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18th November 2021

Genetics Otago Zoom Symposium

Thursday, 18th November 2021

Posters will be on display throughout the day in the foyer, please take time to view these during the breaks. Judges will speak with authors during morning tea break, so please make sure you attend your poster during this break.

8:45am – 9:00am	Coffee		
9:00am – 9:05am	Welcome		*Dr Nathan Kenny and Dr Louise Bicknell*
Session 1			**Chair: Kate Harding**
9:05am – 10:05am	Winds of Change	Keynote Speaker	Prof. Vicky Cameron, *Department of Medicine, Christchurch*
10:05am – 10:20am	The Role of DUF247 in Ryegrass Self-Incompatibility	Selected Abstract	Tyler McCourt, *Department of Biochemistry*
10:20am – 10:35am	Bush genomics: using genetics and genomics tools to discover and understand New Zealand's truffle-like fungi	Invited Speaker	Associate Professor David Orlovich, *Department of Botany*
10:35am –11:05am Tea/coffee break			

Session 2			**Chair: Victoria Sugrue**
11:05am – 11:20am	Characterising host factors involved in jumbo phage infection in *Serratia*	Invited 400–level student	Kate Harding, *Department of Microbiology and Immunology*
11:20am – 11:35am	Tuberculosis or soil bacteria? Detection and authentication of highly degraded *M. Tuberculosis* DNA in bioarchaeological remains from the Pacific	Selected Abstract	Meriam van Os, *Department of Anatomy*
11:35am – 11:50am	Assessing the pharmacogenomic landscape of adverse drug reactions in Aotearoa	Invited Speaker	Dr Simran Maggo, *Department of Pathology and Biomedical Science, Christchurch*
11:50am – 12:05pm	Identification of tumour specific-epigenetic signatures in circulating tumour cells to understand their role in colorectal cancer metastasis	Selected Abstract	*Sai Shyam Vasantharajan, Department of Pathology*
12:05pm – 12:20pm	Replication of a Māori and Pacific-specific Type II Diabetes risk variant within *JAZF1*	Invited 400–level student	Milly Morice, *Department of Biochemistry and Department of Pathology*
12:20pm – 1:20pm Lunch			

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Lab Supply Science Communication Prize:		
1:20pm – 1:30pm	3 Minute Thesis Presentation	Chi Lynch-Sutherland, *Department of Pathology*
	Short Video	Victoria Sugrue, *Department of Anatomy*
	Essay – Did We Get *Tuberculosis* From Seals?#	Meriam van Os, *Department of Anatomy*
	Blog - Lost Worlds Vanished Lives*	Dr Nic Rawlance, *Department of Zoology*

Meriam's Essay can be found on pages 32 – 33 of 'On the GO 2021' available in the room.

*Dr Nic Rawlence (Zoology) started writing his highly popular Lost Worlds Vanished Lives Sciblog (https://sciblogs.co.nz/blogs/lost-worlds/) in 2017 (with 29 blogs to date). In 2021 he has written about various topics including his genetic research into the impact of the last Ice Age on southern beech in New Zealand, the importance of correct metadata when using historical specimens to inform species translocation, why New Zealand had so many giant birds, what can be done about the current extinction crisis, how human arrival resulted in flightless insects (highlighting research conducted by Prof. Jon Waters) and the evolution of kakariki. Some of Dr Rawlence's most popular blogs have been on ancestry DNA testing and the importance of taxonomy. His blogs are shared and read worldwide. They are regularly republished in the Biology Educators of Aotearoa New Zealand newsletter (which is read by science and biology teachers across New Zealand), and Southern Bird, the magazine of Birds New Zealand. An example of his latest blog can be found at the link below, highlighting the importance of how new genetic data can change what we know about the evolution of taonga species: https://sciblogs.co.nz/lost-worlds/2021/09/10/resolving-a-genetic-mash-up-reconstructing-an-accurate-evolutionary-history-of-kakariki/

Scan to visit Lost Worlds Vanished Lives Sciblog

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Session 3:			**Chair: Gracie Kroos**
1:30pm – 2:30pm	The circle of life: connecting cell division with cell fate	Keynote Speaker	Professor Julia Horsfield, *Department of Pathology*
2:30 – 3:00 pm	Understanding active DNA demethylation specificity and function	Selected Abstract	Claudia Davies, *Department of Anatomy*
	LncRNA-targeting antisense oligonucleotides and combination therapy for triple negative breast cancer	Selected Abstract	Kaitlyn Tippett, *Department of Biochemistry*
	Brothers discordant for gout have differing DNA methylation levels in inflammatory genes	Selected Abstract	Tanya Major, *Department of Biochemistry*
	Sex, Brains & RNA	Selected Abstract	Susie Szakats, *Department of Anatomy*
	Investigating the mechanisms during whole-body regeneration of a marine colonial chordate	Selected Abstract	Berivan Temiz, *Department of Anatomy*
	Capturing species-wide diversity of the gut microbiota and its relationship with genomic variation in the critically endangered kākāpō	Selected Abstract	Annie West, *Department of Anatomy*
3:00pm – 3:30pm	**Tea/Coffee Break**		

Session 4			**Chair: Meriam van Os**
3:30pm-3:45pm	Exploring the genetic basis underlying repeated wing loss events in the native New Zealand stonefly Zelandoperla fenestrata	Invited 400–level student	*Gracie Kroos Department of Zoology*
3:45pm – 4:00pm	Leveraging adaptive sampling and nanopore sequencing of environmental DNA for monitoring the critically endangered kākāpō	Selected Abstract	*Dr Lara Urban, Department of Anatomy*
4:00pm – 4:15pm	Sequencing the genome of the New Zealand Brushtail Possum: a platform for biological discovery and pest control	Selected Abstract	*Dr Donna Bond, Department of Anatomy*
4:15pm – 4:30pm	Castration delays epigenetic aging and feminises DNA methylation at androgen- regulated loci	Selected Abstract	*Victoria Sugrue, Department of Anatomy*
4:30 – 5:00pm	Awards and Closing		*Dr Louise Bicknell and Associate Professor Logan Walker*

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Abstracts

Keynote Speakers:

Winds of Change
Professor Vicky Cameron, *Department of Medicine, University of Otago, Christchurch*

This talk is a lighthearted retrospective of my personal observations of the major shifts in scientific research culture in Aotearoa / NZ over the past 40 years. The first half of the talk will focus on the role of women in science, covering some of my own experiences as a young research student in Antarctica, as a working Mum, and mentioning some inspirational people I have worked with along the way. In the second half of the talk I will reflect on the changing concepts of health research over my career, from the exclusive, mostly male, medical establishment of 40 years ago, to an increasingly diverse and inclusive environment. I will end with some personal views as I look out to the horizon.

The circle of life: connecting cell division with cell fate
Professor Julia Horsfield, Department of Pathology, University of Otago

To make a brand new animal from a fertilised egg, two important processes must be integrated. These are (1) cell division for embryo growth, and (2) cell differentiation, in which cells 'decide what to be'. When cells decide their fate, they usually stop dividing and turn on specific 'cell identity' genes. Once an animal is grown, the same cycle of cell growth and cell fate decision is co-opted to renew tissues such as blood, bone and skin. Thus, the circle of cell division and cell fate choice must be precisely coordinated for normal growth. For developing humans, the consequences of disruption to this process are developmental disorders. Later in life, disruption to normal tissue maintenance can cause cancer; a situation where cells proliferate uncontrollably. My research over the last 25 years has centred on trying to understand the connections between cell proliferation and differentiation, and why disrupting the balance of these processes contributes to human disease. This has culminated in our team's pioneering work on the cohesin complex, which not only controls cell division but also switches on genes that are essential for growth and development. The work has led to the identification of the basis of human developmental disorders known as the "cohesinopathies", and identified new therapeutic opportunities in cancer.

Invited Speakers:

Characterising host factors involved in jumbo phage infection in *Serratia*

Harding, K.[1], Malone, L.[1] and Fineran, P.[1,2]. [1]Department of Microbiology and Immunology, University of Otago, Dunedin. [2]Genetics Otago, University of Otago, Dunedin.

Virulent bacteriophages are viruses that can infect bacteria and cause cell lysis. The killing of infected bacterial hosts by phages occurs as a consequence of the lytic cycle. During the lytic cycle more phage particles are formed and contribute to the propagation of phage in a bacterial population. Interactions between phage and bacterial proteins are crucial to the progress of the lytic cycle yet remain highly under characterised. The recently discovered virulent *Serratia* jumbo phage PCH45 is a member of the *Caudovirale* order (i.e. phages with DNA genomes and tail structures). Upon host cell entry, this phage can form a nucleus-like structure which protects its DNA genome from *Serratia* DNA-targeting CRISPR-Cas systems. The transcription of the jumbo phage genome occurs within the nucleus-like structure, but the translation of phage RNA occurs in the host cytoplasm leaving the phage RNA vulnerable to RNA-targeting CRISPR-Cas systems. Interactions between the jumbo phage PCH45 and the host bacterium *Serratia* during phage infection and the biology of PCH45 remain poorly characterised. In 2020, Kyte et al. identified 107 *Serratia* sp. ATCC 39006 genes to be involved in PCH45 jumbo phage infection using Tn-seq screening methods. Over half of these genes were involved in the biosynthesis of flagella, a molecule required for jumbo phage attachment. The remaining genes were involved in host biological activities. In the current study, the role of genes identified to be involved in jumbo phage PCH45 infection was assessed. CRISPRi mediated silencing was used to knockdown the function of each gene by using guide RNAs to deliver dead Cas9 (dCas9) to the genes to block gene expression. The resulting phage infectivity (i.e. ability of phage to infect) and survival of bacterial population (i.e. bacterial resistance to phage infection) during phage PCH45 challenge was assessed. It was discovered that genes involved in distinct host biological functions were involved in jumbo phage infection as the silencing of these genes lead to decreased phage infectivity and increased bacterial population survival. This included genes involved in lipopolysaccharide synthesis, RNA translation, energy synthesis, metabolism, motility, iron cluster formation, virulence factors and the formation of transporters. Swimming ability was found to be decreased in the majority of silenced genes, indicating many of these genes have a role in the movement or biosynthesis of flagella. Lastly, some phage-host interactions occurring between jumbo phage PCH45 and *Serratia* were identified to occur in non-jumbo phages. By understanding the interactions occurring between phages and their hosts we can alter the efficacy of phage infection in the biotechnological application of phage in health and food industry fields.

Exploring the genetic basis underlying repeated wing loss events in the native New Zealand stonefly Zel*andoperla fenestrata*

Gracie Caroline Kroos, Department of Zoology

Ever since insects were able to fly, multitudes have experienced dispersal reductions. Extraordinarily, independent flight losses can occur persistently across insect populations revealing a predictable evolutionary pattern. Less understood are the genomic mechanisms underlying this phenomenon, which can occur through repeated sorting of shared, pre- existing variation or through convergence. Next generation sequencing approaches have been revolutionary to deduce whether genomic causes driving repeated shifts to flightlessness are occurring by way of the same or distinct pathways. The native New Zealand alpine stonefly *Zelandoperla fenestrata* is an excellent organism to test this, as numerous populations have undergone adaptive, independent wing reduction events in response to environmental changes, intimately tied to position of the alpine tree line, creating fully winged, reduced-winged, and vestigial- winged ecotypes distributed along elevational clines. A genotyping-by-sequencing approach was conducted, retaining 47,319 polymorphic variants, across 258 *Zelandoperla fenestrata* specimens from 22 populations throughout Central Otago, many containing sympatric fully winged and reduced-winged ecotypes. Population divergence analyses uncovered that shallow patterns of genomic differentiation prevailed among ecotypes at local scales, suggesting wing loss events were quite recent, as well as deeper divergences at regional scales. Outlier analyses and alignment to an annotated reference genome implicated genomic regions which consistently differentiated ecotypes, with documented roles in wing development in the model organism Drosophila. This included an outlier within the coding region of multidrug resistant protein 4, shared across two adjacent, independently diverged populations, indicating this is a strong wing loss candidate, as well as outliers within 20 kilobases of vein and lost PHD's of trr, distinct to one population. Results demonstrate the same genomic mechanism could contribute to repeated wing loss events among nearby streams, not found at larger scales. The well-supported theory of recent anthropogenic land use changes as the main driver of wing loss events were extended across a wider range of populations. Finally, a novel link was proposed between epiproct length, a morphological trait required for reproduction, and significant isolation observed among co- occurring, sympatric wing ecotypes.

Assessing the pharmacogenomic landscape of adverse drug reactions in Aotearoa

Simran Maggo[1], Jaslyn Kee[1], Leonie Hitchman[1], Paul Chin[2], Matt Doogue[2], Allison Miller[1], Lili Milani[3] and Martin Kennedy[1].
[1]Department of Pathology and Biomedical Science, University of Otago, Christchurch, NZ, [2]Department of Clinical Pharmacology, Christchurch Hospital, [3]Estonian Genome Center, University of Tartu, Estonia.

The Understanding Drug Reactions Using Genomic Sequencing (UDRUGS) study is an initiative from the Carney Centre for Pharmacogenomics to bio-bank DNA and store associated clinical data from patients with adverse drug reactions (ADRs). The aim is to provide a genetic explanation of drug-induced ADRs using methods ranging from Sanger sequencing, array sequencing, exome and whole genome sequencing. The aims of this particular study were to use the Infinium Global Screening Array, Illumina inc. (GSA-array) on a sub-cohort of consented UDRUGS participants to assess the pharmacogenomic variation in a New Zealand (NZ) population with ADRs. AgResearch NZ was contracted to run N=144 (inclusive of 13 laboratory control samples) DNA samples from UDRUGS participants consenting to future research on the GSA-array. Data was delivered digitally and 141 samples successfully passed initial quality screening. Using a pharmacogenomic analysis pipeline published by Reisberg et al (2019), the data of 141 participants was analysed for key pharmacogenetic variants in the CYP2D6, CYP2C19, CYP2C9, DPYD, TPMT and select HLA genes. Overall, as our study population was largely NZ European, we observed allele frequencies similar to those previously published for the European population. However, sub-cohort analysis of 51 participants with ADRs to SSRIs showed an increase in CYP2D6 intermediate (IM) (44%) and poor (PM) (14%) metabolisers compared with expected CYP2D6 frequencies (35% -IM and 6% -PM respectively). Further sub-cohorts are currently being analysed. As the allele frequency of CYP2D6 is largely unknown in Māori and Pasifika populations, we have collaborated with two research groups to preferentially analyse these populations. Using the GSA- array we have shown that the NZ European population has a pharmacogenomic profile as published previously. In a sub-cohort of SSRI-ADRs we noted an increased frequency of CYP2D6 IM and PMs. Further research into CYP2D6 allele frequencies in indigenous populations is currently being undertaken by our research group.

Reisberg S et al (2019) Translating genotype data of 44,000 biobank participants into clinical pharmacogenetic recommendations: challenges and solutions. Genet Med 21, 1345–1354.

Replication of a Māori and Pacific-specific Type II Diabetes risk variant within *JAZF1*

Milly Morice, Departments of Biochemistry and Pathology, University of Otago

Māori and Pacific individuals have a two times greater risk of Type II diabetes (T2D) compared to other population groups in New Zealand1. This provides a unique opportunity to explore the hypothesis that some of this risk is attributed to genetic variation. Genome sequencing of 56 Māori and Pacific individuals led to the identification of a Māori and Pacific-specific non-coding variant, *rs150587514* in the first intron of the *JAZF1* gene. This variant is common in Māori and Pacific people (minor allele frequency (MAF) of >10%) but uncommon in other population groups (MAF <0.1%). *JAZF1* is a gene essential for β-cell function and insulin secretion2. Previous unpublished work in 1,962 Māori and Pacific individuals found that the C risk allele conferred a 1.8-fold greater risk of developing T2D compared to those without the risk allele (OR = 1.83 [95% CI 1.42; 2.36]). The aim of the current work was to validate this association by genotyping an independent cohort of Māori and Pacific individuals to test the replicability of this previously identified association. Additionally, this project aimed to assess the functional role of this variant through assays designed to test enhancer activity. An independent Māori and Pacific cohort were recruited through a partnership with the Ngāti Porou Hauora Charitable Trust. This resulted in a replication cohort of ~600 Māori and Pacific participants. These individuals were genotyped using custom designed Taqman probes in a Taqman genotyping assay. Using these genotypes and corresponding phenotypic data a logistic regression association analysis was conducted between *rs150587514* and T2D, with T2D as the outcome variable. The association between *rs150587514* and T2D risk was replicated, and the direction of effect remained consistent with the original analysis (OR = 2.07 [95% CI 1.27; 3.37]). Importantly, in combination with the original data, the association between *rs1505887514* and T2D was strengthened (OR = 1.73 [95% CI 1.36; 2.19). The meta-analysis of these two cohorts indicates that per *rs150587514* C-risk allele, Māori and Pacific individuals have a 1.7-fold greater risk of developing T2D. These data provide evidence that the population-specific allele of *rs150587514* substantially increases risk of T2D in Māori and Pacific people.

Bush genomics: using genetics and genomics tools to discover and understand New Zealand's truffle-like fungi

David A Orlovich[1], Andy R Nilsen[1], Josie McGovern[1], Sam Lasham[1], Dayna Williams[1], Laura G van Galen[1], Tina C Summerfield[1], Matt J Larcombe[1], Janice M Lord[1], Chris M Brown[2]

[1]Department of Botany and 2Department of Biochemistry, University of Otago

The sequestrate or truffle-like fungi are an iconic part of New Zealand's fungal flora. Many truffle-like fungi evolved from mushroom ancestors, and most form symbiotic associations with trees. They are striking examples of convergent evolution and it is thought that animal dispersal and climate are drivers for selection. I will talk about how we have used molecular phylogenetics to discover many new species and used whole genome sequencing to obtain key molecular markers from 100-year-old type specimens. We are using environmental DNA to discover new species, determine species distributions, find new habitats for rare species, and estimate how many species there are. Using molecular phylogenetics, we discovered many paired species where one is a mushroom and one is a truffle-like fungus, and these species pairs present the opportunity to understand the repeated transition from mushroom to truffle using comparative genomics. Genetics and genomics tools have given unparalleled insights into the unique fungal flora of the NZ bush.

Selected Abstracts:

Sequencing the genome of the New Zealand Brushtail Possum: a platform for biological discovery and pest control

Bond, D.M.[1], Ortega-Recalde O.J.[1], Laird, M.K.[1], Richardson, K.S.[1], Reese, F.C.B.[1], Alexander A.M.[1], Adams A.L.[2], van Heezik Y.[2], Robertson B.C.[2], The Vertebrate Genomes Project[3], Gemmell, N.J. [1], Hore, T.A.[1]
[1]Department of Anatomy, University of Otago, Dunedin, NZ; [2]Department of Zoology, University of Otago, Dunedin, NZ; [3]Vertebrate Genome Laboratory, Rockefeller University, New York, USA

The New Zealand brushtail possum (*Trichosurus vulpecula*) is an invasive marsupial pest that was introduced from Tasmania and mainland Australia in the 19th- and early 20th-century1. In collaboration with the Vertebrate Genomes Project (VGP), we recently completed sequencing of a male brushtail possum joey from the Otago peninsula. The resulting chromosome-level assembly is arguably the best of any marsupial genome to date, a success largely due to optimised VGP protocols involving long-read sequencing2. Structural and functional annotation of the genome was based on RNA-seq data from a wide-range of tissues belonging to 'Sandy', the sequenced individual, as well as extensive liver sampling from Otago possums. Using this RNA-seq data, we have identified novel developmental expression related to marsupial pouch young, confirmed the occurrence of rare imprinted genes, and found the Australian sources of possums from the Dunedin region. By incorporating modern and historical samples, we have tracked possum population changes following pest control on the Otago peninsula. These results are likely to have implications for possum control and eradication strategies in the future.

1.Pracy, L. T. (1962). *Introduction and liberation of the opossum (Trichosurus vulpecula) into New Zealand*. New Zealand Forest Service
2.Rhie, A., McCarthy, S. A., Fedrigo, O., Damas, J., Formenti, G., Koren, S., ... & Jarvis, E. D. (2021). *Towards complete and error-free genome assemblies of all vertebrate species*. Nature, 592(7856), 737-746.

The Role of DUF247 in Ryegrass Self-Incompatibility

Tyler McCourt; Rowan Herridge; Peter Mace; Lynette Brownfield.
Biochemistry Department, University of Otago, New Zealand.

Manipulation of a plants innate ability to inbreed or outcross is often crucial to improving outcomes in crop breeding programs. Inbreeding is useful to fix desirable or purge deleterious traits, while outcrossing allows introduction of genetic variation and heterosis. Perennial ryegrass (*Lolium perenne*) is a plant which has both male and female reproductive structures but is an obligate outcrossing species due to self-incompatibility (SI). Ryegrass possesses a SI system governed by two loci, *S* and *Z*, where fertilisation is inhibited when the *S* and *Z* alleles are matched in both the pollen and stigma. Evidence suggests that a *Domain of Unknown Function 247 (DUF247)* is the gene encoding the male component of SI at the *S* locus in ryegrass. It is also hypothesised that *DUF247* could be the female component of SI at the *S* locus, suggesting two DUF247 proteins may form a homodimer to trigger the SI response. This research aims to test the hypothesis by investigating DUF247's subcellular localisation, expression in reproductive tissues, allelic variation and potential to form dimers. Characterising the genes involved in the reaction will provide future opportunities to manipulate SI for improvement of ryegrass breeding programs, such as by generation of F1 hybrid crops that display uniform traits and benefit from heterosis.

Castration delays epigenetic aging and feminises DNA methylation at androgen- regulated loci

Sugrue, V.J.[1], Zoller, J.A.[2], Narayan, P.[3], Lu, A.T.[4], Ortega-Recalde, O.J.[1], Grant, M.J.[3], Bawden, C.S.[5], Rudiger, S.R.[5], Haghani, A.[4], Bond, D.M.[1], Hore, R.R.[6], Garratt, M.[1], Sears, K.E.[7], Wang, N.[8], Yang, X.W.[8,9], Snell, R.G.[3], Hore, T.A.[1], Horvath, S.[4*]* *https://doi.org/10.7554/eLife.64932*
[1]Department of Anatomy, University of Otago, Dunedin, NZ; [2]Department of Biostatistics, Fielding School of Public Health, UCLA, Los Angeles, USA; [3]Applied Translational Genetics Group, School of Biological Sciences, Centre for Brain Research, The University of Auckland, Auckland, NZ; [4]Department of Human Genetics, David Geffen School of Medicine, UCLA, Los Angeles, USA; [5]Livestock and Farming Systems, South Australian Research and Development Institute, Roseworthy, South Australia, Australia; [6]Blackstone Hill Station, Becks, RD2, Omakau, NZ; [7]Department of Ecology and Evolutionary Biology, UCLA, Los Angeles, USA; [8]Center for Neurobehavioral Genetics, Semel Institute for Neuroscience and Human Behavior, UCLA, Los Angeles, USA; [9]Department of Psychiatry and Biobehavioral Sciences, David Geffen School of Medicine at UCLA, Los Angeles, USA.

In mammals, females generally live longer than males, significantly so for sheep1. Nevertheless, the mechanisms underpinning sex-dependent longevity are currently unclear. Accurate prediction of the chronological age of meat products and the animal they originate from would provide true farm-to-plate traceability and quality assurance for the New Zealand lamb industry, which already commands a premium price in the international market. Furthermore, there is value in be able to predict an animal's rate of biological aging, thus forecasting their expected productivity. Epigenetic clocks are powerful biological biomarkers capable of precisely estimating chronological age and identifying novel factors influencing the aging rate using only DNA methylation data2. In this study, we developed the first epigenetic clock for domesticated sheep (*Ovis aries*) using DNA from 425 New Zealand and Australian Merino sheep. This clock is capable of predicting chronological age with an error of 5.1 months using DNA methylation data at 185 CpG sites. We discovered that castrated male sheep have a decelerated aging rate compared to intact males, mediated at least in part by the removal of androgens. Furthermore, we identified several androgen-sensitive CpG dinucleotides that become hypomethylated with age in intact males, but remain stable in castrated males and females. Comparable sex-specific methylation differences in *MKLN1* also exist in bat skin and a range of mouse tissues that have high androgen receptor expression, indicating it may drive androgen-dependent hypomethylation in divergent mammalian species. In characterising these sites, we identify plausible mechanisms explaining how androgens drive male-accelerated aging in sheep and other mammals, and provide potential improvements to the genetic selection criteria already in place in New Zealand sheep breeding programmes.

Leveraging adaptive sampling and nanopore sequencing of environmental DNA for monitoring the critically endangered kākāpō

Lara Urban, Department of Anatomy, University of Otago

We used environmental DNA to monitor one of the last surviving populations of the critically endangered kākāpō (*Strigops habroptilus*). We established a barcoding protocol to identify the distribution of the species in a highly localised manner based on soil samples. Leveraging nanopore sequencing, adaptive sampling and the high-quality kākāpō reference genome, we then extracted species-specific DNA from these samples. We combined read-based haplotype phasing with known individual genetic variation in the kākāpō population to predict the presence of individuals, and were able to confirm these predictions based on in-depth metadata describing the kākāpō territories. This study shows that individual identification is feasible through adaptive sampling of environmental DNA, with important implications for in- depth monitoring of rare and elusive species, potentially expanding the application of environmental DNA research from monitoring species distribution to inferring fitness-related parameters such as genetic structure and inbreeding.

Tuberculosis or soil bacteria? Detection and authentication of highly degraded *M. tuberculosis* DNA in bioarchaeological remains from the Pacific

Meriam van Os, Hugh Cross, Olga Kardailsky, Catherine Collins, Kate McDonald, Rebecca Kinaston, Melandri Vlok, Richard Walter, Greg Cook, Htin Lin Aung, Lisa Matisoo-Smith, Hallie Buckley, and Michael Knapp.

Mycobacterium tuberculosis has affected human populations for the past several thousand years. But despite the antiquity of the disease, we know little about where the bacteria originated and how it spread across the globe. How and when tuberculosis arrived in the Pacific is particularly unclear. Historical records and extant tuberculosis strains in the Pacific indicate that tuberculosis was introduced by Europeans in the 18th-19th centuries. However, pre-European skeletal evidence consistent with tuberculosis suggests a different story. We hypothesise that early tuberculosis in parts of the Pacific was introduced not by humans, but by marine mammals such as seals. We know from both modern and ancient cases that seal- to-human transmission can occur, and archaeological evidence shows us that people were processing seals at some sites. This study tests this hypothesis by applying ancient DNA techniques to retrieve and analyse tuberculosis DNA from pre-European Pacific bioarchaeological remains. Three criteria have been used to authenticate the pathogen DNA, which includes evenness of coverage, percent identity and haploidy. Using a range of bioinformatic tools, results indicate the presence of species closely resembling *M. tuberculosis* in human and seal remains, but the major challenge is separating the pathogenic reads from closely related soil mycobacteria.

Identification of tumour specific-epigenetic signatures in circulating tumour cells to understand their role in colorectal cancer metastasis

Sai Shyam[1], Michael Eccles[1,2], Sharon Pattison[3], Euan Rodger[1], John McCall[4], Elin Gray[5], Aniruddha Chatterjee[1]

[1] Department of Pathology, Otago Medical School-Dunedin Campus, NZ; [2] Maurice Wilkins Centre for Molecular Biodiscovery, Level 2, 3A Symonds Street, Auckland, NZ; [3] Department of Medicine, Otago Medical School-Dunedin Campus, NZ; [4] Department of Surgical Sciences, Otago Medical School-Dunedin Campus, NZ; [5] Centre for Precision Health, Edith Cowan University, Joondalup, Australia

New Zealand has the highest death rate for Colorectal Cancer (CRC) accounting for 4 deaths/day. Metastasis is responsible for 90% of cancer-related deaths, but if detected early, the prognosis improves substantially. Circulating Tumour Cells (CTC) are metastatic seeds that arise from solid tumours and their detection has the potential to improve CRC management. However, there is limited knowledge of the role of CTCs in metastasis. Recent work in breast cancer has shown loss of DNA methylation in CTCs in stem cell-associated genes (SOX2 and NANOG), contributed to the CTC's metastatic potential. The epigenetic landscape of CTCs remains largely unknown in CRC patients, and this is due to their low number in patient blood and lack of robust methods to enrich for CTCs in a standard laboratory setting. The only FDA- approved method for CTC isolation is CELLSEARCH, which has many limitations such as cost, availability, and the use of only one type of marker for capture (EpCAM). These limitations could be overcome by employing a size-based CTC enrichment method (MetaCell).

We optimised the MetaCell method by spiking healthy blood with ~10,000 HCT116 cells and checking for expression of CTC markers (EpCAM and cytokeratins) by qPCR and immunostaining techniques. Both approaches demonstrated the capability of MetaCell to enrich for CTCs. The MetaCell sensitivity to detect CTCs in blood spiked with clinically relevant cell concentrations (10 cells), showed good recovery rates (~86%) which was comparable to blood spiked with 500 cells (~88%). We have now used MetaCell to enrich for CTCs from 17 CRC patient blood samples with different stages of the disease, and 8/17 patients were found to be CTC-positive (47.05%). We now aim to generate methylomes and transcriptomes from these enriched CTCs to identify the epigenetic signatures that promote CRC metastasis which could further facilitate exploring the role of these markers in response to therapy in patients in the future.

Bus Stop Presentations:

Abstracts with a number will also be presented as a Poster

1. Understanding active DNA demethylation specificity and function

Claudia I. Davies[1], Oscar Ortega-Recalde[1], Cassandra R. Glanfield[1], Andrew H. Wang[1], Matthias Bochtler[4], Tomasz P. Jurkowski[2,3,*] and Timothy A. Hore[1*], [1]University of Otago, Department of Anatomy, Dunedin, NZ; [2]Universität Stuttgart, Abteilung Biochemie, Institute für Biochemie und Technische Biochemie, Allmandring, Germany; [3]Cardiff University, School of Biosciences; Museum Avenue, Wales, UK; [4]International Institute of Molecular and Cell Biology in Warsaw (IIMCB), Poland; and Institute of Biochemistry and Biophysics PAS (IBB), Pawińskiego 5a, Warsaw, Poland*

Epigenetic memory in the form of cytosine methylation is essential for vertebrate development and formation of cellular identity. Active DNA demethylation catalysed by the Ten-Eleven- Translocation (TET) hydroxylases enables developmental potency, both *in vivo* and during the generation of induced pluripotent stem cells. Yet, how TET proteins are targeted to DNA remains unclear. Here, we rescued TET catalytic activity in embryonic stem cells by forced overexpression in a TET-deficient background and calculated the demethylation velocity for CG-containing hexamer sites. In doing so, we found TET has a strong preference for specific CG-containing hexamers, and that this occurred in a strand-specific manner. Notably, preferred TET targets included recognition sites for iconic methylation sensitive and rapid responding transcription factors, such as MYC and JUN/FOS. By comparing to published data, we found dramatic preference for the same motifs during global methylation erasure post-fertilisation and during and germline specification. Together these results imply that the TET catalytic domain is intrinsically selective, and this, in turn, helps shape which sites can hold epigenetic memory, and which tend to lose it.

2. Brothers discordant for gout have differing DNA methylation levels in inflammatory genes

Emily Ewers[1], Amanda Phipps-Green[1], Nicola Dalbeth[2], Lisa K Stamp[3], Tony R Merriman[1,4], Michelle C Thunders[5], Tanya J Major[1]

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Hyperuricaemia is a prerequisite for gout, but only ~30% of hyperuricaemic people will develop clinical symptoms. Logically, this difference in response to high urate levels is (partially) related to differences in immune response (as the instigator of a gout flare). However, traditional genetic studies have struggled to identify these differences. This study aims to assess whether development of gout is related to epigenetic regulation of immune- system related genes. A cohort of 24 men, 12 pairs of full-siblings discordant for gout, had their DNA methylation levels measured using an Illumina EPIC methylation chip. DNA methylation (CpG) sites from 30 innate immune system-related genes, five genes previously investigated in gout epigenetic studies, and four key urate-related genes were selected for analysis. This resulted in a total of 1,807 DNA methylation sites for analysis. A paired t-test was used to compare methylation levels at each CpG site. Significantly different methylation levels were seen between the paired brothers for 63 of the 1,807 CpG sites, covering 30 analysed genes. The most significantly different methylation sites were located within the LTβ (cg00731683: ~16% increase in men with gout, $P = 0.007$) and HIF1α genes (cg27273157: ~10% decrease in men with gout, $P = 0.0005$). LTβ encodes lymphotoxin-beta and is involved in the inflammatory response system via activation of the NF-κB immune pathway. The increase in methylation levels in the group of men with gout could result in lower LTβ expression, which has been shown to, counterintuitively, reduce inflammation. HIF1α encodes hypoxia-inducible factor 1-alpha and is involved in the regulation of inflammatory cell infiltration. HIF1α activity has been implicated in other inflammatory diseases and is upregulated in other forms of arthritis, consistent with the decreased methylation levels observed here. These results may have identified new genes of importance to gouty inflammation.

3. Sex, Brains & RNA

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Biological sex is a major risk factor for disease prevalence, symptoms and severity. Understanding how sex chromosomes and hormones affect disease-causing mechanisms will therefore shed light on the mechanisms underlying largely idiopathic sex-biased neurodevelopmental disorders such as ADHD, schizophrenia and autism. We have explored sex differences in gene and miRNA expression using embryonic mouse brains. At E15.5, RNA-seq showed 272 genes and small RNA-seq showed 109 miRNAs that significantly differed between male and female (n=3/sex, p adj<0.05). RT-qPCR confirmed differential expression of top genes and miRNAs, demonstrating that many miRNAs with pleiotropic functions in neurodevelopment show sex-biased expression. To determine how sex-biased miRNA expression arises in the developing mouse brain, we assayed regulatory regions near sex-biased miRNA genes for Esr2 binding: the main effector of sex steroid estradiol (E_2). ChIP-qPCR evidence of Esr2 binding suggests that sex hormones drive sex-biased miRNA expression. To further explore the relationship between miRNAs and E_2, we have conducted preliminary *in vitro* E_2 manipulation in primary neurons derived from male and female mice. These experiments will tease apart the factors driving sex differences in miRNA expression, enabling us to understand how certain miRNAs differ between the sexes and how they contribute to sex-biased neurodevelopmental disorders.

Investigating the mechanisms during whole-body regeneration of a marine colonial chordate

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Ascidians are colonial chordates living in the intertidal zone as filter feeders. Among these groups, botryllid ascidians like *Botrylloides diegensis* have various interesting characteristics including the ability to undergo whole-body regeneration (WBR). A vascular fragment (>~100 cells) is enough to create a new fully-functional adult within 8-14 days. Previously, we demonstrated that histone deacetylation activity is essential for the completion of WBR. Histone protein modifications regulate transcription factor access to the genomic DNA by changing chromatin structure. Thus, we performed ATAC-seq during the early stages of regeneration. Over 300 differentially accessible regions were found and the functional annotations linked these regions to genes involved in cell proliferation regulation and differentiation, such as *RIG1*. In addition to genome-wide studies, the roles of the chromatin remodellers during WBR including histone and chaperone targets are currently being investigated. We hypothesize that rapid changes to chromatin dynamics are essential for early cell differentiation from progenitor aggregates. Besides, we also carried out single cell RNA-seq from the adult tissue and aiming to get datasets from different points of regeneration to get information on cell fate. We found *NOTCH1* is a marker for stem-like cell population in the adult colony.

4. LncRNA-targeting antisense oligonucleotides and combination therapy for triple negative breast cancer

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Triple-negative breast cancer (TNBC) makes up 15-20% of breast cancer cases and lacks oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 (HER2). TNBC has poor clinical outcomes due to increased risk of metastasis and limited availability of targeted therapies. The standard treatment regimen for TNBC patients is combinations of chemotherapy, radiation therapy and surgery. There are two targeted therapies approved for TNBC patients, poly (ADP-ribose) polymerase inhibitors (PARPi) and immune checkpoint inhibitors, however, these are generally only used in patients with *BRCA1/2* mutation or patients with programme death-ligand 1 (PD-L1) positive tumours, respectively. Resistance to targeted therapies and chemotherapies are a major concern for TNBC patients and is another contributor to poor clinical outcomes. Developing more broadly applicable targeted treatments and overcoming drug resistance would greatly improve patient outcomes. One approach is through combining long non-coding RNA (lncRNA)-targeting therapies with currently available drugs. LncRNAs are an optimal drug target as they are commonly dysregulated in cancers and have cancer-specific expression. LncRNAs are characterised by being longer than 200 nucleotides and not having a significant open reading frame. One method of therapeutically targeting overexpressed lncRNAs is through DNA antisense oligonucleotides (ASOs), which bind via base complementarity and trigger RNase H- dependent degradation in the cell. The lncRNA *MALAT1,* has previously been shown to drive tumour progression in TNBC and has been implicated in chemo-resistance. Here, we are focusing on combination treatments of paclitaxel and olaparib with ASOs targeting *MALAT1*. The overall goal for this study is to identify how lncRNA-targeting therapies can be best combined with the current TNBC standard of care.

Capturing species-wide diversity of the gut microbiota and its relationship with genomic variation in the critically endangered kākāpō

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The gut microbiota plays an essential role in host health that has important implications for the conservation management of threatened wildlife. While factors such as diet, medication, and habitat are known to shape the microbiota, our understanding of the entirety of factors, including the complex role of the host genomic background, remains incomplete. Our research on the gut microbiota of the critically endangered kākāpō (*Strigops habroptilus*), a flightless parrot species endemic to Aotearoa New Zealand, represents, to our knowledge, the first study to describe the gastrointestinal bacterial diversity for virtually an entire species and to assess the relationship between gut microbiota and host genomic diversity in a highly threatened population. Here we report a 16S rRNA gene-based analysis of kākāpō faecal samples representing the gut microbiota for 84% of kākāpō with sequenced genomes. This survey was then leveraged with exceptional metadata to tease apart the impact of host genomic diversity and factors such as sex, diet, antibiotic treatment, disease status, habitat, and time of sampling on the kākāpō gut microbiota. We find evidence of a highly polygenic genomic architecture of the gut microbiota and further identify associations between gut bacterial diversity and functional biological pathways related to intestinal homeostasis, inflammation, immune response and metabolism. This improved understanding of the kākāpō gut microbiota – and its relationship with host genomics – can directly benefit kākāpō management and conservation by providing new insights into the role of the gut microbiome in kākāpō health and disease mitigation. Overall, we anticipate that an integration of microbiome studies in conservation research and management will improve our understanding of how the concept of One Health with its implications for human, animal and environmental welfare can be achieved.

Posters:

5. Investigating the role of immunotherapy in the treatment of metastatic oestrogen receptor positive breast cancer

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Breast cancer is the third most commonly diagnosed cancer type in New Zealand and the second leading cause of cancer-related deaths. Approximately 80% of breast cancer diagnoses present as oestrogen receptor-positive (ER+), with ~10% of these patients developing metastatic ER+ breast cancer (mBC). The estimated five-year survival rate of mBC is between 20 – 30% and is incurable with current treatments. Within the past decade, a rapid increase in the number of promising new therapies, including immunotherapies, has fuelled hope that effective treatment regimens may be identified. However, the almost impossibly large number of potential therapy combinations means that relevant pre-clinical models are required to assess which will be effective in mBC.

To investigate the use of anti-oestrogen therapies in combination with immunotherapies in mBC, trackable ER+ breast cancer-like SSM3 mouse cells were developed. Fluorescently labelled, antibiotic selectable, mammalian expression vectors encoding Antares2 or Firefly luciferase were introduced to SSM3 cells via non-liposomal lipid based or electroporation mediated transfection, respectively. Following propagation of antibiotic-resistant SSM3 populations, fluorescent microscopy and *in vitro* luciferase assays confirmed stable expression of the tagged plasmids. Vector integration did not impact cell proliferation or viability of transfected SSM3 cells, thus making them suitable to replace the SSM3 cell line in future *in vivo* studies. These cells are a key component in developing an ER+ mBC syngeneic 129S6/SvEv mouse model that will be used to track metastasis using bioluminescent imaging and evaluate the efficacy of combined anti-oestrogen and immunotherapies. This mBC mouse model will be one of the first trackable ER+ mBC models in mice with a fully intact immune system that replicates the biological events that lead to metastasis. Results from this investigation will give insight into a new avenue of breast cancer therapy and provide a tool for bridging the gap in available mBC therapies.

6. Cell-specific DNA methylation patterns of leukocytes and their implication for epigenetic analyses of health and disease

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Distinct cell types can be identified by their DNA methylation patterns. Much research over the last decade has focused on DNA methylation changes that occur in cancer or the use of cell-free circulating DNA in the blood plasma to identify cell death following trauma or organ transplantation. However, there has been little research into the differential methylation patterns between leukocytes and other tissues as a detection tool for inflammation in various contexts. We have identified several loci that are fully methylated in leukocytes but virtually devoid of methylation in a range of other mesoderm, ectoderm, and endoderm derived tissues. Additionally, we have created a panel of markers with differential methylation patterns between leukocyte subtypes. We validated these biomarkers using a combination of amplicon bisulphite sequencing and molecular inversion probes with PBMCs, buccal epithelium and lung organoid tissue. Interestingly, our methylation biomarkers have previously been identified as altered in a range of inflammatory diseases, including Alzheimer's disease, glioma, inflammatory bowel disease, and psoriasis. We hypothesise this is due to leukocyte infiltration rather than a feature of the diseased cells themselves. As such, we emphasise the importance of using pure cell populations when undertaking DNA methylation analyses to ensure accurate and interpretable results.

7. Kinetics of Decitabine-induced and TET-induced Demethylation in Pluripotent Cells

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DNA methylation is an epigenetic modification established during cellular differentiation, and can be disrupted in cancer. DNMT1 typically acts to maintain DNA methylation by catalysing the addition of methyl groups on hemimethylated DNA, enabling the transmission of methylation after DNA replication or repair. Decitabine, which is used in some cancer treatments, can be utilised to block DNMT1 activity and drive global DNA demethylation. To investigate claims that decitabine-induced passive demethylation has preference for particular CG sites for demethylation1,2, we undertook low-coverage whole genome bisulfite sequencing on decitabine-treated mouse embryonic stem cells. The kinetics of demethylation at all 256 CG-containing hexamers (nnCGnn) over a 48-hour period were determined, both within and outside of CpG islands (CGI). Once starting methylation levels and genomic location were taken into account, decitabine-induced demethylation of DNA showed no preference to certain CG-containing hexamers, contrary to prior claims, and thus erasure of methylation occurred evenly across the genome. In stark contrast, during active DNA demethylation the TET hydroxylase enzymes differentially target certain CG-containing hexamer motifs for rapid demethylation but leaves others unaffected. Significantly, both those sites most efficiently targeted, and those not affected by TET, appear to bind methylation sensitive transcription factors implying significant regulatory function. The evolution of this TET-target specificity at the vertebrate-invertebrate boundary is currently under further investigation.

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8. Assessing the impact of self-fertility in ryegrass

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The ability to inbreed plants is an important aspect of plant breeding as it provides the ability to purge deleterious alleles and fix beneficial traits. Self-pollination means high performing inbred lines can be developed with selected traits, which can be crossed to create F1 hybrids benefiting from heterosis; plants are more vigorous than either parental line. However, self- incompatible (SI) species rely on outcrossing and selection is done at a population level, making it difficult to select for lines with desired traits. *Lolium perenne* is a forage crop in NZ with a two-loci SI system (*S* and *Z*) that generally prevents self-pollination. *L. perenne* cannot self-fertilise and cannot fully exploit heterosis, leading to lower genetic gains compared to self- compatible (SC) grass crops. It is not uncommon for SI to be overcome, although a rare event in 2-loci systems, three independent SC *L. perenne* populations have been identified. A causal self-fertile (*SF*) locus has been mapped and shown to act separately from the *S* and *Z* loci, enabling self-fertilization. In this project we have introgressed the *SF-locus* from European germplasm into elite NZ germplasm and are using Genotyping-by-Sequencing (GBS) to assess reduction of heterozygosity and phenotypic impacts of inbreeding in *L. perenne*.

9. APOBEC3B-AS1 is associated with the pluripotent stem cell state and the absence of mutagenic APOBEC3 mRNAs

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Sequencing data from tumour samples have implicated APOBEC3 enzymes as a significant source of mutations in cancer. Regulation of mRNA transcripts that encode these mutagenic enzymes has not been well characterised. We hypothesised that the lncRNA *APOBEC3B-AS1* is a functional natural antisense transcript that may be involved in regulation of *APOBEC3* transcripts. *APOBEC3B-AS1* homologues were found in great ape species, but not other closely related primates, indicating recent evolution of this transcript. Using single cell datasets, *APOBEC3B-AS1* expression was detected in late blastocyst cells of developing embryos, associated with TGF-β/Nodal signalling. Cells expressing this transcript were found to be absent for expression for *APOBEC3A* and *APOBEC3B* mRNAs, which encode mutagenic APOBEC3 enzymes. Attempts at isolating the transcript by RT-PCR using cDNA generated from breast cancer cell lines were unsuccessful. The sequence conservation of *APOBEC3B-AS1* exons in extant species implies that these elements may have retained function. Due to the associations with pluripotent stem cells found in the single cell data, we hypothesise that this transcript is expressed to downregulate mutagenic enzymes in stem cell states. Downregulation of this transcript may then lead to increased APOBEC mutagenesis in stem cells, driving cellular heterogeneity and tumourigenesis.

10. Getting to know your shy neighbour: population genomics of kanakana/piharau

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Geotria australis (pouched lamprey, kanakana/piharau) is a culturally and ecologically significant jawless fish that is distributed throughout Aotearoa. Despite its importance, much remains unknown about historical relationships and gene flow between populations of this cryptic species within Aotearoa. To help inform management, we completed the first comprehensive population genomics analysis of kanakana within Aotearoa using targeted gene sequencing (Cyt-b and COI) and genotyping-by sequencing methods (GBS). Utilizing 16,000 genome-wide single nucleotide polymorphisms (SNPs) derived from GBS and sequence data from Cyt-b (766 bp) and COI (589 bp), we reveal low levels of structure across 186 individuals from 10 populations spanning the species range within Aotearoa. F- statistics and STRUCTURE suggest a single panmictic population, and Mantel and EEMS tests reveal no significant isolation by distance. This implies either ongoing gene flow among populations or recent shared ancestry among Aotearoa kanakana. We can now use the information gained from these genetic tools to assist managers with monitoring effective population size, planning possible translocations (and/or propagation programs), and managing potential diseases (including syndromes such as Lamprey Reddening Syndrome).

11. Unraveling the mechanisms involved in the co-regulation of breast cancer associated genes at the 6q25.1 locus

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Breast cancer is the most commonly diagnosed cancer affecting women world-wide. Approximately 80% of cases overexpress the estrogen receptor (ER), suggesting that oestrogen signaling is an important driver of tumorigenesis in these patients. Despite this, the mechanisms through which ER expression is regulated are not well known. ER is encoded by the *ESR1* gene on chromosome 6q. A noncoding variant associated with genetic breast cancer susceptibility, which occurs upstream of *ESR1* at 6q25.1, was identified by Stacey *et al.* in 2010 (Stacey *et al.*, 2010). The rs77275268 variant results in a C to T base-shift within a partial-methylated CpG island, which is a known binding site of the CCCTC binding factor (CTCF). CTCF is a transcription factor, which binds to conserved unmethylated sequences and frequently colocalises with cohesin. It is thought that CTCF, in conjunction with cohesin, play an important role in genome architecture through the maintenance of chromatin loops. An aberration in a CTCF binding site or a change in the methylation status of that site may led to alternate chromatin loops and dysregulated gene expression. The expression of *ESR1* in ER positive tumours is highly correlated with the expression of three upstream genes which encode, *ARMT1*, *CCDC170* and *RMND1*, and this appears to be regulated at a transcriptional level (Dunbier *et al.*, 2011). We hypothesis that the rs77275268 variant could alter the three-dimensional organization of these correlated genes, which may contribute to genetic breast cancer susceptibility. To investigate genetic interactions within the 6q25.1 locus we have performed 4C-seq and ATAC-seq in MCF-7 cells. To determine if these interactions are mediated be cohesin, cells were treated with an siRNA which targets the Rad21 subunit. Preliminary results suggest that Rad21 knockdown leads to alternate interactions within this region, and a downregulation in chromatin accessibility.

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12. Sex-dimorphic regulation of *Lhx9* expression in the bi-potential gonad

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Understanding the genetic networks underlying reproductive system development is crucial for improving diagnosis and management of disorders of sex development. Both the male and female reproductive systems form from a progenitor structure called the genital ridge that remains bi-potential until E11.5 in the mouse. The transcription factor *Lhx9* is essential for genital ridge formation and is expressed in both sexes prior to transcription mediated sex-differentiation initiated by *Sry*. We have characterised the expression of *Lhx9* isoforms in the bi-potential genital ridge and uncovered early signs of sex-dimorphic gene regulation prior to sex differentiation. Targeted bisulfite sequencing analysis of three CGIs associated with *Lhx9* showed that the male gonad was significantly more methylated than the female gonad across two of the three CGIs ($p < 0.0001$). We hypothesised that this differential methylation may regulate expression of the three isoforms of *Lhx9* as well as the binding of other transcription factors in the gonad. ChIP- qPCR showed increased enrichment of transcription factor Wt1 binding in the female E11.5 gonad at the same CGIs previously shown to be less methylated in the female. Together these results point to sex-dimorphic regulation of gene expression in the bi- potential gonad established prior to sex differentiation.