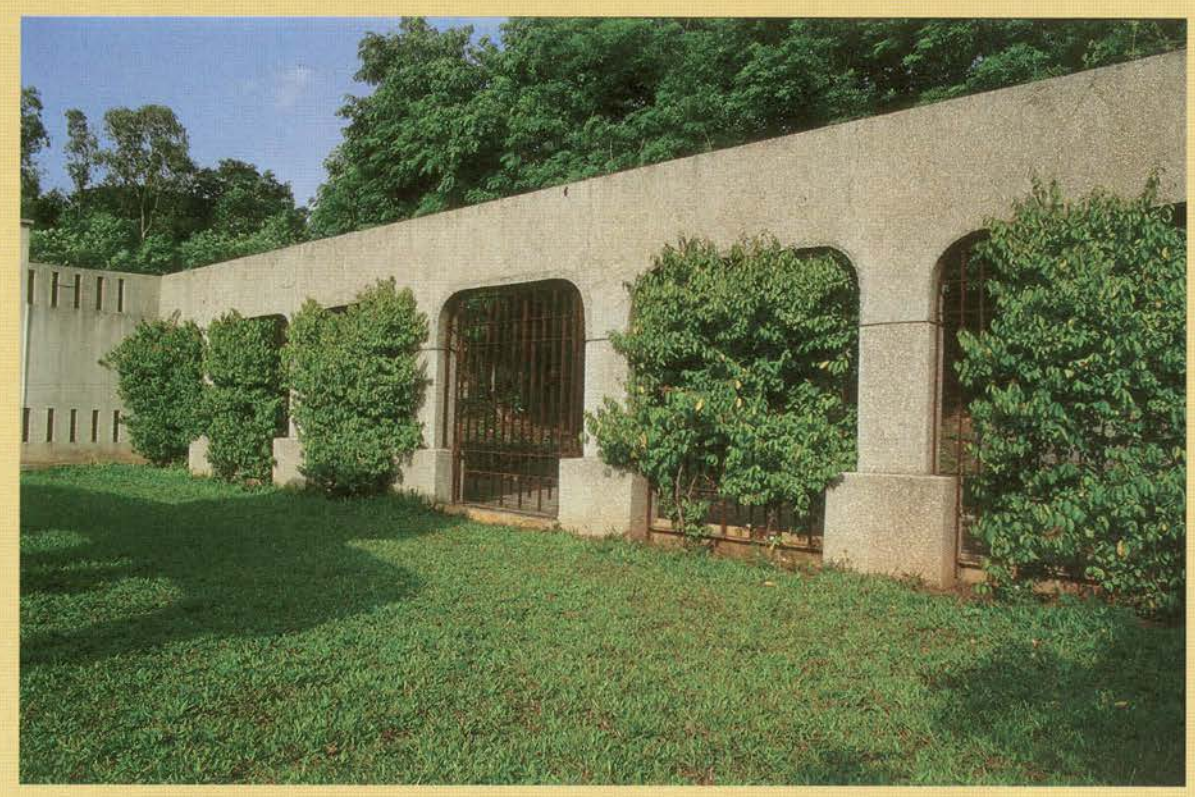
1. Expression and functional characterization of CCPHs of HVS and HHV-8.
2. Expression and functional characterization of deletion mutants of VCP.
3. Generation of a panel of mAbs against VCP that would block the individual functions of VCP.

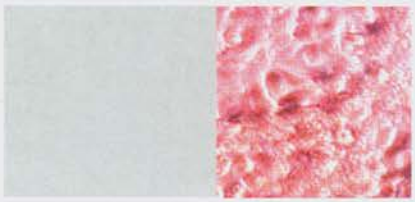
**Table 1**: Functional characterization of anti-VCP monoclonal antibodies.

| mAbs | Isotype | | | Binding to VCP | | | Inhibition of factor I cofactor activity | |
|---|---|---|---|---|---|---|---|---|
| | | ELISA | Western blot | $k_{on}$ (1/Ms) | $k_{off}$ (1/s) | $K_D$ | Fluid phase C3b | Fluid phase C4b |
| NCCS 67.1 | $IgG_1\kappa$ | + | - | $1.33 \times 10^5$ | 0.01 | 76 nM | + | - |
| NCCS 67.2 | $IgG_1\kappa$ | + | - | $2.89 \times 10^5$ | $1.09 \times 10^{-3}$ | 3.7 nM | - | - |
| NCCS 67.3 | $IgG_1\kappa$ | + | - | 714 | 0.016 | 22 µM | - | - |
| NCCS 67.4 | IgMκ | + | - | $4.24 \times 10^5$ | $9.81 \times 10^{-4}$ | 2.3 nM | - | - |




# Chromatin Architecture and Gene Regulation





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## Novel functions of MARs and associated proteins in cellular transformation and HIV LTR mediated transcription

S/MARs (Scaffold/Matrix Associated Regions) are AT rich sequences that occur at every 30 kb in the chromosome. Due to the unique chromatin structure, MARs and MAR binding proteins are necessary for correct chromosomal replication, transcription, condensation and recombination. In recent experiments it has been shown that the alteration of structured chromatin due to abnormal expression of MAR binding protein causes various cancers. Our laboratory focuses on the role of MARs and MAR binding proteins in various cancers, embryogenesis and HIV LTR mediated transcription. We have identified one such MAR binding nuclear protein from mouse thymocytes, SMAR1 that acts as a global transcriptional repressor. It exists in at least two alternatively spliced forms of which the shorter form (deletion of 39aa) is exclusively present in the early progenitor haematopoietic and embryonic cells. Over-expression of alternatively spliced form causes cell cycle arrest in mouse melanoma cells and delays tumour progression in mice. SMAR1 physically interacts with and activates p53. Other studies in our lab show that MAR sequences present upstream of the HIV-LTR promoter causes anti-termination in transcription at a distance. We show a novel mechanism how the MAR sequences alters the chromatin structure allowing HIV Tat dependent and Tat independent transcription in HIV.

### Aims

1. Molecular dissection of SMAR1 protein, identification of nuclear localization and DNA binding domains.
2. Construction of transgenic mice for SMAR1.
3. To elucidate functions of AT-rich MAR sequences as an enhancer of HIV-LTR mediated transcription *in vitro* and *in vivo*.
4. Receptor mediated activation of T-cells by HIV-Tat transactivator: An implication on bystander CD4+ T cells death during HIV pathogenesis.

### Work achieved

#### Antitumorigenic MAR binding protein SMAR1 delays cell cycle progression

We have identified a novel gene SMAR1 that has strong antiproliferative activity (Chattopadhyay et al., 2000, *Genomics*, 68 pp. 93-96). SMAR1 gene is located at human chromosome 16q24 the loss of heterozygosity of this locus causes cancers. By

overexpression of the same protein in the tumorigenic cells and further injection into mice delays tumour formation in mice. Both SMAR1 and p53 has tetramerization domains at the C-terminal end. By immunoprecipitation assays we show that SMAR1 activates and directly binds to p53 indicating a novel mechanism of cell cycle arrest by p53 mediated through direct interaction (Fig. 1). Recently, we have dissected important domains within SMAR1 protein sequence. The nuclear localization sequence (NLS) is present within 245-288 aa (Fig. 2). The DNA binding domain is located just downstream of the NLS spanning from 288-350 aa containing an arginine-rich domain (RRKQR). We are interested to see that specific domain that interacts with p53. The transgenic mice over-expressing SMAR1 has already been constructed. These mice will be studied for the T cell development and the transcriptional down-regulation within the TCRβ region may provide first in vivo function in a context dependent manner. By luciferase assay system in the context of Eβ enhancer and MAR we show that SMAR1 causes a transcriptional repression indicating that in mice probably SMAR1 controls the function of Eβ enhancer that in turn controls the V(D)J recombination in TCRβ locus.

**2. MAR sequences function as an enhancer HIV-LTR promoter**

Chromatin structure alteration plays an important role in integration as well as expression of HIV-1 late genes. We have found a novel MAR sequence from TCRβ+locus+that allows the accessibility for the transcription within a T or non-T cell. This MAR specifically gets induced at the double positive stage of T cell development. We have cloned the same element as a dimer, upstream of HIV- LTR promoter. When this construct is introduced into T cells or other non-T cells, the expression of the foreign genes goes up by 26 fold, indicating that the ATC-rich MAR sequence helps expression of any genes much strongly by recruiting factors which otherwise cannot access or allow transcription (Fig. 3). This experiment was done both in presence and absence of the transactivator protein Tat and we see the overexpression of reporter genes even in the absence of Tat. This is the first *cis* element that allows the gene expression after 1kb of non-specific sequence in HIV genome. RNase protection assays show that the amount of the RNA transcribed is much higher in case of MAR-LTR compared to only LTR promoter, indicating a kinetically strong initiation of transcription mediated through MAR sequences.

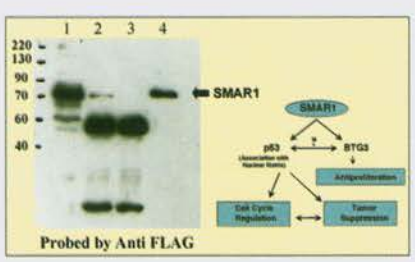


Fig. 1: SMAR1 directly interacts with p53 as shown in lanes 2 and 4. Lower two bands in lanes 2 and 3 correspond to IgH and IgL.

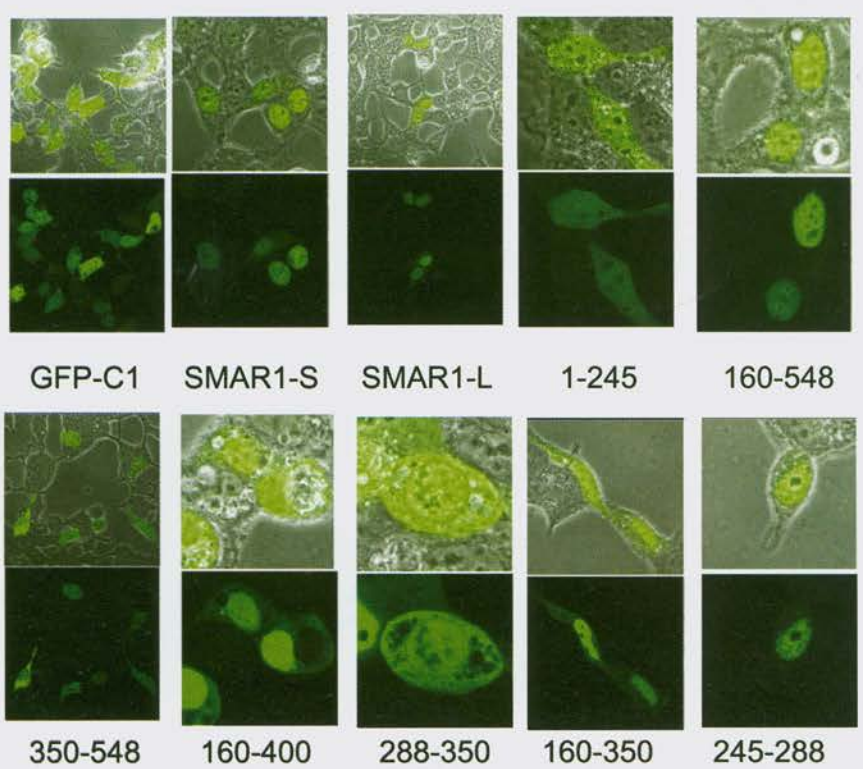


Fig. 2: Nuclear localization signal (NLS) of SMAR1 is located between 245-288 aa.

### 3. Th1 specific activation of T-cells by extracellular Tat

HIV transactivator protein Tat interacts with the nascent transcript transcribed through the 5' LTR promoter. A number of reports suggest that along with its major function as a transactivator, Tat plays critical role in activation of T cells. This bystander activity that mediates rapid depletion of CD4+ T cells causes a rapid loss of immunity during HIV pathogenesis. We have constructed an expression vector of LAMP (lysosomal associated membrane bound protein) fused to Tat, to study how Tat might work through the T cell receptors. We have been able to show the surface expression of Tat along with LAMP by Western blot analysis. This fusion protein was expressed on a heterologous CHO cell line and co-cultured with T cells. We find a strong costimulatory activity exerted by membrane bound Tat that causes proliferation of T cells. By cytokine assays, we see a Th-1 type of immune response induced by extracellular Tat. In future we would like to identify the specific receptor that mediates the signal transduction cascade causing T cell activation.

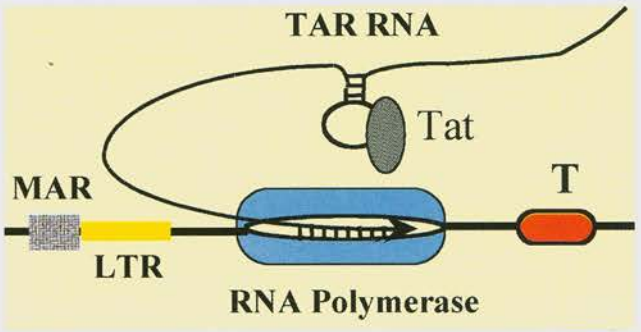


Fig. 4: HIV transcription antitermination. Binding of HIV1 transactivator protein Tat to its recognition site TAR and further interaction to the C-terminal site of RNA polymerase allows it to anti terminate and late genes expression occur.

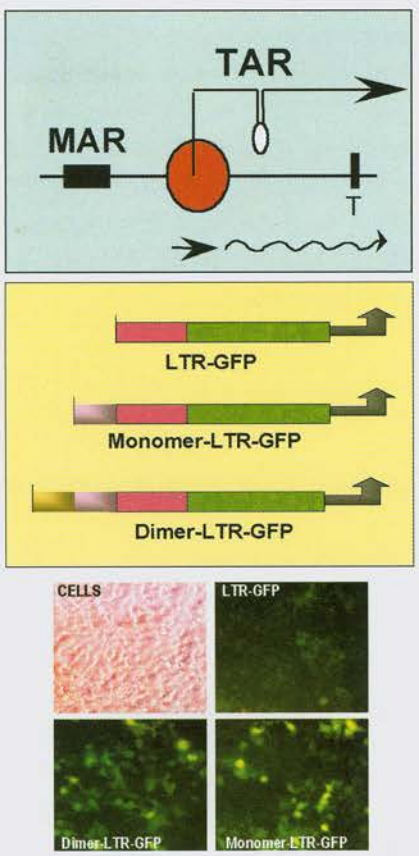


Fig. 3: MAR sequences upstream of HIV1-LTR promoter enhances the transcription at a distance

### Future plan

1. The specific amino-acid/s in SMAR1 responsible for nuclear localization (NLS) and DNA binding will be identified.

2. The transgenic mice over-expressing SMAR1 protein will be studied for the T cell development. These mice will also be used as a model for tumorigenesis.

3. The role of chromatin structure modulation by SMAR1 will be elucidated in both embryonic cells as well as cord blood stem cells.

4. Molecular mechanisms of MARs and its effects in HIV-LTR mediated transcription will be studied in detail. We are interested to understand how MAR sequences allow HIV transcription at distance to express late genes in the absence of transactivator protein Tat using in vitro cell free system.

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

SATB1 is a cell type-restricted protein expressed predominantly in thymocytes and is essential for T cell development. It possesses a MAR-binding domain and homeodomain 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. The dimerization of SATB1 is disrupted due to cleavage by a caspase 6-like protease during T cell apoptosis. Once SATB1 is cleaved, even if the MAR-binding domain and the homeodomain remain intact, it readily dissociates from chromatin *in vivo* concomitant with the cleavage of its target sequences. In signal transduction pathway surface receptors, ion channels and internal signalling molecules combined with kinases act together in a sequential cascade and transmit signals to nuclear receptors which in turn leads to activation of gene(s). Many of these receptors contain PDZ domain(s). 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. This proposed signal transduction pathway is schematically represented in Fig. 1. SATB1 may therefore act as an architectural protein on the nuclear matrix. The consequence of such protein-protein interaction(s) may lead to the activation or repression of SATB1 activity. Examination of the each associated protein may provide insights into the specificity and activity of SATB1 in T-cell development and function. In light of these, we propose to delineate the role of covalent modifications and protein interactions of SATB1 in the regulation of its biological function. The specific questions that we would like to address are: (i) what are the factors (modifications) regulating the MAR-binding activity of SATB1? (ii) How SATB1 specifically recognizes MARs and not any general DNA sequence? (iii) How SATB1 regulates transcription?

### Aims

1. To study the effect of various covalent modifications on the DNA binding activity of SATB1 in vitro and in vivo.

2. To monitor the conformational change(s) in SATB1 that is induced upon binding to BURs.

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

## Work achieved

### Covalent modifications of SATB1

We have obtained data indicating that at least two different types of SATB1 exist in the cell: one that binds DNA and other that doesn't. What is the qualitative difference within these two types of molecules? To separate different species of SATB1 we performed high resolution 2-D gel electrophoresis as described by O'Farrell followed by immunonlot analysis. Interestingly, the immunoblot analysis of 2-D separated proteins from 293 HEK cells indicated presence of an acidic species of SATB1 that is presumably its phosphorylated form. We have observed that different forms of SATB1 are generated due to two distinct mechanisms: one is dependent on phosphorylation, in which case we see spots differing only in their relative pI values. Secondly, alternative splicing also gives rise to at least one additional high molecular weight species of SATB1 (Fig. 2). We are currently analysing the effect of phosphorylation on the DNA-binding activity of these two alternatively spliced forms.

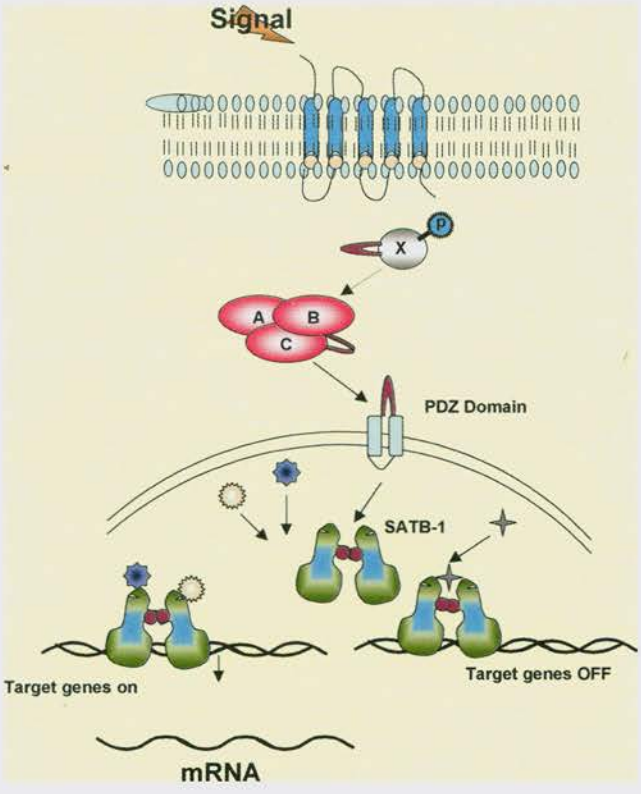



Fig. 1: Schematic representation of the signal transduction pathway involving PDZ domain-containing proteins. SATB1 is depicted in green colour within the nucleus. PDZ domains are indicated in magenta.

### Expression, purification and activity analysis of SATB1

The expression and purification of recombinant proteins is an important step in biochemical analysis. We have constructed several versions of full length and truncated SATB1 for in vitro transcription-translation as well as for over expression in bacterial and mammalian cells. GST-fused MD+HD (the DNA-binding domain of SATB1) and GST alone were prepared. The DNA-binding activity of these proteins was determined by electrophoretic mobility shift assay (EMSA) using a $^{32}$P-labelled WT (25)$_2$ synthetic MAR probe in the presence of excess competitor DNA. The assay results indicated that in vitro translated full length SATB1 bound very strongly to the probe and virtually the entire probe is shifted in the form of a high molecular weight complex typical of SATB1. However, the MD+HD translation product did not exhibit any binding activity. The translation product containing PDZ+MD+HD domains bound as efficiently as the full-length SATB1. This data clearly showed that PDZ domain is required for the DNA binding activity of SATB1 and correlates with the previous observation, which demonstrated that caspase 6-mediated cleavage at the PDZ domain abolishes the MAR-binding activity of SATB1 during early T-cell apoptosis. Thus,

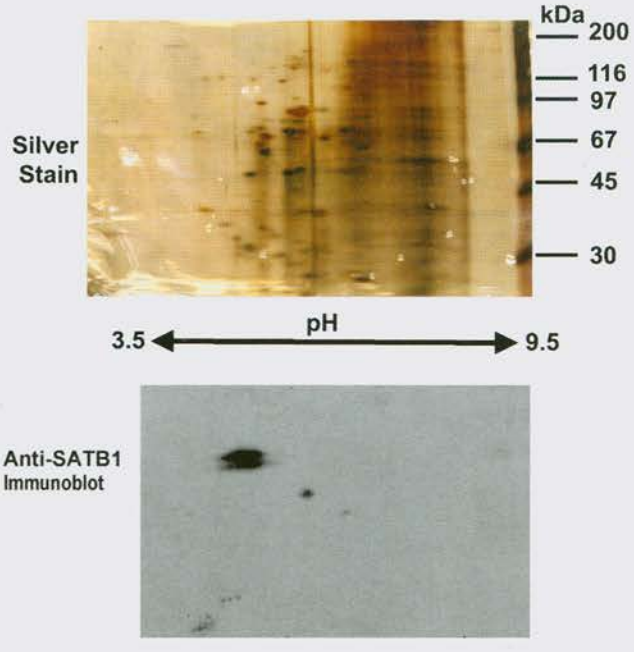



Fig. 2: **High resolution two-dimensional separation of proteins.** To separate different species of SATB1 we performed high-resolution 2-D gel electrophoresis as described by O'Farrell (***Top panel***) followed by immunoblot analysis using anti-SATB1 (***Bottom panel***). Interestingly, the immunoblot analysis of 2-D separated proteins from 293 HEK cells indicated presence of an acidic species of SATB1.

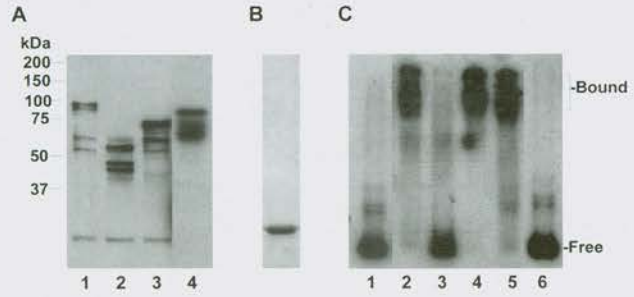



Fig. 3. Fusion with GST restores the DNA-binding activity of SATB1. A. Immunoblot analysis of various protein preparations with polyclonal anti-SATB1. B. Coomassie brilliant blue stained gel picture of glutathione-Sepharose spin column-purified GST. C. Electrophoretic mobility shift (EMSA) analysis of SATB1 using a $^{32}$P-end filled WT $(25)_7$-mer synthetic BUR DNA substrate and in presence of 0.5 μg of double-stranded poly (dI-dC). Samples analyzed were: Lane 1, no protein; lane 2, *in vitro* translated full-length SATB1; lane 3, *in vitro* translated MD+HD; lane 4, *in vitro* translated PDZ+MD+HD; lane 5, GST+MD+HD; lane 6, GST alone. The positions of the free DNA probe (free) and SATB1-DNA complexes (bound) are indicated on the right.

any truncation of SATB1 devoid of its PDZ domain is expected to also lack DNA-binding activity despite containing both MD and HD. Interestingly, GST+MD+HD fusion protein bound very strongly to the Wt $(25)_7$ probe and formed a high molecular weight complex similar to the full length and PDZ-MD+HD forms of SATB1 (Fig. 3). Importantly, the binding affinity of the GST+MD+HD is in the sub-nanomolar range and is comparable to that of native SATB1. GST alone failed to bind the DNA probe under the conditions employed. This result reveals that PDZ function was substituted at least in part, by the GST tag and aided in the proper folding of SATB1 resulting in its native DNA-binding activity. Since expression and purification of GST+MD+HD at milligram scale has been achieved, we will therefore use this fusion protein for the BUR-specificity analyses.

### Search for SATB1-interacting proteins

We have prepared constructs for yeast two-hybrid analysis towards the search for SATB1's interacting partners. We intend to use 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. We have amplified a cDNA expression library and have standardized sequential- as well as co-transformation protocols. The library screening is underway.

### Future plan

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

2. To clone and characterize the alternatively spiced form of SATB1.

3. Perform mutation analysis of the important amino acids in the PDZ domain so as to disrupt interaction with other proteins but not the dimerization of SATB1.

4. Monitor the expression level and splicing status of SATB1 in embryonic stem cells.

# Support Units


CLEAN

## Experimental Animal Facility

The Experimental Animal Facility is an infrastructure 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

**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 F14, F12 and F11 respectively. These colonies are the nuclear colonies for the long-term propagation of an inbred strain.

As reported earlier one mouse came up with spontaneous congenital cataract that was detected in a production colony of BALB/c strain of mice. Breeding studies suggest that this mutation is caused by a gene defect inherited in an autosomal recessive manner. Histology of the mutant lenses during embryonic stage revealed the vacuolation and degeneration of the lens fibre cells. Attempts are being made to separate and propagate the line as a true breeding mutant strain. The mutant colony is currently at **F18** level of inbreeding.

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.

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.

| Sr. No. | Strains/ Species | Animals Procured | Animals Bred | Animals Supplied |
|---|---|---|---|---|
| 1 | **RATS** | | | |
| | Wistar | — | 99 | 119 |
| | Lewis | — | 76 | — |
| 2. | **MICE** | | | |
| | BALB/c | 190 | 6635 | 3087 |
| | C57bl/6 | — | 2312 | 971 |
| | SWISS | 30 | 1206 | 362 |
| | DBA/2 | — | 807 | 48 |
| | Nude (nu/nu) | 164 | 37 | 46 |
| | BALB/c* | — | 334 | 23 |
| | C3H | 20 | — | 20 |
| 3. | **MASTOMYS COUCHA** | — | 47 | — |
| 4. | **RABBIT** (NZW) | — | 8 | 1 |

* **BALB/c with cataract mutation.**
As a part of the health-monitoring program around 40 samples were subjected for haematological screening and around 20 samples for clinical biochemistry.

Histopathology: Over 100 samples have been processed for hispathological examination.

## The Fluorescence Activated Cell Sorter (FACS)

During the period approximately 8000 samples were acquired and analysed. The samples were of surface staining, DNA staining, analysis of GFP fluorescence, calcium uptake. We have also taken samples from other institutes like NARI, Hyderabad University etc. for analyzing samples using our facility.

## The Confocal Laser Scanning Microscope (CLSM)

The confocal laser-scanning microscope (CLSM) is a state of the art laser scanning microscope 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. Our facility has analysed about 800 samples from NCCS and approximately 200 samples came from various other institutes.

## The Automated DNA Sequence Analyser

For catering sequencing needs of the Institute, an ABI 310 Single capillary based machine was purchased. Since its installation in April 2001, more than two thousand samples have been processed for sequencing. This also includes samples from other organizations like National Chemical Laboratory, Pune University, Agharkar Research Institute, Serum Institute etc.

## Transgenic Facility

To master in transgenic and knock-out techniques this year NCCS has procured the entire set up for micro-manipulation and micro-injections by the end of the coming year we will be able to successfully inject embryos and get transgenic mice. This will not only help in understanding immune system better but also to make mouse model systems for understanding tumorigenicity and other diseases.

## The 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 750 volumes per year. During the period of 2001-2002, the Library has added ninety books and 670 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.

## The Computer Centre

The computer section has expanded the Local Area Network and procured 28 new computers to fulfil the requirement of dedicating computers to each scientist with internet connection.

In order to safeguard and continue smooth functioning of Internet server, computer section is maintaining anti-Virus server, in addition to the mail, web, proxy server and Fire Wall server.

To increase the quality of 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 colour laserjet printer.

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, Operating System, Software's and Drivers, Server operating system up-gradation and maintenance, Website up-gradation and maintenance.

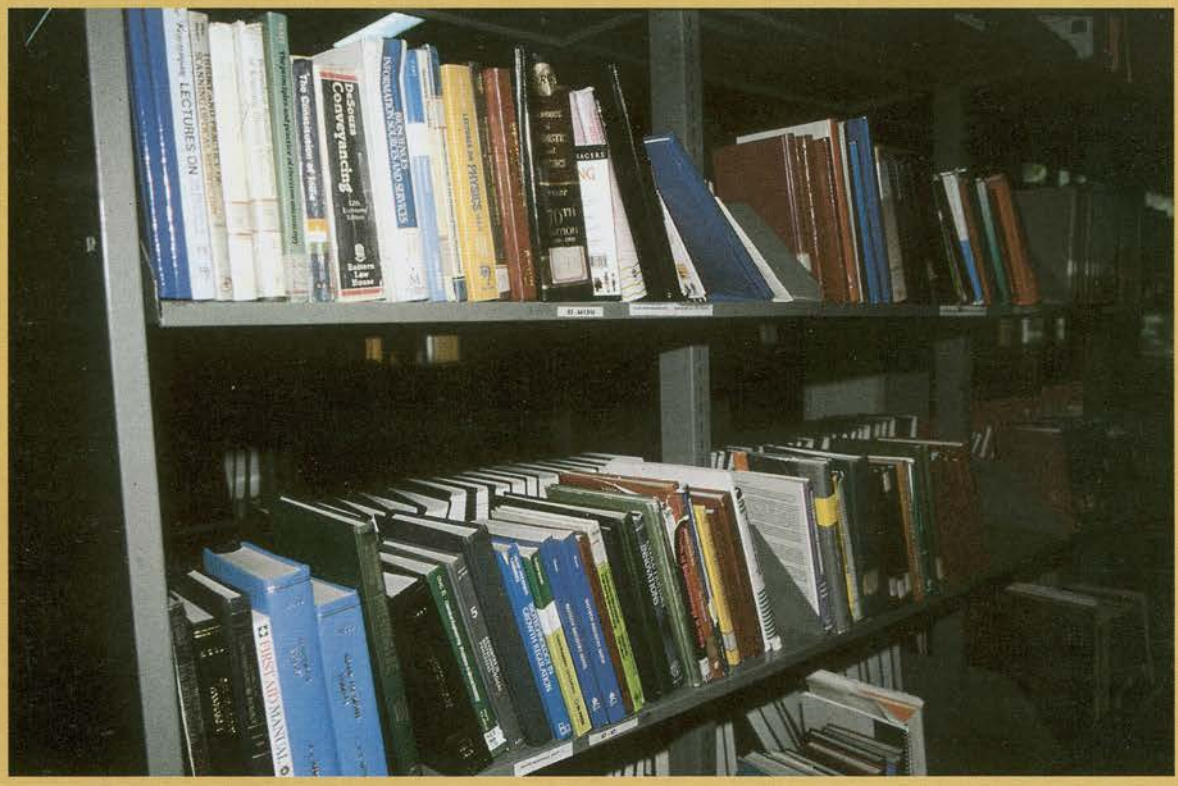

## Publications

## Awards and Honours

## Funds Received

## Seminars Attended

## Committees of NCCS


LECTURES ON
Conveyancing
Butterworths
Law
Books
FIRST AID MANUAL

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## Honors and Membership received by NCCS Scientists

**Gopal C. Kundu**

- Member of the Indian Society of Cell Biology, life member (1999-onwards).

**Musti V. Krishnasastry**

- Elected member for the Guha Research Conference (2001-onwards)

**Ramesh R. Bhonde**

- Member of the Task Force of DBT in Aquaculture and Marine Biotechnology, (2000- onwards).
- Member of the National Academy of Sciences, Allahabad, (1999-onwards).
- Life Member of The Indian Society for Cell Biology, (2001-onwards)
- Life Member of The Indian Society of Developmental Biologists, (1999-onwards).

**Samit Chattopadhyay**

- Member of Maharashtra Academy of Sciences, (2001-onwards).
- Member of governing body committee of Indian Society for the Developmental Biologists (ISDB).

**Debashis Mitra**

- Member of Maharashtra Academy of Sciences, (2001-onwards).

**Arvind Sahu**

- Wellcome Trust Overseas Senior Research Fellowship, The Wellcome Trust, UK (2001-2006).
- Member of the International Complement Society (1991 onwards).

**Sanjeev Galande**

- Received fellowship to partially cover the registration costs of the Stem Cells course organized by Cold Spring Harbor Laboratory, USA (July 30-August 12, 2002).
- **Visiting Student Fellowship to Ajith Mathew J.** Institute of Medical Technology University of Tampere Finland (February 2002-November 2002.)

## Extramural funding received by the NCCS Scientists

**Mohan R. Wani**

- Council of Scientific and Industrial Research (CSIR) project: Isolation, purification and characterization of anti-osteoporotic factor in Indian green mussel (*Perena Viridis*).

**Gopal C. Kundu**

- Department of Science and Technology (DST) project: Role of osteopontin on matrix metaloproteinase-2 expression, cell migration and ECM-invasion in melanoma cells.
- Department of Biotechnology (DBT) project: Role of novel factor in suppression of breast cancer cells migration and metastasis.
- The British Council through the Higher Education Link Program funding Development of breast cancer specific drugs.

**Debashis Mitra**

- DBT Jai Vigyan Mission Project: Immune response to HIV infection towards generation of DNA vaccine.
- Department of Biotechnology (DBT) project: Identification and characterization of anti-HIV compounds in Indian marine bivalves.
- Indian Council of Medical Research (ICMR) project: New natural products as HIV-1 reverse transcriptase inhibitor from the genus *Calophyllum*.
- Council of Scientific and Industrial Research (CSIR) project: Isolation of active compound in the extract of mussels to develop drugs.

**Yogesh S. Shouche**

- Department of Biotechnology (DBT) project: The *Helicobacter pylori* genome prommage: genome sequencing, functional analysis and comparative genomics of the strains obtained from Indian patients.

**Bhaskar Saha**

- Defense Research and Development Organization (DRDO) project: Modulation of toxic shock syndrome by neonatal priming with staphylococcal enterotoxin B (SEB).

**Ramesh R. Bhonde**

- Department of Biotechnology (DBT) project: Studies on the induction of islet neogenesis in vivo and in vitro for its potential role in the treatment of diabetes.

**Vaijayanti P. Kale**

§ Department of Biotechnology (DBT) Project: Fetal liver infusion: further studies on the mode of action of fetal liver cells on the regeneration of adult bone marrow, in vitro evaluation.

**Gyan C. Mishra**

§ Department of Biotechnology (DBT) project: Cancer Immunotherapy: Antigen delivery and vaccination by genetically engineered dendritic cells.

**Samit Chattopadhyay**

§ DBT Jai Vigyan Mission Project: Targeted modulation of gene expression: Inhibition of HIV infection by gene therapy.

**Lalita S. Limaye**

§ Defense Research and Development Organization (DRDO) project: Addition of membrane stabilizes and antioxidants in the conventional freezing mixture to conserve growth factor responsiveness and engraftment potential of the frozen marrow.

**Padma Shastry**

§ Indian Council of Medical Research (ICMR) project: Multi Cellular Spheroids (MCS): A 3D in vitro model for studies on cellular and molecular aspects of tumor progression and metastasis.

**Arvind Sahu**

§ Wellcome Trust, UK project: Structure function analysis of viral homologs of complement control proteins.

## Seminars/Invited talks given by NCCS Scientists at other places

**Vaijayanti P. Kale**

§ Characterization of Stem cells. Presented in the Task Force meeting on 'Stem cell biology and its applications" at Department of Biotechnology, New Delhi on 30th Nov. 2000.

§ Biology and clinical application of cord blood stem cells. Presented in Stem Cell CME at Bharati Vidyapeeth, 21st Dec 2001.

§ Stem cells: research, potential and development. Presented at MIT study circle. 19th Oct 2001

**Lalita S. Limaye**

§ Cord blood stem cells tissue culture and assay techniques. During 'Cord blood stem cells: New vistas for research' at the Department of Pathology Bharati Vidyapeeth, Pune, Dec. 2001

**Nibedita Lenka**

§ Embryonic stem cells and in vitro neurogenesis: A developmental paradigm. INDO-US symposium on "Brain Research", New Delhi, January 10-12, 2002.

§ Targeting and quantitation of es cell derived neural progenitors and *in vitro* neurogenesis at Annual Conference of the Indian Society of Developmental Biologists, Kanpur, February 17-20, 2002.

§ Specification and Characterization of ES cell derived Neural Progenitors and Dopaminergic Neurons at International Conference on Emerging Trends in Biotechnology: "Stem Cells: Technology, Potential & Trends". Hyderabad, May 2-4, 2002

**Gopal C. Kundu**

§ Osteopontin regulates tumor growth and activation of promatrix metalloproteinase-2 through nuclear factor-kB-mediated induction of membrane type 1 matrix metalloproteinase in murine melanoma cells, University of Hull, UK, June 2001.

§ Matrix Metalloproteinase-2: mechanism of activation and its role in cellular invasiveness and tumor growth at international conference on emerging trends in cancer research at JNU, New Delhi, 14th-16th March 2002.

**Padma Shastry**

§ Cell cycle regulatory proteins – Role in health and disease. Modern Education Society's Nowrosjee Wadia College. Pune, 28th Feb 2002.

**Anjali Shiras**

§ Recent advances in Cancer Research. Rani Laxmibai popular lecture series, Pune, 14 November 2001.

**Musti V. Krishnasastry**

§ Activation of protein tyrosine phosphatases by membrane binding Proteins: search for missing links and mechanism. GRC, Coorg, Karnataka, 19th December 2001.

**Ramesh R. Bhonde**

§ Invited to give a talk on "Islet neogenesis and trans differentiation as a means to over come pancreatic insufficiency of insulin and reversal of diabetes", at the annual conference of ISDB held at Kanpur during Feb 17-20, 2002.

§ Invited as a faculty to give a talk on "Islet tissue engineering and newer models of islet neogenesis", at the workshop on Cell Transplantation held at Chennai on March 3, 2002.

§ Delivered a lecture on "Tissue banking and role of stem cells in diabetes" at Department of Zoology, Shivaji University Kolhapur, on 31st January 2002.

§ Delivered a lecture "Biomaterials for drug delivery, islet encapsulation and wound healing" at 2nd SciTech Showcase was held at NCCS on 5th May 2001.

**Yogesh S. Shouche**

§ Invited to give a talk on Ribosomal RNA sequence analysis in phylogeny, taxonomy and diagnostics at CCMB, Hyderabad, September 2001

**Samit Chattopadhyay**

§ Presented talk on MARs and MAR binding proteins: Role in Cancer and HIV Transcription in the conference by Indian Society for the Developmental Biologists (ISDB), at Indian Institute of Technology, Kanpur, 17th to 20th Feb 2002.

§ Presented talk on Human genome organization: dynamics and diseases, A teaching program for 27th Mahabaleswar seminar on Modern Biology, Mahabaleswar, March 23-27, 2002.

**Debashis Mitra**

§ Interaction between HIV-1 proteins Nef and Tat: implication in viral gene expression and replication. Molecular Immunology Forum, Shantiniketan, West Bengal, February 22-24, 2002.

**Bhaskar Saha**

§ Parasite Bioinformatics: Promises and challenges. Symposium on parasite bioinformatics at Centre for Bioinformatics. Pune.

§ Immunobiology of CD28 expression on human neutrophils at London School of Hygiene and Tropical Medicine. London.

## Seminars given at NCCS by visiting scientists

**Dr. Amitabha Mukhopadhya**

National Institute of Immunology, New Delhi.

*Salmonella modulates intracellular trafficking in macrophages for their survival.*

**Dr. Jean Louis Herrmann**

Service de Microbiology, Hospital Saint-Louis Paris, FRANCE.

*Constrained intracellular replication of mycobacterium tuberculosis in human dendritic cells.*

**Tom Slyker**, Program Manager

Proteomics Division, Bio-Rad Lab., USA

*Comparative proteomics of human gastrointestinal pathogens camphylobacter jejuni and helicobacter pylori using The ProteomeWorks™ system.*

**Prof. Colin Ratledge**

University of Hull, Hull, UK

*Iron and mycobacterial virulence.*

**Dr. Harish C. Pant**

Chief, Cytoskeletal Regulatory Protein Section, Laboratory of Biochemistry, NIH, NINDS, Bethesda, Maryland, USA.

*CDK5: Role in nervous system development.*

**Dr. Harry Griffin**

Assistant Director (Science), Roslin Institute, Edinburgh,UK.

*Regulating research on human embryos and embryonic stem cells.*

**Dr. Simon L. Croft**

London School of Hygeine and tropical medicine, UK.

*Chemotherapy of malaria.*

*Chemotherapy of leishmaniasis and trypanosomiasis.*

**Dr. Rajendra N. Damle**

Department of Experimental Immunology, Northshore, L.I.J Research Institute, NY, USA

*Chronic lymphocyte leukemia: analysis of novel subgroups.*

**Dr. Amit Kumar**

National Cancer Institute, NIH, USA

*Molecular mechanism of left right asymmetry in vertebrate development.*

**Dr. U.R. Rao**

Department of Infectious Diseases, Washington University Medical Centre, St. Louis, USA.

*Effect of antibiotics on flaria and walbacia.*

## Participation in National/International Conferences

**XXVth All India Cell Biology Conference held at Indian Institute of Science, Bangalore, and November 1-3, 2001.**

§ **J.M. Chiplonkar and M.R. Vipra**
Analysis of phenotypic changes resembling epithelial mesenchymal transition in human cervical cancer cell line (SiHa).

§ **Suman Raphael and Vaijayanti Kale.**
Disparate expression profiles of caveolin expression in tumor cells.

§ **Lalita Sasnoor, Vaijayanti Kale, Lalita Limaye.**
Improved cryopreservation of hematopoietic cells by the use if membrane stabilizers and bio-antioxidants.

§ **L. Mangasetti, S. Khapli and M.R. Wani**
Interleukin-4 is a potent inhibitor of osteoclastogenesis and bone resorption *in vitro.*

§ **Philip S. and Kundu G.C.**
Oral Presentation: Osteopontin induced expression of matrix metalloproteinase-2 plays significant role in invasiveness and tumor growth using murine melanoma cells.

§ **Khapli S., Mangasetti L., and Wani M.R.**
Oral Presentaion: A role for IL-3 in osteoclast differentiation *in vitro.*

§ **Oral Presentation: Bhonde R.R.**
A new model for induction and modulation of pancreatitis in Balb/c mice.

§ **Oral Presentation: Jayashree S. Ladha, Ajith Mathew, Mitra D.**
HIV-1 induced T-cell apoptosis: studies on differential gene expression.

**International Conference on Emerging Trends in Cancer Research held on 14th-16th March 2002 at JNU, New Delhi**

§ **Rishi Raj Chhipa and Manoj Kumar Bhat**
Increase in cytotoxic activity of antineoplastic agents in presence of doxycycline- its implication on solid tumor cells.

§ **Ravi Shukla, Anjali Shiras and Padma Shastry**
Analysis and expression of S100 proteins S100 A2, A4, A6 and S100B in neuroectodermal tumors.

§ **Sandhya Sitasawad and Tanuja Bankar**
Anti-tumor effect of bittergourd fruit juice on cancer: a preliminary investigation on human breast cancer cell lines.

§ **Varsha Shepal, Suvarna Deshmukh and Anjali Shiras**
Characterization of a novel melanoma clone with transforming potential.

**XIX Annual Meeting of Indian Academy of Neuroscience and International Symposium on Neurodegeneration and Neuroprotection. Calcutta Neuroscience Conference-2002 held at Indian Institute of Chemical Biology 18-20 March 2002**

§ **Jayashree C. Jagtap, Anmol Chandele, B. A. Chopade and Padma Shastry.**
Sodium Pyruvate Protects Against $H_2O_2$ Mediated Apoptosis in Human Neuroblastoma Cell Line.
*Awarded N.S. Parmar Award for Best Poster.*

**CME meeting on Omega 3 Fatty acids in health and disease, 4th January 2002**

§ **Paper Presentation: Bhonde R.R.**
Role of omega 3 fatty acids in diabetes and maintenance of islet cell viability and functionality.

**Yogesh Shouche** attended the Association for Promotion of DNA Fingerprinting and Other DNA Technologies (ADNAT) meeting on Functional Genomics at Hyderabad, Feburary 2002.

Workshop attended by **P. Deshpande** on Immunobiology of parasites at Regional Medical Research Centre, Bhubaneshwar, Orissa, 11th-17th February 2002.

**S. Bapat** attended the All India Technology Managers workshop for IPR and Technology Management, Neemrana, Rajasthan, 12th-15th March 2002.

## Active collaborations of NCCS with other national/international institutions

§ **Debashis Mitra**
**Anil Chatterji**
National Institute on Oceanograpy,Goa.
*Anti-HIV activity in marine bivalves*

**M.K. Gurjar and S.P. Joshi**
National Chemical Laboralory
*Anti-HIV activity in Calophyllum*

§ **G.C. Mishra**
**Prakash Deshpande**
Pr. Pierre-Andre CAZENAVE
Pasteur Institute,Paris, FRANCE
Sylviane PIED, CR,CNRS
*T Lymphocyte response in human malaria pathogenesis.*

§ **G.C. Mishra**
**Paul M. Kaye**
London School of Hygeine and Tropical Medicine
*The role of co-stimulatory molecules in the regulation of anti-leshmanial immune response*

§ **Vaijyanti Kale**
**L.C. Padhy**
Molecular Biology Group
TIFR, Mumbai
*Stromal cell biology*

§ **Gopal C. Kundu**
**Christopher J. Newton**
Medical Research Laboratory
University of Hull, UK

**Subhas Padhye**
Department of chemistry
University of Pune
Pune
*Development of breast cancer specific drugs*

§ **C.G. Naik**
National institute of Oceanography, Goa
*Identification of bioactive compound from marine sources*

§ **Anjali Shiras**
Molecular Biology Group
TIFR, Mumbai
*Transforming genes in melanoma*

§ **Ramesh R. Bhonde**
**Anil Chatterji**
National Institute of Oceanography,Goa
*Production of indian horseshoe crab amoebocytes* in vitro.

§ **Debashis Mitra**
**Ramesh Bhonde**
**Pradeep Parab**
**Anil Chatterji**
National Institute of Oceanography,Goa
*Antiviral compounds from the Indian marine bivalves and their potential role in curing diseases*

§ **Samit Chattopadhyay**
**Satish Totey, Subeer Majumdhar, Neerja Gulati and Arundati Mandal**
National Institute of Immunology, Delhi
*Construction of transgenic mice for SMAR1*

§ **Bhaskar Saha**
**Simon Craft**
London School of Hygeine and Tropical Medicine
Subhas Padhye, Department of Chemistry
University of Pune
Pune
*Screening compounds active against Leishmania*

**Paul Kaye**
London School of Hygeine and Tropical Medicine
*The Role of Co-Stimulatory Molecules in Regulation of Anti-Leishmanial Immune Response*

§ **Mohan Wani**
**Anil Chatterji,** National Institute of Oceanography, Goa
**Kanury Rao,** ICGEB, New Delhi
*Identification of anti-osteoporotic activity in marine bivalves*

§ **Lalita Limaye**
**S.G.A. Rao and R.L. Marathe,** Cancer Research Institute, Mumbai
*Studies on cryopreservation of haematopoietic cells*

§ **Sandhya Sitasawad**
**S. P. Joshi,** National Chemical Laboratory, Pune
*Isolation and characterization of biologically active novel anti-cancer agents from* Momordica Charantia Linn *fruit and evaluation of its anti-cancer efficacy* in vitro *and* in vivo.

§ **Padma Shastry**
**B.A. Chopde,** Department of Microbiology, Pune University
*Effect of antioxidants and sodium pyruvate protects against $H_2O_2$ mediated apoptosis in human neuroblastoma cell line: SK-N-MC*

§ **Debashis Mitra**
**K.N. Ganesh,** National Chemical Laboratory, Pune
*Peptide-nucleic acids and anti-HIV activity.*

# Committees of NCCS

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Hon'ble Minister for HRD and
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Ocean Development,
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New Delhi 110 001

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Asst. Director General (Animal Health),
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10. **Dr. Padma Shastry** — Member
Scientist E,
NCCS,
Pune 411 007

11. **Dr. G.C. Mishra** — Member Secretary
Director,
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National Centre for Biological Sciences,
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   Central Drug Research Institute,
   Chattar Manzil, Post Box No. 173,
   Lucknow 226 001

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   Technical Director,
   Intervet Laboratories Ltd.,
   Briahnagar, Off Pune-Nagar Road,
   Wagholi 412 207

4. **Dr. (Smt.) Rajani Nadgauda** — Member
   Scientist,
   National Chemical Laboratory,
   Pashan Road,
   Pune 411 008

5. **Dr. G.C. Mishra** — Member Secretary
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   Pune 411 007