Pulmonary Hypertension in Lung Disease: a Key Role for Gremlin

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Pulmonary hypertension (PH) is a common complication of many lung diseases. Chronic hypoxia is implicated as the principle stimulus for the development of PH. Recently our lab identified gremlin, a BMP antagonist, as being upregulated uniquely in the pulmonary endothelium in response to hypoxia. The BMPs play an important role in maintaining vessel homeostasis and upregulation of gremlin may block this signalling pathway.

The aim of this study was to further investigate the potential role of gremlin in hypoxic PH. First we confirmed gremlin upregulation in an *in vivo* mouse model of PH by RT-PCR and immunohistochemistry. RT-PCR analysis of the gremlin target proteins (BMP-2, -4 and -7) in hypoxic mouse lungs revealed upregulation of BMP-2. However, immunoblotting showed downregulation of protein in response to hypoxia. Wound healing assays on cultured human pulmonary microvascular endothelial cells (HMVEC) showed that treatment with BMP-2 induced wound healing and this was blocked when gremlin was added. Also BMP-2 induced downstream Smad1/5/8 phosphorylation and Id1 expression in these cells and this could be blocked by gremlin treatment. Immunoblotting of Smad1/5/8 phosphorylation in hypoxic mouse lung tissue showed a significant decrease in response to hypoxia indicating reduced BMP signalling. Finally, we investigated gremlin protein expression in lungs of human patients with PH and found it to be upregulated in these diseased lungs when compared with control patients.

Our findings confirm gremlin upregulation in human PH disease, thus identifying a novel protein that may play an important role in the development of PH, by blocking BMP signalling.

School of Medicine and Medical Science
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Vascular Biology

CDK-dependent phosphorylation of Bim during Taxol-induced cell death

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The mitochondrion plays an important regulatory role during caspase-dependent and caspase-independent cell death, through the release of apoptogenic proteins such as cytochrome C, Smac/Diablo, AIF, Omi/Htra and Endonuclease G from the intermembrane space. Mitochondrial release of apoptogenic proteins is regulated by the Bcl-2 protein family that is made up of both pro-apoptotic and anti-apoptotic members. Post-translational modification of Bcl-2 protein family members, such as phosphorylation and proteolytic cleavage, plays an important part in regulating their activity.[1]
The BH3-only pro-apoptotic family member, Bim, is phosphorylated by the Erk and JNK MAP kinases. Erk phosphorylates Bim resulting in proteasomal degradation of Bim.[2] The JNK MAP kinase phosphorylates Bim directly on serine and threonine residues resulting in its release from microtubules. Furthermore, JNK induces upregulation of Bim through the activation of the transcription factor, c-jun.[3,4]

It has been previously shown that chronic myeloid leukaemia (CML) cells undergo caspase-independent cell death following disruption of the microtubule network by microtubule targeting agents including Taxol. (1 and unpublished results) In this study it has been found that Bim resides in the mitochondria of CML cells. In addition, the two Bim isoforms, Bim EL and L, undergo phosphorylation following treatment with Taxol. Phosphorylation of Bim occurs in a time- and dose-dependent manner and precedes Taxol-induced cell death in CML cells. On further examination it has been found that phosphorylation of Bim EL occurs within 8 hours treatment with Taxol, whereas phosphorylation of Bim L does not occur until 12 hours after treatment. Synchronisation of K562 CML cells by double thymidine block and treatment with Taxol, has revealed that phosphorylation of Bim correlates with the accumulation of cells in G2/M. Pre-treatment of cells with the CDK inhibitors, Flavopiridol and Roscovitine, was found to block the phosphorylation of Bim EL and L upon Taxol treatment.

These results suggest that phosphorylation of Bim at the mitochondrion occurs during mitosis, which may represent an important event that connects cell cycle arrest to the cell death machinery following microtubule disruption.

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SUMO Protein Modification Promotes Glycolysis in Hypoxia

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In hypoxia, eukaryotic cells undergo a shift in metabolic strategy to decreased oxidative phosphorylation and increased glycolysis (the Pasteur Effect). The molecular mechanisms underlying this critical survival response remain poorly understood. Post-translational SUMO-1 and SUMO-2 modification of proteins regulates sub-cellular localization, stability and interactions with other proteins.

Exposure of Hela cells and SUMO-1 overexpressing cells to hypoxia resulted to a global increased in protein sumoylation. Using Tandem Affinity Purification-tagged-SUMO-1 (TAP-tag-SUMO-1) expressing Hela cells and mass spectrometry we identified hypoxia-dependent SUMO-1 modification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a central enzyme in glycolysis. GAPDH has a consensus site for SUMO-1-modificaton between residues 60 and 63 (VKAE) on the exposed surface of the protein in a structural configuration consistent known SUMO-modification sites. Furthermore, GAPDH has two SUMO binding motifs which facilitate interaction with other SUMO-1-modified proteins.

In vivo and In vitro assays confirm GAPDH as a target for SUMO-1 modification. Size exclusion chromatography using whole cells lysates from SUMO-1 overexpressing cells in hypoxia show that SUMO-1 and GAPDH exists in the same high molecular weight fraction. In hypoxia, GAPDH forms complexes, an event enhanced in cells overexpressing SUMO-1 leading us to hypothesize a role for sumoylation in spatial sub-cellular organization of GAPDH into complexes. Finally, overexpression of SUMO-1 and SUMO-2 leads to increased ATP generation and lactate production in hypoxia reflecting enhanced glycolytic capacity.

We propose that SUMO-1 and SUMO-2 protein modification represents a novel adaptive mechanism by which cells respond to hypoxic stress to enhance glycolysis.

A Functional Genomics Screen Identifies SerpinB2 as a Key Mediator of Breast Cancer Progression.

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Metastasis is the terminal event in carcinogenesis and a major contributor to mortality in patients with breast cancer. However, relatively little is known regarding the mechanism of this complex, multi-step process. Previously, we reported a bioinformatic reanalysis of a seminal transcriptomic study of breast cancer progression (O'Brien *et al.* 2007), where we identified a panel of genes associated with good/poor prognosis.

Here, the functional role of these genes in the metastatic process has been examined by RNA interference (RNAi), directed by short hairpin RNA (shRNA). To achieve this, we constructed a breast cancer metastasis-associated lentiviral shRNA library, consisting of 192 hairpins targeting 64 genes (3 hairpins/gene). The shRNA panel was used to generate cell lines exhibiting stable, RNAi-mediated suppression of metastasis-associated target genes. The metastatic/tumourigenic capacity of transduced cells was then evaluated using a range of *in vitro* assays aimed at determining the roles played by individual genes in the progression of breast cancer and dedifferentiation of breast cancer cell lines.

Knockdown of several genes in MDA-MB-231 breast cancer cells demonstrated phenotypic effects in our functional assays, for example alterations in would healing measured by scratch-wound assay, alterations in the ability of cells to form colonies in soft agar, resistance to anoikis, and changes in invasive capacity measured by Boyden chamber assay. However, the most significant effects were seen in cells displaying shRNA-mediated knockdown of SerpinB2. SerpinB2 (or PAI-2) is a key negative regulator of the urokinase plasminogen activator system, one of two central proteolytic systems (along with the matrix metalloprotease system) involved in remodelling of the extracellular matrix and the promotion of invasion/migration of cancer cells. Reduced serum levels of SerpinB2 have long been associated with poor outcome in breast cancer, however, the mechanism behind this remains unclarified. Here, we demonstrate, for the first time, the phenotypic effects of SerpinB2 loss in a well characterised model of breast cancer metastasis, and show that down-regulation of SerpinB2 induces an enhanced migratory phenotype and increased tumourigenic capacity.

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PI:Prof. William Gallagher
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Role of Rab5 in Agonist-Induced Internalization of the human Prostacyclin Receptor

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The human prostacyclin receptor (hIP) undergoes rapid agonist-induced internalization, albeit by a largely unknown mechanism(s) that involves clathrin and dynamin. The aim herein was to investigate the role of Rab5 in mediating Cicaprost-induced internalization of the hIP in human embryonic kidney (HEK) 293 cells. Over-expression of Rab5 significantly increased the level of agonist-induced hIP internalization. Additionally, the hIP co-localized to Rab5 containing endocytic vesicles in response to Cicaprost stimulation and, coincident with this, there was a net translocation of Rab5 from the cytosolic to membrane fraction.

Furthermore, co-immunoprecipitation studies confirmed a physical interaction between the hIP & Rab5. Whilst the dominant negative $Rab5^{S34N}$ did not fully impair hIP internalization nor show decreased interaction, it prevented relocalization of the hIP to endocytic vesicles. Moreover, the GTPase deficient $Rab5^{Q79L}$ significantly increased hIP internalization and co-localized with the hIP in enlarged endocytic vesicles. Taken together, data herein suggest that Rab5 mediates agonist-internalization & trafficking of the hIP. Deletion of the carboxyl terminal domain of the hIP did not inhibit agonist-internalization, Rab5 co-localization or co-immunoprecipitation; however, hIP trafficking was altered.

These data add to our understanding of hIP internalization and of the factors regulating prostacyclin signaling. **Supported by The Wellcome Trust & Science Foundation of Ireland.*

Enhanced intestinal delivery and stability both *in vivo* and *in vitro* of site specific PEGylation of salmon calcitonin to different molecular sizes of PolyPEG®

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Injected and nasal salmon calcitonin (sCT) is used for the treatment of osteoporosis and Paget's disease, but upon oral delivery it is subject to proteolytic attack by various intestinal peptidase enzymes and demonstrates poor intestinal epithelial permeability.

Here, we describe the successful approach and beneficial effects of site specific PEGylation of a PEGylated methacrylate polymer, PolyPEG® (Warwick Effect Polymer Ltd) *via* the N-terminal cysteine-1 of sCT and the effects of increasing molecular weight (MW) of the polymer on the pharmacokinetics and efficacy. The feasibility of the conjugates were evaluated in order to determine: (i) proteolytic resistances to endopeptidases, (ii) *in vitro* bioactivity on the breast cancer cell line T47D which over express calcitonin receptors and (iii) its *in vivo* pharmacokinetics and *in vivo* hypocalcemic efficacy in rats. Results show sCT-PolyPEG® significantly increased resistance to endopeptidase enzymes present in rat intestine, liver and serum. The bioactivities of sCT conjugated to different molecular sizes of PolyPEG®(6.5-40kDa) compared to free sCT were found to be preserved. It was also observed that there is a relationship between the molecular weight of the polymer conjugated to sCT, efficacy and potency. I.V.-administered sCT-PolyPEG® was measured by both total serum calcium and serum ELISA for sCT in rats. All sCT-PolyPEG® derivatives induced a decrease in plasma calcium levels. However, sCT-PolyPEG®$_{40kDa}$ significantly reduced plasma calcium by 30%, which was superior than all other formats. Conjugation of PolyPEG®$_{40kDa}$ also extended the half life of sCT *in vivo* compared to free sCT.

Our findings suggest that site-specific conjugation of sCT to an appropriate size PolyPEG® enhances its pharmacokinetic properties by increasing resistance to both proteolysis. These conjugates reduced systemic clearance of sCT without significantly reducing bioactivity *in vitro* or *in vivo* and they increased the half life of sCT *in vivo*.

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IL-1RI$^{-/-}$ mice are protected against obesity-induced insulin resistance.

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Obesity is associated with a complex systemic pro-inflammatory state implicated in the development of insulin resistance. Infiltration of macrophages into adipose tissue may be an important source of the inflammatory response associated with the development of insulin resistance, and a dysregulated macrophage response may be protective.

This study investigates links between obesity and the pro-inflammatory response using IL-1R1$^{-/-}$ mice, which have a compromised macrophage response. C57BL6 and IL-1RI$^{-/-}$ mice were fed a high-fat diet for 16 weeks. Subsequently, plasma metabolic markers and adipose tissue specific molecular markers of insulin resistance and inflammation were determined.

IL-1RI$^{-/-}$ mice fed a high-fat diet became obese but not insulin resistant, with significantly lower fasting plasma insulin, glucose, HOMA-IR, TNFα and IL-6 concentrations with elevated adiponectin levels, compared to obese C57BL/6 wild-type (WT) mice. Adipocyte GLUT4 and IRS-1 mRNA and protein expression were increased in the obese IL-1RI$^{-/-}$ mice. In contrast, adipose tissue TNFα,IL-6, F4/80, MCP-1, and MIP-1α mRNA levels were lower in obese IL-1RI$^{-/-}$ versus WT mice. Interestingly, adipose tissue phosphorylated (p) ERK, JNK and P38 MAP kinase were also significantly lower in obese IL-1RI$^{-/-}$ mice. Immunohistochemistry showed significantly less F4/80^{+} macrophages in the adipose tissue stroma vascular fraction of obese IL-1RI$^{-/-}$ versus WT mice. Complimentary cell culture analysis demonstrated that IL-1β significantly reduced 3T3-L1 adipocyte GLUT4 and IRS-1 but increased p-ERK expression, an effect which was impeded by IL-1R antagonist.

This study demonstrates a potential functional role for IL-1RI in obesity-induced insulin resistance.

Cyclophilin A is located at the centrosome and functions in the maintenance of genome stability

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Cyclophilin A (cypA) belongs to the evolutionarily conserved peptidyl-prolyl isomerase (PPIase) family which includes the structurally distinct FK-506 binding proteins, the parvulins, and the recently identified PTPA. PPIases are believed to play a role in protein folding and transport, however their true cellular function remains unclear.In recent years the parvulin, pin1, has been shown to regulate the activity of important cell cycle proteins and thus exhibit both tumour promoting and tumour suppressor activity, raising the possibility that pin1 can function as a tumour promoter or as a conditional tumour suppressor in a cell-type-selective manner [1].Recently cypA was found to be overexpressed in pancreatic and lung cancer cells suggesting a possible role during tumourigenisis [2], however its function during tumour development and progression is unknown. In this study we have found that cypA is overexpressed in cells derived from a number of solid tumours including breast, prostate and cervix, and haematopoietic malignancies such as chronic myeloid leukaemia and Jurkat T lymphoma.Investigation into a possible function of cypA has shown that it is localised at the centrosome in interphase haematopoietic cells. During mitosis, cypA concentrates at the spindle poles and migrates to the midbody during cytokinesis. Centrosomal localisation of cypA was confirmed by double staining of cells with anti-cypA and anti-γ-tubulin, which regulates microtubule function during mitosis. Merged images illustrate co-localisation of cypA and γ-tubulin suggesting a potential role for cypA during cell division. In support of this, it was found that leukaemia and lymphoma cells that do not express cypA undergo defective cell division and become multinucleated. Furthermore cypA-/- cells display a weakened mitotic spindle checkpoint in response to the microtubule-disrupting agents, taxol and nocodazole. Measurement of mitotic index has revealed that cells that lack cypA exit mitosis prematurely without completing cytokinesis and subsequently re-enter mitosis leading to the accumulation of cells with >4N. Collectively, this study strongly suggests a novel role of cypA in the maintenance of genome stability and provides valuable information that will help elucidate the precise role of PPIases during tumourigenesis.

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EFFECTS OF MILD HYPOXIC EXPOSURE IN RAT HIPPOCAMAL SLICES; ROLE FOR ADENOSINE A1 RECEPTORS IN THE MAINTENANCE OF LTP

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The majority of brain energy consumption is used by neurons to maintain ionic gradients that are important for conductivity and synaptic function. One of the earliest mechanisms of neuronal responsiveness to hypoxia and ischaemia is a depression of synaptic transmission, mainly triggered by an accumulation of extracellular adenosine induced by the collapse of ATP levels. It is well known that the neuronal protective role of adenosine released after hypoxia/ischemia is primarily achieved by inhibition of an excessive release of glutamate by activating adenosine$_1$ receptors (A_1R), although it has not been investigated if the mechanism may affect long term potentiation (LTP) induced after an hypoxic exposure. Here we report a depression of synaptic transmission in the Shaffer collateral pathway following mild hypoxia exposure (PO_2 37±2 mmHg), which may have a pre-synaptic component (PPF increased by 10.4±3.3% after 30 min in hypoxic exposure when compared to control slices; n=5 $p<0.05$) and is parallel to the drop of oxygen tension. A rapid recovery of synaptic transmission and stable LTP followed re-oxygenation after 120 min of hypoxia exposure. Inhibition of A_1Rs (DPCPX 0.2 μM) reversed the hypoxic induced synaptic depression (86.9±5.2% versus 31.8±11.8% controls; n=5, $p<0.05$) and reduced LTP after re-oxygenation (103.2±14.2 versus 143.7±8.0% in controls; n=5; $p<0.05$). The A_1R antagonist, DPCPX, also reduced the hypoxic dependent reduction of the pharmacological isolated NMDA field EPSP (61.8±2.5% versus 43.8±4.6% controls; n=5; $p<0.05$). These data together demonstrate a primary role for A_1R in reducing synaptic responses during hypoxia, which may involve presynaptic mechanisms. Inhibition of NMDAR activity through A_1R also supports the evidence of a neuroprotective role of adenosine after hypoxia/ischemia. It will also be important to investigate in more detail the mechanisms, which bring about the reduction of LTP observed in DPCPX/hypoxic slices.

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Investigation of EGF receptor signalling in dysfunctional 3D-mammary epithelial acinus assembly induced by JNK inhibition.

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Primary mouse mammary epithelial cells (PMMECs) cultured on an EHS-derived laminin-rich extracellular matrix (ECM) undergo a stereotypic morphogenetic programme of cellular remodelling events that culminates with the formation of polarised 3D hollow structures called acini. We have previously established that the inhibition of JNK with the specific low molecular weight activity blocker, SP600125 and shRNA, leads to formation of dysfunctional spheroid assemblies of surviving, non-polarised cells with packed lumina. We established that the effects of JNK inhibition on acinus formation can be nullified or reversed by EGF receptor-ERK MAP kinase pathway inhibition using an EGFR inhibitor suggesting that JNK activity is required to restrict proliferative and/or cell survival actions driven by EGFR-ERK signalling during normal acinus formation. Investigation of EGFR levels and activation state in response inhibition of JNK showed low expression of total EGFR protein along with high levels of auto-phosphorylation. This may indicate that sustained activation of the EGFR requires receptor internalisation and recycling. During normal acinus formation laminin in the supporting extracellular matrix causes activation of focal adhesion proteins including FAK, paxillin and Src kinase. Treatment with the JNK inhibitor blocks this activation. We have found that a Src kinase inhibitor, as seen with the JNK inhibitor, causes a decrease in the expression of total EGFR along with an increase in the phosphorylation at Try 1173. These results would suggest that JNK is working through Src to regulate EGFR phosphorylation, its activation state, turn-over and signalling capacity.

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Area: C) Mechanisms of Disease / Diabetes, vascular biology and cancer

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Increased Intestinal Permeability Induced by the Antimicrobial Peptide Melittin: *Ex Vivo* and *In Situ* studies

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One of the limiting factors in oral drug delivery is the physical barrier between the gastrointestinal lumen and bloodstream. By targeting the tight junctions that join the epithelium together, it is possible to increase the absorption across the epithelium of the small and large intestine. The antimicrobial peptide, melittin, has been shown previously to increase the absorption of marker compounds across cultured intestinal monolayers (1) and rat colonic mucosae (2) mounted in Ussing chambers. This study focused on the capacity of melittin to increase permeability across excised rat gastrointestinal mucosa as well as human colonic mucosa *ex vivo*. Melittin (10-50 μM) was shown to increase the permeability of two separate flux markers (mannitol and FD4) in rat (colon and ileum) and human colonic mucosa mounted in Ussing chambers over 120 minutes (Table 1). The concentrations of melittin required to increase fluxes across human colon did not attenuate the ion transport capacity of the tissue since carbachol-stimulated short-circuit current was unchanged, suggesting that function was not reduced. Perfusion of melittin through rat jejunum also led to a 2-fold increase in the flux of mannitol across the epithelium ($P< 0.01$). Furthermore, the peptide did not induce gross histopathological changes to the mucosae in any of the studies. The data presented in this study demonstrates that melittin is an effective enhancer of transmucosal permeability across a number of representative models of the intestinal epithelium.

Table 1

P_{app} *(cm/s)*	*Control Mannitol*	*Melittin Mannitol*	*Control FD-4*	*Melittin FD-4*
Human Colon	0.6 ± 0.1	1.8 ± 0.3 * [3]	n/a	n/a
Rat Colon	0.6 ± 0.1	3.2 ± 0.5 * [1]	8.1 ± 0.8	22.1 ± 6.1* [2]
Rat Ileum	n/a	n/a	9.8 ± 2.1	34.0 ± 4.8* [2]

Melittin concentrations were 10 μM (1), 20 μM (2) and 50 μM (3) (n= 3-6), $P<0.01$versus control fluxes.

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Rapid Characterization of Cancer Cells and Oncogenes with Phenotype MicroArrays

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Abstract:

Phenotypic characterization of mammalian cells is commonly limited by the need for engineered reporter genes, expensive target-specific reagents such as antibodies or oligonucleotides, cell lysis, and multiple readouts. Biolog has recently developed a novel 96-well plate-based platform (Phenotype MicroArraysTM) for rapid analysis of 1000+ metabolite utilization and chemosensitivity phenotypes employing a universal colorimetric readout that can be used with living cells. To exemplify the use of this approach for discovery research in oncology, we profiled two isogenic mouse embryonic fibroblast cell-lines that differed by the presence or absence of an oncogenic *ras*-gene using six of the twelve Phenotype MicroArray panels available from Biolog. Expression of the *ras* gene was associated with 16 loss-of-function phenotypes related to utilization of different metabolites (including pyruvate, various sugars, sugar phosphates, sugar methyl esters and glucosides) and 18 gain-of-function phenotypes related to an increase in resistance to various anti-cancer drugs or salts such as NaCl and CaCl2. Loss of pyruvate metabolism could be indicative of the Warburg effect, commonly seen in primary tumor cells, but the other metabolic differences are open to interpretation, as they do not appear to have been previously reported in the literature. The metabolic changes induced by *ras* and other oncogenes identified by using Phenotype MicroArrays could eventually lead to better design of selective screens for drugs that target cancer cells *in vivo*.

IRS-2 signalling during Kidney Fibrosis in Diabetic Nephropathy.

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(C) Mechanisms of Disease / Diabetes, vascular biology and cancer

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Insulin is a hormone which requires a family of adaptor molecules, called insulin receptor substrate (IRS) proteins, to transmit its signal within cells. Mice lacking IRS-2 develop type 2 diabetes and defects in organs such as the brain and retina. Our hypothesis is that mice lacking IRS-2 will display signalling defects in the kidney that contribute to diabetic nephropathy in vivo.

In vitro experiments to investigate the role of IRS-2 in TGF-β-mediated fibrosis in diabetic kidney disease were carried out. TGF-β stimulation causes cells to undergo epithelial-mesenchymal like changes leading to tubular dysfunction. siRNA was utilised to knockdown IRS-2 expression and the effect of TGF- β -induced EMT was examined. The FOXO family and FOXO regulated genes e.g. MnSOD and Bim, which are downstream of TGF- β and IRS-2 have been analysed. Stimulation of HK-2 cells by TGF- β caused a significant decrease in the expression of MnSOD and Bim.
Our in vivo strategy involved analysing pre and post diabetic time points for IRS-$2^{+/+}$, IRS-$2^{+/-}$ and IRS-$2^{-/-}$ C57Bl/6 mice. Male IRS-$2^{-/-}$ mice become spontaneously diabetic at 8-10 wks. We detected evidence of renal kidney damage with microalbuminuria and an increased albumin-creatinine-ratio in IRS-$2^{-/-}$ vs. wild-type mice. Significant alterations in the expression of genes which are implicated in TIF and EMT were also detected. Evidence suggests that downregulation of FOXO regulated genes occurs in the IRS-2-/- mice compared to wild-types.
Together these approaches will aim to elucidate a signalling pathway centering on IRS-2 in diabetic nephropathy.

Assessing cardiac function in mouse embryos using high frequency ultrasound.

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Aim: To assess the cardiac function of the mouse embryos using Doppler, m-mode and b-mode ultrasound functions. We assessed cardiac function of embryos using the myocardial performance index (MPI index), which indicates both systolic and diastolic function and is heart rate and ventricular morphology independent. Isovolumetric relaxation time (IVRT) and Isovolumetric contraction time (ICT) were calculated to assess diastolic and systolic function respectively. Percent fraction shortening (% FS) was also calculated to indicate systolic function of the left ventricle.

Methods: Using the high resolution ultrasound probe with a frequency of 55 MHz (Visualsonics, Toronto, CA) cardiac function of mouse embryos from E10.5 to E18.5 were assessed. C57Bl/6j normal pregnant mice were anaesthesised using isoflurane gas and following hair removal, an ultrasound probe was passed over the maternal abdomen and the cardiac function of the embryos close to the maternal skin were analysed throughout gestation. Mice were scanned on days E10.5, E12.5, E14.5, E16.5 and E18.5 at the same time of day. Doppler was used to measure embryonic cardiac IVRT, ICT and myocardial performance index. M-mode was used to evaluate embryonic left ventricular size and wall dimensions.

Results: Results are shown on table below; at each gestational age 32 embryos were examined.Fetal heart rate increased with gestational age, MPI and ICT values remained stable with gestation. Embryonic diastolic function (IVRT) appeared to decline as gestation progressed, perhaps due to an increasingly compliant ventricle with embryonic maturation.

	E10.5	E12.5	E14.5	E16.5	E18.5
Fetal Heart rate BPM	171 +/- 10	168 +/- 7	178.6+/- 11.6	179.5+/- 11	227.9 +/- 22.7
TEI index	0.73 +/- 0.1	0.84 +/- 0.2	0.89 +/- 0.1	0.69 +/- 0.1	0.72 +/- 0.1
IVRT ms	56.6 +/- 16.6	80.2 +/- 20.4	61.43 +/- 5.1 ms	43.70 +/- 9.5	35.65 +/- 6.9
ICT ms	44.4 +/- 10.2	56.7 +/- 6.0	37.75 +/- 4.4 ms	36.29 +/- 9.2	23.14 +/- 8.8
E/A	0.27 +/- 0.1	0.17 +/- 0.02	0.23 +/- 0.05	0.32 +/- 0.1	0.35 +/- 0.1
Left ventricle shortening fraction. %	-	85.1 +/- 0.5	62.9 +/- 3.3	48.29 +/- 0.4	57.73 +/- 8.9

Conclusion: There is little data available on the normal values of indices of cardiac function in mouse embryos. This technique of ultrasound investigation using a high frequency ultrasound probe provides clear images of high resolution (spatial resolution of 30 μm) of the developing embryonic heart. This normative data will provide a basis with which to compare mutant models of pathophysiological diseases.

POTENTIAL SELECTIVE ROLE FOR CXCR7/CXCL12 SIGNALLING IN THE LUNG IN RESPONSE TO HYPOXIC STRESS.

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PI: Professor Paul McLoughlin, Category C) Mechanisms of Disease / Diabetes, vascular biology and cancer

Chronic lung diseases impose a heavy burden of death and disability worldwide. Regional hypoxia in the lung occurs in virtually all patients with significant pulmonary disease. While classically thought of as a secondary event in disease progression, there is now an emerging consensus that vascular loss and damage can be an initiating event in certain disease situations. We therefore undertook to identify novel genes involved in pulmonary vascularisation.

Affymetrix gene chip experiments showed, for the first time, that a recently identified CXCL12 receptor, namely CXCR7, was upregulated in response to hypoxia in human lung endothelium. TaqMan analysis of a panel of tissues from mice housed in either normoxia or hypoxia (10%, 48hrs), showed a >2-fold increase in CXCR7 mRNA, only in lung tissue. Immunohistochemistry demonstrated that CXCR7 protein was also markedly increased in the hypoxic murine lung and in human lung disease *in vivo*. We also demonstrate that expression of the CXCR7 ligand, CXCL12 (a potent secreted pro-angiogenic chemokine) is uniquely high in the human pulmonary endothelium (>15 fold) when compared to the systemic endothelium. ELISA confirmed that CXCL12 protein is significantly elevated in the plasma of patients with chronic hypertensive lung disease.

These novel results suggest that signalling via the pro-angiogenic CXCR7/CXCL12 pathway is selectively expressed in the lung in response to hypoxic stress. Future studies will identify the potential involvement of this pathway in the development of increased pulmonary vascular resistance and pulmonary hypertension in patients with chronic lung disease and following exposure to high altitude.

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Novel Titanocene analogues induce apoptosis in prostate epithelial cells by initiating a DNA damage response

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PI/Research Group: RWG Watson
Thematic Area: Mechanisms of Disease/Diabetes, vascular biology and cancer

Introduction: Treatment options for locally advanced metastatic prostate cancer are extremely limited with Docetaxel (Taxotere®) being the standard chemotherapy but only providing a three month survival advantage. The objectives of this study are to investigate novel titanocene analogues as possible alternative chemotherapies for advanced disease. The primary aims are to investigate the apoptotic effects of these novel titanocene analogues on prostate cells and to examine their mechanisms of action.
Methods: PwR-1E and PC-3 cell lines were grown in optimal conditions and treated with titanocene analogues at different doses and times. Apoptosis and viability were assessed by propidium iodide staining and flow cytometry. Cellular uptake and DNA binding of Titanium was measured by atomic absorption spectroscopy. Alkaline single cell gel electrophoresis was carried out using the Trevigen CometAssay™ kit to assess DNA damage. To confirm a DNA damage response Replication Protein A (Ser 4/8) and p53 (ser 15) phosphorylation were assessed by western blotting. Knock-down of p53 was achieved by si-RNA and assessed by western blotting.
Results: PwR-1E and PC-3 cells undergo apoptosis in a dose dependent manner following treatment with a range of titanocene analogues as determined by PI DNA staining. In addition to inducing apoptosis, these compounds also have a long term effect on cell survival as demonstrated by the clonogenic assay. These compounds enter both cell lines and bind to DNA as confirmed by atomic absorption spectroscopy. These results confirm a correlation of increased Titanium-DNA binding and apoptotic responses. The differential apoptotic response between the PwR-1E and PC-3 cell lines correlates with the uptake of Titanium into the cells and consequently the level of DNA binding.
The titanocene compounds induce DNA damage in both cell lines as shown by the formation of 'comet tails' of DNA fragmentation upon single cell gel electrophoresis and the phosphorylation of Replication Protein A and p53. However induction of apoptosis by the titanocene compounds is not p53 dependent as demonstrated by knock-down of p53 by si-RNA in the PwR-1E cell line and no expression in the PC-3 cells.

Conclusion: These pre-clinical studies demonstrate for the first time that these novel titanocene analogues induce apoptosis in prostate cancer cell lines. Further evaluating the mechanism of action will indicate their appropriate clinical use in different stages of prostate cancer development.

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The effect of alleic deletion of Gremlin on vascular complications associated with a mouse model of type 1 diabetic nephropathy.

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Gremlin is a bone morphogenic protein antagonist and displays increased expression in models of renal fibrosis, including diabetic nephropathy (DN). Gremlin has been shown to promote vascular smooth muscle cell proliferation and migration, and has also been implicated in the pathogenesis of diabetic retinopathy. With this knowledge our aim was to investigate the role of Gremlin in the systemic complications of diabetes.

Our study investigated the role of Gremlin in DN in wild type (WT) and Gremlin heterozygote (GH) mice. The study was evaluated at three time points: 18, 27 and 33 weeks of hyperglycaemia. The first two time points were utilised to study the potential systemic effects of deletion of Gremlin in the diabetic state. At time of harvest the retina, posterior cup and aorta were isolated. Histological slides were prepared from the thoracic aorta and the intima-medial (IM) area was calculated. Retina and abdominal aorta specimens were used for extraction of RNA.

Results from lipid studies indicated a decrease in total cholesterol, HDL and LDL, and a rise in the triglyceride level in the WT and GH diabetic groups. GH mice displayed lower basal HDL, TG and total cholesterol compared to WT. Evaluation of the histological data indicated no obvious atheromatous plaque formation in either group. The increase in the IM area in the thoracic aorta of the GH diabetic group at 18 and 27 weeks was lower than that observed in the WT diabetic group. We have evaluated the expression of genes including Gremlin, fibronectin (FN), oncofetal fibronectin (OnfFN), embryonic form of myosin heavy chain and End 1.
Kidneys from our study exhibit altered gene expression as a result of diabetes and deletion of the Gremlin allele. We are currently studying the effect this state may have on another tissue susceptible to microvascular injury, the retina.
These results may indicate a potential role that Gremlin has in the pathological development of micro and macrovascular complications of type 1 diabetes.

RET variation in the aetiology of Vesicoureteric Reflux

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Vesicoureteric reflux (VUR) is the retrograde flow of urine from the bladder towards the kidneys and is a major cause of renal failure and hypertension. Primary VUR is a developmental anomaly of the vesicoureteric valves and commonly occurs along with other developmental anomalies of the urinary tract in the same individual or other members of the same family.

The cause of VUR is unknown but it often runs in families and may be inherited as an autosomal dominant in most cases. Some of the genes already known to be involved in urinary tract development are also involved in other developmental processes and therefore their mutation is liable to cause multiple anomalies and is unlikely to result in isolated VUR. *RET* is such a gene. Some mutations of *RET* result in multiple endocrine neoplasia, and others in Hirschsprung disease (defective intestinal innervation).

However, a group in Quebec found that a single nucleotide polymorphism (SNP) in *RET*, which changes an amino-acid (p.Gly691Ser), is greatly increased in VUR, with a heterozygote frequency of 69% as against 29% in the healthy Quebec population. We present the results of a study of this SNP in VUR patients and healthy controls in the Irish population and discuss the implications.

The search for genes involved in Vesicoureteric Reflux

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Mechanisms of Disease

Vesicoureteric reflux (VUR) is the retrograde flow of urine from the bladder towards the kidneys. It is common in young children and is a major cause of renal failure and hypertension, though the condition resolves in some as they grow.

Primary VUR is a developmental anomaly of the vesicoureteric valves and is part of a spectrum of developmental anomalies of the urinary tract. Though a few genes are known whose mutation causes VUR in addition to defects of other organs (such as renal-coloboma syndrome, and branchio-oto-renal syndrome), the cause of isolated VUR is unknown, but genetic studies so far suggest that it is highly genetically heterogeneous. A genome scan that we performed on 129 Irish families highlighted 10-15 regions of the genome that appeared to show linkage to the disorder, including 2 regions yielding non-parametric lod scores >2.5.

We investigated the genes and non-coding regulatory elements in these regions to develop a priority list of places in which to search for possible pathogenic mutations, and present the results of our search so far.

Characterisation and manipulation of chemotherapeutic resistance in hormone-refractory prostate cancer

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Thematic Area: Mechanisms of disease/diabetes, vascular biology and cancer

Introduction

Docetaxel (Taxotere®) is the most effective chemotherapeutic agent in metastatic hormone-refractory prostate cancer. However the development of acquired resistance to docetaxel often occurs and is a significant clinical problem, but the cause of this resistance remains unclear.

Objectives

To determine the gene changes which occur in the emergence of resistance to docetaxel in the progression of prostate cancer.

Methods

We generated two docetaxel-resistant prostate cancer cell lines by repeated exposure of PC-3 cells to docetaxel over a six month period, mimicking the emergence of resistance in a clinical setting. We then confirmed their resistance to docetaxel by assessing apoptosis. We performed low density arrays to identify candidate genes associated with docetaxel resistance. Gene expression changes were then validated at a protein level with western blotting.

Results

Gene expression from low density arrays demonstrated up-regulation and down-regulation of many genes involved in apoptosis, chemotherapy resistance, cell cycle and DNA repair. As a number of genes had changed significantly, we targeted pathways rather than individual genes, in an attempt to re-sensitize these cell lines to docetaxel. We identified the up-regulation of a number of genes regulated by NKκB. Inhibition of NFκB pathway resulted in a re-sensitisation of these resistant cells to apoptosis.

Conclusions

Understanding the molecular profile of the emerging docetaxel resistant phenotype will allow us to identify targets for therapeutic manipulation in hormone and chemoresistant metastatic prostate cancer.

An investigation into the function of the hereditary spastic paraplegia protein, spartin. Exploration of spartin interacting proteins

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Hereditary spastic paraplegia (HSP) is a general name for a cluster of inherited neurodegenerative disorders, which manifest the same clinical features: progressive spastic paralysis and weakness of the lower limbs. Troyer Syndrome is an autosomal recessive form of complicated HSP. It was lately discovered to be caused by a frameshift mutation (1110delA) in the novel SPG20 gene. The exact function of spartin remains unknown, however research suggest a potential role in endocytosis, vesicle trafficking or mitochondrial dysfunction. Previously it was revealed that spartin is targeted to mitochondria.

The aim of this project is to explore further the function of this protein and to characterise spartin-interacting proteins. An expression vector pCMV-TAP-SPG20 was constructed by subcloning full length human spartin protein coding sequence from pLP-ECFP-SPG20 plasmid into pCMV-TAP. The resulting vector posesses spartin cDNA in frame with N-terminal TAP-tag (Tandem Affinity Purification tag). HEK 293T cell line was transfected with pCMV-TAP-SPG20 plasmid. TAP-spartin protein and all prospective interactors were purified using Tandem Affinity Purification (TAP). The protein extracts were resolved on SDS-PAGE gels and appropriately prepared gel slices were analysed by mass-spectrometry. From the list of potential interactors 5 were picked for further analysis. Ongoing immunoprecipitation experiments will help to confirm particular inetractions.

Immunofluorescent staining of HEK 293T, pCMV-TAP-SPG20 and pCMV-TAP empty vector transfected cells confirmed information about spartin mitochondrial localisation. Western Blot analysis with anti-spartin rabbit IgG performed on high-purity mitochondria isolated from HEK 293T cells also revealed the presence of the spartin specific band.

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Applying Statistical feature selection to Proteomics and Metabolomic data in the identification of Biomarkers for Prostate Cancer presence and progression

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PI: Prof. William Watson. / Surgical group

Thematic Area: Mechanisms of Disease/ Diabetes, vascular biology and cancer.

Background and Hypothesis

Prostate cancer (PCa) is a highly prevalent disease in men and the second leading cause of male cancer deaths. The diagnostic biomarker PSA does not provide enough confidence in the routine screening for the presence of PCa. Panels of potential biomarkers in the serum and urine might well represent a more reliable tool for monitoring PCa that can lead to appropriate treatment strategies. The aims of this study are to identify panels of biomarkers for multiple diagnostic purposes by applying appropriate statistical approaches to serum Proteomics and Metabolomics data.

Methods

2-D DIGE and NMR were deployed to explore the Proteomics and Metabolomics changes in serum samples collected from cancer and BPH control patients as part of the Prostate Cancer Research Consortium BioResource. Two statistical feature selection methods were developed in the selection of potential biomarkers that aim to classify cancer and non-cancer patients, different Gleason grades and the presence of Extracapsular-Extension. The features lists were evaluated through cross-validation.

Results

The cross-validation results based on LDA, QDA, PCR and PLSR give comparable performance for different panels of biomarkers. The features selected from Stepwise t-test feature selection method are superior than coefficients based method.

Conclusion

Panels of potential biomarkers have been identified using different feature selection methods. These panels of potential biomarkers provide higher sensitivity and specificity for multiple diagnostic purposes. The identity of these protein spots and metabolites are under experimental investigation, which will provide further insights towards a better understanding of prostate cancer identification and treatment.

Evaluation of a novel signature panel of prostatic secretory proteins as a prognostic biomarker panel for prostate cancer

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Introduction & Objectives:

Prostate cancer (PCa) is a leading cause of morbidity and mortality in men. However, no single molecular biomarker capable of reliably predicting PCa stage or prognosis exists. Our proteomic discovery programme and systematic review revealed possible panels of proteins.

Aims are to correlate serum expression of VEGF-A, VEGF-D, IGF-I, IGFBP-3, PEDF and CD14 in the healthy state, Benign Prostatic Hyperplasia (BPH) and differing Pca grades.

Material & Methods:

BPH, Gleason Score (GS) 5 organ-confined (OC), and GS 7 non-OC PCa serum samples were obtained from the PCa Research Consortium. Age-matched healthy serum was received from the Tyrol PCa Screening Group. Protein levels were measured using commercial ELISA's.

Results:

Total PEDF and CD14 are under-expressed in the cancer groups, compared to controls (ANOVA $p<0.01$ and 0.05 respectively). Spearman's Method shows positive correlations (coefficient >0.5) between IGF-I/IGFBP-3 ($p<0.05$) and VEGF-D/Unbound PEDF ($p<0.01$) in controls (n=10). Within BPH patients (n=15) there is positive correlation between VEGF-D/IGF-I ($p<0.05$) and negative correlation between VEGF-A/IGFBP-3 ($p<0.01$) and VEGF-A /Total PEDF ($p<0.01$). Within GS5 cohort (n=14) there are positive correlations between VEGF-A/VEGF-D ($p<0.01$) and IGF-I/Unbound PEDF ($p<0.05$); and negative correlations between VEGF-A/CD14 ($p<0.05$) and VEGF-D/CD14 ($p<0.01$). There is a positive correlation between IGFBP-3/Unbound PEDF ($p<0.05$) in GS7 patients (n=12).

Conclusions:

Differential correlations between a signature panel of molecular biomarkers are evident. Statistical manipulation of these, combined with patient's clinical details may reveal a novel nomogram to aid in PCa stratification. This new prognostic tool would act as a pre-operative adjunct in the prediction of post Prostatectomy outcomes.

Gene–nutrient interactions in the metabolic syndrome: the role of adiponectin.

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The LIPGENE project (Diet, genomics and the metabolic syndrome: an integrated nutrition, agro-food, social and economic analysis) aims to understand gene–nutrient interactions. Specifically how dietary fat exposure might modulate associations between polymorphisms (SNPs) and risk factors of the metabolic syndrome (MetS), in a well characterised cohort of 486 patients with MetS.

Four SNPs within the adiponectin gene (ACDC: rs266729) and its 2 receptors (ADIPOR1: rs10753929, rs10920533 and ADIPOR2: rs6489323) showed significant associations ($P \leq 0.006$) with fasting insulin levels when levels of plasma saturated fatty acids (SFA) were taken into account. Levels of circulating adiponectin are reduced in MetS, and this adipokine may be an important regulator of insulin sensitivity and inflammation. The most informative of the SNPs were combined to look at the effect of carrying a multilocus genotype at baseline. The effect of genotype on responsiveness over the intervention period was also examined to determine the relationship between changes in levels in SFA and changes in fasting insulin.

This potential gene-nutrient interaction provides further evidence towards the emerging role of adiponectin in obesity and related disorders. It also suggests that 'personalised' nutrigenomic approaches that account for environmental and genetic risk may be applicable in disease management. The results demonstrate that certain genotypes may give rise to more modifiable phenotypes that are more sensitive to levels of SFA, whereby they modify insulin, a key biomarker of MetS. It is possible that such individuals might respond more positively to dietary interventions, reducing the need for drug treatment, or slowing disease progression.

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Quantitative automated Aperio image analysis of two key checkpoint proteins, MAD2 and BRCA1 in breast cancer.

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The checkpoint proteins, Mitotic Arrest deficient 2 (MAD2) and breast cancer susceptibility gene 1 (BRCA1) are crucial cell cycle checkpoints in the cell that ensure accurate chromosomal segregation during mitosis (McGrogan et al, 2007). Clinically, the roles played by these checkpoint proteins have implications for Taxol® resistance as correct functioning of the Spindle Assembly Checkpoint (SAC) is crucial for an appropriate response to anti-mitotics, such as Taxol. Indeed studies indicate that decreased mitotic checkpoint function lead to Taxol resistance (Sudo et al, 2004; Chabalier et al, 2006). Therefore, objective quantification of MAD2 and BRCA1 in formalin-fixed paraffin embedded (FFPE) material may predict patient response to treatment and lead to more patient-tailored treatment regimes. The objective of the current study was to develop an automated IHC quantification for MAD2 (BD Transduction Labs) and BRCA1 (Calbiochem) using the Aperio system, and to determine the manual vs. automated percentage positive staining of these proteins in a cohort of 39 primary breast cancers (defined as either Taxol responders or Taxol non-responders). An automated score was generated for each antibody by applying a specifically trained nuclear algorithm to selected annotated tumour areas within each tissue section and validating the automated score with a manual count of the same areas. Within this cohort of patients, four different staining patterns were observed for each antibody these were [1] Nuclear, [2] Nuclear and Cytoplasmic, [3] Perinuclear and [4] Negative. Good correlation between manual and automated scores was achieved. Interestingly, there were differing MAD2 staining patterns associated with Taxol responders and non-responders. Increased perinuclear and cytoplasmic localization of MAD2 was observed within the Taxol non-responder group. In contrast in the Taxol responder group, MAD2 expression was predominantly confined to the nuclear and occasional perinuclear localization with little or no cytoplasmic localization. The significance of these results remains to be elucidated on an extended tissue microarray breast cancer platform of Taxol chemoresponsive/ non-responsive cases. TMAs will be stained with MAD2 and BRCA1. The trained nuclear algorithm will be applied to generate data to help further define the significance of these checkpoint proteins in predicting response to Taxol therapy in breast cancer patients.

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SELECTIVE PRIMING OF NEUTROPHIL SURFACE ADHESION MOLECULE EXPRESSION IN OBSTRUCTIVE SLEEP APNOEA

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Mechanisms of Disease / Diabetes, vascular biology and cancer

Rationale: OSAS is a major independent risk factor for cardiovascular disease; yet the underlying mechanism(s) of this association remains unclear. We have previously observed that OSAS patients have elevated numbers of circulating neutrophils, thus leading us to hypothesise a role for the neutrophil in the pathobiology of OSAS. Neutrophil adhesion to endothelium is mediated by the surface adhesion molecules L-selectin and CD11b/CD18. The aim of this study was to assess neutrophil adhesion molecule expression/responsiveness in OSAS.

Methods: Neutrophils from 27 OSAS patients and 11 non-OSAS controls were directly assessed by Fluorescence-activated cell sorter (FACS) analysis for surface expression of L-selectin and CD11b before and after stimulation with interleukin 8 (IL-8) or f-Met-Leu-Phe (fMLP). Neutrophils were also isolated from normal subjects and the response to multiple cycles of intermittent hypoxia was examined in an *in vitro* model.

Results: Basal CD11b expression was lower in OSAS patients compared to controls ($p=0.0278$). Basal levels of L-selectin were comparable between both groups. Following stimulation with fMLP, expression of CD11b by neutrophils from OSAS patients was enhanced when compared with controls ($p=0.005$). There was no difference in response between groups following stimulation with IL-8 ($p=0.709$).

There was no difference in the response of isolated neutrophils to intermittent hypoxia in our in vitro model compared to normoxic and sustained hypoxic controls.

Conclusion: Neutrophils in OSAS are not activated but are primed. The selective neutrophil priming shown adds to the mounting evidence that OSAS promotes a systemic pro-inflammatory state which potentiates cardiovascular risk.

Differential Cell Adhesion within an Isogenic Model of Melanoma Progression Under Shear Flow Conditions Using a Microfluidic Cell-Based Assay

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(C) Mechanisms of Disease / Diabetes, vascular biology and cancer

Steps in the metastatic process include intravasation, survival in circulation, arrest in a specific organ, extravasation, growth and secondary tumour formation.

In this study, we modelled survival in circulation, adhesion to endothelial cell-derived proteins, as well as to endothelial cells, using Cellix's VenaFluxTM Platform, to determine if the steps in extravasation differed in an isogenic melanoma cell line model of progression. The isogenic cell line model series was comprised of the poorly tumourigenic parental cell line, WM793, and its derivatives, WM793-P1, WM793-P2 and 1205-Lu (the latter obtained from a spontaneous metastasis to the lung). We found that WM793, WM793-P1 and WM793-P2 cells did not adhere to VCAM, ICAM or fibronectin, whereas 1205-Lu cells adhered to VCAM under defined flow conditions. To examine this further, we applied a decreasing gradient shear stress of 5, 2, 1, 0.5, 0.25 and 0.1 dyne/cm2, which resulted in increasing adhesion of 1205-Lu cells to VCAM at shear stresses lower than 2 dyne/cm2. GFP-labelled WM793 and 1205-Lu cells were flowed over endothelial cells at defined shear stress; again, adhesion could only be observed in case of 1205-Lu.

The VenaFluxTM platform is a useful and reliable *in vitro* screening tool for key cellular phenotypes relevant to metastasis.

The role of macrophage accumulation in adipose tissue in the development of obesity-induced insulin resistance

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Macrophages are a heterogeneous population of cells that play a role in the innate immune response. Recent studies have shown that macrophages are key cells in the development of obesity, wherein there is progressive infiltration of macrophages into the adipose tissue. These adipose tissue macrophages (ATMs) are referred to as classically activated, or M1 macrophages [(1)]. They release pro-inflammatory cytokines creating an inflammatory response contributing to insulin resistance [(2)]. In lean individuals macrophages are predominantly in an M2-polarization or alternatively activated state. The IL-1 receptor type 1 (IL-1R1) is responsible for transmitting the pro-inflammatory effects of IL-1. The aim of this study was to determine the number and activation status of ATMs using flow cytometry in wild-type and IL-1R1$^{-/-}$ mice. C57BL/6 wild-type (WT) and IL-1R1$^{-/-}$ mice with a C57BL/6 background were fed a high-fat diet (45% EN fat) for 12 weeks. At weeks 0 and 6 epididymal adipose tissue (EAT) stromal vascular cells (SVC) were isolated, labelled with antibodies for macrophage markers F4/80, CD11B and CD11C and analysed by flow cytometry. F4/80^{+}CD11B^{+}CD11C^{+} are associated with M1, while F4/80^{+}CD11B^{+} indicate M2 macrophages [(3)].

During the development of obesity WT mice had significantly more EAT SVF F4/80^{+}CD11B^{+}CD11C^{+} cells at week 6 compared to the IL-1R1$^{-/-}$ mice ($P < 0.05$). Whilst this study is on-going these results imply that impairing IL-1 signalling decreases the macrophages ability to switch from an M2 to an M1-polarization state. This reduces the infiltration of M1 macrophages into the EAT, thereby lowering the pro-inflammatory response and attenuating the progression of insulin resistance.

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The Tumour Suppressor Maspin, and the Squamous Cell Carcinoma Marker SCCA-1 Bind the Glycosaminoglycan Heparin

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Abstract:
Serpins (serine protease inhibitors) are a superfamily of proteins exhibiting a diverse range of functions including plasma protease regulation, hormone transport and inhibition of angiogenesis. They possess a conserved tertiary structure, with a C-terminal loop (RCL) as the functional group required for protease inhibition. The glycosaminoglycan, heparin has been well documented as a cofactor for inhibitory activity of plasma serpins. Interestingly, the heparin-binding ability of antithrombin lll and kallistatin has recently been shown to be crucial for their anti-angiogenic activity.

In this study we investigated the ability of the cancer related serpins, maspin (serpin B5) and SCCA-1 (serpin B3) to bind heparin. Maspin is a tumour suppressor serpin downregulated in breast and prostate cancer, and is a known angiogenesis inhibitor. SCCA-1 was initially identified as a tumour marker protein in cervical carcinoma. It can inhibit lysosomal cathepsins and it has been shown to protect keratinocytes from UV-induced damage.

We have expressed maspin and SCCA-1 as His-tagged proteins in *E.coli* and purified them by immobilized metal ion affinity chromatography. We have shown that they bind to heparin agarose and that elution requires high concentrations of salt or addition of free heparin. Chemical modification of maspin and SCCA-1 by biotinylation greatly reduced their affinity for heparin, indicating the importance of lysine residues for heparin binding. We have shown that addition of heparin shifts the electromobility of maspin and SCCA-1 in a native PAGE gel. Finally, we have shown that only heparin and heparin sulphate, but not other glycosaminoglycans, can elute maspin and SCCA-1 from a heparin agarose affinity matrix. These results indicate that in spite of possessing a negative overall pI, these serpins appear to specifically bind heparin, and we propose that this interaction may be important for their physiological function.

TLR agonist induced changes in BRIN-BD11 clonal β-cell metabolism, signal transduction and insulin secretion

A. Kiely, P. Newsholme

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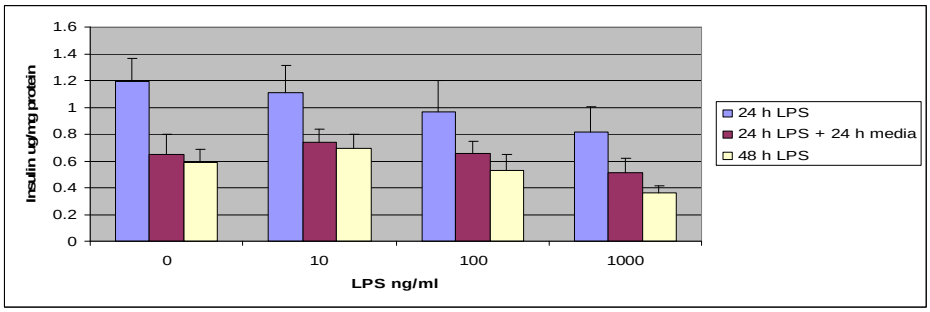
Insulin resistance associated with obesity and Type 2 diabetes is commonly associated with changes in muscle, adipose tissue and liver signal transduction pathways. Evidence for involvement of Toll like receptors in insulin resistance has been recently published. TLR4 and TLR2, whose agonists include lipopolysaccharides and saturated fatty acids, are implicated in altered patterns of signalling in animal models of insulin resistance and obesity. Indeed loss of function mutations in the TLR4 receptor had a beneficial effect on the insulin signalling pathways in adipose, muscle and liver tissues in mice and improved insulin action.

We have demonstrated a small but significant reduction in insulin secretion associated with 24hr exposure to the TLR4 agonist LPS, and an increase in IR□ expression.

We also found exposure to LPS resulted in a reduction in the level of activated Akt which could potentially impair the viability of the β-cell after longer periods of incubation. However, the concentrations of LPS used in this study did not impair cell viability during the period of our experiments.

Here's the graph again from earlier for chronic insulin release.



Phil, if you look at the graph, after 24 or 48 hours in the presence of 1000ng/ml LPS there is a significant decrease in chronic insulin secretion compared with control. After 24 hours in LPS followed by 24 hours in media alone, this decrease is no longer significant. However, the difference between the red bar and the yellow bar at 1000ng/ml is only p=0.07. I'm thinking recovery.

Metabolic effects of high levels of L-arginine on a clonal pancreatic β-cell line during a pro-inflammatory cytokine exposure.

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Thematic Area: (C) Mechanisms of Disease / Diabetes, vascular biology and cancer

We propose that L-arginine may act as a critical modulator of β-cell secretory and protective responses. BRIN-BD11 cells were incubated in the absence or presence of a non-lethal pro-inflammatory cytokine cocktail (Kiely, 2007) with 1.15 or 5mM L-arginine. After 1-hr and 24-hr incubation, cells and an aliquot of the media was removed and used for quantitation of insulin and metabolites (urea, nitrites, glucose, glutamate, glutamine, lactate and glutathione). After the 24-hr incubation, the cells were stimulated acutely (20min) with 16.7 mM glucose and 10 mM alanine, and insulin determined using an ELISA kit.

Cell viability was assessed by WST-1. Insulin secretion decreased with 5mM of L-arginine (2.4μg/mgProtein/24hr/1.5x10^5 from cells with 1.15mM arginine to 2.082.4μg/mgProtein/24hr/1.5x10^5 with 5mM). In the group treated with the cytokine cocktail for 24hr the subsequent acute stimulated, insulin secretion level was reduced but this effect was attenuated in the presence of 5mM of arginine (control: 0.09μg/mgProtein from cells incubated in 1.15mM arginine and 0.2μg/mgProtein from cells incubated in 5mM; cytokine treated group: 0.03μg/mgProtein from cells incubated in 1.15mM arginine and 0.28μg/mgProtein from cells incubated in 5mM). Production of GSH was higher in the 5mM groups (0.59μmol/mgProtein – control group; 0.29 – cytokine group) against 0.28μmol/mg protein and 0.15 – on the 1.15mM control and cytokine group, respectively. GSSG levels were: 0.16μmol/mgProtein – 1.15mM control group; 0.49 – 1.15mM cytokine group; against 0.021μmol/mgProtein – 5mM control group and 0.26 – 5mM cytokine group. No changes in cell viability were observed after 24hr.

We report that arginine demonstrated protective effects in chronic incubation conditions with cytokines.

Claudin-4 is an independent prognostic marker in breast cancer and may be associated with tamoxifen resistance

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The role of tight junctions in breast tissues is traditionally thought to be in maintaining polarity and barrier function. However, claudin-4, a tight junction protein, has been shown to be overexpressed in breast tumours, which generally lose polarity as they progress. Previous studies which have shown this looked at small cohorts, and had no information on clinicopathological characteristics or survival. We examined claudin-4 expression in a large cohort of invasive breast tumours with extensive clinical information in order to determine its usefulness as a prognostic and predictive biomarker in breast cancer.

We stained a breast tissue microarray containing 512 tumours, using a claudin-4 antibody. These tumours were scored both manually and using an automated algorithm. Claudin-4 expression was found to correlate with breast cancer specific survival (BCSS) ($p = 0.0029$) and recurrence-free survival (RFS) ($p = 0.0252$) in the entire cohort. Claudin-4 also correlated positively with grade and Her2, and negatively with ER. Analysis of ER positive patients alone revealed that claudin-4 was associated with BCSS in the tamoxifen-treated cohort, but not in the tamoxifen-untreated cohort. Multivariate analysis revealed that claudin-4 was a predictor of BCSS in the entire cohort (HR 2.00; 95% CI 1.04-3.87; $p = 0.039$) and also independently predicted BCSS in ER positive, tamoxifen treated patients (HR 4.01; 95% CI 1.43-11.29; $p = 0.008$).

These findings show that claudin-4 is a poor prognostic marker in breast cancer. This was verified in three independent datasets. Claudin-4 may also be a marker for tamoxifen resistance, as it is more significantly associated with poor prognosis in tamoxifen treated patients than untreated, even when adjusted for other clinical variables. Functional studies are currently underway in order to elucidate the role of Claudin-4 in breast cancer.

Breast Cancer Biomarkers: A Proteomic Approach to Biomarker Discovery

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Breast cancer is the most common cancer in women today. The mortality in this disease is primarily caused by metastasis to other organs and tissues. To address this issue, research has been driven toward finding biomarkers and mediators of progression to metastasis.

Here, we have performed 2D gel analysis coupled with mass spectrometry on a human isogenic cell-line model of breast tumour cell invasion. In this model, we use the Hs578T line and a derivative line (Hs578T I8) which has been created with increased propensity for invasion in vitro, as well as acquired tumourigenicity in vivo. The whole cell extracts and secretome of this model have been analysed using 2D-DIGE as well as a label-free relative quantitative MS approach. The gel and MS data analyses were performed with Progenesis Samespots and Spectrum Mill respectively. From the large number of differentially expressed proteins we filtered for candidatesthat would be more probable to be found in accessible body fluids such as serum.

This was achieved by cross referencing candidate markers with known plasma proteins as well as looking at predicted secreted proteins. Using these techniques, we have observed a cohort of differentially expressed proteins potentially linked to breast cancer progression and may have clinical utility as biomarkers. We are currently exploring the role of these factors in breast cancer in more detail.

Hypoxic-induction of Type I Transglutaminase in Primary Human Lung Small Airway Epithelial Cells: Potential Role in Barrier Function.

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Hypoxia is implicated in many lung diseases but the responses of primary human lung epithelial cells to hypoxia have not been fully examined. Identifying novel hypoxia effector genes will hopefully lead to a broader understanding of how hypoxia contributes to disease pathogenesis.

Type I transglutaminase was initially reported to be exclusively expressed in the skin. It is directly involved in the formation of the cornified cell envelop of terminally differentiated epidermis by cross-linking protein beneath plasma membranes. The expression of TGM1 in other tissues has not been greatly studied but has been reported in epithelial and endothelial cells, where it is suggested to play a role in maintaining cell junctions and barrier function. In this study, we examined the mechanism of TGM1 gene transcription in response to hypoxia.

Hypoxia-responsive genes were identified in primary human small airway epithelial cells using DNA microarray. Exposure to 3% oxygen for 24hr induced a 12 fold increase in TGM1 expression. TGM1 gene expression was confirmed using QPCR and showed significant induction as early as 6hr after exposure to hypoxia. We report that TGM1 mRNA and protein are expressed in human lung epithelial cells and conclude that hypoxia is a potent inducer of TGM1 expression. The hypoxia-responsive region was mapped to a HIF-1 responsive element within the promoter region of the TGM1 gene. Inhibition of HIF-1 using siRNA abolished hypoxia-induced promoter activation of the TGM1 gene. Ongoing studies are investigating the cellular significance of this regulation in human lung epithelial cells.

Theme: mechanisms of disease

From genome to interactome – proteomic investigation of LRRFIP1, a novel regulator of platelet function in health and disease

Iain C. Macaulay, Jyoti Khadake, Philippa Burns, Isabelle I. Salles, Marie N. O'Connor, Desmond J. Fitzgerald (PI), Willem H. Ouwehand (PI), Alison H. Goodall (PI), Patricia B. Maguire (PI) on behalf of the Bloodomics Consortium.

Platelets are anucleate fragments of their precursor cells, megakaryocytes, and play a critical role in hemostasis. In addition to this, platelets have a well characterized role in coronary artery disease and myocardial infarction (MI).

Recent genome wide association studies have identified novel association signals for MI. To search for additional genes implicated in MI, the Bloodomics consortium has used a systems biology approach based on analysis of human platelets. This approach identified three novel genetic associations with platelet function and MI, including a non-synonymous coding SNP in the *LRRFIP1* gene. However, little is known of the functional role the LRRFIP1 protein might play in platelets. To elucidate this role, we performed comprehensive proteomic analysis of LRRFIP1 co-immunoprecipitates (Co-IPs) to identify the LRRFIP1 interactome in resting and activated platelets.

Thirty-six unique LRRFIP1 interacting proteins were identified following stringent proteomic analysis of LRRFIP1 co-IPs, of which 15 were detected in IPs from both resting and activated platelets. The platelet LRRFIP1 interactome includes Flightless-I and Drebrin, for which the interaction was confirmed by IP/Western blotting. Functional classification of the interacting proteins, coupled with *in silico* expansion of the LRRFIP1 interactome using the IntAct database, strongly suggests a role for this protein in the regulation of the platelet cytoskeleton and signaling in platelet adhesion and thrombus formation. Indeed, morpholino based knock-down experiments in *Danio rerio* support this proposed functional role, suggesting a biologically plausible mechanism by which polymorphisms of the *LRRFIP1* gene may be associated with variations in platelet function and onset of disease.

Affiliated school SBBS

Molecular discrimination between hereditary and sporadic colorectal cancer.

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The familial cancer Hereditary Non-polyposis Colorectal Cancer (HNPCC) is characterised by microsatellite instability (MSI) in the tumour DNA. However approximately 15% of non-familial, sporadic colorectal cancers also display MSI. Differentiating these two groups is important in clinical practice. An oncogenic V600E hotspot mutation in BRAF, a kinase-encoding gene from the RAS/RAF/MAPK pathway has been reported to occur with high frequency in sporadic MSI colorectal cancers but never in HNPCC.

Method: Real-Time PCR is a highly sensitive and specific methodology for detecting DNA point mutations and it may be an alternative to direct sequencing, particularly for screening in a clinical setting. We investigated 28 sporadic MSI colorectal cancer cases and 16 HNPCC cases using DNA extracted from formalin-fixed paraffin-embedded tissue. For each case tumour and matching normal tissue was assessed. The V600E mutation of the *BRAF* gene was detected using ABI PRISM 7900HT Sequence Detection System. The PCR analyses were performed in the hybridization probe mode (Taqman).

Results: Of the 28 sporadic cases, 16 (57%) showed the BRAF mutation. None of the 16 HNPCC cases had the BRAF mutation. All the matching normal tissues from both sporadic and HNPCC cases were BRAF wild-type.
The finding of the BRAF mutation in a microsatellite unstable colorectal cancer would indicate that it is unlikely to be a familial cancer.This test assists discrimination of microsatellite unstable colorectal cancers into hereditary and sporadic groups which is important for clinical care.

Conway PI: Dermot Leahy.
Theme: Diabetes, vascular biology and cancer.

Hydrophobicity of the α-helical peptide, melittin, is a factor in both its cytotoxicity and intestinal permeating properties

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The oral delivery of biopharmaceutical drugs is restricted by the barrier function of the intestinal epithelium. These poorly absorbed drugs are often formulated as invasive injectables at greater inconvenience to the patient and, although considerable research effort has been placed on designing novel drug delivery systems, the oral route remains the Holy Grail in the delivery of these class III biopharmaceutical preparations. The use of absorption promoters is one approach that has been widely studied in order to increase the transmucosal flux of poorly absorbed drugs. However none have been licensed for use as enhancers. In previous studies we showed that melittin, a cationic amphipathic peptide, enhances the transmucosal flux of polar molecules across a number of *in vitro* (cell culture models), *ex vivo* (isolated rat and human colonic mucosae) and *in situ* (rat instillation) permeability models. Melittin forms channels in the plasma membrane of mammalian cells, which has been attributed to the peptides hydrophobicity. In the current study we examined the functional role of hydrophobicity in the peptide's ability to enhance the transmucosal flux of paracellular flux markers. Structural modification to melittin by specific amino acid replacement caused a reduction in hemolytic activity ($IC_{50\ (rat\ erythrocytes)}$ 0.8μM-to->20μM) and cytotoxicity against absorptive enterocytes ($IC_{50\ (Caco\text{-}2\ cells)}$ 2μM-to>20 μM). Reduction of the peptide's hydrophobicity by amino acid substitution significantly attenuated the drop in TEER and the transmucosal movement of a 4 kDa flux marker, FD-4, across Caco-2 monolayers induced by native melittin (Table 1). The data presented in this study provides useful SAR-based data for the design of a novel group of permeability enhancing peptides to increase oral bioavailability of biotech drugs with minimal local mucosal damage.

Melittin analogue	Permeability coefficient ($\times 10^{-7}$ cm/s)	Fold Increase
Control	0.4	-
Melittin (isolated)	9.1	20
Melittin (synthetic)	9.4	21
Analogue-1	0.2	0
Analogue-2	2.7	6
Analogue-3	0.64	1.5
Analogue-4	0.57	1.3

Table 1. FD4-flux across Caco-2 monolayers

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A predictive random forest based model of nephrotoxicity in HK-2 cells

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Thematic area of research: C) Mechanisms of Disease / Diabetes, vascular biology and cancer - PI: Dr. Peadar Ó 'Gaora

Abstract:

To design, develop and successfully bring a new drug onto the market is a time-consuming process requiring vast resources of time, personnel and money. A potential drug candidate may pass the early stages of drug development only to fail at the later stages of a clinical trial due to an unforeseen toxic effect. Therefore, the ability to detect potential toxicity at an earlier stage in drug development would be of great benefit. We investigated the changes in gene expression associated with renal toxicity using Affymetrix microarrays. The transcriptomic response of HK-2 cells exposed to 9 different toxic chemicals over a range of doses and times was measured. Unsupervised clustering of the resultant gene expression changes revealed three different gene signals associated with toxic exposure. A machine learning technique, Random Forests, identified 40 genes or potential biomarkers of renal toxicity in HK-2 cells based on the 3 different gene signals associated with toxic exposure. From the gene expression change of these 40 genes we built a random forest classifier which was capable of categorising known compounds from external sources with a high degree of accuracy (85-99%).

This study suggests that it is possible to predict nephrotoxicity of a potential new drug at an early stage of its development based on gene expression changes in cell culture.

Role of PKB/Akt activation and protein phosphorylation in TGFβ-induced Diabetic nephropathy

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Transforming growth factor-beta (TGFβ) is the primary cytokine driver of epithelial-mesenchymal transition (EMT) in kidney epithelial cells. In this project the activation of protein kinase B (PKB/Akt) in kidney epithelial cells in response to TGFβ stimulation was examined in order to elucidate the role of PKB/Akt in kidney fibrosis during diabetic nephropathy.

Time course study of TGFβ-induced PKB/Akt activation during EMT via Western blotting and in vitro kinase assays was examined and the effects of wild-type and mutant phosphatase and tensin homologue deleted on chromosome 10 (PTEN) was used as a tool to inhibit PKB/Akt activation. Human Kidney Cells (HK-2) were transfected with PTEN (wt) and mutant PTEN (C124S) as tools to inhibit PKB/Akt activation followed by TGFβ stimulation showing that TGFβ is able to increase phosphorylation through Ser473 site. Preliminary data using TaqMan assay has shown that TGFβ increases expression of E-Cadherin, one of the epithelial markers through PTEN and PTEN (C124S).

TGFβ-induced protein phosphorylation was detected by Western blot probing for phospho-tyrosine and phospho-serine protein residues time course stimulation in HK-2 cells and NRK52E renal epithelial cells. Similar phosphorylation effects were also observed when the experiment was performed using mesangial cells. Total phosphorylation analysis was performed using Phos-tag, detecting all phospho-peptides present. It was also shown that TGF-β induced Smad3 phosphorylation is not PI3-Kinase or PKB/Akt dependent process. These results demonstrate that PKB/Akt activity is important in TGFβ mediated EMT in kidney epithelial cells and inhibition of PKB/Akt isoform expression using siRNA constructs and epithelial cells lacking PKB/Akt on TGFβ-mediated EMT will bring us a step closer to determining the downstream phospho-protein targets of PKB/Akt that are involved in driving EMT during renal fibrosis in DN.

Epigenetic suppression of CTNNA3 and its nested gene LRRTM3 in Urothelial Carcinoma of the Bladder (UCB).

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Mechanisms of Disease / Diabetes, vascular biology and cancer

LRRTM3 is normally neuronally expressed and is located within the largest intron of CTNNA3 suggesting co-evolution of these two genes. LRRTM3 is entirely located within CTNNA3, but is transcribed in the opposite direction and is therefore a nested gene. Moreover, CTNNA3 is a developmentally imprinted gene, with preferential expression of the maternal allele, while LRRTM3 is not imprinted.

Taqman® QRTPCR employing the relative quantity method was used to determine the mRNA levels of CTNNA3 and LRRTM3 in a series of UCB cell lines (HT1376, RT4, T24, TCCSUP, RT112, CAL29). The demethyl transferase inhibitor 5-aza-2'deoxycytidine (DAC) was used for the chromatin modifying treatments of TCCSUP.

We demonstrate that CTNNA3 and LRRTM3 are co-ordinately expressed in these UCB cell lines. In TCCSUP, mRNA levels of CTNNA3 and LRRTM3 are minimal. However, following DAC treatment, CTNNA3 and LRTMM3 demonstrated increased mRNA expression by 4 and 7 fold respectively. Two CpG islands identified in the promoter region of CTNNA3 (*MethPrimer*) show no evidence of DNA methylation following sodium bisulphite modification and sequencing. Therefore, the increased expression of CTNNA3 following DAC treatment suggests indirect effects of this drug on this region such as the demethylation of transcription factors or transcription factor binding sites common to both genes.

Tissue-specific transcriptomic signatures to characterizethe effect of feeding a diet enriched with conjugated linoleic acid – adipose tissue, liver and skeletal muscle.

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School of Public Health and Population Science

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Conjugated linoleic acid (CLA) is a dietary fatty acid that is commonly found in dairy, beef and lamb, and has been shown to be a potent bioactive nutrient with anti-diabetic and anti-inflammatory properties (Moloney*et al.,* 2007). It is not clear, however, if CLAhas variable effects across the major insulin-responsive tissues, or if these anti-diabetic properties can be traced to a common global response.Using adipose, liver and muscle tissue fromgenetically obese and insulin resistantmice which were fed a diet rich in CLA, we used gene set enrichment analysis (GSEA) of microarray data to interpret gene expression changes in response to CLA supplementation. Results from our analyses indicatea modest gene expression response in adipose tissue (26 genes; $p<0.05$), despite dramatic expression changes in liver and muscle(1458 and 810 genes, respectively; $p<0.05$). The GSEA of gene sets representing metabolic pathways revealed 63 pathways that were affected across all tissues ($p<0.05$) but, interestingly, discordantpatterns of change between tissues. The magnitude and direction of changewas generally comparable in liver and muscle, however a number of key pathways thought to be linked to progression of the metabolic syndrome – such as MAPK and T-cell signaling (Crunkhorn*et al.,* 2007; Taleb*et al.,* 2007), and regulation of actincytoskeleton (Das &Rao, 2007) - were up-regulated in adipose, and down-regulated in liver and muscle. These results indicate that single-tissue analyses are insufficient, and perhaps misleading, as a means to draw global conclusions on the effects of CLA in insulin-responsive tissues.

MJM is a recipient of the IRSCET Postgraduate research scholarship. The project,funded by the Department of Agriculture & Food, Food Institutional Research Measure (FIRM), is entitled 'Nutrigenomic Technologies & Functional Food Development'.

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Assessment of glucokinase activator GKA50 on pancreatic beta cell function and viability in clonal beta cell line BR1N-BD11.

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Background: Central to the pathophysiology of type two diabetes mellitus is a failure of the pancreatic beta cells to secrete appropriate amounts of insulin. Therapeutic strategy for type 2 diabetes focuses on increasing the insulin secretory capacity of beta cells. The beta cell enzyme glucokinase has emerged as a promising target for a novel anti-diabetic agent. We have tested glucokinase activator GKA50 with respect to clonal beta cell (BRIN-BD11) metabolism, insulin secretion and cellular integrity.

Materials and Method: The metabolic and insulin secretory action of GKA50 was determined using basal (1.1mM) and stimulatory (16.7mM) glucose + alanine (10mM) conditions. Insulin secretion was determined by ELISA. The effects of GKA50 on cell viability was assessed using the WST-1 cell viability assay.

Results: Experiments have shown that micromolar and submicromolar concentrations of GKA50 are potent activators of glucokinase and increase glucose stimulated insulin secretion in BRIN-BD11 cells in a dose dependent manner becoming significant at 10μM GKA50 ($p<0.05$). Insulin secretion was further enhanced by the presence of 10mM alanine. No significant difference in viability was found between non GKA50 stimulated control cells and cells cultured with GKA50, even at elevated concentrations (100μM) of the drug.

Conclusion: Increased activation of glucokinase increases cellular metabolism and insulin secretion. Our results highlight the importance of glucokinase in metabolic regulation and stimulus secretion coupling in pancreatic beta cells. Glucokinase activator drugs offer the possibility of effective pharmacological control over glycaemia and may be a novel therapeutic approach to the treatment of type two diabetes mellitus.

Creation of a Digital Slide and Tissue Microarray Resource from a Multi-Institutional Predictive Toxicology Study in the Rat: An Initial Report from the PredTox Group

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Affiliated School: School of Biomolecular and Biomedical Science, **PI:** Prof. William Gallagher,
Thematic Area: (C) Mechanisms of Disease/Diabetes, vascular biology and cancer

Abstract

The widespread use of digital slides has only recently come to the fore with the development of high-throughput scanners and high performance viewing software. Here, the use of this technology in the creation of a comprehensive library of images of preclinical toxicological relevance is demonstrated. The images form part of the ongoing EU FP6 Integrated Project, Innovative Medicines for Europe (InnoMed). The primary aim of this project is to assess the value of combining data generated from 'omics technologies (proteomics, transcriptomics, metabolomics) with the results from more conventional toxicology methods, to facilitate more informed decision making in preclinical safety evaluation. A library of 1,709 scanned images was created of full-face sections of liver and kidney tissue specimens from male Wistar rats treated with 16 proprietary and reference compounds of known toxicity; additional biological materials from these treated animals were separately used to create 'omics data, that will ultimately be used to populate an integrated toxicological database. In respect to assessment of the digital slides, a web-enabled digital slide management system, Digital SlideServer™ (DSS), was employed to enable integration of the digital slide content into the 'omics database and to facilitate remote viewing by pathologists connected with the project. DSS also facilitated manual annotation of digital slides by the pathologists, specifically in relation to marking particular lesions of interest. Tissue microarrays (TMAs) were constructed from the specimens for the purpose of creating a repository of tissue from animals used in the study with a view to later-stage biomarker assessment. As the PredTox consortium itself aims to identify new biomarkers of toxicity, these TMAs will be a valuable means of validation.

The use of tissue microarray to validate if two protein biomarkers, CDCA7 and RFC4, associate with melanoma prognosis

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Background: Malignant melanoma is an aggressive disease, which displays increasing prevalence and is very difficult to predict. Early melanomas can be cured with surgical resection, however advanced lesions can prove difficult to treat and have very poor prognosis. Advances in "-omic" profiling have uncovered myriad putative genes associated with increased or decreased survival. However, these have not been extensively validated in a protein or in-vivo model. In this project, we utilized Tissue microarray (TMA) to evaluate two proteins of interest, namely CDC-A7 and RFC4. These two genes were found to be associated with 4 year patient survival via DNA microarray analysis on fresh frozen in a study performed by our collaborator, Winnepenninckx et al.
Aim: To use TMAs to validate the expression of two potential biomarkers, CDC-A7 and RFC4 via immunohistochemistry (IHC), and determine if expression correlates with clinical parameters and survival of melanoma patients.

Materials and Methods: For this study, a TMA using formalin fixed paraffin embedded melanoma samples from St. Vincents University Hospital was created. To create the TMA, each melanoma H&E slide was reviewed by a pathologist and the tumour areas were marked. Using a manual arrayer, 4x1mm cores were taken from the marked area, and placed into a new recipient block. In all, 230 malignant melanomas are represented on the final TMA. A database that included all clinical and survival information for each patient was also constructed. To check the TMA was constructed correctly, the TMA block was cut into 5µm sections and verified using H&E and s100 (a common marker for melanoma). Before IHC for the two proteins was performed, the specificity of the antibodies was checked by western blotting analysis and by IHC on cell line arrays.

Results: Single bands, of the correct size, for both CDC-A7 and RFC4 antibodies was detected by western blot, indicating the antibodies were specific for the proteins. In addition, both were optimized for IHC staining, followed by analysis on the melanoma TMA. Currently, these TMAs are being graded by two pathologists, and once the results are known, the statistically software program SPSS will be used to determine if expression correlates with clinical variables, disease free survival or overall survival.

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Elucidation of the role of Connective Tissue Growth Factor in diabetic kidney: *in vitro* and *in vivo* approaches

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PI: Dr. Derek Brazil, Category: C. Diabetes, vascular biology and cancer

Connective tissue growth factor (CTGF) has been implicated in the pathogenesis of diabetic nephropathy (DN) as both a driver of renal damage and a molecular marker of disease progression. The aim of this project was to evaluate the modalities of CTGF promoter activation using *in vitro* and *in vivo* models of DN.

Three cDNA fragments of the mouse CTGF promoter were isolated and sub-cloned into the pGL3 luciferase plasmid. All three promoter fragments were responsive to TGF-β1 stimulation. The largest of these promoter fragments (3024 bp) was then characterized and selected for transgenic mouse generation. To facilitate inducible gene expression, we selected the rtTA2s-M2/tTS^{kid} (IRES-M2) plasmid to construct our CTGF promoter mouse. The ability of CTGF to drive the tetracycline-dependent rtTA transcriptional element leading the activation of a reporter gene in *trans* was verified prior to transgenic mouse generation.

Following pronuclear microinjection of the CTGF promoter plasmid, one male founder was obtained. The CTGF promoter transgene was found to be transmitted in the germline to both male and female offspring. The novel line was crossed with a $(TetO)_7$-LacZ reporter line, and type I diabetes was induced by injection of streptozotocin. Mice were harvested at different time points and CTGF gene activation was followed in the kidneys using β-galactosidase staining as a measure of CTGF promoter activity.

CTGF levels will be correlated with standard indices of renal damage such as creatinine clearance and microalbuminuria. These experiments will provide novel insights into the temporal and spatial activation of CTGF in DN.

Adipocyte size is a better indicator of metabolic health than body mass index in severe obesity

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PI/Research Group: Prof Donal O'Shea, Obesity Research Group

Introduction:

A subgroup of obese individuals seems to be protected from insulin resistance (IR), diabetes, dyslipidaemia and non-alcoholic fatty liver disease (NAFLD).

It has been proposed that in the obese state, the problem may not be having too many adipocytes, but having too few adipocytes, which are all too full, leading to lipid deposition elsewhere which causes IR. Adipocyte size may be a key determinant of an individual's ability to accommodate excess calories, and so affect the severity of lipid and glucose 'overflow' and consequent IR.

Methods:

We have examined adipocyte size, clinical data and liver histology for 42 severely obese patients who have undergone bariatric surgery. Liver biopsies were assessed for degree of NAFLD as this may be considered an additional feature of the metabolic syndrome. From this group, 23 patients were separated into BMI-matched metabolically 'healthy' or 'unhealthy' groups, based upon standard cut-off points for blood pressure, lipid profile and fasting glucose.

Results:

Within this extremely obese population, adipocyte size, and not Body Mass Index (BMI), correlated with fasting insulin and HOMA ($r=0.903$, $p=0.0004$), and degree of NAFLD ($R^2=0.846$, $p=0.001$). Adipocyte size was significantly lower in the healthy group (77.74µm +/- 12.2) compared to the unhealthy group (99.97µm +/-8.2; $p<0.0001$).

Conclusion:

Adipocyte size is a better indicator of metabolic health than BMI in severely obese individuals and may be a key factor in the development of type 2 diabetes and NAFLD in this patient group.

Vascular Targeted Photodynamic Therapy with BF2-Azadipyrromethene (ADPM) Therapeutics: Mechanism Studies *In Vitro* & *In Vivo*

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Name of P.I.: Prof. William Gallagher, Thematic area: Mechanisms of Disease / Diabetes, vascular biology and cancer

Photodynamic therapy (PDT) is a treatment modality for a range of diseases including cancer. We have developed novel non-porphyrin PDT agents, BF2-tetraaryl-azadipyrromethenes (ADPMs), which display excellent photochemical and photophysical properties. *In vitro* mechanism studies have revealed that PDT using our lead candidate ADPM06 induces apoptosis through activation of caspase 3, 7 and PARP cleavage. Additionally, ADPM06- mediated PDT may elicit an anti-angiogenic effect as demonstrated using endothelial cell tube forming assays.

Critically, studies using dynamic PET and MRI have allowed us to explore ADPM06 mechanism of action of *in vivo*. We have postulated that ADPM06 is predominantly retained in tumour vasculature within the first few minutes following administration. Thus, using a short drug-light interval, we have sought to elicit a tumour vasculature-specific response. To test this hypothesis directly, dynamic PET with continuous I.V. infusion of 18F-FDG was performed over 4hrs. Following initial tracer uptake, rats bearing mammary carcinoma tumours were treated by parenteral administration of ADPM06, followed immediately by tumour illumination. Light treatment resulted in decreased 18F-FDG tumour uptake over time. Such behaviour is compatible with a vascular targeting response to therapy. The extent of inflammation, vascularisation and necrosis was monitored by MRI and compared with PET recovery data. Correlating transient metabolic effects during tumour PDT derived from real-time PET imaging and tumour response follow-up data from MRI provides a valuable tool to better characterise PDT mechanism of action in vivo.

Our data continue to show the ADPM family to be an exciting new class of therapeutic photosensitiser. Elucidation of drug mechanism of action may help to inform the design of future clinical trials.

Hypoxia Leaves its mark on the Epigenome

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Alterations in the tissue microenvironment of the aging prostate, such as increasing levels of hypoxia, impact normal prostate cells by inducing the expression of pro-survival proteins that may promote cancer development. Such changes may be attributable to the impact of hypoxia on the patterns of epigenetic modifications. The objective of this study was to measure global changes in epigenetic modifications in normal prostatic cells as a result of chronic hypoxia.

PWR-1E prostatic cells were grown under normoxic and hypoxic conditions (sublines derived following over 30 passages at 10%, 3% and 1% O_2). Flow cytometry and immunofluorescence were used to measure changes in epigenetic modifications using FITC- labelled anti-AcH3K9, anti-5'Methylcytidine and anti-pan Histone H3 antibodies.

Low density microarrays previously demonstrated significant alterations in the proliferation and apoptotic phenotype of the chronic hypoxic cultured PWR-1E sub-cells. Our results have identified concurrent disruption of global epigenetic profiles, which include a significant H3K9 hyperacetylation ($p=0.0001$) and DNA hypermethylation ($p=0.001$) relative to PWR-1E cells cultured in a normoxic environment. Interestingly, chronic hypoxia was also associated with an enlargement of the PWR-1E nuclei, suggesting that a genome-wide de-condensation of nuclear chromatin may occur as a result of hypoxia-induced hyperacetylation. Furthermore, treatment with Decitabine showed a significant reduction in global DNA methylation that closely resembled normoxic levels. Our current investigations are focused on determining if alterations in the activity of the epigenetic modifying enzymes HAT, DNMT1 and DNMT3b are responsible for hypoxia-induced epigenetic aberrations.

The identification of these hypoxia-induced epigenetic alterations may ultimately contribute to the initial development of prostate cancer and represent important targets for detection and manipulation in early stage disease.

Mathematical modelling of efflux of P-glycoprotein substrates across cultured human epithelial monolayers: sink versus non-sink conditions

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Apparent permeability coefficients (P_{app}) generated using Caco-2 monolayers grown on permeable filters enable reasonable prediction of passive flux of orally administered drugs in man. As a major intestinal efflux transporter, P-glycoprotein (P-gp) may significantly reduce the bioavailability of drugs with low solubility. P_{app} values determined *in vitro* also identifies P-gp substrates and inhibitors in cells expressing MDR1. The standard model used to determine P_{app} is based on Fick's law: $P_{app} = dQ/dt \times (1/(A.C_0))$.

It has been criticised for incorporated assumptions which may reduce the accuracy of the true P_{app}. In particular, if basolateral-to-apical flux is significant (10% in 2 hours), sink conditions on the apical receiver side may not be maintained, resulting in back-diffusion which can produce mathematical error (1). Therefore various mathematical models have been developed to more accurately determine P_{app}. The primary aim of this investigation was to compare the basolateral-to apical (BL-to-AP) P_{app} ($P_{app,BA}$) of rhodamine-123 (Rh-123), a well established P-gp *substrate*, determined using sink and non-sink conditions across human in vitro cultured intestinal epithelial Caco-2 monolayers and the use of mathematical modelling to reduce experimental error. Secondly, the IC_{50} of the potent anti-parastic agent and P-gp *inhibitor*, ivermectin (added bilaterally, concentration range: 0.01-1.0 μM), was determined using the Papp equation under sink and non-sink conditions for Rh-123 flux. Despite BL-to-AP fractional transport of ≥ 10% Rh-123 over a 2 hour period, indicating presence of non-sink conditions, the experimental P_{app}'s did not differ between sink and non-sink. Mathematical modelling corrected for certain assumptions, reduced data variability and shifted the data towards a common P_{app}.

Importantly, ivermectin's IC_{50} did not differ significantly between sink and non-sink conditions. Mathematical modelling may therefore improve the accuracy of P_{app} determination, at least for P-gp substrates. The use of non-sink conditions when ranking P-gp inhibitor potencies using Rh-123 is valid and accurate data can be calculated and modelled.

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Elevated BNP production indicates a fibrotic process in a hypertensive population

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Name of PI: John Baugh
Research area: (C) Mechanisms of Disease / Diabetes, vascular biology and cancer

Cardiac fibrosis contributes to the development of diastolic dysfunction (DD) in a hypertensive population. We have demonstrated previously that peripheral serum levels of certain markers of collagen turnover, including MMP-2, TIMP-1, PINP, CITP and PIIINP, correlate with the degree of myocardial fibrosis and DD. However in clinical practice these assays are not practical due to low specificity. B-type natriuretic peptide (BNP) has been associated with increased risk of diastolic heart failure and has also been correlated with the degree of DD on echocardiography. The aim of this study was to elucidate whether BNP levels correlate with biochemical markers of myocardial fibrosis in a hypertensive population with normal ejection fraction.

Coronary sinus (CS), aortic root (AR), and peripheral serum was collected from 41 hypertensive patients with normal systolic function. BNP levels were measured in all three serum sources. CS serum MMP-2 and TIMP-1 levels were quantified using ELISA. CS MMP-2 activity was also assessed using gelatin based zymography. CS serum PINP, CITP and PIIINP were quantified using radioimmunoassay.

The BNP value from the AR was subtracted from CS BNP to give the central production of BNP from the myocardium. This level correlated significantly with increased CS levels of MMP-2 ($r=0.43$, $p=0.007$), TIMP-1 ($r=0.33$, $p=0.045$), PINP ($r=0.50$ $p=0.003$), CITP ($r=0.46$, $p=0.005$) and PIIINP ($r=0.47$, $p=0.004$). Importantly, peripheral BNP levels also correlated significantly with the central levels of these markers.

BNP levels significantly correlate with central levels of markers of collagen turnover. These findings support the hypothesis that BNP is associated with increased myocardial collagen turnover in an at risk population. We thus propose that BNP is an attractive surrogate marker of myocardial fibrosis.

Exon junction based detection of alternative splicing in Mass Spectrometry data

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Alternative splicing is an intrinsic regulatory mechanism has been reported to occur in 75% of all human genes. Splice variants have also been found or implicated in various diseases including cancer. Isoform expression can be tissue specific or developmentally regulated. The detection of these variants will lead to a better understanding of the human genome and will possibly lead to the identification of disease specific isoform as these can be potential biomarkers of susceptibility to a disease, disease progression or response to treatment.

We have adopted a proteomics approach to the identification of exon skip events which are the most common form of alternative splicing. We have created a database representing all hypothetical cassette exon skip junction peptides which are the translated nucleotide sequences crossing an exon-exon boundary. This database houses 307,030 peptides that were created from 22,680 protein-coding genes from the human genome.

We have identified alternative splicing events in platelets by comparing a comprehensive set of mass spectrometry spectra of platelet proteins against the alternative splicing database. We are currently in the process of validating several splice events in genes which play a role in platelet biology.

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- Thematic research area: (C) Mechanisms of Disease / Diabetes, vascular biology and cancer

Cancer: a bridge too far

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Background: The spindle assembly checkpoint system ensures accurate chromosome segregation during mitosis. The mitotic arrest deficiency protein 2 (MAD2) is a key player in this process, maintaining the chromosomal stability.

A prerequisite of chromosomal instability is anaphase bridge formations which represent lagging chromosomes that cannot resolve following anaphase, contributing to genomic instability catastrophe.

Objective: The objective of this study was to elucidate if MAD2 expression influence cell cycle kinetics and the frequency of anaphase bridge formation.

Methods: MCF-7 cells were cultured in the presence and absence of 100 nM Paclitaxel for 24 hours. In addition a MAD2 knockdown MCF-7 line was created using a siRNA approach and similarly treated. MAD2 expression was determined by WB. Cell cycle kinetics were analyzed by flow cytometry. Anaphase bridges were visualized using haemotoxylin and percentages of bridges were expressed over total cell number. Cell senescence was assessed by ß-galactosidase staining and proliferation was measured by colony forming assay.

Results: The MAD2 knockdown line showed a complete reduction in MAD2 expression and displayed a 3 fold higher level of anaphase bridges compared to the untransfected and scrambled controls. Cells displaying high anaphase bridge formations correlated with a G2/M phase accumulation and there was also a significant increase of the percentage of polyploid cells. MAD2 depleted cells were growth arrested and a significantly high percentage of them were senescent. There was no effect on anaphase bridge numbers following paclitaxel treatment.

Conclusion: Here we report the first causative link between MAD2 expression and anaphase bridge formations which will ultimately enhance genomic instability.

Investigation into the protective role of phosphorylated hsp27 against atherosclerosis using 2D-DIGE

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Area of research: Mechanisms of Diseasae; Vascular biology

Vascular expression of hsp27, specifically diphosphorylated hsp27, is associated with protection against transplant atherosclerosis or cardiac allograft vasculopathy (CAV). Here we investigate whether hsp27 exerts a similar protective role in non-transplant atherosclerosis. Hsp27 expression was examined in coronary arteries from 7 patients with Ischaemic heart disease (IHD), 10 with dilated cardiomyopathy (DCM) and 7 controls (unused donors for heart transplant). Western blotting of total hsp27 demonstrated no significant difference between IHD, DCM and control. However, vessels from IHD had significantly lower phosphorylated hsp27 at Ser82, 78 and Ser15 ($p=0.02$, 0.008 and 0.004 respectively) compared to controls. No significant difference was observed in DCM. Immunohistochemistry of control vessels demonstrated that hsp27 was located within smooth muscle cells, and endothelial cells. However, the phosphorylated forms were predominantly located within the endothelial cells. Hsp27 is known to stabilise the actin cytoskeleton within cells and here it may help to stabilise the endothelial barrier.

To understand this protective role of hsp27, vessels were analysed by 2D DIGE to determine possible interacting or downstream effected proteins. Vessels from IHD, DCM and control patients were labelled with Cy5 and separated on 2D SDS PAGE. A pooled sample was labelled with Cy3 and used in each gel as a reference. Differential protein expression was determined by Progenesis Same spots and PG240. A total of 93 unique spots were found to be differentially expressed among the 3 groups with a p value of <0.05. These spots are being identified by MALDI TOF MS/MS.

Stereological Quantification of Intra-Acinar Vessel Length by Lumen Diameter Distribution

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Previous pulmonary hypertension studies frequently report changes in mean lumen diameter to account for changes in pulmonary vascular resistance. However, the resistance of the vascular bed is not a unique function of mean diameter. A given mean diameter may arise from a vascular bed composed of similarly sized vessels arranged in parallel or very large and very small vessels arranged in series, with these two arrangements having different effects on resistance. To address this, we report a technique for assessing the lumen diameter of intra-acinar blood vessels and the length of vessel in each diameter category.

The left lungs of rats (n=19) were isolated post mortem and fixed under standard airway and intra-vascular pressure conditions. A systematic random sampling strategy was employed to generate isotropic uniform random sections. Using an unbiased counting frame, vessels were randomly selected for measurement of diameter and calculation of length. The length of vessel in each specific diameter category was computed and vessel lumen volume calculated. To validate this method we independently assessed total vessel lumen volume using standard point-counting.

The mean lumen volume (±SD) calculated on the basis of vessel length and diameter ($0.0574\pm0.0098cm^3$) and point-counting ($0.0586\pm0.0117cm^3$) were not significantly different. The variances were also similar.

The finding of similar mean volumes and variances using the two approaches demonstrates the validity of the length-diameter approach, as both have similar bias and precision. The information gained on the length and pattern of blood vessel distribution may provide new insights into the pathogenesis of pulmonary hypertension.

Funding: Health Research Board Ireland and PRTLI
P.I.: Prof Paul McLoughlin
Category: (C) Mechanisms of Disease/Diabetes, Vascular Biology and Cancer

Effects of feeding high glycaemic meals in the last trimester of pregnancy on offspring birthweight and postnatal growth in sheep

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Experimental evidence indicates a direct relationship between maternal glucose levels and infant birth weight in humans. We investigated the effects of feeding hyperglycemic meals in the third trimester of pregnancy in the ewe on offspring health and development.

Ewes were assigned to one of two treatments: 100 ml of propylene glycol (PG) twice/day (n=50) or 100 ml of water twice/day (control(C)) (n= 52). On D 110 and D 140 of gestation blood samples were collected from a sub sample of ewes (n=12/group) to determine the ewe's glucose and insulin response propylene glycol or water. Lambs birthweight, body dimensions and a ponderal index was calculated as weight/height^3. Lambs were weighed every eight weeks from birth until slaughter at a live weight of about 40 kg. Administration of propylene glycol resulted in elevated plasma glucose and insulin concentrations for two hours post administration compared with control ewes. Lambs (C: n=80; PG: n=70) born to ewes fed high glycaemic meals had a higher birth weight (PG: 5.27 ± 0.11 kg; C: 5.01 kg ± 0.09 kg, p=0.032) and ponderal index (p=0.043), higher plasma glucose concentrations (PG: 3.88 ± 0.29 mM/l; C 2.87 ± 0.17 mM/l, p= 0.001) and reached a similar (p>0.05) slaughter weight (20.0-20.6 kg) at an earlier age (PG: 166.0 d ± 6.75; C: 183.4 d ± 7.02, p=0.039) when compared to controls.

In conclusion, feeding high glycaemic meals to ewes in the third trimester of pregnancy resulted in offspring with a heavier birth weight and faster growth rates in early postnatal life.

The effects of dietary fat modification on insulin sensitivity in subjects with the metabolic syndrome: insights from the LIPGENE Dietary Intervention Study. By **A.C. Tierney**[1], J. McMonagle[1], D.I. Shaw[2], J.A. Lovegrove[2], H. Lovdal Gulseth[3], C. Defoort[4], E. Blaak[5], J. López Miranda[6], A. Dembinska-Kiec[7] , B. Karlström[8], B. Vessby[8], W. Saris[5], D. Lairon[4], C.A. Drevon[3] and H.M. Roche[1], [1]*Nutrigenomics Research Group, UCD Conway Institute, University College Dublin, Dublin, Republic of Ireland,* [2]*Hugh Sinclair Unit of Human Nutrition, School of Food Biosciences, University of Reading, Reading UK,* [3]*Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway,* [4]*INSERM, 476, Nutrition Humaine et lipids, INRA, 1260, Université de la Méditerranée Aix-Marseille 2, Marseille, France,* [5]*Maastricht University, Maastricht, The Netherlands,* [6]*Lipid and Atherosclerosis Unit, School of Medicine, University of Cordoba, Cordoba, Spain,* [7]*Department of Clinical Biochemistry, Jagiellonian University Medical College, Krakow, Poland and* [8]*Department of Public Health & Caring Sciences/Clinical Nutrition and Metabolism, Uppsala University, Uppsala, Sweden*

Evidence suggests that dietary fatty acid composition affects insulin sensitivity. The LIPGENE Human Dietary Intervention Study is a multi-centre pan–European randomised controlled trial with the aim of determining the relative efficacy of reducing dietary SFA consumption, by altering the quality and reducing the quantity of dietary fat, on metabolic risk factors of the metabolic syndrome (MetS)

Free-living subjects (*n* 417) with the MetS received one of four dietary treatments for 12 weeks: high-fat (HF), saturated fat (SFA)-rich (HFSFA) diet; HF monounsaturated fat (MUFA)-rich (HFMUFA) diet; low-fat (LF) high-complex-carbohydrate (HCC; LFHCC) diet; LFHCC diet with 1.24 g long-chain *n*-3 PUFA/d (LF*n*-3PUFA). Dietary compliance pre-, mid- and post intervention was assessed from 3 d weighed food intakes. An intravenous glucose tolerance test (IVGTT), determined insulin sensitivity (S_I) and acute insulin response to glucose (AIRg) pre- and post intervention. HOMA-IR, a measure of insulin resistance (Matthews *et al.*, 1985) and quantitative insulin sensitivity check index (QUICKI), a measure of insulin sensitivity (Katz *et al.*, 2000) were measured as common surrogate measures.

Dietary fat modification had no significant effect on insulin sensitivity (S_I), or any of the other IVGTT variables, HOMA-insulin resistance or QUICKI. The effect of the four dietary treatments was determined in volunteers with a habitual high- or low-fat intake pre-intervention, defined as being above or below the median (36 % total energy from fat). S_I was significantly lower following the HFSFA diet (P=0.021) in subjects with a habitual low-fat intake pre-intervention. The sensitivity to SFA was more applicable to females for whom S_I was reduced following the HFSFA diet. HOMA-IR was also reduced in females following the HFMUFA diet (P<0.05). In females with an habitual high fat diet (>36% energy), AIRg improved following the HFMUFA diet (P<0.05).

The study has provided interesting data in relation to the effect of dietary fat modification on modifiable metabolic risk factors associated with S_I. The effectiveness of dietary fat modification has been shown to be dependent on pre-intervention dietary fat intake. Furthermore, altering dietary fat seems to be more effective in females.

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Generation of a Targeted Nanoparticle Inhibitor angiogenesis

Authors: ***Roy van der Meel***[1], *Raymond Schiffelers*[2], *William M. Gallagher*[1], *Annette T. Byrne*[1]

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Abstract

Angiogenesis is the sprouting of new blood vessels from pre-existing ones. It is now well accepted that tumor vascularization is critical for cancer progression. A growing body of evidence indicates the involvement of Rho GTPases in the angiogenic pathway. Rho GTPases regulate a large number of cellular functions[1]. Rac-1 is a Rho GTPase that plays an important role in angiogenesis[2]. We have therefore hypothesized that Rac-1 represents an attractive target for inhibition of tumor angiogenesis and growth, and have sought to employ a gene silencing (RNAi) approach to inhibit Rac-1 function using *in vitro* and *in vivo* models.

For proof of principle studies *in vitro*, we have used electroporation, facilitating siRNA transduction in endothelial cells. HUVECs were electroporated and silencing efficiency determined by Western Blotting. *In vitro* effects of Rac-1 knockdown on angiogenesis (tube formation assay), migration (scratch wound healing assay), invasion (Boyden Chamber assay), proliferation (MTT assay) and endothelial cell permeability (barrier function assay) were assessed. *In vivo* delivery strategies are further required to obtain efficient therapeutic application of siRNA in the target tissue[3]. Non-viral targeted nanoparticles are attractive systems to deliver siRNA into cells. One of these systems involves a self assembling cationic polymer conjugated with RGD coupled poly-ethylene-glycol. RGD targets integrins upregulated at sites of neovascularization[4].

Thus, we have attempted to deliver Rac-1 siRNA to tumour endothelial cells *in vivo* packaged in nanoparticles following parenteral injection. Early data suggests that Rac-1 represents an attractive new angiogenesis target. Studies are ongoing to fully determine this activity.

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Effects of prolonged exposure to high glucose on glucose metabolism and metabolomic profiles in pancreatic beta cells.

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Mechanisms of disease/diabetes

Long term exposure to high levels of glucose has been shown to have detrimental effects on β-cell function. The current study investigated the effects of high glucose levels on metabolic pathways to enhance our understanding of the mechanisms of glucotoxicity.

BRIN-BD11 cells were cultured under the following conditions for 20 h: (1) 5 mM (2) 11.1 mM or (3) 25 mM glucose. The cells were then incubated for 2h with 14 mM glucose and extracts prepared. ^{1}H and ^{13}C NMR spectra were acquired and analyses were performed on both targeted metabolites and untargeted global metabolomic profiles.

There was a significant decrease in glucose stimulated insulin secretion from 35.1 ± 1.2 to 29.5 ± 2.5 ng/mg protein ($P<0.03$) and a significant decrease in glucose uptake from 37.7 ± 4.9 to 32.6 ± 4.8 µmol/mg protein ($P<0.05$) in the high glucose treated group indicating mild glucotoxicity. Principle component analysis (PCA) of the ^{1}H NMR spectra showed a distinct separation between the high glucose treated group and the control group. Preliminary interrogation of the corresponding loadings plot identified alanine and aspartate as being decreased and GABA and glycine as being increased in the high glucose treated group. There was also a significant decrease in the amount of ^{13}C label at glutamate C4 from 33.8 to 27.7 nmol/mg protein ($P<0.05$) in the high glucose treated group indicating changes in fluxes through pyruvate carboxylase(PC) and pyruvate dehyrdogenase(PDH).

Results show that exposure to high glucose causes changes in metabolic flux into the TCA cycle and production of certain key amino acids. The novel combination of metabolic flux and metabolomic analyses in this study gives an enhanced understanding of the underlying mechanisms of glucotoxicity.

Elevated BNP is associated with exaggerated fibroproliferative responses in patients at risk of developing cardiac fibrosis and diastolic heart failure

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Cardiac fibrosis, characterised by increased fibroblast proliferation and collagen deposition, contributes to the development of diastolic dysfunction (DD) in a hypertensive population. B-type natriuretic peptide (BNP) has been associated with increased risk of diastolic heart failure and has also been correlated with the degree of DD on echocardiography

The aim of this study was to determine whether elevated serum BNP levels are associated with the expression of mitogenic factors and exaggerated fibroproliferative responses in patients that are at risk of developing DD.

Peripheral serum from 49 hypertensive patients was categorized according to BNP levels; low BNP<100pg/ml (n=30), high BNP≥100pg/ml (n=19). Extending from this population coronary sinus serum was also taken from 33 hypertensive patients and categorized as high BNP (n=7) and low BNP (n=26). Serum deprived normal human primary cardiac fibroblasts were incubated with 2% patient serum for 120 hours. Proliferation was quantified using CellTitre-Glo assay. Serum levels of the mitogens aldosterone and TGFβ were quantified using ELISA.

Serum from hypertensive patients with an elevated BNP induces increased cardiac fibroblast proliferation when compared to serum from patients with a normal BNP reading. BNP also significantly correlated with fibroblast proliferation level ($p<0.01$; $r=0.54$). There was no significant difference in quantified serum levels of aldosterone or TGFβ between the high and low BNP groups. Further work is required to identify the mitogenic factors within the serum of this at risk population.

THE PROBLEM OF RESISTANCE TO THROMBOLYTIC THERAPY FOLLOWING ACUTE MYOCARDIAL INFARCTION – A PROTEOMIC APPROACH

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RATIONALE: Thrombolytic therapy (TT) following acute myocardial infarctions (AMI) in only effective in approximately 70 % of patients. Understanding of the mechanisms of failed thrombolysis may allow development of novel pharmacological lysis facilitating strategies, both as primary therapy for AMI and as part of a facilitating strategy prior to primary angioplasty.

METHODOLOGY: We have undertaken a preliminary proteomic analysis of plasma from 5 patients presenting with AMI who underwent primary angioplasty, and were presumed to be TT-sensitive ('responders'), and 5 patients who failed to reperfuse with TT and underwent "rescue" angioplasty ('non-responders'). Plasma samples were depleted of albumin and immunoglobulin G, and proteins were labeled using the Differential in Gel Electrophoresis technique. This approach utilizes CyDyes and permits standardization for quantification of protein levels across a series of gels through the use of a 'pooled' sample created from all samples in the experimental set. RESULTS: Approximately 250 protein spots were selected for analysis and a total of 16 proteins were found to display statistically significant differential abundances between the two groups – 9 proteins increased and 7 decreased in 'non-responders' samples compared to 'responders', respectively.

CONCLUSIONS: Several proteins (including fibrinogen, clusterin, and alpha 1-antichymotrypsin) have been tentatively identified and may have biological relevance. For example, there is growing evidence to suggest that different isoforms of fibrinogen may modulate the thrombolytic response, while less clusterin in plasma from 'non-responder' patients suggests a role for complement mediated cytolysis. Identifications are being confirmed empirically using mass spectrometry.

Investigation of the Absorption Enhancing Effect of Ethanol in Rats

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Thematic area: (C) Mechanisms of disease

When taken in moderation, alcohol is known to reduce the incidence of coronary heart disease. High concentrations and chronic intake can cause morphological and functional damage of the gastrointestinal (GI) mucosa and also lead to liver disease. One of the major functions of GI epithelium is to provide a barrier to keep internal tissue from damaging agents. Tight junctions (TJs) play an important role in this. The disruption of TJs allows an increase in paracellular penetration, which can potentially cause mucosal injury and also may promote permeability increases.

The aim of this study was to use a rat *in vivo* single-pass perfusion model to investigate whether low levels (≤ 10%) ethanol can result in promotion of the paracellular permeation of mannitol across rat jejunum. We also confirmed the possible involvement of the metabolic energy in EtOH effects by comparing the apparent permeability of mannitol in the presence and absence of 3 mM sodium azide, an agent that reduces ATP levels. The potential toxicity in tissue was evaluated by lactate dehydrogenase (LDH) assay. Results showed that 1). EtOH perfusate at concentrations of 7.5% and above significantly increased the permeability of [^{14}C]-mannitol in the upper rat jejunum; 2). In terms of LDH release, at 7.5 and 10 %, there was no significant difference compared to vehicle-treated groups; 3). Metabolic inhibitor sodium azide prevented the EtOH-induced enhancement of mannitol permeability.

These results suggest that 7.5 and 10 % EtOH is relatively non-cytotoxic to the intestine following a single short term exposure, but at the same time paracellular permeability was increased and this may require energy. 40% EtOH boosted permeability but was cytotoxic. Concentrations of 7-10% EtOH may be present on a repeated basis in the intestines of alcohol drinkers.

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