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RNA Bath 2019

Friday 14th June 2019
Lecture Theatre 3WN 2.1.
University of Bath, Claverton Down,
Bath, BA2 7AY

Keynote Speaker:
Prof Nicola Gray, University of Edinburgh
'RNA-binding protein dysfunction: phenotypes and mechanisms'

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	TIME	SPEAKER	INSTITUTION	TALK TITLE
Welcome and Introduction: Michael Ladomery & Sebastian Oltean	9.55-10.00			
Session 1 Chair: Mark Lindsay	10.00-10.15	Tareg Belali	University of the West of England	WT1 activates the transcription of SRPK1 in cancer cell lines
	10.15-10.30	Arinzechukwu Ude	University of the West of England	Role of Exosomes in Chemotherapy-induced Bystander Effect
	10.30-10.45	Danny Legge	University of Bristol	Epigenetic inactivation of the epithelial splicing regulator ESRP2 in Wilms' tumour
	10.45-11.00	Jean-Michel Carter	University of Bath	FICC-Seq: A method for enzyme-specified profiling of uridine modification in cellular RNA
	11.00-11.20	Jon Rock	Lexicon	The QuantSeq product family: The power of 3' sequencing. Why less is more!
Tea/Coffee	11.20-11.40			
Session 2 Chair: Lorna Harries	11.40-11.55	Benjamin Lee	University of Exeter	Dietary restriction in ILSXISS mice is associated with widespread changes in splicing regulatory factor expression levels
	11.55-12.10	Keith Vance	University of Bath	The MITF-SOX10 regulated long non-coding RNA DIRC3 is a melanoma tumour suppressor
	12.10-12.25	Emma Denham	University of Bath	A stand-alone sponge RNA regulates the levels, processing and efficacy of the key Gram-positive riboregulator of central metabolism RoxS

	12.25-12.40	Ben Jenkins	University of Exeter	An RNA-interference dependent 'physiological cost' to host fitness for endosymbiont elimination in a nascent phototrophic endosymbiosis
Lunch	12.40-13.30			
Session 3 **KEYNOTE LECTURE** Chair: Sebastian Oltean	13.30-14.30	Nicola Gray	University of Edinburgh	RNA-binding protein dysfunction: phenotypes and mechanisms
	14.30-.14.45	Ian Blagbrough	University of Bath	Non-viral delivery of siRNA by lipopolyamines achieving quantitative gene silencing
	14.45-15.00	Susanne Wijesinghe	University of Birmingham	Joint inflammation in obese patients with osteoarthritis: The role of long non-coding RNAs
	15.00-15.15	Nicola Jeffery	University of Exeter	Cellular stressors may alter islet hormone cell proportions by moderation of alternative splicing patterns
	15.15-15.30	Lisa Hobson	University of the West of England	Targeting the ERG Oncogene with Splice-switching Oligonucleotides in Prostate Cancer
Tea/Coffee	15.30-16.00			
Session 4 Chair: Michael Ladomery	16.00-16.15	Francesca Carlisle	University of Exeter	CDK11A and CDK11B: the significance of small differences
	16.15-16.30	Swati Mahapatra	University of Bath	Highly Conserved Protein SpoVG Binds RNA in vivo and has Pleiotropic Functions in Bacillus subtilis
	16.30-16.45	Shahna Haque	University of Exeter	Differential expression of circRNAs with ageing

	16.45-17.00	Pamela Fernandez	University of Exeter	Alternative splicing of the apoptosis gene Bcl-x in the diabetic glomerular endothelium
Concluding remarks Michael Ladomery & Sebastian Oltean	17.00-17.10			

WT1 activates the transcription of SRPK1 in cancer cell lines

Tareg Belali[1*], Stefan Roberts[2], and Michael R. Ladomery[1].

[1]Faculty of Health and Applied Sciences, University of the West of England, Coldharbour Lane, Bristol BS16 1QY, United Kingdom
[2]Biomedical Sciences Building, Bristol University, University Walk, Clifton, Bristol, United Kingdom

[*]Tareg.Belali@uwe.ac.uk; Michael.Ladomery@uwe.ac.uk

The SRPK1 gene encodes a protein kinase that phosphorylates SR protein splice factors modifying their ability to regulate alternative pre-mRNA splicing. SRPK1 expression is elevated in cancer[1]. We previously showed that WT1 represses SRPK1 transcription in glomerular podocytes[2]. However whether or not WT1 regulates SRPK1 transcription in cancer cells is not known.

We transfected PC3 prostate cancer and K562 leukemic cell lines with WT1 and BASP1-expressing plasmids; BASP1 is a WT1-interacting co-suppressor[3]. The effect of WT1 on SRPK1 transcription was measured using a Luciferase reporter[2]. SRPK1 transcription was activated by WT1, but not when the WT1 binding site was mutated. This was not the case in BASP1-expressing K562 cells or in PC3 cells transfected with a BASP1-expressing vector. Therefore BASP1 turns WT1 into a transcriptional repressor of SRPK1.

The fact that WT1 can both activate (in prostate cancer and leukemic cell lines) and repress (in podocytes) the same target gene (SRPK1) is not a surprise, as the effect of WT1 on its target genes depends on the co-expression of transcriptional co-factors such as BASP1[4]. We suggest that WT1 contributes to the transcriptional activation of SRPK1 in cancer cells, thereby altering the alternative splicing of key cancer-associated genes.

1. Bullock N & Oltean S (2017). The many faces of SRPK1. J Pathol 241: 437-440.
2. Amin EM, Oltean S, Hua J, Gammons MV, Hamdollah-Zadeh M, Welsh GI, Cheung MK, Ni L, Kase S, Rennel ES, Symonds KE, Nowak DG, Royer-Pokora B, Saleem MA, Hagiwara M, Schumacher VA, Harper SJ, Hinton DR, Bates DO, Ladomery MR (2011). WT1 mutants reveal SRPK1 to be a downstream angiogenesis target by altering VEGF splicing. Cancer Cell 20: 768-780.
3. Toska E, Campbell HA, Shandilya J, Goodfellow SJ, Shore P, Medler KF, Roberts SG (2012). Repression of transcription by WT1-BASP1 requires the myristoylation of BASP1 and the PIP2-dependent recruitment of histone deacetylase. Cell Rep 2:4 62-69.
4. Toska E & Roberts SG (2014). Mechanisms of transcriptional regulation by WT1 (Wilms' tumour 1). Biochem J. 461:15-32.

Role of Exosomes in Chemotherapy-induced Bystander Effect

Arinzechukwu Ude, Dr. Michael Ladomery And Dr Ruth Morse

University of the West of England (UWE), Bristol, UK
Centre for Research in Biosciences, UWE Bristol, UK

Donor cell leukaemia (DCL) is a complication where transplanted haematopoietic stem cells become malignant in the recipient whilst the donor remains healthy. We hypothesised that chemotherapy treated mesenchymal stem cells (MSC) of the bone marrow produce a bystander effect during transplantation via exosomes trafficking microRNAs. Bystander effect occurs when treatment signatures or biological effects are induced in unexposed cells which are in close proximity to the directly exposed cells, via intercellular communication. Exosomes are small 50-100nm extracellular vesicles that play an integral role in intercellular communication via uptake of lipids, microRNAs, mRNAs and proteins by recipient cells. The MSC cell line, HS-5, was treated with and without clinically relevant dose of mitoxantrone (500ng/ml) for 24 hours. Treated HS-5 cells were then fixed with 2% paraformaldehyde, negatively stained with uranyl acetate and examined using transmission electron microscopy (TEM) to see if these cells release exosomes. Furthermore, exosomes were isolated from the treated and untreated cells, and also examined under TEM. Treated cells were then co-cultured with a bystander TK6 cells for 24 hours and RNA was isolated from treated HS-5 cells, conditioned media, bystander TK6 cells and exosomes to assess their microRNA profiles. Our current data suggest that HS-5 cells release exosomes in response to chemotherapy. Certain microRNA signatures, such as miR-21-5p, miR-16-5p, miR-155-5p, miR-146a-5p, miR-24-3p, miR-20a-5p, miR-19b-3p, miR-103-3p, miR-30d-5p and miR-30e-5p were also upregulated in the bystander TK6 cells. For future work, we will assess if these upregulated microRNAs can also be found in the conditioned media and exosome to confirm their release and transport from HS-5 cells to bystander TK6 cells.

Epigenetic inactivation of the epithelial splicing regulator ESRP2 in Wilms' tumour

Danny Legge[1], Whei Moriarty[1], Sebastian Oltean[2], Karim Malik[1] and Keith Brown[1]

[1]School of Cellular and Molecular Medicine, Biomedical Sciences Building, University Walk, Bristol. BS8 1TD.

[2]Institute of Biomedical and Clinical Sciences, University of Exeter Medical School, Heavitree Road, Exeter. EX1 2LU.

Our genome-wide analysis of DNA methylation had previously identified Epithelial splicing regulatory protein 2 (ESRP2) as a gene that is frequently hypermethylated in Wilms' tumour (WT; a common childhood kidney cancer), with associated downregulation of expression. ESRP2 encodes an RNA splicing factor that is essential for epithelial differentiation, a process that is deregulated in WT development.

To investigate the function of ESRP2 in WT development, we generated a WT cell line (WiT49 E200L) with doxycycline (Dox)-inducible expression of ESRP2, along with control cells transfected with empty vector (WiT49 V200). ESRP2 induction following Dox addition was confirmed by PCR and immunoblotting. Alternative splicing of known ESRP2 targets FGFR2 and ENAH was observed following induction of ESRP2 in E200L cells but was not identified in V200 control cells. Furthermore, colony formation was significantly inhibited by around 50% upon induction of ESRP2. Importantly - and in agreement with colony forming data - cell growth was reduced by around 50% following 6 - 8 days of ESRP2 induction in E200L cells.

Finally, to identify any ESRP2-induced alternative splicing events or gene regulation that may be important in WT development or progression, we performed RNA-Seq analysis in E200L cells treated with DMSO or Dox for 96 hours. Analyses show enrichment of genes involved in proliferation and cell division among the differentially spliced genes. Data generated from this study has the potential to highlight novel alternative splicing events that could potentially be targeted for more effective therapies for Wilms' tumour in the future.

FICC-Seq: A method for enzyme-specified profiling of uridine modification in cellular RNA

Jean-Michel Carter[1], Warren Emmett[2,3], Igor Ruiz de los Mozos[2], Annika Kotter[4], Mark Helm[4], Jernej Ule[2], Shobbir Hussain[1*]

[1] Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath, BA2 7AY, UK; [2] The Francis-Crick Institute, 1 Midland Road, London, NW1 1AT, UK; [3] University College London Genetics Institute, Gower Street, London, WC1E 6BT, UK; [4] Johannes Gutenberg-Universität, Institut für Pharmazie und Biochemie, Staudinger Weg 5, 55128 Mainz, Germany.

Methyl-5-uridine (m5U) is one the most abundant non-canonical bases present in cellular RNAs, and is formed via catalytic conversion of uridines. In yeast, the modification is known to be present at U54 of tRNAs where the conversion is catalysed by the methyltransferase Trm2. Although the mammalian enzymes that catalyse m5U formation are yet to be identified via experimental evidence, based on sequence homology to Trm2, two candidates currently exist, TRMT2A and TRMT2B. Here we developed a genome-wide single-nucleotide resolution mapping method, Fluorouracil-Induced-Catalytic-Crosslinking-sequencing (FICC-Seq), in order to map the relevant enzymatic targets in cellular RNA. We demonstrate that TRMT2A is responsible for the majority of m5U present in human RNA, and that it ubiquitously targets U54 of cytosolic tRNAs. By comparison to current methods, we show that FICC-Seq is a particularly robust method for accurate and reliable detection of relevant enzymatic target sites. We anticipate that the method will be useful for mapping of other uridine modification types, such as pseudouridine, in an enzyme-specified fashion in similar future studies.

Dietary restriction in ILSXISS mice is associated with widespread changes in splicing regulatory factor expression levels.

Benjamin P. Lee[1], Lorna Mulvey[2], Greg Barr[1], Jemma Garratt[1], Emily Goodman[1], Colin Selman[2], Lorna W. Harries[1].

[1]Institute of Biomedical and Clinical Sciences, University of Exeter Medical School, UK, EX2 5DW

[2]Institute of Biodiversity Animal Health & Comparative Medicine, University of Glasgow, UK, G12 8QQ

Dietary restriction (DR) is widely regarded as a robust intervention to extend lifespan and improve health outcomes in a wide range of species. However, substantial variability in the beneficial response has been observed, both between and within species. While much progress has been made in elucidating the pathways involved in the response to DR, the precise mechanisms underlying the reported variation in effect are still not well characterised. mRNA splicing regulatory factors have been implicated in the pathways involved in the increased longevity seen with DR in C. elegans and have also been shown to be associated with lifespan itself in mice and humans. Here we have measured expression levels of splicing factors in 3 tissues from recombinant inbred strains of mice displaying differential lifespan responses to DR in an effort to determine whether these splicing factors could contribute to the mechanistic basis of DR-induced lifespan response.

Our results show that patterns of splicing factor expression are highly tissue specific, and that widespread differences are apparent in long-term DR conditions in brain, whereas heart and kidney show more changes with short-term DR, suggesting systematic differences in homeostatic response. Subsequent statistical interaction analyses indicate that a subset of these splicing factors could have mechanistic involvement in the divergent lifespan response to DR observed in these mouse strains.

This study provides further evidence that regulation of alternative splicing is fundamental to the mechanisms of lifespan modulation in response to DR and gives some insight into the differential tissue response.

The MITF-SOX10 regulated long non-coding RNA DIRC3 is a melanoma tumour suppressor

Elizabeth A Coe[1], Jennifer Y Tan[2], Michael Shapiro[1], Pakavarin Louphrasitthiphol[3], Andrew R Bassett[4], Ana C Marques[2], Colin R Goding[3], and Keith W Vance[1]

[1] Department of Biology and Biochemistry, University of Bath, Bath, BA2 7AY, UK

[2] Department of Computational Biology, University of Lausanne, 1015 Lausanne, Switzerland

[3] Ludwig Institute for Cancer Research, University of Oxford, Oxford, OX3 7DQ, UK

[4] Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridgeshire, CB10 1SA.

The MITF and SOX10 transcription factors regulate the expression of genes important for melanoma proliferation, invasion and metastasis. Despite growing evidence of the contribution of long noncoding RNAs (lncRNAs) in cancer, including melanoma, their functions within MITF-SOX10 transcriptional programmes remain poorly investigated. Here we identified 245 candidate melanoma associated lncRNAs whose loci are co-occupied by MITF-SOX10 and that are enriched at active enhancer-like regions. We show that one of these, Disrupted In Renal Carcinoma 3 (DIRC3), is a clinically important MITF-SOX10 regulated tumour suppressor. DIRC3 depletion in human melanoma cells leads to increased anchorage-independent growth, a hallmark of malignant transformation, whilst melanoma patients classified by low DIRC3 expression have decreased survival. DIRC3 is a nuclear lncRNA that activates expression of its neighbouring IGFBP5 tumour suppressor through modulating chromatin structure and suppressing SOX10 binding to putative regulatory elements within the DIRC3 locus. In turn, DIRC3 dependent regulation of IGFBP5 impacts the expression of genes involved in cancer associated processes. Our work indicates that lncRNA components of MITF-SOX10 networks are an important new class of melanoma regulators and candidate therapeutic targets that can act not only as downstream mediators of MITF-SOX10 function but as feedback regulators of MITF-SOX10 activity.

A stand-alone sponge RNA regulates the levels, processing and efficacy of the key Gram-positive riboregulator of central metabolism RoxS

Adam Callan-Sidat[1], Josie Mckeown[1], Gergana Kostova[2], Juan R. Hernandez-Fernaud[3,] Tauqeer Alam[1], Andrew Millard[1,4], Chrystala Constantinidou[1], Ciarán Condon[2], Sylvain Durand[2] and Emma L. Denham[1,5]

1 - Warwick Medical School, University of Warwick, Coventry, UK
2 - Institut de Biologie physico-chimique, Université Sorbonne Paris Cité, Paris, France
3 – School of Life Sciences, University of Warwick, Gibbet Hill Road, Coventry, UK
4 – University of Leicester, Leicester, UK
5 - Department of Biology and Biochemistry, University of Bath, Bath, UK

Metabolism operates as a highly integrated network that creates the energy and building blocks necessary for all cellular processes. Therefore, it is important that this process is optimally regulated. Bacteria have evolved elegant systems to enable rapid proliferation to take place when a preferred carbon source is available. This is well understood at the level of transcriptional regulation, but it was only relatively recently that the role of post-transcriptional factors began being recognised. RoxS has been identified as the key riboregulator of metabolism in Gram-positive species of bacteria and has been studied in molecular detail in Bacillus subtilis and Staphylococcus aureus. RoxS targets many mRNAs that encode proteins involved in metabolism. During a high-throughput approach to globally map pairwise RNA interactions in vivo using AMT crosslinking we identified a sRNA interacting with RoxS. We named this sRNA RosA (Regulator of sRNA A) and have shown that it acts as a RNA sponge under conditions where a preferred carbon source is not available. RosA is a highly processed sRNA that effects the fitness of the bacterium. RosA not only acts to regulate the level of RoxS, but also its processing and efficacy. This additional post-transcriptional regulation adds a new level of control in correctly balancing the metabolic state of the cell.

An RNA-interference dependent 'physiological cost' to host fitness for endosymbiont elimination in a nascent phototrophic endosymbiosis

B. H. Jenkins[1], D. Milner[1], F. Maguire[2], G. Leonard[1] and T. A. Richards[1]

[1] Living Systems Institute, University of Exeter, UK
[2] Dalhousie University, Nova Scotia, Canada

Endosymbiosis between two or more ancestral cells was responsible for the subsequent complexity of eukaryotic cellular evolution, via acquisition of the mitochondria and plastid organelles. Paramecium bursaria and its green algal endosymbiont Chlorella spp. represent a nascent facultative endosymbiosis, offering a unique insight into the cell-cell interactions in emergent endosymbiotic systems. Closely interacting organisms may be subject to sRNA cross-talk, yet the existence of such mechanisms in an endosymbiotic system have yet to be investigated.

Through RNAi feeding, we demonstrate a host Dicer-dependent cost to host fitness for endosymbiont elimination. We additionally show that host gene expression may be impacted by as little as 23 nt of endosymbiont transcript with >90% sequence homology, via delivery of a double-stranded or single-stranded (sense) chimeric construct. RNA seq. further reveals that knock-down of host Dicer leads to a reduction in the relative abundance of endosymbiont derived 23nt sRNA released during endosymbiont elimination. These findings provide evidence that endosymbiont derived sRNA is processed by host RNAi factors and can impact host gene expression via RNAi 'collisions' between homologous endosymbiont and host mRNA. An endosymbiont sRNA derived cost to host fitness incurred during endosymbiont elimination has important implications for endosymbiotic control of the cell-cell interaction, putatively demonstrating appropriation of host sRNA processing by an endosymbiont for the first time.

Non-viral delivery of siRNA by lipopolyamines achieving quantitative gene silencing

Ian S. Blagbrough,* Abdelkader A. Metwally, and Charareh Pourzand
Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, UK
* prsisb@bath.ac.uk

We have designed, prepared, and evaluated new cationic lipid delivery vectors, demonstrating that N^4-linoleoyl-N^9-oleoyl-1,12-diamino-4,9-diazadodecane (LinOS), based on the symmetrical polyamine spermine conjugated with two different long-chain fatty acids (linoleic and oleic acids), is an efficient non-viral vector. LinOS, on direct mixing with siRNA, self-assembles into siRNA lipoplexes, resulting in efficient non-toxic siRNA delivery and quantitative gene silencing in vitro.

LinOS was prepared by the stepwise coupling of the activated fatty acids (DCC/DMAP) to N^1,N^{12}-diphthalimido-protected spermine and then hydrazine deprotection. Self-assembled siRNA lipoplexes were prepared by adding ethanolic LinOS/Chol mixtures to siRNA in serum-free OptiMEM. HeLa cells stably expressing EGFP were transfected in DMEM containing 10% FCS, in 24-well plates. Delivery of Alexa Fluor 647-tagged siRNA against EGFP and reduction in EGFP expression were evaluated by FACS 48 h post-transfection when HeLa cell-line viability (94%) was evaluated by measuring the reducing capability of the transfected cells using alamarBlue®. LinOS/Chol (molar ratio 1:2) lipoplexes (particles of 113 nm) at N/P = 3.0, reduced stably expressed reporter gene EGFP to 20%, comparable to TransIT TKO, quantified by FACS.

These results show that N^4,N^9-di-fatty acid conjugates of spermine are excellent non-viral vectors for siRNA mediated gene silencing. LinOS more than delivers as an efficient, non-toxic, non-viral vector for siRNA gene silencing in HeLa cells stably expressing GFP even in the presence of FCS. Such self-assembled lipoplexes delivering siRNA achieving non-toxic quantitative silencing with sequence specific gene knock-down means that lipopolyamine non-viral vectors have potential as medicines in a variety of difficult to treat diseases.

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Joint inflammation in obese patients with osteoarthritis: The role of long non-coding RNAs

Susanne N. Wijesinghe, University of Birmingham

Historically, osteoarthritis (OA) has been recognised as a non-inflammatory disease of the cartilage. As such, research has focused on understanding the mechanisms of cartilage degeneration. However, there is now substantial evidence to support the notion that synovial inflammation (synovitis) is a significant driver of OA pathology (1). Importantly, many patients who suffer with OA are obese and we recently reported that obese OA patients exhibit a more inflammatory phenotype with greater levels of pro-inflammatory cytokines in the synovial fluid, compared to normal-weight OA patients (2). Despite this, the cellular mechanisms that mediate OA synovitis and its contribution to OA pathogenesis are poorly understood.

Here, we identify long noncoding RNAs (lncRNAs) associated with the inflammatory phenotype of synovial fibroblasts from obese OA patients and explore their expression and function. Obese OA fibroblasts exhibited an inflammatory transcriptomic phenotype, with increased expression of pro-inflammatory mRNAs including cytokines and chemokines, compared to normal-weight OA. Sequencing analysis found 20 lncRNAs differentially expressed between obese OA and normal-weight OA fibroblasts, including MALAT1, CARMN and MIR155HG. These novel lncRNAs were rapidly induced in response to pro-inflammatory cytokines and adipokines in OA synovial fibroblasts. Knockdown of MALAT1 in OA synovial fibroblasts decreased the expression of BCL-6, IL-6 and CXCL8 and inhibited synovial fibroblast proliferation. Additionally, single cell RNA sequencing (scRNAseq) of synovial fibroblasts from normal weight and obese hip OA patients identified high expression of MALAT1 lncRNA in synovial fibroblasts subsets with attributed functions including proliferation, fibrosis and inflammation. The “inflammation-associated” lncRNA MALAT1 may be a central regulator of inflammation in the obese OA synovial joint, driving OA joint degeneration.

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2) Pearson et al., Scientific Reports 2017. doi: 10.1038/s41598-017-03759-w.

Cellular stressors may alter islet hormone cell proportions by moderation of alternative splicing patterns

Nicola Jeffery[1], Sarah Richardson[1], David Chambers[2], Noel G. Morgan[1], Lorna W. Harries[1].

[1]Institute of Biomedical and Clinical Sciences, University of Exeter Medical School, Barrack Road, Exeter, UK, EX2 5DW

[2]Wolfson Centre for Age Related Diseases, Kings College London, London, UK, WC2R 2LS

Changes to islet cell identity in response to type 2 diabetes have been reported in rodent models but are less well characterised in humans. We assessed the effects of aspects of the diabetic microenvironment on hormone staining, total gene expression, splicing regulation and the alternative splicing patterns of key genes in EndoC-βH1 human beta cells. Genes encoding islet hormones (SST, INS, GCG), differentiation markers (FOXO1, PAX6, SOX9, NKX6.1, NKX6.2) and cell stress markers (DDIT3, FOXO1) were dysregulated in stressed EndoC-βH1 cells, as were some SRSF splicing activator and HNRNP inhibitor genes. Whole transcriptome analysis of primary T2D islets and matched controls demonstrated dysregulated splicing for approximately 25% of splicing events, of which genes themselves involved in mRNA processing and regulation of gene expression comprised the largest group. Approximately 5% of EndoC-βH1 cells exposed to these factors gained somatostatin positivity in vitro. An increased area of somatostatin staining was also observed ex vivo in pancreas sections recovered at autopsy from donors with type 1 or type 2 diabetes (9.3% for T1D and 3% for T2D respectively compared with 1% in controls). Removal of the stressful stimulus or treatment with the AKT inhibitor SH-6 restored splicing factor expression and reversed both hormone staining effects and patterns of gene expression. This suggests that reversible changes in hormone expression may occur during exposure to diabetomimetic cellular stressors, which may be mediated by changes in splicing regulation.

Targeting the ERG Oncogene with Splice-switching Oligonucleotides in Prostate Cancer

Lisa Hobson, University of the West of England

Abstract

The ERG oncogene was first identified in 1987; it resides on chromosome 21 and encodes a 486 amino acid ETS family transcription factor. Misregulation of this gene is linked to a number of diseases including prostate cancer where aberrant expression of the gene is associated with higher severity and a worse clinical outcome. Alternative splicing occurs within the ERG gene which contains 12 different exons; of particular interest is the oncogenic cassette exon 7b. Splice-switching oligonucleotides (SSOs) were designed to target the 3' or 5' of both the oncogenic cassette exon 7b and the constitutively spliced exon 4 and tested in the MG63 cell line. Both of the 3' targeting SSOs showed a higher efficiency in inducing skipping of the target exons with the exon 4 3' SSO showing the greatest efficiency at both the RNA and protein level. Exclusion of these exons resulted in an increase in apoptotic events and a decrease in migration and invasion in vitro, with exon 4 skipping giving rise to the most notable differences. These results suggest that the splice-switching oligonucleotides can be used to effectively induce skipping of ERG exons with notable downstream effects on cellular function as a result of disrupted ERG expression.

CDK11A and CDK11B: the significance of small differences

Francesca Carlisle, Steve West

Living Systems Institute, College of Life and Environmental Sciences, University of Exeter

CDK11 is one of several cyclin-dependent kinases (CDKs) known to participate in transcription regulation. Unlike many other CDKs, the nuclear p110 CDK11 isoform has a substantial N-terminal domain with several protein interaction motifs in addition to its kinase domain. Interestingly, the CDK11 gene is duplicated in humans to produce closely related CDK11A and B proteins. While several roles for CDK11 in mRNA metabolism have been proposed, its function and whether there are specific functions for the human A and B forms is poorly understood. To address this, we employed CRISPR/Cas9 gene editing to generate human cell lines specifically lacking CDK11A or B. Surprisingly, cells completely depleted for CDK11B were nonviable whereas CDK11A-depleted cells were growth-impaired but viable. Strikingly, this difference was accompanied by substantially different gene expression changes relative to wildtype strongly suggestive of isoform-specific roles in transcription. To dissect the underlying function(s) of CDK11A and CDK11B, we performed proximity-dependent biotinylation with CDK11A versus CDK11B. In addition to highlighting divergence in the proteins' interactomes, these experiments identified a novel role for CDK11B in the DNA damage response. Our data further characterise the functions of this enigmatic kinase and uncover differences between its two closely related human forms

Highly Conserved Protein SpoVG Binds RNA in vivo and has Pleiotropic Functions in Bacillus subtilis

Swati Mahapatra[1,4], Niki Christopoulou[2], Pedro Arede Rei[2] and Sander Granneman[2,]

Juan H Fernaud[3], Tauqeer Alam[1], Chrystala Constantinidou[1], Emma L Denham[4,1]

1Warwick Medical School (University of Warwick, UK),

2Centre for Synthetic and Systems Biology (SynthSys) (University of Edinburgh, UK),

3Proteomics Research Technology Platform (University of Warwick, UK),

4Department of Biology and Biochemistry (University of Bath, UK)

RNA-binding proteins (RBPs) have important roles in post-transcriptional gene regulations. Hfq is a highly conserved, widely studied RBP in the Enterobacteriaceae, but has minimal roles in Gram-positive bacteria. SpoVG is a highly conserved protein, known to bind RNA in vitro. Deletion of spoVG in Listeria monocytogenes results in pleiotropic properties and we have shown that similar is true for Bacillus subtilis.

Using UV cross-linking, immunoprecipitation and sequencing (CLIP-Seq), we have shown SpoVG binds RNA in vivo. We have monitored the ΔspoVG mutant for common B. subtilis phenotypes and have shown that the strain exhibits reduced motility, changed antibiotic sensitivity and production, and altered pellicle and biofilm formation. We have monitored the transcriptome and proteome of the ΔspoVG mutant, and have found many of the genes and proteins involved in these processes to be altered. Hence, we present SpoVG as a novel RBP in B. subtilis with diverse regulatory functions.

Differential expression of circRNAs with ageing

SF Haque and DR L Harries [1]

[1]Univeristy of Exeter, UK

CircRNAs are an emerging class of non-coding RNA, formed by the backsplicing of a downstream exon to an upstream exon. CircRNAs are thought to modulate gene expression by sequestering RNA binding proteins directly affecting transcription of genes or sponging other non-coding RNAs such as miRNAs to modulate miRNA-dependent gene expression.

We aimed to generate peripheral blood circRNA profiles from 20 pooled young (age 21-26yrs) and 20 pooled old age (71-85 yrs) human donors to identify those which were associated with age. Firstly, we selected 15 age-related circRNAs to assess in relation to human ageing phenotypes in 364 samples from the InCHIANTI study of Aging. Secondly, we compared expression levels of these circRNA in young and senescent human primary fibroblasts, astrocytes, endothelial cells and cardiomyocytes. Finally, we correlated circRNA expression with median strain lifespan in six mouse strains of different longevities.

We found 2024 circRNAs were expressed in peripheral human blood. 15 circRNAs taken forward for analysis, four were associated with parental longevity and one was associated with markers of frailty. Furthermore, all circRNA tested demonstrated differential expression in senescent or senescing human primary cells of different tissues. Those that were conserved between mouse and man also demonstrated correlations between expression and lifespan in rodent models.

Our results indicate that circRNAs have potential to be involved in ageing and longevity pathways in man and mouse and signal a need for further research to determine the utility of blood-borne circRNAs as future disease markers or predictive factors for human age-related disease.

Alternative splicing of the apoptosis gene Bcl-x in the diabetic glomerular endothelium

Pamela Fernandez, Megan Stevens, Sebastian Oltean
Institute of Biomedical & Clinical Sciences, College of Medicine & Health, University of Exeter Medical School, Exeter, UK

Background: Alternative splicing (AS) is a ubiquitous mechanism that diversifies gene expression by enabling mRNA to give rise to different protein isoforms. Previous RNAseq studies carried out on glomerular endothelial cells (GEnCs) in our lab found that exposure to a diabetic environment resulted in the disruption of Bcl-x AS; alternative 5' splice site usage in exon 2 yield two isoforms: the pro-apoptotic short Bcl-xS and the anti-apoptotic large Bcl-xL isoform.
Aim: We aim to determine how Bcl-x splicing is regulated in the diabetic glomerular endothelium.
Methods: GEnCs were exposed to the following conditions: 5 mM glucose (normal glucose control), 5 mM glucose + 25 mM mannitol (osmotic control) or a "high glucose soup" (30 mM glucose, 1 ng/ml TNFα, 1 ng/ml IL6 and 100 nM insulin), for 1 to 7 days. We then performed RT-PCR to assess Bcl-x splicing at the mRNA level. We further analysed Bcl-x splicing at the protein level with Western blotting.
Results: RT-PCR analysis showed a trend towards an increase of Bcl-xS in diabetic environment compared with controls after 7 days of treatment. There was also an increase in the Bcl-xS/Bcl-xL ratio at the protein level after 7 days. In addition, expression of the splice factors PTBP1 and SRSF1 were dysregulated and IL6 expression was upregulated when exposed to the diabetic environment, all of which have been previously implicated in Bcl-x splicing regulation.
Conclusion: Dysregulation of Bcl-x AS was found to promote the Bcl-xS isoform in the presence of a diabetic environment in GEnCs. Further analysis of Bcl-x AS will provide an essential insight into the pathogenesis of diabetic nephropathy

Poster Presentations

Single cell expression profiling identifies pigment cell differentiation trajectories from unexpectedly multipotent intermediate pigment progenitor cell

Tatiana Subkhankulova[1], Masataka Nikaido[1], Artem Kasianov[2], Leonid Uroshlev[2], Gerardo Aquino[3], Hartmut Schwetlick[4], Tatjana Sauker-Spengler[5], Andrea Rocco[3], Jonathan Dawes[4], Vsevolod Makeev[2], Robert N. Kelsh[1]

[1] Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK

[2]Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow 119991, Russia

[3] Department of Microbial and Cellular Sciences, University of Surrey, Guildford GU2 7XH, UK

[4] Department of Mathematical Sciences, University of Bath, Bath BA2 7AY, UK

[5] MRC Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DS

The neural crest (NC) is a major model for understanding how multipotent progenitors generate a balance of derivative cell-types. There is long-standing controversy over whether NC fate choice proceeds via a Direct Fate (no intermediates) or Progressive Fate Restriction (partially-restricted intermediates) model. Zebrafish NC generates three distinct pigment cell types (melanocytes, iridophores and xanthophores), proposed to share a common cellular origin, a partially-restricted *ltk*-expressing chromatoblast, and a bipotent melanoiridoblast. Fate-mapping of *ltk*-expressing cells confirms their multipotency, but reveals their unexpected peripheral neuron potential. NanoString expression profiling of single NC-derived cells identifies a likely course of differentiation of pigment cells via intermediates. These intermediates again identify a highly multipotent partially-restricted intermediate in vivo, a finding supported by single cell qRT-PCR profiling. Surprisingly, we find no evidence for a bipotential melanoiridoblast. Together these data give us a novel and unexpected view of the mechanisms of pigment cell development *in vivo*, with the implication that pigment cell-types may differentiate directly from a multipotent (not bipotent) progenitor state. We propose a novel Cyclical Fate Restriction mechanism consistent with these data and which goes some way towards reconciling the Direct and Progressive Fate Restriction models. We use a theoretical approach to assess the feasibility of such a process, proposing that multipotent states may be characterized by dynamically evolving levels of expression of *ltk* and other fate specification genes in a cyclical network of inhibition, similar to the synthetic genetic regulatory network known as the ‘repressilator’ constructed by Elowitz & Leibler (Nature, 2000).

RNA Methylation in Diabetes Induced Endothelial Damage Contributing to Limb Ischemia

Walid Sweaad, Aranzazu Chamorro-Jorganes, Marie Besnier, Costanza Emanueli

Abstract

Diabetes mellitus induced hyperglycaemia impairs endothelial cell function leading to microvascular rarefaction, macrovascular disease and compromised post-ischemic reparative neovascularization. Novel regenerative therapies for diabetic vascular complications are urgently needed. RNA methylation, specifically on the RNA adenosine base at the nitrogen-6 position (m^6A), is emerging as a new layer of gene expression regulation. The functional roles of m^6A methylation in EC biology and diabetic microvascular complications remains completely unexplored. Here, we reveal a reduction in the expression of Methyltransferase like 3 (METTL3), the active catalytic subunit of the methyltransferase complex, in ECs cultured under conditions mimicking diabetes and ischaemia *in vitro*. Total m^6A RNA methylation levels were also decreased under these conditions. Utilising a mouse model of limb ischemia, we show a reduction in endogenous METTL3 expression in ECs isolated from ischemic muscles of diabetic mice. Moreover, we found that METTL3 depletion decreases EC proliferation, survival and migration and compromises the angiogenic capacities of cultured ECs. Collectively, our data thus far suggests a novel role for m^6A modifications in mediating physiological angiogenesis. We also reveal a diabetes induced dysregulation of m^6A. Regulatory components of the m^6A machinery, such as METTL3, could therefore represent novel therapeutic targets for vascular complications in diabetic patients.

MicroRNA (miRNA) activity and regulation are of increasing interest as new therapeutic targets

Emmad Manni, University of Exeter

MicroRNA (miRNA) activity and regulation are of increasing interest as new therapeutic targets. Traditional approaches to assess miRNA levels in cells rely onRNA sequencing or quantitative PCR. While useful, these approaches are based on RNA extraction and cannot be applied in real-time to observe miRNA activity with single-cell resolution. We developed a green fluorescence protein (GFP)-based reporter system that allows for a direct, real-time readout of changes in miRNA activity in live cells. The miRNA activity reporter (MiRAR) consists of GFP fused to a 3? untranslated region containing specific miRNA binding sites, resulting in miRNA activity-dependent GFP expression. Using qPCR, we verified the inverse relationship of GFP fluorescence and miRNA levels. We demonstrated that this novel optogenetic reporter system quantifies cellular levels of the tumor suppressor miRNA let-7 in real-time in single Human embryonic kidney 293 (HEK 293) cells. Our data shows that the MiRAR can be applied to detect changes in miRNA levels upon disruption of miRNA degradation pathways. We further show that the reporter could be adapted to

monitor another disease-relevant miRNA, miR-122. With trivial modifications, this approach could be applied across the miRNome for quantification of many specific miRNA in cell cultures, tissues, or transgenic animal models.

The GNG12-AS1/DIRAS3 locus: how a lncRNA impacts the chromatin architecture of the area

Giuseppina Pisignano[1*], Stephen Richer[1], Valeriya Malysheva[2], Stefan Schoenfelder[2], Cameron Osborne[3], Adele Murrell[1]

[1] Dept Biology and Biochemistry, University of Bath, Claverton Down, Bath, BA2 7AY, United Kingdom
[2] Babraham Institute, Cambridge, CB22 3AT, United Kingdom
[3] King's College London, Guy's Hospital, London, SE1 1UL, United Kingdom

Long non-coding RNAs (lncRNAs) are important epigenetic regulators of gene expression. Emerging evidence suggests that their expression affects the surrounding chromatin organization and the activity of neighbouring genes; however, the molecular mechanisms that underpin this regulatory crosstalk are poorly understood. Imprinted genes are an excellent model system for examining lncRNAs and their roles in coordinating the expression of multiple genes within a gene cluster. We are using the GNG12-AS1/DIRAS3 locus to inspect how chromatin architecture is affected by lncRNA transcription and how this impacts on the epigenetic regulation of nearby genes. DIRAS3 is an imprinted tumour suppressor gene embedded within the GNG12-AS1 lncRNA. Inhibition of GNG12-AS1 transcription, upregulates DIRAS3. Conversely, when DIRAS3 is up-regulated, GNG12-AS1 expression is reduced.

To analyse the effect of the lncRNA on the chromatin architecture we have performed allele- specific capture-HiC which has yielded insights into how DIRAS3 and GNG12-AS1 are topologically organized, and identified domain structures that may dictate their reciprocal transcription. Next we are addressing the potential of RNA polymerase (RNAP) collision/pausing to resolve how the GNG12-AS1 transcription interferes with the convergent transcription of DIRAS3 gene. We are carrying out Nuclear Run-On together with DRIP and RNAPII Chromatin Immunoprecipitation to map the engaged RNAPII pausing and assess the re-annealing of nascent transcripts to the DNA template (R-loops formation).

This multiple approach in studying the epigenetic regulation of a key gene affected by the neighbouring lncRNA, will elucidate the role of lncRNAs in the regulation of adjacent genes, especially in a complex mechanism such as imprinting and cancer mediated impairment.

Long Non-coding RNAs Are Central Regulators of the IL-1β-Induced Inflammatory Response in Normal and Idiopathic Pulmonary Lung Fibroblasts

Marina R. Hadjicharalambous[1], Benoit T. Roux[1], Carol A. Feghali-Bostwick[2], Lynne A. Murray[3], Deborah L. Clarke[4†] and Mark A. Lindsay[1]

[1]Department of Pharmacy and Pharmacology, University of Bath, Bath, United Kingdom
[2]Division of Rheumatology and Immunology, Department of Medicine, Medical University of South Carolina, Charleston, SC, United States
[3]RIA IMED Biotech Unit, AstraZeneca, Gothenberg, Sweden
[4]MedImmune, Cambridge, United Kingdom

There is accumulating evidence to indicate that long non-coding RNAs (lncRNAs) are important regulators of the inflammatory response. We have employed next generation sequencing to identify 14 lncRNAs that are differentially expressed in human lung fibroblasts following the induction of inflammation using interleukin-1β (IL-1β). Knockdown of the two most highly expressed lncRNAs, IL7AS and MIR3142HG, showed that IL7AS negatively regulated IL-6 release whilst MIR3142HG was a positive regulator of IL-8 and CCL2 release. Parallel studies in fibroblasts derived from patients with idiopathic pulmonary fibrosis showed similar increases in IL7AS levels, that also negatively regulate IL-6 release. In contrast, IL-1β-induced MIR3142HG expression, and its metabolism to miR-146a, was reduced by 4- and 9-fold in IPF fibroblasts, respectively. This correlated with a reduced expression of inflammatory mediators whilst MIR3142HG knockdown showed no effect upon IL-8 and CCL2 release. Pharmacological studies showed that IL-1β-induced IL7AS and MIR3142HG production and release of IL-6, IL-8, and CCL2 in both control and IPF fibroblasts were mediated via an NF-κB-mediated pathway. In summary, we have cataloged those lncRNAs that are differentially expressed following IL-1β-activation of human lung fibroblasts, shown that IL7AS and MIR3142HG regulate the inflammatory response and demonstrated that the reduced inflammatory response in IPF fibroblast is correlated with attenuated expression of MIR3142HG/miR-146a.

Conservation and diversification of small RNA pathways in a sibling species of *C. elegans*

Vicky Hunt, University of Bath

Small RNA (sRNA) pathways are well characterised in the nematode Caenorhabditis elegans and understanding these pathways has led to important developments in genetic manipulation methods. However, we know little about these pathways in other nematodes. Here, we have investigated sRNAs in the fig nematode C. inopinata, the closest known relative of C. elegans. Most sRNA pathways and their associated sRNAs are conserved between these two species. However, C. inopinata has lost the ERGO-1 pathway which is thought to be involved in regulating the expression of tandemly duplicated genes and pseudogenes. Orthologues of genes coding for the ERGO-1 Argonaute protein and other genes involved in the ERGO-1 pathway are not present in the C. inopinata genome. This loss is likely to be the result of high levels of transposase activity in C. inopinata. The 26G small interfering RNAs associated with the ERGO-1 pathway are also absent in C. inopinata and the sRNA profiles in these nematodes are similar to C. elegans ergo-1 mutants. To understand the conservation and diversification of the ERGO-1 pathway in nematodes more widely we have identified ERGO-1 pathway orthologues across diverse nematode species. We find that most genes associated with the ERGO-1 pathway are absent from nematodes outside of the Caenorhabiditis clade. We conclude that the ERGO-1 pathway characterised in C. elegans is unique to the Caenorhabidtis clade of nematodes but has been lost in C. inopinata. This work also highlights the importance of studying non-model organisms to understand the variation and diversification of sRNA pathways.