

Thursday April 7

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## PREMIER LECTURE SERIES

**Location: PFC G07**



**PL5 09.00-09.40**

**Professor Thomas Cremer**  
*Department Biology II, Ludwig  
Maximilians University, Munich,  
Germany*

### FROM CHROMOSOME TERRITORIES TO A FUNCTIONAL NUCLEAR ARCHITECTURE

Thomas Cremer, Heiner Albiez, Andreas Bolzer, Daniela Köhler, Katrin Küpper, Stefan Müller, Michaela Neusser, Lothar Schermelleh, Heinrich Leonhardt, Babett Wagler, Roman Zinner, Marion Cremer, Irina Solovei  
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The chromosome territory - interchromatin compartment (CT-IC) model argues for a functional organization of the cell nucleus. Chromosome territory (CT) arrangements show profound differences between certain cell types. In the spherically shaped lymphocyte nuclei gene dense CTs are preferentially located in the nuclear interior, while gene poor CTs are preferentially located towards the nuclear periphery. In fibroblast nuclei with their flat-ellipsoidal shape, however, CTs of small chromosomes - independent of their gene density - are located towards the nuclear center and CTs of large chromosomes towards the nuclear rim. Studies of other primates indicate an evolutionary conservation of gene density correlated and size correlated, radial arrangements in the respective cell types. Using defined BACs in combination with multicolor 3D FISH for gene dense and gene poor chromosomal sub-regions, we have started to define the 3D architecture of individual CTs and their topological interactions with each other and the IC. In addition, we have established a procedure to manipulate chromatin condensation and the width of the IC in nuclei of living cells in a fully reversible way. Induced chromatin condensation

(ICCond) with a concomitant enlargement of the IC occurs rapidly, when living cells are exposed to a hyper-osmotic medium. Cells, which were kept up to 30 min in the ICCond state, before they were released to normal osmotic conditions, retained their viability. Exposure of cells to several, short cycles of ICCond and decondensation yielded highly reproducible patterns of condensed and normal chromatin textures. During ICCond individual CTs could still be delineated as distinct objects using chromosome painting. Interestingly, neighboring CTs were not fully separated from each other, but formed a 3D higher order chromatin network. The contact sites of chromatin from different CTs have not yet been identified. Splicing speckles, nuclear bodies and several proteins, described as markers for a nuclear matrix, were detected within the enlarged IC. Nascent transcripts, pulse-labeled immediately prior to ICCond with BrUTP, were detected at the surface of condensed chromatin streaks lining the enlarged IC. This and other experiments argue that for transcription genes must be positioned in perichromatin regions lining the IC or on chromatin loops expanding into the IC. Transcription as well as DNA replication was inhibited during ICCond, but continued as soon as the normal chromatin condensation state was restored. We propose that CTs and the IC form two separate, yet structurally and functionally closely associated nuclear networks. Border zones of chromatin domains, also called perichromatin regions, line the IC and provide essential sites for transcription, co-transcriptional splicing, DNA replication and DNA repair.

#### Biography

Thomas Cremer was born in 1945. From 1964 to 1970 he studied human medicine at the University of Freiburg (Germany). In the early 70<sup>th</sup> he built a laser-uv-microbeam apparatus together with his brother Christoph Cremer, presently full Professor of Applied Optics at the University of Heidelberg. Their early microbeam studies with living cells yielded compelling evidence for chromosome territories in nuclei of mammalian cells. Since 1974 T.C. has led his own research group first at the University of Freiburg i. Br. and since 1978 at the University of Heidelberg. 1978 the brothers published the first construction proposal for a laser confocal scanning fluorescence microscope to enable 3D-imaging of cells.

In 1978 Professor Cremer was a guest researcher in the laboratory of Michel W. Berns, University of California, Irvine. From 1986 - 1988 he worked as a visiting Professor at Yale, Medical School, New Haven, together with Laura Manuelidis and David C. Ward. During this time he contributed essentially to the development of FISH-protocols for the visualization of individual chromosomes in human cells. Thereafter he worked as a lecturer of human genetics at the University of Heidelberg. In 1996 he became Professor and Chair of Anthropology and Human Genetics in the Faculty of Biology, University of Munich. Major topics of research: development of FISH tools for interphase cytogenetics and studies of higher order chromatin arrangements in the cell nucleus, as well as chromosome and nuclear architecture evolution; comparative genomic hybridization (CGH) and matrix (or array) CGH; experimental studies of the functional compartmentalization of the cell nucleus led to the formulation of the chromosome territory – interchromatin compartment model (with C. Cremer and P. Lichter). Since 1998 the Cremer laboratory has made major efforts to develop approaches for studies of higher order chromatin arrangements and their dynamics in nuclei of living cells.



**PL6 09.40-10.20**

**Dr Nick Gilbert**  
*MRC Human Genetics Unit,  
 Edinburgh, UK*

## **CHROMATIN ARCHITECTURE OF THE HUMAN GENOME**

Human cells package their DNA into chromatin, a nucleoprotein complex. In chromatin the DNA is wrapped 1.75 times around a nucleosome, consisting of an octamer of histone proteins. Under physiological salt conditions nucleosome arrays adopt the conformation of a 30nm fibre. Cellular processes such as gene transcription, DNA replication and repair occur in this environment and the modulation of the chromatin structure is central to their regulation. The structure of the chromatin fibre is best understood at the level of the nucleosome and its modifications such as acetylation and methylation. However, beyond the nucleosome itself there are other states of chromatin that will influence how the underlying DNA sequence is read. Transcriptionally active and silent regions of the genome are often considered to have "open" and "closed" chromatin structures respectively.

However, biophysical evidence for different chromatin fibre structures which might equate with these concepts has been lacking. Cancer is a disease of aberrant gene expression. Since gene expression is regulated by chromatin structure it is important to understand how the chromatin structure can affect gene expression.

In normal lymphoblastoid cells we have undertaken the first analysis of chromatin fibre structure across the human genome. By sucrose sedimentation we separated compact and open chromatin fibre structures. Their distribution was analysed by hybridisation to metaphase chromosomes (low resolution) and genomic microarrays (high resolution). A whole genome microarray assembled from clones, spaced at ~1Mb intervals, from the "golden path" used in the sequencing of the human genome and a chromosome 22 tiling-path array with an average resolution of 78kb were used. By utilising genomic microarrays of known sequence we can now relate known genomic features and gene expression levels to the underlying chromatin structure.

We have shown that compact chromatin fibres originate from some sites of heterochromatin (C-bands), and G-bands (euchromatin), whilst open chromatin fibres correlate with regions of highest gene density, but not with gene expression. Inactive genes can be in domains of open chromatin, and active genes in regions of low gene density can be in compact chromatin. We have also shown that chromatin fibre structure impacts on further levels of chromatin condensation as regions of open chromatin fibres are cytologically decondensed and have a distinctive nuclear organisation. We would suggest that domains of open chromatin create an environment that facilitates transcriptional activation whilst an aberrant alteration in chromatin structure will cause widespread misregulation of genes.

### **Biography**

Having studied biochemistry at The University of Edinburgh, Dr Gilbert spent a short period in Cambridge working on "cancer gene therapy". He completed his PhD in Edinburgh studying the higher-order structure of mammalian heterochromatin. He continues to working chromatin research at the MRC Human Genetics Unit with a specific interest in mammalian higher-order chromatin fibre and how its conformation is modulated.

### *References.*

- [1] Gilbert, N. and Allan, J. (2001). Distinctive higher-order chromatin structure at mammalian centromeres. *Proc. Natl Acad. Sci. USA* 98, 11949-11954.
- [2] Gilbert, N., Boyle, S., Sutherland, H., de Las Heras, J., Allan, J., Jenuwein, T., Bickmore, W. A. (2003)

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- [3] Gilbert, N., Gilchrist, S., Bickmore, W. A. (2004) Chromatin organisation in the mammalian nucleus. *Int. Rev. Cytol.* (in press).
- [4] Gilbert, N., Boyle, S., Fiegler, H., Woodfine, K., Carter, N. P., Bickmore, W. A. (2004) Chromatin architecture of the human genome: gene-rich domains are enriched in open chromatin fibres. *Cell* (in press).



**PL7 10.20-11.00**

**Dr Tom Misteli**

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## FUNDAMENTAL PRINCIPLES OF NUCLEAR ARCHITECTURE IN CANCER

Genomes are expressed in the context of the architectural framework of the cell nucleus. The spatial and temporal organization of genomes and of nuclear functions is central to the controlled expression of genomes. Defects in nuclear architecture contribute to disease including cancer. We are exploring various fundamental principles of nuclear organization and their role in cancer formation. One of the most striking organizational features of the mammalian cell nucleus is the non-random spatial arrangement of chromosomes. Chromosomes occupy preferential radial positions with respect to the center of the nucleus and they are positioned in particular patterns relative to each other. This relative positioning of chromosomes and gene loci has an important role in genome stability and formation of chromosomal translocations as observed in cancer cells. We have proposed that proximal positioning of chromosomes increases their probability of forming translocations. In support, we have found preferential proximal positioning of translocation-prone gene loci and chromosomes in mouse and human systems in comparison to non-translocating loci. Consistent with a role of spatial proximity in determining translocation partners, we have found evidence for tissue specificity of spatial genome organization within the interphase nucleus. A second fundamental nuclear process involved in cancer formation is chromosome segregation. Defects in mitotic division results in aneuploidy. We are characterizing novel components

of the mitotic division machinery and are testing their potential for formation of aneuploidy and tumor formation. These investigations of the fundamental cell biological principles of nuclear architecture and genome function are leading to insights into disease mechanisms and the development of novel diagnostic and therapeutic strategies.

### Biography

Tom Misteli heads the Cell Biology of Genomes Group at the National Cancer Institute, NIH. His laboratory investigates the fundamental cell biological principles of nuclear architecture and how they contribute to disease. Dr. Misteli is a member of several editorial boards and has received several awards for his research contributions. He is currently a fellow of the Keith R. Porter Endowment for Cell Biology.

**11.00-11.30 COFFEE AND NETWORKING**

## FREE PAPER SESSION 3 (Parallel I)

**Location: PFC G07**

**011 11.30-11.45**

### HISTONE ACETYLATION INDUCES CHROMATIN REORGANIZATION AND ALTERED GENE EXPRESSION IN PROSTATE NEOPLASIA

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*Introduction.* We have previously shown that reorganized chromatin and concurrent global H3K9 hypoacetylation is associated with the development of prostate cancer in human tissue. Acetylation of H3K9 may therefore be functionally involved in the epigenetic regulation of chromatin patterns and associated alteration in gene expression involved in prostate neoplasia. The aim of this study was to investigate the role of H3K9 acetylation in prostate cancer using Trichostatin A (TSA).

**Materials and Methods.** PNT1A (Sv40 immortalised normal prostate cell line), LNCaP (Lymph node metastatic), and DU145 (Brain metastatic) were treated with TSA at concentrations of 12ng/ml and 100ng/ml and cells were harvested after 24 hours. Cell cycle alterations and apoptosis were assessed using flow cytometry. Quantitative densitometric and spatial alterations in chromatin phenotype and H3K9 acetylation status (monoclonal histone H3(acetyl K9) antibody) induced by TSA were determined by high resolution digital texture analysis. Flow cytometry was also adopted to detect changes in AcH3K9 status across the cell cycle in response to TSA treatment, using both single and dual parameter methods. Nuclear localization of AcH3K9 and differential expression associated with cell cycle phase was confirmed by confocal microscopy. DNA expression microarray analysis using a human 30K array was assayed to determine alterations in gene expression in response to altered acetylation.

**Results:** TSA induced a global increase in H3K9 acetylation as shown by flow cytometry, western blotting and quantitative immunohistochemistry. At high TSA doses (>50ng/ml), this hyperacetylation was associated with cell cycle arrest in G2 and apoptosis, with 'normal' PNT1A cell line showing more sensitivity and greater disturbance of the cell cycle at lower concentrations. This appeared to be related to the pretreatment global levels of H3K9 in these cells. While very low TSA doses (12ng/ml) did not induce obvious cell cycle alterations, global chromatin reorganization was evident by digital imaging, suggesting that chromatin phenotype was sensitive to minor changes in global acetylation status. H3K9 acetylation was not evenly distributed across the cell cycle. Analysis of untreated cells, dual labeled for AcH3K9 and DNA content (propidium iodide), and analysed using dual parameter flow cytometric analysis showed significantly higher levels of AcH3K9 and distinct cellular sub-populations in G2/M cell cycle phase. This is the first recorded use of flow cytometry to assess genome-wide acetylation status in cells. Interestingly, the increased acetylation and reorganised chromatin architecture induced by TSA was also associated with significant downregulation of numerous genes by DNA microarray analysis.

**Conclusion.** The disruption of histone acetylation by TSA has a major impact on cell cycle regulation in prostate cancer. However, normal prostate cells are more sensitive to inhibition of deacetylating enzymes. Precise analysis of histone acetylation status is reliant on cell cycle phase and flow cytometry now provides a means to disentangle concurrent alterations in histone acetylation and cell cycle modifications induced by compounds such as TSA. Further analysis of chromatin texture on sorted

G1 cells may help to unmask the complex role of H3K9 acetylation in the remodelling of chromatin in the development of prostate cancer.

**012 11.45-12.00**

**DISCOVERY OF NOVEL, ANTI-APOPTOTIC GENES ASSOCIATED WITH PROSTATE CANCER PROGRESSION**

Ms Sinead Walsh, Ms Ann Maria McCrohan,  
Dr Amanda J. O'Neill, Prof John M. Fitzpatrick,  
Dr R. William G. Watson

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**Introduction.** The progression of androgen-dependent prostate cancers to metastatic, androgen-independent disease is associated with a resistance to cell death/apoptosis. Disruption of the apoptotic pathway is one mechanism by which these tumours evade cell death. We hypothesise that the reduced rate of apoptosis in prostate cancer is due, in part, to the over-expression of anti-apoptotic proteins. The aims of this study are to identify up-regulated genes that are involved in prostate cancer progression and apoptotic resistance.

**Materials and Methods.** Data was compiled from published papers which used microarray techniques to compare the gene expression profiles of normal prostate tissue to different stages of prostate cancer. From the list of significantly up-regulated genes, and using bio-informatic web sites, genes relevant to the apoptotic pathway were selected. Primers and probe-sets were designed to validate the over-expression of selected genes using TaqMAN real time PCR in both prostate cell lines and primary prostate epithelial cells. Cell lines used were: androgen-dependent LNCaP, and androgen-independent PC3 and DU145 cell lines. Primary prostate tumour and benign tissue was collected from patients (n=10) undergoing radical prostatectomy, and epithelial and stromal cells were cultured separately.

**Results.** List of over-expressed genes in prostate cancer studies: DEEPEST (mitotic spindle coiled-coil related protein), Prostate specific membrane antigen 1 (PSMA-proteolysis and peptidolysis), Serine Protease 8 (Prostasin -proteolytic enzyme found in seminal fluid) Hepsin (serine protease), Serpin B6 (serine or cysteine proteinase inhibitor). Expression analysis in prostate cancer cell lines show that DEEPEST is most highly expressed in

androgen-independent DU145 cells. DEEPEST is expressed up to 4-fold higher in primary tumour epithelia compared to matched benign epithelia. Expression of the other genes is currently being validated.

*Conclusions.* Using numerous published microarray studies, we have identified genes that are consistently over-expressed in the development and progression of prostate cancer. The novel gene, DEEPEST has been validated in clinically relevant material, and now represents an important target for manipulation in the treatment of apoptotic-resistant prostate cancer.

### 013 12.00-12.15

#### **COPY NUMBER AND EXPRESSION ANALYSIS OF PROSTATE CANCER CELL LINES AND XENOGRAFTS BY MICROARRAYS**

Ms Outi Saramaki, Dr Kati Porkka, Prof Robert Vessella, Prof Tapio Visakorpi

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*Introduction.* The use of cDNA-microarrays has become a standard rather than an exception for profiling the expression of genes in cancer. Comparative genomic hybridisation (CGH) to cDNA-slides is useful in screening for amplified or deleted genes in cancer. Its advantage compared to metaphase CGH is its better resolution (300-2000bp vs. 20 Mb). The use of cDNA microarrays allows a rapid analysis of association of gene copy number and expression.

*Materials and Methods.* We used a microarray consisting of approximately 16000 cDNAs to screen five prostate cancer cell lines and thirteen prostate cancer xenografts (the LuCaP-series) for copy number and expression changes. The copy number data was also compared to previously obtained metaphase CGH data.

*Results.* Array-CGH seemed to discover the same copy number aberrations as metaphase CGH (agreement 80%,  $\kappa=0.49$ ). Array-CGH also found several novel amplicons and deletions. These included, for example, amplicons at 3cen-q13 and 19p13 in PC-3 and 1q21-q22 and 22q11 in DU145 as well as deletions at 14cen-qter in PC-3 and 22q12-qter in DU145. Due to its better resolution, array-CGH also narrowed down the regions of previously implicated aberrations. The global gene copy number was strongly associated with the expression of the genes ( $p<0.0001$ ) indicating the

influence of gene dosage on the expression. Of the individual genes, the expression of about 30 genes correlated significantly with the gene copy number ( $p<0.05$ , Mann-Whitney U-test). These included genes from chromosomal regions 9p13 and 16p, which have not previously been implicated in prostate cancer.

*Conclusions.* A combination of copy number and expression analyses from microarray experiments is efficient in screening for putative target genes of chromosomal copy number alterations. CGH to cDNA-microarrays can also be used to identify novel aberrations and to define the boundaries of known aberrations.

### 014 12.15-12.30

#### **PROSTATE CANCER CELLS ARE SENSITISED TO TRAIL-INDUCED APOPTOSIS BY THE CHEMOPREVENTIVE AGENT RESVERATROL**

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*Introduction.* Apoptosis is initiated through binding of ligands such as TRAIL to death receptors on the cell surface. TRAIL is a promising agent for cancer therapy due to its ability to trigger apoptosis in a variety of tumour cell lines, but not most normal cells. However, many tumour cell types, including some prostate cancer cell lines, exhibit resistance to TRAIL. Resveratrol, found in grapes and red wine, is a potential cancer chemopreventive agent. Studies demonstrate resveratrol-mediated sensitisation to chemotherapeutic agents, including TRAIL, in a range of cancer cell lines. The aims of this study were to demonstrate the differential sensitivity of prostate cancer cell lines to TRAIL and to investigate whether resveratrol could sensitise resistant cells to TRAIL-mediated apoptosis.

*Methods.* PWR-1E, PC-3 and DU145 cell lines were grown in optimal conditions. Apoptosis and viability were assessed by propidium iodide DNA staining and flow cytometry. Caspase-3-like activity was measured using fluorescently labelled substrate.

*Results.* PC-3 and DU145 cell lines are resistant to TRAIL-mediated apoptosis, while PWR-1E undergo apoptosis in a dose-dependent manner following TRAIL treatment. Preincubation with resveratrol resulted in a time-dependent increase in apoptosis following TRAIL treatment in PC-3 and DU145 cells. Sensitisation to

TRAIL involved induction of caspase-3 like activity in PC-3 and DU145 cells.

*Conclusion.* This study demonstrates that androgen independent prostate cancer cell lines are resistant to TRAIL-mediated apoptosis. We have shown for the first time that resveratrol sensitises PC-3 and DU145 cells to TRAIL by a mechanism that involves the induction of caspase activity. Dietary intake of resveratrol may represent a strategy for enhancing susceptibility to apoptosis in prostate cancer.

#### 015 12.30-12.45

##### **REGULATION OF THE CELL CYCLE PROGRESSION OF PC-3, HUMAN PROSTATIC CELLS AND A549, HUMAN LUNG CELLS TREATMENT BY MAJOR PAPRIKA CAROTENOIDS ELEMENTS CAPSANTHIN, CAPSOURBIN AND BETA-CAROTENE**

Ms Harukuni Tokuda, Dr Fumio Enjo, Dr Takashi Maoka, Dr Kooichi Mochida, Ms Junko Takasasu, Dr Hoyoku Nishino

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Ripe fruits of red paprika (*Capsium annuum* L.) are used widely as vegetables and food colorants, which are good source of carotenoid pigments. The red carotenoids in paprika are mainly capsanthin and capsourbin having a 3-hydroxy k-end group, and showed strong quenching activity of single oxygen by free radical. Capsorubin, final metabolite of beta-carotene in paprika, had potent growth inhibitory effects in vitro against A549 and PC-3 cell in medium contain 10% of FBS. This was associated with increased level of p27 protein and decrease of cyclin A, cdk2, cdc2 through no significant changes were seen in the level of cyclin B1, cyclin D1, p38, MEK, NF-kB and beta-catenin. These results suggested that treatment of capsourbin cause growth inhibitory by reduction of cyclin A without changing MAP kinase, NF-kB pathway, nor suppression of beta-catenin integretion into nucleus. We studied, for the first time, one of the elements capsourbin, in paprika extracts as chemopreventive agents.

#### 016 12.45-13.00

##### **A 3D-ORGAN CULTURE OF HUMAN BENIGN AND TUMOURAL PROSTATIC EXPLANTS**

Dr Sandra Papini, Prof Anna DeMatteis, Dr Daniela Campani, Prof Cesare Selli, Prof Roberto Paolo Revoltella

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*Introduction.* We studied the profile and immunophenotype of gland cells from benign and tumoural prostate, using 3D-organotypic cultures preserving stromal and epithelial organization present in vivo.

*Materials and Methods.* Small explants were cultured on sponges for 3-4 weeks and the luminal and basal epithelium and cellular responses to 4,5α-dihydrotestosterone (DHT) stimulation were studied. In addition, basal cells from normal or benign fragments or from the sponge were isolated by enzyme-digestion and expanded on feeder fibroblasts. Markers: 34 bE12, basal cells; p63, progenitor epithelial cells; PSA secretory cells; Androgen Receptor (AR), luminal-secretory cells.

*Results.* In normal and benign fragments, with DHT, the gland tissue could be successfully maintained even after 3 weeks in culture. Without DHT, samples showed a progressive loss of luminal cells and a strong progressive basal cell (BC) hyperplasia. BC proliferated with retention of the lumen or forming nests with squamous differentiation, lining the fragment surface and migrating to the underlying sponge. After 3-4 weeks, from normal or benign samples, we isolated selectively primary prostate epithelial basal cells (over 90% 34Eb12 +, over 65% clone p63 +). The tumour glands were maintained up to 14-21 days. In certain cases the tumour glands lost the secretory cells (in 7-14 days), especially in the central zone of the fragment; these cells were replaced with basal cells (HMwCK+). Peripheral nests of cancerous cells were also observed (AR +, PSA + HMwCK-). After 14-21 days we also found a mixed cell population, composed by cuboidal (HMwCK+, PSA-) and columnar cells (HMwCK-, PSA-) both in the glands and lining the fragment. The addition of DHT, apparently, didn't affect the behaviour of the tumoural cell compartment.

*Conclusions.* Organotypic cultures should be useful in studying regulation of proliferation and differentiation of

prostatic progenitor cells and the tumoural cell compartment within their natural microenvironment.

**FREE PAPER SESSION 4  
(Parallel II)  
Location: PFC G06**

**017 11.30-11.45**

**GROWTH-PROMOTING EFFECTS OF  
THYROID-STIMULATING HORMONE ON  
HUMAN MELANOMA CELLS**

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*Introduction.* We have reported a high prevalence of hypothyroidism in the cutaneous melanoma population, suggesting that the pathologic hormonal environment of hypothyroidism promotes melanoma growth. The objective of this study was to test the hypothesis that thyroid-stimulating hormone (TSH), which is elevated in the circulation of hypothyroid individuals, stimulates the growth of melanoma cells.

*Methods.* Immunohistochemistry was used to examine the expression of TSH receptors in human melanoma tumors and melanoma cells lines. The activation of these receptors by TSH was analyzed by a cAMP immunoassay. Proliferation was assessed by uptake of bromodeoxyuridine. Expression array technology was used to detect melanoma genes regulated by TSH.

*Results.* TSH receptors were detected in 19/20 primary melanomas and on all melanoma cell lines. Melanoma cells responded to TSH by cAMP expression and proliferation in a dose-dependent fashion. Cyclin D3 gene expression was increased over ten-fold in response to TSH. However, cytoplasmic cyclin D3 protein levels declined, suggesting a shift to the nuclear compartment.

*Conclusions.* Melanoma cells have phenotypic features similar to thyrocytes: they carry TSH receptors, respond to TSH by cAMP production, and proliferate in response to TSH through a mechanism involving D cyclins. We conclude that TSH promotes melanoma growth in vitro,

raising the possibility that this hormone may promote tumor progression in vivo. Clinical studies are warranted to further examine the association of hypothyroidism and elevated TSH levels with outcomes of melanoma patients.

**018 11.45-12.00**

**AUTOCRINE GROWTH-STIMULATION BY  
GRANULOCYTE-MACROPHAGE COLONY-  
STIMULATING FACTOR (GM-CSF) IN HUMAN  
GLIOMA AND DERIVED CULTURES**

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*Introduction.* The present work was performed in an attempt to support the autocrine role of granulocyte-macrophage colony-stimulating factor (GM-CSF) in malignant gliomas

*Materials and Methods.* We studied the expression of GM-CSF and its receptor (GM-CSF.R, a and b subunits) genes in brain gliomas with different malignant grading and in established cell lines derived from these tumors.

*Results.* We studied the expression of 16 human brain gliomas with different tumor grading and demonstrated high level mRNA expression and protein production of both GM-CSF and GM-CSF.R exclusively in the highest-grade tumors. Five glioma lines showing a homogeneous staining for their astrocytic origin were isolated. They were tumorigenic when injected to athymic mice. They produced GM-CSF constitutively, detected by positive immune- staining and ELISA in cell lysates. Two lines (AS-1 and PG-1), isolated from patients with glioblastoma multiforme, secreted active GM-CS into their conditioned media , tested by 14-day-human BM clonogenic assays. They constitutively co-expressed GM-CSF and its receptor genes. Cell proliferation was enhanced in response to rhGM-CSF, the response being concentration-dependent and specifically inhibited with anti-CM-CSF Abs in the medium. Different AS-1 clones, isolated after subsequent passages in vitro and transplantation into athymic mice, demonstrated higher tumorigenic capacity with

increasing passages in vivo. In the presence of rhGM-CSF, 3H-Th uptake and cell proliferation were stimulated in late-stage high-malignant clones bearing appropriate GM-CSF.R, whereas no stimulatory effect was detected in early-stage clones expressing GM-CSF.R. The addition of inflammatory cytokines (e.g. IL-1 $\beta$ , TNF $\alpha$ , IFN $\gamma$ , GM-CSF ) induced gene activation for several cytokines and functional proteins (e.g. G-CSF, VEGF, NGF, GM-CSF-itself and GM-CSF.R) indicating that these cells could undergo dynamic changes in response to environmental stimuli.

*Conclusions.* These results imply that GM-CSF has an effective role in the evolution and pathogenesis of malignant gliomas

#### 019 2.00-12.15

##### **CENTROSOME ABERRATIONS IN THE ADENOMA-CARCINOMA SEQUENCE OF COLORECTAL CARCINOMAS ANALYSED BY SYNTACTIC STRUCTURE ANALYSIS**

Dr Gian Kayser, Dr Ulrike Gerlach, Dr Axel Walch, Dr Roland Nitschke, Dr Sabine Haxelmanns, Prof Klaus Kayser, Prof Ulrich Hopt, Prof Martin Werner, Dr Silke Lassman

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*Aim.* Numerical and qualitative centrosome aberrations have been described for many solid tumors, including prostate, breast, pancreas and GI-carcinomas. In this study we investigated numerical and structural centrosome aberrations in the adenoma-carcinoma sequence of colo-rectal carcinomas in order to learn more about its occurrence and influence on carcinogenesis.

*Material and Methods.* A total of 76 specimens (normal colon mucosa =21; low grade intraepithelial neoplasia n=27, high grade intraepithelial neoplasia n=16 and invasive adenocarcinomas n=33) were investigated. Centrosomes were labeled through immunofluorescence staining against  $\alpha$ -tubulin (clone GTU-88, Sigma). Staining was evaluated by recording three-dimensional image stacks using a Zeiss LSM510 confocal microscope and further numerical and structural

data analysis was performed by self-written programs based on the DIAS software package. The NCSS-software was used for statistical analysis with the regarding to histological type of the lesion and number of centrosomes per cell.

*Results.* The mean number of centrosomes in normal colon epithelial cells was 0,8775 signals per cell. Already low grade intraepithelial neoplasias showed a highly significant increase of centrosome signals per cell (mean 1,787,  $p < 0,0001$ ). In high grade intraepithelial neoplasia a further increase of signals to 2,268 occurred, and was similar to the values measured in invasive carcinomas (2,267 signals per cell). A significant increase of structural entropy from normal mucosa (3.956) via low (6.393) and high grade intraepithelial neoplasia (5.745) to invasive carcinoma (6.856) was observed ( $p < 0.001$ ). In concordance with this the minimum spanning tree (MST) of the centrosome signals also shortened significantly (normal: 38.78; LGIN: 26.00; HGIN: 26,968; invasive carcinoma: 28.08;  $p < 0.001$ ).

*Conclusion.* Our results indicate that centrosome aberrations occur as an early event in the adenoma-carcinoma sequence of colo-rectal carcinomas.

#### 020 2.15-12.30

##### **RELATION BETWEEN CHROMATIN TEXTURE AND PHENOTYPE IN ACUTE LEUKEMIAS**

Prof Konradin Metzke, Miss Rosana C Silva, Dr Randall L Adam, Prof Neucimar J Leite, Mrs Fernanda G Pereira, Prof Irene Lorand-Metze

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*Introduction.* The chromatin structure of leukemic blasts is a fundamental hallmark for morphological diagnosis in routine cytology. Changes of chromatin arrangement as well as the expression of cytoplasmic or membrane proteins reflect the degree of cellular differentiation. The aim of our study was to correlate chromatin texture features with the expression of membrane and cytoplasmic proteins in blasts of patients with acute leukemia.

*Materials and Methods.* 32 patients with acute lymphatic (ALL) and 31 patients with acute myeloid leukemia

(AML) entered the study. Diagnosis was based on cytologic features, phenotype (determined by flow cytometry) and cytogenetic analysis. In each patient texture analysis was done on gray-scale transformed digitalized images of 100 cells of routinely stained May-Grünwald-Giemsa preparations. We measured granulometric features (using the gray level height of the basins as filter parameter) as well as Shannon's entropy and the fractal dimension in images after application of thick and thin contour detection. Antigen expression was quantified by mean fluorescence intensity (10 000 cells per patient ; paint-a-gate software).

**Results.** In ALL there was a negative correlation between the expression of immunoglobulins (light chains ) and the number of granulometric residues at lower height of the basins (  $-0,54$ ;  $p=0,005$ ). Furthermore we found a positive correlation between the CD20 expression and the fractal dimension after morphologic gradient filtering ( $r= 0,39$ ;  $p=0,036$ ). In AML patients the number of granulometric residues at lower height of basins were inversely correlated with CD7 ( $r = -0,50$  ;  $p=0,004$  ) and myeloperoxidase expression ( $r= - 0,42$  ;  $p=0,036$ ). A positive correlation between CD45 expression and the number of granulometric residues at higher basins ( $r = 0,45$ ;  $p=0,038$ ) was seen.

**Conclusion.** In leukemic blasts, changes of the chromatin structure may express differences in maturation measured by quantitative expression of antigens routinely used for diagnosis.

Supported by FAPESP, FAEP, CNPq.

## 021 12.30-12.45

### **BCR-ABL KINASE DOWN-REGULATES CCN3 EXPRESSION IN CHRONIC MYELOID LEUKAEMIA**

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**Introduction.** Chronic Myeloid leukaemia (CML) is characterized by expression of the constitutively active

Bcr-Abl protein tyrosine kinase. We have previously shown reduced expression of the growth regulator, CCN3, as a result of Bcr-Abl kinase activity in the murine FDCP-Mix haematopoietic stem cell line, using microarray technology. In this study we have examined CCN3 expression in human CML cell lines and in primary human CML cells at diagnosis and following treatment.

**Methods.** Real-Time PCR was used to monitor mRNA expression of BCR-ABL and CCN3 in the human CML cell lines K562, KU812, LAMA and also in normal bone marrow (NBM) and in 3 human CML patient samples taken at diagnosis and following response to treatment. Western blot analysis and confocal microscopy were used to detect Ccn3 protein expression.

**Results.** BCR-ABL mRNA expression was strong in the human CML cell lines whilst CCN3 expression was weak or absent. CCN3 mRNA expression in BCR-ABL negative cells (HL60), and in NBM was 30-fold higher than expression in the CML cell lines. Treatment of K562 cells with the Bcr-Abl tyrosine kinase inhibitor, Imatinib (1 micromolar, 96 h) resulted in a 5-fold reduction in BCR-ABL expression and a concomitant 4-fold increase in CCN3 mRNA. Primary human CML cells at diagnosis showed a four-fold lower expression of CCN3 mRNA in comparison to NBM ( $p=0.015$ ). Patients responding to treatment showed that as BCR-ABL expression declined (mean decrease 15-fold) CCN3 expression had increased (mean increase 19-fold) and was comparable with levels found in NBM. Differential expression of Ccn3 protein at diagnosis and in response to treatment, was also confirmed by confocal microscopy and Western blotting.

**Conclusion.** Dysregulation of CCN3 expression has not previously been recognized in CML. CCN3 and BCR-ABL expression appear to be inversely related. Loss of CCN3 expression is consistent with the CML phenotype.

## 022 12.45-13.00

### **A STRATEGY FOR DEFINING BIOLOGICALLY RELEVANT LEVELS OF P53 PROTEIN EXPRESSION IN CLINICAL SAMPLES**

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Numerous studies have investigated the value of p53 immunohistochemistry as a diagnostic, prognostic or predictive marker in diverse malignancies. However the literature is confused and in places contradictory. Furthermore the literature pays little attention to pivotal aspects of p53 biology nor basic principles of immunochemistry. Consequently conceptual and methodological flaws exist in much of the literature. Here we highlight one such issue, the effect of varying antibody concentration on the immunohistological detection of p53 expression. We demonstrate that the signal obtained from p53 immunohistochemical studies in proliferative endometrium, endometrioid carcinoma and serous carcinoma is fundamentally dependent on this critical parameter. The proportion of positive tumour cell nuclei in these exemplar conditions varied dramatically with the antibody concentration employed. Such data have diverse implications. First, the p53 labelling index is not reliable in clinical practice without attention to such issues. Second, comparisons between studies are not valid since different antibody concentrations (as well as a variety of other parameters which may affect immunostaining results) have been employed. Thirdly, standardisation between laboratories in terms of immunohistochemical staining with p53 and other antibodies is necessary if clinical utility is to be attached to these markers. Finally we propose a simple strategy for determining the optimal p53 antibody concentrations to be used that might resolve some of these issues. A key issue for the future will be to relate such immunochemical data to the specific mutation spectra of p53 which may fundamentally determine key aspects of protein stabilization and other biologically important phenomena.

|                    |                                      |
|--------------------|--------------------------------------|
| <b>13.00-14.00</b> | <b>LUNCH AND NETWORKING</b>          |
| <b>14.00-15.30</b> | <b>POSTER VIEWING AND TRADE SHOW</b> |
| <b>15.30-16.00</b> | <b>COFFEE AND NETWORKING</b>         |

## **FREE PAPER SESSION 5**

**Location: PFC G07**

**023 16.00-16.15**

### **THE ROLE OF MIF1/HERP IN REGULATION OF HYPOXIA-MEDIATED CELL DEATH**

Theo van Laar, Carrol Terleth, Ron Schouten, Avi Shvarts, Paul van Diest, Elsken van der Wall  
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*Department of Toxicogenetics, Leiden University Medical Center, Leiden, The Netherlands*

There is a large overlap between proteins induced by hypoxia (low oxygen) and stress in the endoplasmic reticulum (ER-stress). ER-stress is caused by the accumulation of malformed proteins in the endoplasmic reticulum and results in activation of the unfolded protein response pathway (UPR).

Previously we have identified the Mif1/Herp gene as a target for UPR. The Mif1 promoter contains at least two ER-stress responsive elements (ERSE). The Mif1 protein contains a N-terminal ubiquitin-like domain (Ubl), suggesting a role for Mif1 in protein degradation. Proteins missing the proper tertiary structure are degraded in response to ER-stress by the ER-Associated protein Degradation (ERAD) mechanism.

Here we present evidence that Mif1 indeed might associate with the 26S proteasome. First, we show that a tagged version of Mif1 associates with a tagged version of S5a, a subunit of the 19S regulatory complex of the proteasome. Furthermore, we identified in a yeast-2-hybrid screening TBP1, another subunit of the 19S regulatory complex, as a Mif1-binding protein.

Recently, Hrd1 has been identified as an Ubiquitin E3-ligase involved in ERAD. We show here that a tagged version of Hrd1 co-immunoprecipitates with a tagged version of Mif1, again suggesting a role for Mif1 in ERAD.

In addition to induction by ER-stress, Mif1 is strongly activated in response to hypoxia. Hypoxia induces apoptosis in many cell types. Abrogation of hypoxia-mediated apoptosis is thought as one of the late steps in the development of a tumor. Hypoxia-mediated apoptosis is counteracted by the Ubiquitin E3 ligase Hrd1.

Currently, we are investigating the role of Mif1 in the regulation of hypoxia-mediated cell death through its association with Hrd1.

#### 024 16.15-16.30

##### **THE INFLUENCE OF HYPOXIA IN PROMOTING MALIGNANT PROGRESSION IN PROSTATE CANCER CELLS**

Dr Karl Butterworth, Dr Helen McCarthy,  
Dr Jenny Worthington, Dr Tracy Robson,  
Prof Stephanie McKeown

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*Introduction.* The malignant progression of prostate tumors is characterized by the development of a hormone refractory phenotype. There is increasing evidence that tumor hypoxia can influence this process in many tumour types including prostate. The aim of this study was to investigate whether growth in hypoxic conditions would lead to the emergence of a hormone refractory phenotype in LNCAP cells.

*Materials and Methods/Results.* Initially we investigated the hypoxia tolerance of log phase LNCAP cells in an atmosphere of 0.1% oxygen: 94.9% nitrogen: 5% CO<sub>2</sub> in a hypoxic workstation. LNCAPs were surprisingly hypoxia resistant. Exposure for 24 hours at 0.1% oxygen caused almost no loss of cells. Exposure for 72h was effective at killing all the cells. To investigate the effect of intermittent hypoxia on LNCaP growth, log phase cells were grown in the 0.1% oxygen atmosphere initially for 48 hours, every week for 6 months when an increase in growth rate was noted. These cells were harvested and designated LNCaP-H1. The cells were injected into a female SCID mouse and were capable of forming a tumour, suggesting that the cell line was androgen independent. Implanted parental cells did not grow. When compared with the parental cell line the LNCaP-H1 cells exhibit features which are in line with a more malignant hormone refractory phenotype.

*Conclusions.* Our model suggests that hypoxia can cause malignant progression in prostate cancer cells and more importantly we have shown that the selected cells are androgen-independent; the LNCaP-H1 cells can grow successfully in charcoal stripped medium. We hypothesize that low oxygen tension may select for a

more aggressive phenotype, provide a mutagenic microenvironment for cellular dedifferentiation or trigger the expression of stress response genes which facilitate malignant progression.

#### 025 16.30-16.45

##### **EPIGALLOCATECHIN GALLATE INDUCED PROSTATE CANCER CELL APOPTOSIS IS NOT ALTERED BY HYPOXIA DESPITE ITS EFFECTS ON MITOCHONDRIAL ACTIVITY AND OXIDATIVE ENVIRONMENT**

Dr Colm Morrissey, Dr Amanda O'Neill, Mr Kevin Cunningham, Dr Cormac Taylor, Prof John M. Fitzpatrick, Dr R. William G. Watson

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*Introduction.* Antioxidants may reduce tumorigenesis in prostate cancer due to their chemopreventative properties, however they have also been studied from a chemotherapeutic standpoint. Epigallocatechin gallate (EGCG) a potent antioxidant has been shown to induce apoptosis in prostate cancer cells in vitro. The aims of this study were to determine if a hypoxic environment alters the effects of EGCG on metastatic prostate cell number and apoptosis as well as mitochondrial activity and oxidative cellular changes.

*Methods.* Two androgen independent prostate cancer cell lines, PC-3 and DU-145 were cultured to confluence and treated with different concentrations of EGCG in hypoxic and normoxic conditions. Cell number and apoptosis was assessed using crystal violet assays and flow cytometric analysis. The effect of EGCG on mitochondrial activity was assessed using the MTT assay and MnSOD expression by western analysis. The influence of hypoxia and EGCG on the oxidative environment and reactive oxygen species (ROI) production in PC-3 and DU-145 cells was measured by flow cytometry and luminometry using DCFH-DA.

*Results.* Despite EGCGs anti-oxidant potential, it was shown to increase ROI generation in normoxic conditions in the PC-3 and DU-145 cells, which was

significantly inhibited in an hypoxia environment. Associated with the increase in ROI, EGCG decreased MnSOD expression in normoxic conditions which again was inhibited and in fact increased in a hypoxia environment. Despite the ability of hypoxia to change the cells mitochondrial and ROI response to EGCG it had no effect on blocking its apoptotic inducing effects, but longer exposure of 48 hours hypoxia, induced apoptosis in both cell lines.

**Conclusions.** The apoptotic inducing effects of EGCG are not influenced by changes in the hypoxia environment of the tumour, despite changes in redox environment, mitochondrial activity and MnSOD levels. This demonstrates that EGCG induced apoptosis is independent of mitochondrial disruption and manipulation. Where the hypoxic environment might influence functional activity it did not alter the fundamental mechanisms of cell death induced by EGCG.

## 026 16.45-17.00

### DEVELOPMENT OF SINGLE CHAIN LLAMA ANTIBODIES AGAINST THE HYPOXIA INDUCIBLE FACTOR 1-ALPHA PROTEIN USING PHAGE DISPLAY

Groot AJ, Gort EH, Westerlaken EJ, Verheesen P, Van der Groep P, Van Laar T, Verlaan I, van der Wall E, van Diest PJ, Shvarts A  
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**Introduction.** Hypoxia is a powerful trigger of angiogenesis and associated with carcinogenesis, tumour progression and resistance to therapy. In mammalian systems, the heterodimeric basic helix-loop-helix (bHLH)-PAS transcription hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ) has emerged as the key regulator of responses to decreased oxygen concentrations and is over expressed in many solid tumours, including premalignancies, which is associated with aggressive clinical behavior. Levels of HIF-1 $\alpha$  are tightly regulated by oxygen concentration. For the functional and diagnostic studies on the HIF-1 $\alpha$  and its domains we have chosen to generate Camelidae derived specific antibodies against the HIF-1 $\alpha$  protein.

The Camelidae (camel, dromedary and llama) possess in addition to conventional antibodies a repertoire that is devoid of light chains, the so-called heavy-chain antibodies (HCAs or VHH). These single domain fragments are the smallest intact antigen-binding fragments known to man. In 1990 McCafferty et al. showed that antibody fragments could be displayed on the surface of filamentous phages, called phage-display. After displaying an antibody fragment on the protein surface of the phage, antigen specific phages can be selected. This technique makes it possible to select phages and thereby antibodies, from a phage display library, that bind almost any antigen.

**Methods.** A naïve library, constructed at Unilever Research, and provided by Prof. C.T. Verrips of the UU, of non-immunized llamas and dromedaries was selected to construct antibodies that could recognize the human HIF-1 $\alpha$  protein.

**Results.** So far, five different VHH antibodies recognizing epitopes in the oxygen-dependent degradation domain (ODDD) within the protein were identified.

**Conclusions.** The small size of these VHH antibodies (15kDa) makes them a very useful tool for in vitro and in vivo intracellular functional analysis of the HIF-1 $\alpha$  protein. At this moment we are testing the potential of these antibodies as molecular biological tools and for diagnostic purposes.

## PREMIER LECTURE SERIES

**Location: PFC G07**



**PL8 17.00-18.00**  
**PLOEM DISTINGUISHED**  
**LECTURE**

**Professor Steve Pennington**  
*Proteomics, Conway Institute for Biomolecular and Biomedical Research, University College Dublin, Ireland*

## INTEGRATED PROTEOMIC STRATEGIES FOR BIOMARKER DISCOVERY

Many biomarker discovery strategies share a common requirement for the rapid identification and quantification of proteins – however biomarkers are often likely to represent proteins with diverse physicochemical properties. This means that proteomic strategies for biomarker

discovery require a range of integrated strategies. Two-dimensional gel electrophoresis (2-DE) and multi-dimensional liquid chromatography (LC) based methods have emerged as powerful approaches for discovery based protein expression profiling but each has significant limitations. 2-DE can resolve several hundreds of proteins simultaneously but is not well suited for the separation of membrane proteins - an extremely important class of proteins of particular interest to the pharmaceutical industry. It also has limitations for the analysis of the protein components of serum. Multi-dimensional LC potentially has the advantage of higher sensitivity but as yet is not *routinely* applied to the separation and quantification of very complex mixtures of proteins - and certainly not in high throughput. This presentation will include a description of the use of 2-DE and isotope coded affinity tag approaches for biomarker (and target) discovery in pancreatic cancer and drug toxicity.

### Biography

Stephen R Pennington has been appointed to the Professorship of Proteomics in the Conway Institute of Biomolecular and Biomedical Research. Stephen graduated from Imperial College of Science and Technology (University of London) with a joint honours degree in Chemistry and Biochemistry (upper second) before completing a PhD in Biochemistry at the University of Cambridge. During his PhD he was awarded an Elmore Medical Research Fellowship. It was during this fellowship that his interests in the regulation of the mammalian cell cycle began, the subject of his research that was continued when he moved to the Department of Human Anatomy & Cell Biology at the University of Liverpool to take up a post as a Wellcome Trust funded lecturer. Subsequently he was a University Lecturer and then Senior Lecturer at the University of Liverpool. During this period his research used two-dimensional gel electrophoresis (2-DE) combined with mass spectrometry that served as the foundation for the evolution of the exciting field of 'Proteomics'. Stephen anticipated this evolution and in 1997 published an important review of emerging proteomics strategies and edited a book on proteomics that brought together the leading international proteomics experts. Stephen has also been active in the practical implementation of collaborative proteomics projects and set up a multi-user proteomics facility at the University of Liverpool that now contains state-of-the-art instrumentation and software for 2-DE gel running, image analysis, spot cutting, automated protein digestion, MALDI and electrospray mass spectrometers. The facility is being used to support a series of collaborative research programmes in biomedical sciences that Stephen continues to support and be involved in. Stephen and his group are also inter-

ested in the development of novel proteomics technologies and have received a Sir Henry Wellcome Commemorative Award for Innovative Research to support some of this work. More recently they have been working on a novel antibody screening strategy that supports the generation of antibodies for incorporation into protein arrays. This project is part of a multi-disciplinary consortium, the Centre for BioArray Innovation, that was initiated in October 2001 with a £2.4million grant from the BBSRC. Stephen's reputation in the field of proteomics is such that a number of the key commercial organisations in the field consult with him regularly and increasingly bring their state-of-the-art instrumentation into his laboratory. He also regularly gives presentations in the UK, Europe, the US and Asia. He was recently invited to participate on two lecture tours of Asia where he gave presentations on the multi-user proteomics facility in Taiwan, South Korea, Hong Kong and mainland China. Stephen's vision for the development of proteomics in the Conway Institute is to use his expertise and experience to develop a Proteomics Research Centre which will bring the very latest state-of-the-art instrumentation and support to apply proteomics to clinical research projects and to support translational projects by bringing research results to the clinical setting.

### 19.30-LATE ISCO GALA DINNER (BELFAST CITY HALL)



The City Hall stands in the centre of Donegal Square in the heart of downtown Belfast, dominating the City's

principal shopping area and adding a distinctive outline to the Belfast skyline. The City Hall, whose influence radiates outward from the heart of the city, is a magnificent piece of Classical Renaissance architecture dating back to 1898. The ISCO2005 Gala Dinner will be a memorable experience for all of those attending the conference.



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