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Exploring the Role of FGFR2c in the Pathogenesis of Craniofacial Birth Defects

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Craniosynostosis is a common feature of craniofacial birth defects. It is characterised by premature fusion of the cranial sutures in the developing calvarium. One of the causes of craniosynostosis is associated with pathogenic FGFR2 signalling, and is caused by mutations in the *FGFR2* gene which are thought to be activating in nature. In particular, mutations in the IIIc isoform (e.g. FGFR2c-C342Y) contribute to several craniofacial abnormalities including human Crouzon syndrome. However, little is known about how FGFR2c signalling regulates suture patency. This study aims to uncover the downstream effects of pathogenic FGFR2c signalling involved in craniofacial abnormalities using two mouse models. To address its role in calvarial development, conditional overexpression of Fgfr2c in a novel transgenic mouse reveals bones derived from the neural crest lineage were reduced in size. Contrary to expectations, craniosynostosis was absent. Moreover, these mutants exhibit global skeletal hypoplasia and cleft palate. The complexities of FGFR2c signalling highlight the need to uncover novel downstream targets. Ongoing work includes RNAseq transcriptome analysis of coronal sutural mesenchyme acquired using laser captured microdissection from the Crouzon mouse. Uncovering novel biomarkers will therefore improve the aetiology of FGF signalling in normal craniofacial development and its related pathologies.

Super-resolution imaging and 5C reveals *Shh*/ZRS spatial proximity and limb ZPA-specific co-localisation

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Limb-specific expression of *Shh* is regulated by the long-range (~1 Mb) ZRS enhancer. Murine activation of *Shh* occurs from E10 until E11·5 within the zone of polarizing activity (ZPA) at the distal posterior limb bud and this restricted spatiotemporal expression domain is essential for the correct formation of the autopod. To investigate the role of higher-order genome organisation in the long-range regulation of *Shh* we determined the chromatin conformation over a ~2Mb region in expressing and non-expressing tissues by fluorescence in situ hybridization (FISH) and chromosome conformation capture carbon copy (5C).

FISH combined with conventional and super-resolution light microscopy identified significantly greater *Shh*/ZRS co-localisation in ZPA cells compared to other limb regions. Intriguingly, median spatial distances between *Shh* and the ZRS were consistently shorter than distances measured between either and a neural enhancer located nearly midway on the intervening genomic region in expressing and non-expressing cells. Analysis of E11·5 anterior and posterior limb bud cell populations by 5C identified a topologically associating domain (TAD) over the genomic region from *Shh* to the ZRS and enriched interaction between the gene and a locus ~20 kb from the ZRS, which following 5C analysis of non-limb tissues we suggest is a constitutive conformation.

The role of Gas1 in murine salivary gland development

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Salivary gland (SG) are highly branched organs that arise as a thickening of the oral epithelium that invaginates in the underlying mesenchyme. SG development requires reciprocal interactions between the oral epithelium and neuronal cells. The neuronal precursors coalesce around the epithelial bud stalk around E12 giving rise to the submandibular parasympathetic ganglion and its axonal projections extend towards the epithelial endbuds. Gas1 is a key component of Shh signalling which is crucial for SG development. Gas1 has recently been shown to have a key role in axonal guidance. The aim of this project is to address the role of Gas1 in SG development. We identified that *Gas1* is expressed in SG epithelium and mesenchyme since early developmental stages. Interestingly, *Gas1* null mice display hypoplastic SGs with defects in branching. In addition, disrupted innervation was observed accompanied by ganglion hypoplasia and misplacement. These data suggests a critical role of Gas1 in epithelial patterning and SG innervation.

Elucidating the Mechanisms of WT1 Glomerulopathy

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Podocytes play a key role in maintaining glomerular permselectivity. Podocyte injury may lead to proteinuria, focal segmental glomerulosclerosis (FSGS) and diffuse mesangial sclerosis (DMS). DMS and FSGS are associated with mutations in Wilm's tumour 1 (*WT1*) as well as in ectopically Notch activated developing and differentiated podocytes of the murine glomerulus. My project investigates the mechanisms underlying mammalian *Wt1* glomerulopathy and whether podocyte Notch activation modifies transcriptional responses to *Wt1* as a pathomechanism of podocyte injury.

An inducible transgenic mouse model of *Wt1* glomerulopathy ($CreERT2^{+/-};Wt1^{fl/f}$) was analysed in early and late glomerulosclerosis; Day 4 (D4) post-tamoxifen induction (P.I), TEM revealed focal foot process (FP) effacement associated with normal glomerular morphology on light microscopy in $Cre(+);WT1^{fl/fl}$ (mutants) compared to normal FPs in $Cre(-);Wt1^{fl/fl}$ (controls) transgenic mice. Urine albumin/creatinine ratio (UA/UC) was significantly higher in mutants by D12 P.I. FSGS was noted at D5 P.I. and global glomerulosclerosis was evident by D8 P.I. to D12 P.I. A significant increase in Podoplanin positive, Caspase-3 positive glomerular cells in mutants was observed by D5, indicating apoptosis. Furthermore, cleaved Notch-1 and Hes1 were expressed in podocytes of D5 P.I. mutant glomeruli. Glomerular RNAseq analysis at D4 P.I revealed increased expression of *Ascl1*, a transcription factor known to induce the Notch ligands, Delta and Jagged. Future work will validate *Ascl1* function in FSGS and similar mechanisms will be addressed in human WT1 glomerulopathy.

Apoptotic Signalling in Salivary Gland Lumen Formation

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Salivary glands (SG) are essential for the maintenance of oral health by providing lubrication and antimicrobial protection to the mucosal and tooth surfaces. Saliva is delivered to the oral cavity by a multifunctional ductal system, which are formed as solid tubes that undergo cavitation to create lumens during development. Apoptosis has been suggested to play a role in this cavitation process along with changes in cell polarity. We observed that apoptosis occurs from the very earliest stages of mouse SG development, much earlier than previously reported. Apoptotic cells were found in the centre of the first epithelial stalk at early stage E12·5 using both TUNEL staining and Cleaved Caspase-3 immunofluorescence. The presumptive lumen space was highlighted by the co-localisation of a predictive lumen marker, Cytokeratin 7 (K7). At E14·5, as lumens started to form throughout the glands, apoptotic expression

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decreased while K7 remained positive. *In vitro* inhibition of all caspases in E12·5 and E13·5 SG resulted in wider ducts compared to the controls and a defect in lumen formation. In contrast no such defect was observed at later stage E14·5. Our data indicates that apoptosis is involved during early stages of gland formation (E12·5 onwards) and appears important for shaping the forming ducts.

Cell-matrix interactions and cell dynamics of neuroepithelial bending during mouse spinal neural tube closure

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During spinal neurulation, the neuroepithelium elevates to bring together the apposing neural folds, with bending at the median hinge point (mode 1), and at paired dorsolateral hinge points (modes 2 and 3). We found that transition from mode 1 to modes 2/3 is characterized by a more than 2-fold increase in cell number in the dorsal neuroepithelium. This cell number increase exceeds the predicted cell number from cell cycle analysis, leading us to hypothesize that neuroepithelial cells may translocate from ventral to dorsal regions in the elevating neural fold. We tested this hypothesis by vital fluorescent dye labelling in whole embryo culture, and demonstrated that cells from the mid-ventral neural plate eventually reach the most dorsal regions during the onset of dorsolateral bending. According to our model, the neuroepithelium acts as a biphasic structure, with low cell density ventrally and high cell density dorsally, suggesting that dorsolateral bending occurs via buckling of the neuroepithelium at the transition point between the two regions.

We are also investigating the potential driving mechanism underlying this translocation by studying the role of the extracellular matrix (ECM) and their integrin receptors, at both mRNA and protein level, and reconstructing 3D-networks of fibronectin and laminin using Apotome technology.

An angiogenic niche regulates embryonic hindbrain neurogenesis

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Neurons are born throughout life in two germinal regions of the adult rodent brain that are associated with blood vessels, and blood vessel growth and vessel-derived factors are thought to regulate adult neurogenesis. However, it is not known whether a vascular niche also regulates developmental neurogenesis. Here, we introduce the mouse embryo hindbrain as a powerful model to study the relationship between neural progenitor cell (NPC) behaviour and growing blood vessels. We show that a subventricular vascular plexus (SVP) extends through the germinal zone populated by NPCs, and that the peak in NPC mitosis follows a surge in SVP growth. Agreeing with a spatial and a temporal relationship of vessel growth and neurogenesis, mice genetically defective in SVP formation have a precocious peak in NPC mitoses that is followed by a premature loss of proliferative activity and impaired hindbrain growth. We conclude that hindbrain vasculature regulates the pattern of NPC division.

Analysis of 3D embryo data for the International Mouse Phenotyping Consortium

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The goal of the International Mouse Phenotyping Consortium (IMPC) is to functionally characterise every mouse gene using standardised phenotyping tests. Around 35% of the analysed knockout lines are embryonic/perinatal lethal or subviable. To determine the cause of lethality, embryos are imaged using a variety of 3D imaging modalities.

Manual annotation of aberrant morphology is not feasible due to the large quantities of data that will soon be submitted, and subtle phenotypic effects could easily be missed. Automated analysis methods have already been developed and are in use by the IMPC. This current work is aimed at creating a lightweight, and fast automated pipeline that will be easy for developmental biologists to set up and use on a standard desktop PC. By registering mutants and wild types towards a population average, it can identify abnormalities using intensity differences between images as well as morphometric and texture-based analysis. Current work includes testing the robustness

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of the pipeline and analysing the effect of varying the number of specimens on the ability to detect known phenotypes. This is especially important for large scale projects as production and imaging costs can result in low numbers of embryos.

Mosaic activating mutations in *GNA11* and *GNAQ* cause Phakomatosis Pigmentovascularis and extensive dermal melanocytosis

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Birthmarks are often overlooked, however they can be a sign of underlying genetic disorders. Dermal melanocytosis (Mongolian Blue Spots) usually fade, but are occasionally large, permanent and associated with extra-cutaneous abnormalities. Appearance together with vascular birthmarks is the hallmark of Phakomatosis Pigmentovascularis (PPV), a group of conditions which can be associated with ophthalmological and neurological symptoms as well as overgrowth and malignant complications. In this study we have identified that extensive dermal melanocytosis and PPV are caused by activating mutations in two genes that encode Gα subunits of heterotrimeric G-proteins, *GNA11* and *GNAQ*. Mutations were identified in the DNA of affected skin biopsies but not in collected blood, suggesting these conditions are post-zygotic mosaic disorders similar to McCune-Albright and Sturge-Weber syndromes. Human cell line expression studies using mutant $GNA11^{R183C}$ and $GNA11^{Q209L}$ identified activation of the downstream p38 MAPK signalling pathway, and the p38, JNK and ERK pathways respectively. Transgenic mosaic zebrafish models expressing mutant $GNA11^{R183C}$ using the *mitfa* promoter mirrored the human phenotype showing extensive dermal melanocytosis. This genetic information will allow more accurate diagnosis of this subset of common birthmarks by identifying infants most at risk of serious complications.

Genome Defence in Hypomethylated Developmental Contexts

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DNA methylation is one of the major epigenetic mechanisms in mammals, and is essential in repressing retrotransposons throughout mammalian development. Yet during normal mouse embryonic development some cell lineages become extensively DNA hypomethylated and it is not clear how these cells maintain retrotransposon silencing in a globally hypomethylated genomic context.

Hypomethylation in multiple contexts results in the consistent activation of only one gene in the mouse genome - *TEX19·1*. Thus if a generic compensatory mechanism for loss of DNA methylation exists in mice, it must function through this gene. $TEX19{\cdot}1^{-/-}$ mice de-repress retrotransposons in the hypomethylated component of the placenta and in the mouse germline, and have developmental defects in these tissues. Here we show that TEX19·1 functions, at least in part, through physically interacting with KAP1, a transcriptional co-repressor of retrotransposons. $TEX19{\cdot}1^{-/-}$ ES cells have reduced levels of KAP1 bound retrotransposon chromatin and reduced levels of the repressive H3K9me3 modification at these loci. Furthermore, these subsets of retrotransposon loci are de-repressed in $TEX19{\cdot}1^{-/-}$ placentas. Thus, our data indicates that mouse cells respond to hypomethylation by activating expression of *TEX19·1*, which in turn augments compensatory, repressive histone modifications at retrotransposon sequences, thereby helping developmentally hypomethylated cells to maintain genome stability.

Germline and Maternal Pathways in the Transmission of Paternal Experience

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Paternal environmental experiences can predict developmental outcomes in subsequent generations of offspring. These effects occur even in the absence of paternal care, suggesting that environmentally-induced epigenetic changes within the paternal

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germline are inherited. Though epigenetic marks may be associated with paternal transmission, the interplay between paternal effects and maternal influences on offspring development may be an important consideration the emergence of complex phenotypes. In the present study we explored the effect of chronic paternal food restriction (FR) in C57BL/6 mice on offspring behavioral development. We show that offspring phenotype is dependent upon whether offspring were generated through NM or ET. For example, while offspring of FR fathers who were derived through ET show reduced growth rates, memory impairments and reduced sucrose consumption, some of these phenotypes were absent in FR offspring generated through NM. We propose that though some paternal FR effects may be generated though germline transmission, compensatory maternal investment induced in FR-mated females likely accounts for at least some of the observed effects. Therefore, germline inheritance of environmentally-induced factors cannot be the exclusive mechanism driving the transmission of paternal experience.

The Fine Tuning Role of HIRA in The Differentiation Of Mouse Embryonic Stem Cells Into A Cardiogenic Mesoderm

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HIRA is a histone chaperone which deposits H3·3 in a replication independent manner. Mesp1Cre conditional HIRA mutants displayed several cardiac phenotypes. It was described in other cell types that HIRA interact with important chromatin remodellers essential for heart development, BRG1 and WHSC1. To study early cardiac mesoderm, we assessed these interactions in differentiating mouse embryonic stem cells (mESCs). No interaction between HIRA and WHSC1 or BRG1 was identified in undifferentiated mESCs. Then these cells were differentiated for fifteen days by Hanging drop method. Real time PCR was performed to assess the sequential gene expression of specific cardiac markers. Brachyury, Mesp1 and Nkx2·5 were expressed at days 3, 5 and 7 of differentiation respectively. We identified an interaction of HIRA with WHSC1 and BRG1 only at day 15 of differentiation when markers of primitive cardiomyocytes were upregulated. We performed a HIRA ChIP at day 5, 10 and 15 and found an enrichment of HIRA at a common enhancer of *Tnni2/Tnnt3* (TLT) site only at day 15. In conclusion, we found both an interaction of HIRA with WHSC1 and BRG1 associated with an enrichment of HIRA at the *TLT* site only in 15 days differentiated mESCs, when cardiac markers are expressed.

The role of Hippo signalling in pituitary development and stem cells

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The pituitary controls major physiological processes such as growth, metabolism, fertility and the stress-response. Abnormal development and disruptions in the pituitary stem cell pool can lead to disorders such as hypopituitarism or tumours. Multiple signalling pathways regulate pituitary development but the role of the Hippo-YAP/TAZ cascade is currently unknown. In multiple tissues, the Hippo kinase cascade influences organ size through regulation of proliferation and apoptosis, and has roles in determining stem cell potential. Low Hippo activity is associated with high nuclear accumulation of effectors YAP/TAZ and promotion of stem cell activity, whereas high activity degrades YAP/TAZ and restricts tissue growth.

Using a sensitive mRNA *in situ* hybridisation method (RNAscope) we identified expression of Hippo pathway components throughout murine pituitary development. The highest levels of inactive YAP was seen in regions of commitment where growth becomes restricted, whereas total YAP protein shows high levels in the nuclei of pituitary stem cells. We have carried out tissue-specific knockout of YAP, without revealing gross phenotypic anomalies, however loss of TAZ leads to increased apoptosis, reduced proliferation and causes mild hypoplasia at birth. To overcome compensation we are generating double mutants and will study the effects on pituitary development and long-term potential of pituitary stem cells.

Isolating and characterising human cone photoreceptors from pluripotent stem cell cultures

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Retinal dystrophies are a major cause of blindness, featuring the degeneration and loss of photoreceptor cells. As cone photoreceptors are crucial for colour vision and high visual acuity, their loss is most detrimental for vision. Retinal differentiation of human pluripotent stem cells (hPSCs) serves as a renewable source of photoreceptors to be transplanted into the retina to restore vision. However, the recapitulation of *in vivo* cone development within *in vitro* culture systems has yet to be demonstrated and the human cone photoreceptor transcriptome remains undefined. This project aims to assess similarity between human foetal cones and hPSC-derived cones.

A 3D hPSC-derived retinal differentiation culture system was established that generates optic vesicles and photoreceptors. Cone-specific marker expression was explored in the human foetal retina and hPSC-derived retinal cultures via qPCR and immunofluorescence. Human foetal retinae showed higher gene expression and earlier protein onset of cone markers, however some cone transcripts showed comparable expression *in vivo* and *in vitro* between 12–14weeks. Human foetal cones were labelled and isolated via viral transduction of a fluorescent reporter gene, driven by a cone gene promoter. This reporter also labels hPSC-derived cells and is being used to evaluate cone genesis and for comparative molecular profiling.

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Regulation of Stem to Progenitor Transition in the MSC Niche

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Adult mesenchymal stem cells (MSCs) provide a source of cells for tissue growth and repair. Mouse incisors grow continuously, a process achieved by stem cells residing at their apical end that provide an ideal model to study MSCs and their niche *in vivo*. We show here that expression of Ring1b, Thy1 and Celsr1 in the MSC niche correspond to fast cycling progenitors, slow cycling stem cells and quiescent cells respectively in the mouse incisor. Ring1a/b double knock out mice show dramatically reduced proliferation of progenitors and an increase in apoptosis of slow cycling cells. Gene microarrays reveal the CDK inhibitor Cdkn2a as significantly up-regulated following Ring1 deletion. ChIP-seq data identifies binding sites of Ring1b on the Cdkn2a gene promoter, suggesting a direct target of Ring1b. We identify specific CBX proteins as components of the PRC1 complex with Ring1a/b in the MSC niche. Co-immunoprecipitation and flow cytometry show the co-localization of Ring1b with CBX7 and H3K27me3. Our data suggest that Ring1b interacts with CBX7 and binds to trimethylated H3K27 to repress Cdkn2a expression in progenitor cells to regulate their proliferation, and indirectly acts to maintain the stem cell population.

HIRA is required for heart development and directly regulates *Tnni2* and *Tnnt3*

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Constitutively null *Hira* embryos all die between embryonic day (E) 6·5 and 10·5 exhibiting a range of phenotypes. Embryos surviving to E10·5 presented with heart defects. A conditional allele was therefore employed to examine the role of *Hira* during heart development. Conditional ablation in the cardiogenic mesoderm (*Mesp1Cre*) led to surface oedema, ventricular septum defects (VSD) and embryonic lethality. By RNAseq, we identified upregulation of troponins *Tnni2* and *Tnnt3* and decreased expression of *Epha3,* a gene necessary for fusion of the interventricular septum with the endocardial cushions. In addition, Immunostaining of Troponin C (TnC) emphasised a disorganisation of the contracting meshwork of myofibril. ChIPseq experiment revealed that HIRA binds to GAGA rich DNA sequence in the embryonic heart and is enriched at the common enhancer of *Tnni2/Tnnt3 (TLT)*. Furthermore, *in vitro* and *in vivo* experiments revealed that HIRA interacts with WHSC1 a protein thought to play a major role in Wolf-Hirschhorn syndrome, which also interacts with NKX2·5[1], a major cardiac transcription factor that binds the same regulatory *TLT* site as HIRA[2]. Altogether, this work gives evidence for a specific requirement of HIRA in mesodermal cardiac progenitors during heart development. HIRA influences contractility, troponins expression and the endothelial to mesenchymal transition in the cardiac cushions.

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