Analysis of Autophagy Regulation:
Discussion of recent research and new technologies

Thursday, 03 October 2013
Cineworld: The O2, London, UK

This meeting will present and discuss current research into autophagy regulation including new flow cytometric and imaging assays and approaches which available to study this regulation. This event has CPD accreditation.This event is part of the **2013 Flow Cytometry Forum** – www.FlowCytometry2013.com

Meeting Chair: **Dr. Gary Warnes,** Blizard Institute, Barts & The London School of Medicine & Dentistry, UK

Who Should Attend

Flow cytometry specialists
Biotech and Pharma Industry: CEOs, Chief Scientists, Group Heads, Senior and Junior Scientists, Research Managers
Academic and Research Institutes: Group and Lab Heads, Postdoctoral Scientists and Research Students

The Deadline for abstract submissions for oral presentation has now passed. Abstracts for *poster presentation only* can be submitted up to two weeks before the event. **You can download the instructions for authors at www.euroscicon.com/AbstractsForOralAndPosterPresentation.pdf**

9:00 – 9:45 **Registration**

9:45 – 10:00 **Introduction by the Chair:** *Dr. Gary Warnes,* Blizard Institute, Barts & The London School of Medicine & Dentistry, UK

10:00 – 10:30 **Flow Cytometric Measurement of Cell Organelle Phagy**
*Dr. Gary Warnes***,** Blizard Institute, Barts & The London School of Medicine & Dentistry, Queen Mary University
The mechanism of organelle autophagy is little understood. We employed a range of autophagy inducing agents to determine the relative preference for the type of organelle-phagy caused by rapamycin, chloroquine, nutrient and low serum starvation. Organelle autophagy of mitochondria and Endoplasmic Reticulum (ER), termed mitophagy and ER-phagy was determined flow cytometrically by the employment of organelle mass probes, MitoTracker Green (MTG) and ER Tracker Green (ERTG). Relative changes in linear scaled median fluorescence intensity (MFI), were compared to control cells to determine the degree and type of organelle-phagy induced by different inducers of autophagy. These flow cytometric organelle phagy assays can be used by researchers to study the autophagic process further in terms of cell function.

10:30 – 11:00 **Autophagy and neurodegeneration**
Professor David C Rubinsztein, Professor of Molecular Neurogenetics, Wellcome Trust Principal Research Fellow, Deputy Director, Cambridge Institute for Medical Research, Honorary Consultant, University of Cambridge, UK
I will describe our recent studies that implicate the plasma membrane as a source for autophagosomes, before focussing on the roles of autophagy in neurodegeneration. We showed that the autophagy inducers reduced the levels of mutant huntingtin and related neurodegenerative disease-associated proteins. These compounds ameliorated the toxicity of these proteins in cells and in vivo. While autophagy induction is protective in models of various neurodegenerative diseases, certain other conditions, including lysosomal storage disorders, are associated with compromised autophagy. I will review these data and then describe how impaired autophagy compromises cellular processes, including the ubiquitin-proteasome system.

11:00 – 11:30 **Speakers' photo then mid-morning break and trade show/poster viewing**
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11:30 – 12:00 **A role for Rab8 and autophagy in the regulation of synapse growth**
Dr Sean T Sweeney, University of York, UK
We have developed a model of Frontotemporal dementia (FTD) in Drosophila based on ESCRTIII dysfunction. In a screen for enhancers and suppressors of the FTD phenotype we identified Rab8. Mutations in Rab8 have overgrown neuromuscular synapses by a factor of 100%. Examination of Rab8 mutants revealed endosomal dysfunction and accumulation of autophagosomes. Within the dysfunctional endosome we have identified signaling events organizing the prolonged activation of TGF-beta and JNK/AP-1 signaling generating synapse overgrowth. Autophagic activity is also necessary for the generation of synaptic overgrowth observed. The novel events we describe are likely to be critical to neuronal atrophy in FTD.

12:00 – 12:30 **Autophagy as a barrier to viral and non-viral gene delivery**
Professor Tom Wileman, University of East Anglia, UK
There is great interest in the development of viral and non-viral gene therapy vectors to replace defective genes associated with specific illnesses. Our work shows that viruses and non-viral gene delivery vectors can activate autophagy resulting in delivery to autophagosomes. Autophagy provides a powerful means of killing intracellular viruses by delivering them to lysosomes for

degradation, and at the same time slows release of genes into cells. Authophagy has therefore evolved as an efficient defence against viral infection, but becomes a major barrier to the development of gene therapy vectors.

12: 30 – 13: 30 **Lunch and trade show/poster viewing**

13:30– 14:20 **Question and Answer Session**
Delegates will be asked to submit questions to a panel of experts. Questions can be submitted before the event or on the day

14:20 – 15:00 **Oral Presentations:**

14:20 – 14:30 **17 BETA-ESTADIOL AND PROGESTERONE ENHANCE EXPRESSION OF AUTOPHAGIC GENES IN BOVINE MAMMARY EPITHELIAL CELLS.**

M. Gajewska, K. Zielniok, A. Majewska, T. Motyl
Department of Physiological Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences (SGGW), Nowoursynowska 159, 02-672 Warsaw, Poland, e-mail: malgorzata_gajewska@sggw.pl

14:30 – 14:40 **INDUCTION OF AUTOPHAGY IN CHRONIC MYELOID LEUKAEMIA FOLLOWING TREATMENT WITH TYROSINE KINASE INHIBITORS MAY CONTRIBUTE TO DISEASE PERSISTENCE**

A. Mukhopadhyay, G.V. Helgason, M. Karvela, E. Allan, R. Mitchell, T.L. Holyoake
Paul O'Gorman Leukaemia Research Centre, College of Medical, Veterinary & Life Sciences, Institute of Cancer Sciences, University of Glasgow

14:40 – 14:50 **THE CORE AUTOPHAGY PROTEIN ATG4B IS CRITICAL TO THE SURVIVAL OF LEUKEMIC STEM/PROGENITOR CELLS AND PREDICTS CLINICAL OUTCOMES OF CML PATIENTS TREATED WITH IMATINIB THERAPY**

Katharina Rothe[1,2], Kevin B.L. Lin[1], Hanyang Lin[1,3], Amy Leung[4], Hui Mi Wang[1], Mehrnoush Malekesmaeili[1], Ryan Brinkman[1], Donna Forrest[3,5], Sharon Gorski[4], Xiaoyan Jiang[1,2,3]
[1]Terry Fox Laboratory, BC Cancer Agency; [2]Department of Medical Genetics, University of British Columbia; [3]Department of Medicine, University of British Columbia; [4]Genome Sciences Centre, BC Cancer Agency; [5]Leukemia/BMT program of BC, BC Cancer Agency, Vancouver, BC, Canada

14:50 – 15:00 **DYNAMIC ASSOCIATION OF THE ULK1 COMPLEX WITH OMEGASOMES DURING AUTOPHAGY INDUCTION**

E. Karanasios, E. Stapleton, M. Manifava, T. Kaizuka, N. Mizushima, S. A. Walker and N. T. Ktistakis
Signalling Programme, The Babraham Institute, Babraham Research Campus, Cambridge, CB22 3AT, UK

15:00 – 15:30 **Afternoon Tea**

15:30– 16:00 **Molecular mechanisms of mammalian autophagy**
Dr Sharon A. Tooze, London Research Institute, UK
Autophagy, a highly conserved cell survival pathway essential for cell health and homeostasis, is a membrane-mediated lysosomal degradation process that can be acutely induced. Induction by amino-acid starvation has been fundamental in the identification of the 36 autophagy-related (Atg) genes first in yeast, and more recently in mammals. Formation of autophagosomes requires the concerted effort of at least 18 Atg proteins, initiated by the activity of the ULK complex and the PI3-kinase complex including Beclin 1. I will discuss our recent findings about key protein-protein interactions and membrane contributions from a variety of subcellular compartments that drives autophagosome formation.

16:00 – 16:30 **Autophagy in host-pathogen interaction**
Dr Agnes Foeglein, MRC Laboratory of Molecular Biology, Cambridge, UK
Cells deploy autophagy to protect their cytosol against infection. For efficient delivery to autophagy invading pathogens are specifically recognized by NDP52 and other cargo receptors. I will discuss the interplay between autophagy and pathogens with special emphasis on how cells restrict the proliferation of bacteria in their cytosol, how professional cytosol-dwelling bacteria avoid such attack, and how viruses even appropriate autophagy.

16:30 – 17:00 **Chairman's summing up**

Keywords: autophagy, Flow Cytometric & Image Analysis, LC3-II, organelle phagy, Necrobiology, Apoptosis, Flow cytometry, apoptosis, Image Stream, T cells, Immunosenescence, Wnt, colorectal cancer, GMP-compliant, cytotoxicity Humira, ADCC, CDC, Cell death, proliferation dyes, cell imaging, cell morphology, Flow cytometry, benchtop, quantitative imaging, 10-12 colour flow cytometry, synapse, neuromuscular, autophagy, Frontotemporal Dementia, endosome,ULK1/2, WIPI2, autophagosome, viruses, liposomes and lipoplex, gene delivery, cell-autonomous immunity, Salmonella, NDP52, Galectin-8, autophagy; neurodegeneration; treatment

Registration Website: www.regonline.co.uk/autophagy2013

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About the Chair
Gary Warnes interest in flow cytometry started at St. Mary's in 1986, analyzing T-cell subsets. Then set up a new flow cytometric T-cell subset service at St.Thomas' Hospital. Completed a PhD investigating the immunosuppression of HIV-ve haemophiliacs at St.Thomas' Hospital. Post-doctoral position, investigated the regulation of Tissue Factor expression by immune co-stimulatory molecules in sepsis. Then managed the Flow & Imaging Core Facilities at the MRC Clinical Science Centre at Hammersmith Hospital. Worked with Derek Davies at Cancer Research UK. Currently managing the Flow facility at the Blizard Institute, Queen Mary University.

About the Speakers
David Rubinsztein is a Wellcome Trust Principal Research Fellow and Professor of Molecular Neurogenetics at the University of Cambridge, where he is Deputy Director of the Cambridge Institute for Medical Research. Rubinsztein has been an author on more than 290 scientific papers, including recent studies in Nature Chemical Biology (2007, 2008), Molecular Cell (2009,2011,2012), Cell (2010,2011, 2012,2013), Science Translational Medicine (2010), and Nature Cell Biology (2010, 2011). He was awarded the Graham Bull Prize for Clinical Science by the Royal College of Physicians in 2007. Rubinsztein is a Fellow of the Academy of Medical Sciences and a member of EMBO.

Agnes Foeglein, is originally from Hungary, but by now (more or less) German after growing up in Bavaria. She did her undergraduate degree in Erlangen in Molecular Medicine. Being interested in infectious diseases, Agnes came to Cambridge to carry out a PhD on Influenza virus and has now joined the Randow lab to work on cytosolic immunity.

Sean T Sweeney, gained his PhD in the Department of Genetics, University of Cambridge with Dr Cahir O'Kane. In his PhD he developed tetanus toxin light chain as a tool for the targeted transgenic silencing of neurons. This study also revealed the critical role for synaptobrevin at the synapse. He then studied with Prof. Grae Davis at UCSF as a Wellcome Prize Travelling fellow and published studies on a number of mutants involved in membrane traffic at the synapse. Since setting up as a PI he has focused on membrane traffic and endosome function at the synapse within the context of neurodegeneration, using Drosophila as an experimental system.

Sharon A. Tooze has been interested in understanding organelle biogenesis starting at the European Molecular Biology laboratory (EMBL) in Heidelberg, Germany and continuing at the London Research Institute, Cancer Research UK. Since 2004, her interests have been focused on understanding how cells make autophagosomes, and the process of autophagy, in mammalian cells. Her lab identified several autophagy proteins, and is continuing to reveal their function and regulation. Autophagy is fundamental for cell survival and death. A molecular understanding of the process and how it is regulated will provide insight into the role of the autophagy pathway in human diseases.

Tom Wileman trained in cell biology and immunology at Washington University and Harvard Medical Schools in the USA between 1982 and 1994. He was Assistant Professor at Harvard before moving to the Institute for Animal Health (Pirbright, UK) in 1994 as Head of Immunology to study the cell biology of virus infection. His recent studies have focussed on autophagy and have shown that some viruses activate autophagy during cell entry and that this pathway is also activated by the cationic polymers used as gene delivery vectors. His collaborative work with Kostas Kostarelos at UCL, has shown that non-viral gene delivery vectors are captured within specialised autophagosomes called tubulovesicular autophagosomes that slow gene delivery into cells.

ORAL AND POSTER PRESENTATIONS

17BETA-ESTADIOL AND PROGESTERONE ENHANCE EXPRESSION OF AUTOPHAGIC GENES IN BOVINE MAMMARY EPITHELIAL CELLS.

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Mammary gland undergoes cycles of remodelling based on successive growth and involution of secretory tissue. Involution occurs after the period of functional activity (lactation) and serves to regress the mammary gland to the state of development similar to the one prior pregnancy. In dairy cattle involution is typically overlapped by next pregnancy and it is induced by termination of milking in order to rebuilt the secretory tissue before the next lactation. During the dry period bovine mammary epithelial cells (MECs) undergo stress connected with milk stasis and deprivation of nutrients and biologically active compounds, which are extensively used by simultaneously developing foetus. At this time high concentrations of pregnancy hormones occur in the mammary gland. Our previous studies have shown that bovine mammary tissue from dry period contains a high number of MECs with autophagic vacuoles. Since autophagy is regarded as a temporary survival mechanism providing an alternative energy source during stress induced by starvation it can play an important role during the regenerative involution in dairy cattle. Our further *in vitro* studies on bovine mammary epithelial cell line BME-UV1, demonstrated that reduction of the content of foetal bovine serum (FBS) in culture medium (from standard 10% to 0.5%) induced formation of cleaved form of autophagic marker LC3-II, and decreased phosphorylation of mTOR kinase, indicating induction of autophagy in these cells. Thus, we used the FBS deprivation as an *in vitro* model of involution of bovine MECs. Additionally, we noted that in the

presence of 17β-estradiol (E2), or progesterone (P4) the level of LC3-II was further increased. Therefore, the aim of the present study was to evaluate the role of E2 and P4 in regulation of autophagic genes in BME-UV1 bovine MECs. The expression of chosen autophagic genes: *beclin1, Atg3, Atg5, LC3B* was evaluated after 2, 4, 6, 12 and 24h of culture in experimental medium (0.5% FBS) with or without addition of sex steroids (E2, 1nM; P4, 5ng/ml), using real time PCR. Furthermore, the levels of proteins encoded by the investigated genes were determined in the same experimental conditions after 2, 6, 12, 24 and 48h incubation, by immunoblotting. Confocal microscopy was used to show the redistribution of steroid receptors (ER and PR) in cells after hormones addition. The results showed that reduction of FBS content increased the expression of all investigated autophahgic genes from the earliest time point (2h incubation in experimental medium). Addition of E2 to the 0.5% FBS medium caused a significant enhancement of expression of *beclin1, Atg3, Atg5, LC3B* genes. In case of LCB the increase in expression was noted already after 6h incubation, whereas the remaining genes showed a clear change after 12h and 24h, in comparison to the experimental conditions. Immunoblot analysis revealed that in the presence of E2 the levels of beclin1, Atg5, LC3B proteins were also elevated in comparison to control (0.5% FBS), especially in the later time points of incubation (between 12 and 48h), although the results were less pronounced. The level of Atg3 protein remained unchanged during the whole experiment. In the case of progesterone the significant impact on expression of investigated autophagic genes was not noted, when the results were compared with 0.5% FBS conditions, however P4 significantly enhanced the expression of *beclin1, Atg3, Atg5, LC3B* genes when compared with the levels observed in standard growth conditions (medium with 10%FBS). Furthermore, the levels of beclin1, Atg5, and LC3 proteins were increased when E2 or P4 were added to the growth medium. These results indicate that 17β-estradiol and progesterone are not only involved in MECs proliferation during development of bovine mammary gland, but also actively regulate the induction of autophagy in these cells. However, it seems that during involution the role of 17β-estradiol in regulation of autophagic genes is more significant. Thus, in cattle autophagy may serve as an important process in preventing extensive cell death during dry period, and its induction by sex steroids may enable easier regeneration of the mammary gland prior next lactation.

Induction of autophagy in chronic myeloid leukaemia following treatment with tyrosine kinase inhibitorS May contribute to disease persistence

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Treatment of Chronic Myeloid Leukaemia (CML) has been revolutionised by the introduction of c-ABL specific Tyrosine Kinase Inhibitors (TKIs). These drugs are capable of inhibiting constitutively active BCR-ABL kinase resulting from reciprocal translocation of long arm of chromosome 9 and 22 generating the Philadelphia chromosome (Ph) [t(9;22)(q34;q11)]. However, minimum residual disease persistence, caused by CML stem cells that survive TKI treatment, is an ongoing problem in CML pharmacotherapy. Autophagy has been shown to play an essential role in normal haematopoietic stem cells and can be utilised by cancer cells to escape death in response to chemo- and radio-therapies. We used electron microscopy to confirm the presence of double membrane bound autophagy vesicles in primary CD34+ CML cells. Following TKI treatment, CML cells, including primitive CD34+38- cells (Bellodi et al., 2009) showed increases in lipidated LC3b-II protein, monitored by Western blot analysis or immunofluorescence (subsequent quantification by AutomeasureTm revealed a significant increase in LC3b-II puncta in treated cells) indicating induction of the autophagy process. Increase in autophagy "flow" following TKI treatment was confirmed by using CML cells stably expressing mRFP-GFP-LC3 protein. The lysosomotrophic drug Hydroxychloroquine (HCQ) can inhibit lysosomal degradation of autophagosomes and thus inhibit normal autophagy flow (shown by the mRFP-GFP-LC3 system). The survival role of autophagy in CML cells was therefore examined following HCQ treatment in addition to knockdown of the essential autophagy proteins ATG5 and ATG7. This revealed that TKI-induced autophagy protects CML cells from TKI-induced death. Of clinical importance, HCQ treatment led to near complete elimination of persistent CML stem cells when combined with TKI treatment in long term stem cell assay. Following the success of the *in vitro* study, CHOICES, (CHlorOquine and Imatinib Combination to Eliminate Stem cells), a randomised Phase II trial is ongoing to evaluate the response to autophagy inhibition in patients (ClinicalTrials.gov Identifier: NCT00049569). In conclusion, TKI-induced autophagy has been shown to be one of the mechanisms for survival of persistent CML stem cells which can be eliminated by combination therapy.

Bellodi, C., Lidonnici, M. R., Hamilton, A., Helgason, G. V., Soliera, A. R., Ronchetti, M., Galavotti, S., Young, K. W., Selmi, T., Yacobi, R., *et al.* (2009). Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in Philadelphia chromosome-positive cells, including primary CML stem cells. J Clin Invest *119*, 1109-1123.

The core autophagy protein ATG4B is critical to the survival of leukemic stem/progenitor cells and predicts clinical outcomes of CML patients treated with imatinib therapy

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Although autophagy is a well-studied catabolic process that takes place at basal levels in the majority of mammalian cells, its role in the regulation of hematopoiesis and pathogenesis of leukemia have yet to be fully explored. Previous studies have shown that imatinib mesylate (IM) induces autophagy in CML and that this process is critical to the survival of leukemic stem/progenitor cells upon IM therapy. However, it is still not known if the autophagy process differs at basal levels between CML patients and healthy individuals and if pretreatment CML cells contain unique autophagy characteristics that could be predictive of patients' clinical outcomes. We have now demonstrated for the first time that several key autophagy genes, and their protein products, are differentially expressed in CD34+ subpopulations obtained at diagnosis from chronic phase CML patients who were retrospectively classified, after initiation of IM therapy, as IM-responders (n=14) and IM-nonresponders (n=14), as well as normal, healthy individuals (n=10). The cysteine protease ATG4B, a crucial enzyme of the autophagic process, was found to be the most highly expressed gene and protein in CD34+ hematopoietic stem/progenitor cells, with 110-fold higher expression than BECLIN-1. Interestingly, the transcript levels of ATG4 family members, ATG5 and BECLIN-1 were significantly increased in CD34+ CML cells compared to CD34+ normal bone marrow cells (p<0.01). Importantly, the transcript and protein levels of ATG4B were significantly higher in CD34+ CML cells from subsequent IM-nonresponders vs. IM-responders (P=0.014); this finding was further confirmed using a logistic regression model analysis (P<0.05). In addition, transcript levels of several

ATG genes, including ATG4B, were also higher in the stem-cell enriched CD34+CD38- population from IM-nonresponders than the same cells from IM-responders. Exposure to IM in vitro consistently elevated the transcript levels of ATG4B further, and induced autophagic flux in CD34+ CML cells. Strikingly, knockdown of ATG4B in IM-resistant K562 and CD34+ CML cells reduced the formation of colonies (CFC) compared to a scrambled control; CFC numbers were further decreased upon IM-treatment. Moreover, deregulated expression of ATG4B and ATG4D in CD34+ CML cells inversely correlated with transcript levels of miR-34a and miR-152, miRNAs predicted to target ATG4B and ATG4D, respectively. This study thus identifies ATG4B as a potential biomarker in treatment-naïve CML stem/progenitor cells that may be useful for predicting therapeutic response and indicates that ATG4B may be a potential drug target in CML stem cells.

DYNAMIC ASSOCIATION OF THE ULK1 COMPLEX WITH OMEGASOMES DURING AUTOPHAGY INDUCTION

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Induction of autophagy requires the ULK1 protein kinase complex and the Vps34 lipid kinase complex. PI3P synthesised by Vps34 accumulates in omegasomes, membrane extensions of the ER within which some autophagosomes form, whereas the ULK1 complex is thought to target autophagosomes independently of PI3P, and its functional relation to omegasomes is unclear. Here we show that the ULK1 complex colocalizes with omegasomes in a PI3P-dependent way. Live imaging of ULK1 complex, omegasomes and LC3 establishes and annotates for the first time a complete sequence of steps leading to autophagosome formation as follows: Upon starvation, ULK1 forms puncta associated with the ER and sporadically with mitochondria. If PI3P is available, these puncta become omegasomes. Subsequently, the ULK1 complex exits omegasomes and autophagosomes bud off. If PI3P is unavailable, ULK1 puncta are greatly reduced in number and duration. Atg13 (a component of the ULK1 complex) contains a region with affinity for acidic phospholipids, required for translocation to punctate structures and autophagy progression.

Aggregation of misfolded proteins and their clearance by autophagy; relevance to neuropsychiatric diseases

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Modified amyloid cascade hypothesis, states that toxic intracellular protein oligomers/ aggregates, cause damage to neurons, which leads to lower neuronal activity, impaired LTP and finally, to their death (thus neurodegeneration). Protein misfolding and subsequent aggregation is the leading cause of most known dementias. In certain dementias patients show profound changes in behaviour accompanied with psychiatric symptoms. On the basis of the observation that progressive myoclonic epilepsies (PMEs) and neurodegenerative diseases share common features of neurodegeneration, we previously proposed autophagy as such a possible common impairment (Polajnar & Žerovnik, 2011, Trends Mol. Med.). Here, we argue along similar lines for some neuropsychiatric diseases. In both depression and schizophrenia, (temporary) decline of memory and concentration are common. Finally, brain may physically shrink, thus qualifying to neurodegeneration. Leaving aside genetic causes (abnormalities in multiple genes, SNPs or their copy numbers), we firstly assume that psychological and environmental stress translates into cellular stress. Secondly, we propose that cellular stress in the form of ROS or general metabolic imbalance/ acidification as well as post-translational modifications (such as methylation or tyrosine residues oxidation) can cause proteins to misfold and aggregate; diminishing and finally overwhelming degradation and chaperone machineries. This could by the proposed toxic effect of oligomers on membranes lead to influx of Ca2+, culminating in common cell death signalling pathways. Wnt signalling and mTOR signalling pathways have been reported for the neuropsychiatric diseases. It is well known that inhibition of the mTOR pathway by rapamicine enhances autophagy. On the other side, some drugs such as carbamazepine, which are claimed to act as authopagy enhancers also positively influence mood, which could mean that enhancing autophagy is beneficial in such diseases. We are aware that no protein inclusions such as amyloid plaques in Alzheimer's disease have been shown for neuropsychiatric cases. The reason may be that they are temporarily. We drive implications from possible gain in toxic aggregates function to maybe prevent or augment treatment of depression and schizophrenia.

17-BETA-ESTRADIOL AND PROGESTERONE REGULATES AUTOPHAGY DURING FUNCTIONAL DEVELOPMENT OF ALVEOLAR STRUCTURES FORMED BY BOVINE MAMMARY EPITHELIAL CELLS CULTURED IN 3D SYSTEM

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Mammogenesis and all consecutive stages of mammary gland physiology are under strict control of sex steroids. From the early phase of formation of a rudimentary system of ducts during embryogenesis, to the expansion of the glandular epithelium occurring at puberty and final functional development during gestation, 17-beta estradiol (E2) and progesterone (P4) mediate their biological responses mainly by their specific receptors (ERα, PR). Their critical role in mammary gland development has unequivocally been confirmed. In general, ER signalling is essential for ductal morphogenesis, while PR signalling is critical for lobulo-alveolar development.We have previously reported that E2 and P4 also regulate the process of autophagy during acini development by bovine mammary epithelial cells (BME-UV1) cultured in three-dimensional (3D) system. Autophagy, observed in the centre of developing acini, occurs after polarization of cells is completed and precedes apoptosis, determining the proper development of mammary alveoli. In this study we used 3D culture model to elucidate the mechanism of autophagy regulation by E2 and P4 during formation of alveoli-like structures by bovine BME-UV1 mammary epithelial cells grown on extracellular matrix (ECM) components. Firstly, we investigated whether autophagy-related genes (Atg) are regulated by sex steroids. Using Western-blot and RT-PCR analyses of autophagic markers: Atg5, beclin1, Atg3, LC3, we demonstrated that steroid hormones E2 and P4 upregulate the expression of Atg5 and Atg3 genes, and this effect is greater after addition of both steroids together. Additionally, an increased level of cleaved, lapidated LC3 protein (LC3II) was observed in the presence of both sex steroids, although a more pronounced effect was evoked by E2. Secondly, we examined the possible nongenomic pathway by which E2 and P4 could regulate autophagy via membrane-bound receptors, causing stimulation of cytoplasmic signalling pathways such as MAPK and PI3K/Akt. These pathways however, constitute an important sensor in cell metabolism and could be also regulated in relation to nutrient and energy availability. Our results showed, that PI3K/Akt and MAPK pathways were activated in BME-UV1 mammary epithelial cells, but any additional effect of E2 and P4 treatment was not observed. Our observations confirmed that sex steroids 17-beta estradiol and progesterone play a major role

in mammary gland development, not only by regulating mammary epithelial cells proliferation, but also by enhancement of Atg genes expression, and induction of autophagy during the process of mammary alveoli formation.

CLEARANCE OF DYING RETINAL PIGMENT EPITHELIAL (RPE) CELLS BY PROFESSIONAL AND NON-PROFESSIONAL PHAGOCYTES AS *IN VITRO MODEL FOR AGE-RELATED MACULAR DEGENERATION (AMD)*

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Purpose: Retinal cells die throughout our lifetime by different cell death modalities including apoptosis, anoikis, autophagy and necrosis. Inefficient removal of the dying cells by professional and non-professional phagocytes can result in cellular debris formation and disturbed tissue homeostasis. We aimed to study the clearance of autophagic dying RPE cells by these phagocytes serving as a model for dry and wet type of AMD, respectively. Methods: Autophagic cell death was induced by serum deprivation and H_2O_2 co-treatment in ARPE-19 and primary human RPE (hRPE) cells. Annexin-V FITC/PI flow cytometric assay was used to determine the cell death rate, while autophagy detection was achieved by Western blot quantification of LC3 II/LC3 I ratio and p62 expression, transmission electron microscopy (TEM) and fluorescence microscopy of GFP-LC3 transfected RPE cells. The clearance of autophagic dying cells by non-professional (living ARPE-19/hRPE cells) and professional (human blood monocyte-derived macrophages) phagocytes were quantified using flow cytometry. Results: An increasing percentage of phosphatidylserine positive or dying RPE cells was observed in a time- and concentration dependent manner upon H_2O_2 treatment. Paralelly, an induction of autophagy could be detected within 2hrs of treatment with 1mM H_2O_2 using TEM, LC3/p62 expression and GFP-LC3 transfection assays. *In vitro* phagocytosis assays found that autophagic dying cells can be efficiently and increasingly engulfed by both professional and non-professional phagocytes over time. Conclusions: The clearance of autophagic dying ARPE-19 and hRPE cells can be used as *a* model for studying both dry and wet type of AMD *in vitro*, as well as for testing future pharmacological agents for treating this disease.

Stimulation of Autophagy as a Potential Treatment for Chondrodysplasia Caused by Collagen X Mutations

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Introduction: Metaphyseal chondrodysplasia type Schmid (MCDS) is a disorder resulting from mutations in type X collagen. Recent studies have revealed that ER stress plays a central role in the MCDS disease mechanism. Previous work on the Col10a1.pN617K mutation has shown that treatment with the autophagy stimulating drug Carbamazepine (CBZ) reduces ER stress levels in vitro. The aim of this study is to investigate whether CBZ is able to reduce ER stress levels produced by other known MCDS- causing collagen X mutations and also to clarify the mechanisms involved.Materials and methods:HeLa cells were transfected with one of four full length his-tagged human collagen X constructs expressing MCDS-causing mutations (N617K/Y598D/G618V/NC1 del 10) and treated in the presence or absence of CBZ (20 μM). RNA and protein was isolated 24 hours after transfection. The levels of ER stress markers BiP, CHOP and spliced XBP1 were determined by real time qPCR. Collagen X expression levels were monitored using an anti-his antibody. To determine the mechanism by which CBZ was acting, HeLa cells were transfected with the collagen X constructs and treated in the presence of absence of CBZ for 16 hours to allow for protein expression. After 16 hours lysosomal (autophagy) inhibitors (leupeptin 100 μM /pepstatin 1 μM) or a proteasomal inhibitor (PSII 10 μM) were added to cells for a further 8 hours. Protein was then extracted from cell layer and analysed by western blotting for collagen X protein.Results and Discussion: Treatment with CBZ caused a reduction in intracellular collagen X protein levels for all four mutations tested, accompanied by significant reductions in mRNA levels of BiP, CHOP and spliced XBP1, indicative of a reduced level of ER stress in the drug-treated cells. The CBZ-induced reduction in intracellular collagen X protein levels appeared to be entirely due to increases in proteasomal rather than autophagic degradation. CBZ is a known stimulator of autophagy and how this activity leads to increased proteasomal degradation of the mutant collagen X resulting in reduced ER stress requires further investigation.

Cross-talk between autophagy and apoptosis in long-term cultures of blood neutrophils

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Neutrophils, known as professional phagocytes, have the shortest half-life amongst leukocytes and are constitutively committed to apoptosis. Significant basal autophagy was shown in neutrophils and is generally considered as a protective mechanism. Moreover, autophagy and apoptosis may cooperate to modulate cell death or survival. Here we investigated whether autophagy is involved in neutrophil survival in long-term cultures. For this purpose, freshly isolated purified CD14-/CD15+/CD66b+/CD63+ blood neutrophils were followed up to 10 days in culture without growth factors using light, time-lapse and confocal microscopy. As expected, after 24 hrs the most of cultured neutrophils had typical apoptotic morphology (nuclear condensation, cell shrinkage) and were Annexin+/PI-. During the next 48-72 hrs, cellular debris (apoptotic bodies, nuclear materials including chromatin fibers) was note. Additionally large neutrophils with aberrant morphology (vacuolated cytoplasm and de-condensed nuclei) having a mixed apoptotic and autophagy phenotype (Annexin+/PI-/LC3B+) were observed. Thereafter they developed into Annexin-V negative giant cells with large vacuoles and engulfed cellular residues in various stages of disintegration. These cells display extended lifespan and vastly enlarged size, exhibited a neutrophilic phenotype (CD14-/ CD66b+/CD15+/CD63+) and were CD68+. Of note, they internalized latex beads, zymozan and oxLDL followed by increased Nox2 expression and ROS production. Lysosomes, LC3B redistribution into punctuated structures and LC3B-coated vacuoles were clearly visualized in these giant phagocytes by confocal microscopy. Furthermore, zymozan and oxLDL induced recruitment of LC3B to autophagosomes. Critically, inhibition at the early stages of autophagy by 3-methyladenine (3-MA) was found to suppress giant phagocytes development. We proposed that activation of the autophagy machinery in some apoptotic neutrophils may lead to cell adaptation and survival resulting in their transformation into long lived phagocytic giant cells. An intensive LC3B aggregation and accumulation during zymosan and oxLDL internalization may indicate on the involvement of an autophagy-related mechanism in phagocytosis and oxLDL uptakes that are critical features of atherogenesis.

EXAMINATION OF COMMON GENE TARGETS FOR β-CATENIN AND NFkB PROTEINS IN IMATINIB SENSITIVE AND RESISTANT K562 CELL LINES

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NFkB is a transcription factor which plays essential role in various cellular processes such as inflammation, immunity, cell proliferation and apoptosis. NFkB activity has been shown to increase in solid cancers, chronic myeloid leukemia and drug resistant leukemia. On the other hand, activation of Wnt/β-catenin pathway has also been shown to be important in progression of CML and acquired drug resistance in CML. As it is known that various transcription factors bind to their target genes to induce their expression, in this study we searched for common target genes for NFkB and β-catenin proteins. We performed chromatin immunoprecipitation (ChIP) assay in K562s (sensitive) and K562r (IMA resistant) cell lines for NFkB and β-catenin proteins. Then, we designed primers for iNOS and MDR genes and performed PCR assay with our immunoprecipitated product. Our results showed that both NFkB and β-catenin proteins bind to iNOS and MDR genes. In conclusion, existence of these common gene targets for NFkB and β-catenin proteins may be an indicator of their interactions in the promotor region.

EFFECT OF NILOTINIB ON INFLAMMATORY MARKERS IN LPS/IFNγ ACTIVATED RAW 264.7 MACROPHAGES

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Nilotinib is a novel tyrosine kinase inhibitor which is used for treatment of BCR–ABL-positive chronic myelogenous leukemia (CML). In earlier studies, Nilotinib has been shown to exhibit antifibrotic or antiinflammatory activities. RAW 264.7 macrophages produce various inflammatory mediators such as nitric oxide, IL-1β, IL-6 and TNF-alpha upon activation with LPS and IFN gamma. Therefore this stimulated macrophage cell line has widely been used as an inflammation model for development of anti-inflammatory agents. In this study, we examined the effect of Nilotinib on inflammatory markers in LPS/IFNγ activated RAW 264.7 macrophages. Griess reaction, MTT assay, RT-PCR and western blot were used to determine nitrite levels, cell viability, iNOS gene expression and NFkB protein expression respectively. It was found that nilotinib pretreatment of RAW 264.7 macrophages before stimulation inhibited nitrite production, iNOS gene expression and translocation of NFkB p65 protein from cytosol to nucleus dose dependently ($p<0.05$). These results show that Nilotinib exhibits antiinflammatory actions in an in vitro activated macrophage cell model and therefore may be beneficial for inflammatory diseases characterized with high NO production.

INTERACTION OF NFKB AND AKIRIN PROTEINS IN IMATINIB RESISTANT AND SENSITIVE K562 CELL LINES.

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Akirin is a highly conserved novel protein which is thought to play important role in immunity by acting in parallel with NFkB. It is thought that Akirin which is known to function in immune response pathway regulates the transcription of NFκB dependent genes critically by binding to NFκB via an undefined protein. NFkB activation plays important role in developing drug resistance in cancer and targeting this pathway may be beneficial for treating cancer and reversing resistance. Since imatinib resistant K562 cell line also exhibits high NFkB activity we chose this cell model to investigate the interactions between Akirin and NFκB proteins and examined if Akirin protein binds to NFκB protein in imatinib resistant and sensitive K562 cell lines. For this purpose, first cells were lysed and nuclear extracts were prepared. Then, co-immunoprecipitation (co-IP) was carried out in nuclear cell lysates according to the manufacturer's instructions. We determined that NFκB protein didn't show direct physical interaction with Akirin protein in both imatinib resistant and sensitive K562 cell lines. In conclusion, it is suggested that an adapter protein could be responsible for the binding of Akirin to NFkB.

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