Stem cells and tissue regeneration

1- **The role of FGFR-4 in skeletal muscle homeostasis and regeneration**

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Fibroblast growth factor receptor 4 (FGFR-4) is specifically expressed in skeletal musde development and adult muscle stem cells (satellite cells). However, its exact role in skeletal muscle remains unknown. Our aim is to assess the effects of Fgfr4 deletion in a ubiquitous versus a conditional knock out model to dissect the muscle-specific role of FGFR-4. Analysis of skeletal muscle in Fgfr4 knockout mice show loss of muscle mass, increase of scapular brown adipose tissue and changes in lipid compositions in the muscle, highlighting its metabolic importance. Cardiotoxin injury model in this knockout have failed to recapitulate the regeneration defect reported previously. Additionally, we have developed a conditional inducible deletion model that selectively knocks out Fgfr4 in satellite cells. RNA-seq analysis of these satellite cells may elucidate the signaling role of FGFR-4. Understanding these external signals may provide us with new tools to enhance regeneration in muscle ageing and disease.

2 **Early inflammatory signals drive neural stem cell activation during zebrafish spinal cord regeneration**

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Unlike mammals, the zebrafish can functionally recover from spinal cord injury (SCI). Neural regeneration in the zebrafish spinal cord is driven by the activation of quiescent neural stem cells called ependymal cells. Ependymal cells in mammals also act as stem cells after injury but fail to give rise to neurons. We have shown that ependymal cells in the zebrafish give rise to a lineage of transit amplifer progenitors (TAP) that rapidly migrate to the lesion site and contribute to neurogenesis. The signals that activate ependymal cells are largely unknown. We have shown that suppressing the immune response following SCI blocks spinal cord regeneration while stimulating the immune response enhances regeneration. To investigate the role of inflammation on ependymal cell activation we treated zebrafish with immunosuppressants and immunostimulants and quantified ependymal cell proliferation using Edu (5-Ethynyl-2'-deoxyuridine) labelling experiments. We found that temporal treatment using the glucocorticoid dexamethasone, an immunosuppressant, directly reduced ependymal layer proliferation, suggesting that early inflammation provides cues for ependymal cell activation. Furthermore, dexamethasone treatment at a later time point directly reduced TAP activation following injury. RNA-sequencing analysis of lesioned zebrafish identified prostaglandin (PGE) signaling as a candidate inflammatory pathway involved during neural regeneration. Interestingly, inhibition of prostaglandin signalling, using a PGE2 inhibitor and EP4 receptor antagonist independently reduced ependymal layer proliferation, demonstrating that prostaglandin signaling is required for ependymal cell activation. Taken together, these results suggest that early inflammation plays a significant role in orchestrating the activation of ependymal cells and TAP's that are required for neural regeneration.

3 **Characterising the Role of Tsc22d3 in Germline Stem Cell Function**

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Transforming growth factor-β1 stimulated clone-22 (Tsc-22) domain family (Tsc22DF) proteins act as pleiotropic cell regulators and contain conserved leucine zipper and tuberous sclerosis complex (TSC)-box motifs. In mouse and human, the family is encoded by four different loci – Tsc22d1, Tsc22d2, Tsc22d3 and Tsc22d4. TSC22DF members are known to be involved in diverse physiological functions including regulation of cellular growth, development, tumour suppression and inflammatory responses.
Within the adult testis, spermatogonial stem and progenitor cells (SSPCs) have self-renewal capacity and produce mature gametes via spermatogenesis. Regulation of SSPC maintenance and differentiation is critical for tissue homeostasis and fertility. Previous studies based on a constitutive knockout model have demonstrated that Tsc22d3 (also known as Gilz) plays an essential role in SSPC maintenance and male fertility. However, the mechanisms by which Gilz regulates SSPC functions are poorly understood. To better define GILZ function in adult SSPCs, we developed an inducible Gilz knockout model. Strikingly, acute deletion of Gilz results in exhaustion of self-renewing population within a week post-tamoxifen treatment, suggesting the importance of GILZ in adult germline stem cell maintenance. We therefore propose to investigate the role of GILZ and mechanisms regulating germline stem cell function.

Organogenesis

4- **Identification of novel epigenetic regulators of lung development**

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The development of the lung is a highly regulated and complex process that is not fully characterised. Lung development starts at embryonic day (E) 9.5 in the mouse and E26 in the human. Although there have been studies into the development of the lung, many of the mechanisms regulating lung organogenesis are still unclear. In recent years, the importance of epigenetic regulators in embryogenesis has been established but epigenetic control of lung morphogenesis is largely underexplored. We aim to identify epigenetic modifiers that are critical for lung development and to establish their mechanism of action.

We have developed a novel E11.5 epithelial lung cell culture system that allows us to keep primary cells in culture for 14 days. Using this platform, we performed a short-hairpin RNA (shRNA) knockdown screen, targeting 200 genes (~10 hairpins/gene) that are involved in enzymatic epigenetic regulation. DNA sequencing at three time points enabled us to identify an increase or decrease in representation of the hairpins over time. The top genes identified in the

screen were validated using in vitro and ex vivo culture systems to determine their role on lung progenitor activity and branching morphogenesis.

These experiments led to the identification of Aurora kinase B (Aurkb) as the most interesting candidate gene. Aurkb exerts a dual role as a regulator of cell cycle and as an epigenetic regulator through phosphorylation of histones. Reduction of Aurkb in vitro abrogates growth of lung epithelial progenitor cells and chemical inhibition of Aurkb leads to defects in lung branching morphogenesis as assessed by ex vivo culture assays. Disruption of Aurkb either by short-hairpin RNA or by chemical inhibition causes defects in cell cycle and an accumulation of cells in G2/M of the cell cycle. We have obtained a mouse with a conditional allele of Aurkb, to specifically address the role of Aurkb in the developing lung epithelium in vivo.

Given that Aurkb is often overexpressed in lung cancer, future work in our lab aims to define the role of Aurkb in cancer and lung organogenesis. Knowledge gained from studies in the embryo can help us understand how developmental genes can become dysregulated later in life leading to disease. Our work highlights the power of interrogating lung development to provide insight into lung diseases and to potentially reveal novel therapeutic targets.

5 **Elk1 in Congenital and Late Onset Cardiac Disease: at the Heart of the Matter**

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Elk1 is an ETS Class I, TCF subfamily transcription factor known as a well- established downstream effector of the MAPK pathway. Recent *in-vitro* evidence places it in the context of the cardiogenic transcription factor network, although its *in-vivo* role in cardiogenesis remains unexplored.

We provide the first *in-vivo* evidence of Elk1 in cardiogenesis using a zebrafish mutant with disrupted DNA binding domain (*elk1* $^{-543/-543}$) and cardiac defects including valve displacement/elongation and hypertrophic/hyperplastic changes. *elk1* $^{-543/-543}$ are predisposed to early embryonic death, with high incidence of heart looping defects and accelerated growth among survivors.

RNA-seq at 6dpf provides insights into the basis of heart defects, indicating upregulation of MAPK pathway genes and downregulation of *trim63a*, encoding a homeostatic protein involved in reducing muscle mass, dysregulation of which is associated with Hypertrophic Cardiomyopathy (HCM) in humans.

MAPK upregulation is commonly associated with HCM although the fundamental basis of this relationship is not completely understood. We provide mechanistic insight, suggesting MAPK perturbations could converge via the TCFs at a (putative) *trim63a* enhancer, downregulating *trim63a* to mediate HCM.

Early developmental RNA-seq indicates loss of Elk1 function causes downregulation of tumor suppressor genes. We hypothesize this promotes embryonic survival via non-optimal pathways,

and fundamentally underlies observed defects.

The sum of changes in *elk1* $^{-543/-543}$ mimic a group of congenital syndromes known as "RASopathies" in humans. Our data provides important insights into the time line of molecular events underlying RASopathies/MAPK pathway defects and their relationship to molecules imperative in heart patterning and homeostasis.

Functional Genomics

6 **Generating a repertoire of cardiac regulatory elements in development and disease**

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Congenital Heart Diseases (CHDs) are the biggest killer of kids under one year of age affecting about 1% of new-born babies (more than 2,000 every year in Australia alone). The only known treatment is invasive surgery or heart transplantation.
Strikingly, in 80% of cases, the cause of CHD is unknown – even in cases where there is a clear family history and the genes appear to be normal. Hence, we hypothesise that CHD is a so-called "enhanceropathy" where erroneous gene regulation is causing heart defects.
We aimed to identify the cardiac-specific regulatory landscape in the developing zebrafish. Zebrafish have a simple, yet similar heart to humans, they generate large quantities of offspring that is transparent and hence easy to screen.
We successfully created the first cardiac cell type-specific repertoire of regulatory elements (REs) by performing ChIP-seq (Chromatin immunoprecipitation followed by high-throughput sequencing) on a small number of sorted cardiomyocytes using an H3K4me1-antibody that indicates enhancer regions.
Our zebrafish dataset of cardiac-specific regulatory elements will be useful for cross-species comparisons to answer questions about heart evolution but also for clinical studies to identify ultra-conserved REs from human to fish as candidates for mutations in congenital heart diseases. Moreover, our dataset will be a great resource for researchers to create new cardiac-specific reporter lines.

Evolution and Development

7 **Molecular mechanisms that generate muscle fibre type diversity during vertebrate evolution.**

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Vertebrate skeletal muscle is composed of a complex array of muscle fibres that differ in physiological type, size, and organisation, depending on mode of locomotion. In the zebrafish (*Danio rerio*, teleost), axial muscle formation includes the formation of distinct epaxial and

hypaxial muscle groups separated by a horizontal myoseptum. Muscle pioneer cells (MP, slow muscle fibre precursors) are medially aligned to this myoseptum in each myomere, of which they define structure and function. Signalling factors including Hedgehogs, BMPs, and FGFs, function in regulating high expression levels of the transcription factor *engrailed2a*, the earliest marker for MPs. It is unclear how evolutionarily broad this MP developmental model stands amongst vertebrates, therefore understanding molecular signals involved in muscle fibre type determination within vertebrate model (zebrafish) and non-model basal gnathostome species (Epaulette shark (*Hemiscyllium ocellatum*, elasmobranch), and Elephant shark (*Callorhinchus milii*, holocephalan)), will provide evolutionary insight into vertebrate muscle development and diversity. Preliminary *in situ* hybridisation data from our lab shows a dorso-ventral broad *en1* myotome expression pattern in Elephant shark and Paddlefish, similar to chick and mouse data, however unlike that of zebrafish. This suggests differences in *engrailed* myotome expression regulation may exist between vertebrate taxa.

Cell adhesion, migration and guidance

8 **Pushing the envelope: Novel zebrafish models for investigating neutrophil nuclear dynamics in vivo**

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Nuclear plasticity is a key determinant of cellular biomechanics and function. Neutrophils, highly migratory and plastic leukocytes, have distinct nuclear morphology that is believed to reflect an extreme functional requirement for nuclear plasticity as they migrate rapidly through tight tissue spaces. Neutrophils are characterised by multi-lobulated nuclei, with specific composition of lamins in the nuclear envelope. Lamin intermediate filaments form the nuclear lamina, a structural meshwork supporting the nucleus and known to affect nuclear stiffness. Unlike other cell types, neutrophils down-regulate Lamin A/C and Lamin B1 whilst maintaining high levels of Lamin B2; suggesting that this lamin composition imbues greater nuclear flexibility. The current project will investigate how nuclear composition and dynamics influence neutrophil flexibility and mobility. Zebrafish reporter lines will be used for 4D *in vivo* imaging of neutrophil nuclear plasticity. To delineate the nuclear envelope, *mpx:eGFP*-Lamin transgenes are being introduced into the Tg(*mpx:H2Bcerulean-mko2CAAX)* line, adding a green-fluorescent nuclear envelope to the blue chromatin and red cytoplasmic fluorophores of this line. Confocal and lattice lightsheet microscopy will be employed to capture neutrophil migration, phagocytosis, and NET release, and morphometric analysis will assess the functional effects of perturbed lamin expression. Preliminary confocal imaging of Tg*(mpx:eGFP-laminB2;H2Bcerulean-mko2CAAX)* confirms delineation of the neutrophil nuclear envelope. Furthermore, effective Crispr/Cas9 gene targeting has been achieved for *laminB1, laminB2,* and *lbr,* and established mutant lines will be used to examine the requirement of lamins and Lbr in neutrophil nuclear plasticity and function. As Lamin B receptor (*LBR*) mutations in humans cause neutrophil hypo-lobulation (the Pelger-Huët anomaly), an *LBR* zebrafish mutant will be made to alter neutrophil nuclear lobulation and assess its impact on cell function. This project aims to provide the most detailed descriptive and functional analyses of neutrophil nuclear dynamics to date.

Signaling pathways in embryonic development

9 **Function of Aspartate Beta-Hydroxylase in zebrafish embryo**

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Hedgehog signalling is considered one of the most mutated pathways in cancer, and the secretion of Hedgehog must be strictly controlled to ensure normal development. The secretion process, however, remains unclear since we do not know how such a strongly hydrophobic morphogen like hedgehog protein can be secreted to target distant cells. To find proteins that are involved in Hedgehog secretion, we performed tandem affinity purification followed by mass spectrometry and isolate proteins that can bind Scube2, an essential factor in hedgehog secretion. In the screen, aspartate beta-hydroxylase (ASPH) was isolated. This enzyme can hydroxylate domains on several factors required for Hedgehog secretion. We hypothesize that ASPH could facilitate the interaction between Hedgehog protein and proteases to release the morphogen on the membrane.

The project started with examining the expression pattern of *asph* in zebrafish embryos. In addition to published expression patterns, we found that this gene was expressed in regions that give rise to muscle progenitors. We also tested if there was a correlation between Hedgehog signalling and *asph*. The levels of *asph* transcript were higher, and the expression domain was expanded when Hedgehog signalling was inhibited. Unexpectedly, we found a second *asph* gene, which could be the orthologue of mammalian ASPH in zebrafish. We are examining the function of this gene by analysing *asph* mutant phenotypes. At the moment, we observed that muscle fibers crossed somite boundaries in *asph* mutant, suggesting a role of this gene in muscle development. *In vitro*, the data indicates that ASPH and Scube could co-localize on the membrane, which is in line with our hypothesis.

In conclusion, my results allow a possibility for ASPH function in muscle development. It can contribute to the model that Hedgehog is secreted as a soluble monomer, without any lipid residues.

10 **Additive Effects of the Endocannabinoid and Retinoic Acid Pathway in Bone Formation in Zebrafish**

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Osteoporosis is a metabolic bone disease with huge implications on personal and societal morbidity. It results in an imbalance between the cells of the bone remodelling cycle, where the bone forming cells, osteoblasts, are reduced and the bone-resorbing cells, osteoclasts, are increased. Current therapeutics for osteoporosis manage symptoms and prevent further deterioration, but none act to reverse the imbalance. The cannabinoid (CB) and the retinoic acid (RA) pathways, have already been demonstrated to alter osteoblast gene transcription and bone mineralization, for example, CB receptors, and mice treated with CB antagonists had increased bone growth. We hypothesised that modulation of these two pathways could modify bone metabolism in developing vertebrates. Zebrafish embryos and adult medaka were

exposed to chemical treatments modulating these pathways, and their effects on bone formation, osteoblast gene expression, and osteoclast activity were investigated. The RA pathway was shown to affect bone formation. In particular, this study demonstrates a varying effect on osteoblast gene expression depending on the timing of exposure, whereby up-regulating the RA pathway decreases early osteoblast gene expression and up-regulates late osteoblast gene expression. Additionally, the CB pathway was shown to modulate bone metabolism, whereby down-regulating the CB pathway leads to increased bone formation and osteoblast gene expression. Finally, the study demonstrated an additive effect on bone metabolism when embryos were treated with a combination of treatments modulating these pathways together. For the first time in any vertebrate, this study demonstrates that modulating the RA pathway and CB pathway together affects bone formation, osteoblast gene expression, and osteoclast enzyme activity. In addition, it provides evidence that the RA and CB pathways could be modulated together in order to develop a novel therapeutic for osteoporosis, which would aim to increase bone formation, increase osteoblast number and decrease bone formation.

11 **The influence of Bisphenol A exposure on lipid usage during embryogenesis**

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Bisphenol A (BPA) is a chemical compound commonly found in polycarbonate plastics. It has been banned by many countries, especially in the production of baby products due to its potential metabolic effects; however it is still used in Australia. Currently, the involvement of BPA in lipid deposition and adipogenesis during early development is unknown. We investigated if direct BPA exposure at physiological doses (nano molar range) could alter lipid deposition or composition in zebrafish embryos. The quantity of lipid deposition was observed via Oil Red-O staining (ORO), a stain that specifically binds to neutral lipids and triglycerides, which was quantified using a new technique we developed based on optical density. We confirmed that BPA exposure led to a 2-fold increase in ORO staining; demonstrating for the first time BPA significantly increases lipid deposition during embryogenesis in vivo. To measure the changes in specific lipid species, lipidomics analysis was performed to analyse the changes across 24 lipid classes. Lipidomics analysis revealed that in BPA exposed embryos there was significant up regulation in the classes of diglycerides and phosphatidylinositols (PIs). Phosphatidylinositols are involved in cell and lipid signalling; hence we wanted to elucidate the downstream targets of PIs and its links to BPA induced obesity. Therefore we investigated the effect on PI synthesis after BPA exposure, as well as the changes in lipidogenesis in embryos that were exposed to a combination of BPA and LY294002, a potent inhibitor of phosphoionositide 3-kinases (PI3K). The effect of BPA on various enzymes involved in the PI3K pathway, such as class 1, 2 and 3 PI3Ks, was also explored to determine if the action of BPA is mediated by the PI3K-AkT pathway in lipidogenesis during embryogenesis, a pathway that plays a crucial role in survival and growth in response to extracellular signals. This work will demonstrate how BPA exposure during early life can affect lipid usage in the developing embryo.

12 **Zebrafish as a model of retinopathy under high-glucose exposure: Long-term implications of embryonic retinopathy extending into adulthood**

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Retinopathy, a major complication of diabetes mellitus causes progressive damage to the retina that ultimately leads to partial or total vision loss. Hyperglycaemia associated with diabetes appears to be a critical factor that initiates the onset of retinopathy by damaging the blood vessels in the retina, which leads to the leakage of blood and other fluids, and the release of angiogenic growth factors like VEGF. This initiates a cycle of events that lead to the formation of leaky and fragile retinal blood vessels, haemorrhages, retinal detachment and vision loss. Diabetic individuals have a high risk of developing diabetic retinopathy during their lifetime. However, the impact of hyperglycaemia due to conditions like gestational diabetes on foetal retinal development and the long-term implications extending into adulthood remains unknown. We aim to address this gap in knowledge with a novel model of retinopathy using zebrafish (Danio rerio). Briefly, Wild type (WT) and Tg (fli1:EGFP) X Tg (mpeg:DsRed) embryos were exposed to 0, 4 and 5% D-glucose in E3 media at 3 hours post fertilisation (hpf). The environment of the zebrafish embryos was alternated between a 0% D-glucose, and 4 and 5% D-glucose exposure every 24 h until 5 days post-fertilisation (dpf). Histological and confocal analysis confirms the detrimental effects of high glucose on embryonic retinal development as indicated by changes in retinal cell layer thickness and blood vessel leakage, hallmarks of retinopathy. Similarly, Tg (shh:GFP) and Tg (gfap:GFP) embryos exposed to glucose show marked reduction in retinal ganglion cells and glial cells in the retina. In addition, Tg (fli1:EGFP) embryos were exposed to glucose until 5 dpf using the established method of alternating glucose exposure and raised until adulthood (3.10 mpf) under standard conditions and fed a normal diet to study the long-term effects of embryonic glucose exposure on adults. Tg (fli1:EGFP) adults showed significantly greater BMI, Fat and retinal angiogenesis as compared to unexposed controls at 3.10 mpf. These data highlight that the developing retina is vulnerable to the effects of high levels of glucose with the detrimental effects extending into adulthood. Therefore, this novel model of retinopathy will find immense use in testing new therapeutics, and identifying novel biomarkers that will allow screening for at-risk population groups for timely therapeutic intervention.