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Mechanisms leading to mosaicism in human preimplantation embryos

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Fluorescent *in situ* hybridization (FISH) has been used to study chromosomes in human preimplantation embryos and high levels of chromosomal mosaicism have been detected. Mosaicism could arise through several mechanisms including abnormal cell divisions (mitotic non-disjunction or anaphase lag), failure of cytokinesis or endoreduplication. The FISH procedure has been criticized as it is prone to failure. In this study a two-probe per chromosome FISH procedure was developed to investigate mosaicism and ensure high FISH efficiency. Three-colour FISH was performed in three rounds. In the first and second rounds different probes were used for chromosomes 1, 11 and 18. In the third round probes were used for chromosomes X, Y and 18. Two groups of embryos were studied on day 5 of development: embryos grown in cleavage medium and embryos grown in blastocyst medium. Fourteen embryos were examined in cleavage medium and 13 were mosaic. To date five embryos have been examined in blastocyst medium and all were mosaic. In 95% of cases results were consistent for each chromosome in the different rounds of hybridization. The type of mosaicism in most cases was diploid/aneuploid mosaics. Chromosome loss was the predominant mechanism, followed by chromosome gain, with few examples of mitotic non-disjunction.

Aneuploidy in immature human oocytes isolated from preantral follicles

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Human oocytes and embryos commonly have missing or extra chromosomes (aneuploidy). Many of these embryos fail to develop, implant and reach term. However, 0·3% of newborns have a chromosomal abnormality, making aneuploidy the leading genetic cause of physical and mental disability. It is generally assumed that aneuploidy arises solely following resumption of meiosis, during anaphase I and II, and that oocytes arrested in prophase I of meiosis in the primordial pool are chromosomally normal. We have used fluorescence *in situ* hybridization (FISH) to test the hypothesis that immature primary oocytes are chromosomally normal (diploid) and that no errors arose during earlier germ cell mitosis during fetal life. Primordial and primary follicles were isolated enzymatically and mechanically from ovarian biopsies taken, with informed consent, from women undergoing surgery. Oocytes were dissected from the follicles and spread on microscope slides. Using FISH, 457 oocytes from 37 patients were analysed for aneuploidy of chromosomes 13, 21 and X (chromosomes implicated in early embryonic loss and miscarriage). Rather than the expected two signals, 11% of analysed oocytes had three signals for one or more chromosomes. Six per cent, 4% and 2% of the oocytes had three signals for chromosome 21, X and 13, respectively. The extra signals could represent either trisomy (which would have arisen during germ cell mitosis) or prematurely separated sister chromatids. Either interpretation would predispose the oocyte to further aneuploidy following resumption of meiosis I.

Eighteen per cent of the oocytes had one or more single signals, which could be due either to monosomy or to overlapping FISH signals in the prophase oocytes, where homologous chromosomes are closely associated. In conclusion, the primordial pool in humans contains primary oocytes (which have not yet resumed meiosis) which are aneuploid. Whether these aneuploid oocytes are eliminated by atresia, or reach preovulatory stages, remains unknown.

Mechanisms of maternal aneuploidy: FISH analysis of human oocytes and polar bodies

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Constitutional aneuploidy occurs in at least 5% of recognized pregnancies, with apparent preferential involvement of the X chromosome and the smaller autosomes. Molecular cytogenetic investigations of cleavage-stage embryos have revealed anomalies affecting all sizes of chromosomes. The aim of the study was to investigate the variety of anomalies arising during maternal meiosis I by analysing unfertilized oocytes and polar bodies to gain insight into aneuploidy mechanisms. Sequential fluorescence *in situ* hybridization (FISH) analysis was carried out with specific probes derived from eight chromosomes, representing all sizes. Only imbalance due to a gain of a whole chromosome or chromatid, represented by extra signals, was counted to avoid artefact. Data were obtained on 236 eggs from 124 patients of average age 32·5 years (range 22–44 years). Ten patients (average age 32·6 years) had abnormal eggs. The abnormality rate for oocytes and polar bodies was close to 4% for each. Fourteen hyperhaploidies were found, seven involving additional single chromatids. The abnormalities affected chromosomes 13, 16, 18, 21 and X, but not chromosomes 1, 9 or 12. Evidence of several mechanisms leading to aneuploidy was provided. These include: classical whole univalent non-disjunction; chromatid pre-division prior to anaphase I, leading to imbalance detected at metaphase II; gonadal mosaicism for a trisomic cell line; and preferential involvement of the smaller chromosomes.

Quantitative RT-PCR of *TSC2* transcripts reveals mRNA degradation following cryopreservation in the human preimplantation embryo

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Although embryo cryopreservation is widely applied in *in vitro* fertilization (IVF) programmes its effect on embryonic mRNA expression is not known. Quantitative reverse transcription polymerase chain reaction (RT-PCR) was used to determine the expression levels of the *TSC2* gene in fresh and stage-matched thawed single human embryos. *TSC2* is a tumour suppressor gene involved in cell cycle regulation. To assess the impact of temperature changes on mRNA levels during thawing, two different clinical protocols, P1 and P2, were compared. Human oocytes and embryos used in this project were donated with informed consent by patients undergoing IVF at the Wolfson family clinic. Day 2 (D2) frozen embryos had 50% fewer *TSC2* transcripts ($P<0{\cdot}001$) in comparison with the fresh embryos when applying P1 ($n=20$), and 25% fewer with P2 ($P<0{\cdot}01$) ($n=18$). There was no significant difference in the *TSC2* transcript levels between fresh and frozen D3 human embryos with either thawing protocol ($n=20$). After culturing D2 thawed (P1) embryos for an additional day they showed levels of mRNA comparable with fresh D3 embryos ($n=18$). Quantitative RT-PCR reveals cryopreservation has detrimental effects on *TSC2* mRNA levels; however, the embryo appears able to compensate for this mRNA loss by D3 of *in vitro* culture.

Mapping of *teetering* (*tn*), a gene underlying absence seizures in the mouse

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Teetering mice were described in the 1960s as showing progressive ataxia starting at 3–4 weeks of age, culminating in paralysis and premature death at 5–6 weeks. The *tn* mutation is autosomal recessive and was mapped to the distal end of mouse chromosome 11. More recently, teetering mice have been shown to display bilaterally synchronous spike and wave discharges on electroencephalograms, identifying teetering as one of a group of mouse models of absence

epilepsy (J. Noebels, personal communication). We aim to identify the *tn* gene using a positional candidate approach. A (B6C3Fe-*a*/*a*-*tn* x CAST/Ei) F_1 intersubspecific intercross has yielded over 500 F_2 teetering offspring (*tn*/*tn*), which have been typed for microsatellite markers on distal chromosome 11. Results from this screen have localized *tn* to a 1 cM region between *D11Mit104* (79 cM) and *D11Mit69* (80 cM), in a region showing conserved synteny with human 17q25.3. Novel polymorphic microsatellites and single nucleotide repeats (SNPs) have been used to narrow the critical region to 1 Mb between the genes for neuronal pentraxin (*Nptx1*) and phosphodiesterase 6G (*Pde6g*). Genes within the region are being evaluated as candidates using northern blotting and sequencing. In addition, we are further characterizing the pathology of teetering brain using immunohistochemical methods. It is hoped that the identification of *tn* will provide further insight into the genesis of absence seizures.

Clinical and molecular characterization of frontotemporal dementia linked to human chromosome 3

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Frontotemporal dementia linked to chromosome 3 (FTD-3) is an autosomal dominant pre-senile dementia. It is associated with a large Danish pedigree that spans six generations with over 1000 known individuals. Early onset (below 65 years) includes changes in personality, disinhibition, dyscalculia and hyperorality, progressing to a non-fluent aphasia, speech disturbances and dystonic posturing. There is generalized cerebral atrophy with occasional frontal predominance and, in one patient, a reduction in widespread cortical blood flow. Through haplotype analyses we have restricted the dementia locus to a 4 cM region within chromosome 3. Using the current human genome sequence, candidate genes are being sequenced to identify any mutation that segregates with the disease, with particular attention being given to genes expressed in the brain. High-resolution fluorescence *in situ* hybridization (FISH) technology and Southern blot analysis are being used to identify genomic differences and chromosomal anomalies. Comparison of gene expression from FTD-3, Alzheimer and non-neurodegenerative brains will indicate aberrations in molecular pathways and complement the proteomic studies that are under way to investigate disruption to protein expression levels. In addition the development of an information extraction program will provide a novel *in silico* approach to obtain previously undiscovered molecular components and pathways from the literature relating to FTD-3.

Mutation of the forkhead gene *FOXF2* causes iris hypoplasia and glaucoma

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Glaucoma is a major cause of blindness worldwide. Glaucoma families manifesting anterior segment dysgenesis have been mapped to 6p25. In a proportion, mutations in the forkhead transcription factor gene *FOXC1* or duplications of the 6p25 region have been identified. A six-generation family segregating iris hypoplasia and glaucoma maps to 6p25, but there is no *FOXC1* mutation or a duplication event. In the duplication region is a closely related member of the forkhead gene family, *FOXF2*. We show that *FOXF2* is expressed during human and mouse fetal eye development and expression in the iris, trabecular meshwork and retina in adult human eye. Analysis of eyes of *Foxf2*+/− heterozygous mice revealed some abnormalities that corroborate the gene's involvement in eye development. We screened the *FOXF2* gene and found a 3 bp insertion (GGC) in the AD2 transactivation domain of *FOXF2*, which segregated with disease throughout the family. Transactivation assays using luciferase reporter gene demonstrated that mutant *FOXF2* acts as a dominant-negative allele over the wild-type allele. Reduced transactivation was not due to an effect in DNA binding activity, as shown by electrophoretic mobility shift assays. For the first time we present evidence that a dominant-negative *FOXF2* allele underlies an iris hypoplasia and glaucoma phenotype.

Recessive screen at *Del(13)Svea36H*

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A recessive, two-generation, screen for mouse mutants that affect postimplantation embryonic development is under way. ENU-induced mutations are recovered in the region of the Del13Svea36H (Del36H) deletion on chromosome 13. This cytogenetically visible deletion removes a 13 Mb region of MMu13 that includes the satin locus (*Foxq1*). Mice that are homozygous null for *Foxq1* have a glossy coat and are distinguishable from their littermates at 10 days after birth. This locus is used to follow the mutagenized chromosome throughout the recessive screen. In the screening protocol, male mice with a satin coat are mutagenized and crossed to wild-type BALB/C females. F1 male progeny are then crossed to female carriers of Del36H and the prevalence of progeny with a satin coat used to indicate those lines that potentially carry a new recessive lethal mutation. Once the presence of a mutation is confirmed, the time of lethality and the associated embryonic phenotype is investigated, and recombination mapping is performed to further localize the mutation within the deleted region. In parallel with this mutagenesis experiment, a BAC contig across the entire deleted region has been constructed and the finished sequence of this contig has been generated and annotated. The continuing genomic analysis of this region has facilitated the mapping and cloning of the isolated mutants. Results from the screen and the analysis of mutant phenotypes will be presented.

Fine-mapping of a quantitative trait locus affecting skeletal size in mice

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Mapping quantitative trait loci (QTLs) with sufficient precision to identify the underlying genes has proved extremely difficult. However, our experimental system promises the potential to achieve the necessary resolution thanks to a relatively large effect size and a trait that is easily measured. We are investigating a QTL affecting tail length originally identified in an F_2 population of C57BL/6J × DBA/2J mice. By testing a large population of progeny from parents that are recombinant within the region of the QTL (*c.* 1000 offspring from 61 parents recombinant within a 13 cM interval), we have refined its position to a region of approximately 3 Mb (~1·2 cM) containing an estimated 30 genes. Preliminary results indicate that this QTL has a general effect on skeletal size, affecting the length of the humerus, femur, tibia, mandible, scapula, pelvic girdle and a tail bone. However, no significant effect was found on the number of bones in the tail or on the dimensions of the ulna, skull or first vertebra.

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Microarray analysis of transcriptional differences between wild-type and mutant CNS in the calcium channel mutant, *ducky*

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The mouse mutant ducky (*du*), a model for absence epilepsy, is characterized by spike-wave seizures and ataxia. Mutations in the *Cacna2d2* gene were found to underlie the phenotype in the original *du* strain and its allele, du^{2J}. *Cacna2d2* encodes the $\alpha2\delta2$ voltage-dependent calcium channel subunit. The $\alpha2$ and δ chains are derived from proteolytic cleavage of the gene product, the δ subunit acting as a membrane anchor for the extracellular $\alpha2$ subunit. Both mutations are predicted to result in the loss of the full-length protein and hence the δ subunit. *Cacna2d2* mRNA is strongly expressed in cerebellar Purkinje (P) cells, and *du*/*du* mice have abnormalities in their P cell dendritic tree. In addition, co-expression of the truncated protein with the $Ca_v2.1/\beta_4$ channel combination *in vitro* leads to a reduction in current density. To aid elucidation of the function of $\alpha2\delta2$ we are performing microarray analysis at three different stages of development in du^{2J}/du^{2J} mice: embryonic, postnatal and adult. We expect that those transcripts identified as up- or down-regulated in the mutant mice will act up- or downstream of $\alpha2\delta2$, either via the calcium channel as a whole, or directly through the subunit. We have identified a number of candidate transcripts, including a putative homeobox transcription factor.

Confirmation of these differences in expression between wild-type and *du*^{2J} CNS using QPCR is under way, and will be presented.

Isolation and characterization of novel genes involved in inner ear development

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The developing inner ear is first evident as bilateral thickenings of the surface of the head ectoderm – the otic placode, from which all epithelial structures of the inner ear will develop. In the mouse, cells from the otic placode proliferate and invaginate to form the otic cup. By 9·5 days post-coitum (dpc), otic cup closure occurs to form the otic vesicle. From 10·5 to 13·5 dpc the cells in the otic vesicle undergo complex morphogenetic movements and start being specified to one of a variety of structural, sensory or neuronal cell fates. We are particularly interested in isolating and identifying those genes which are required to control and execute the early developmental programme of the inner ear. The 10·5 dpc otic vesicle provides a rich source of transcripts at a developmental time point where specification of otic lineages has been initiated. I have screened a normalized mouse 10·5 dpc otic vesicle library. Housekeeping genes were eliminated, novel genes were identified by sequencing and expression analysis in the 10·5 dpc embryo was performed by *in situ* hybridization. Subsequently, a small number of these novel genes were chosen for further study on the basis of restricted expression pattern in the developing otic vesicle.

The genetic variation of the small mucin gene *MUC7*

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The mucins are a heterogeneous group of glycoproteins which share the common feature of having a central domain of tandemly repeated sequence (TR) showing VNTR-type polymorphism. MUC7, a small mucin principally expressed in saliva, has been shown to have anti-fungal activity and interacts with bacteria. *MUC7* has two common TR alleles, with five or six repeats. Previous studies have indicated that MUC7 plays a role in predisposition to asthma and possibly other chest diseases. The five-repeat allele is under-represented in patients with asthma (Kirkbride *et al*. (2001). *Eur. J. Hum. Genet*. **5**, 347–354). It seems unlikely that the small relative reduction in size of the five-repeat allele is responsible for the protective effect, but rather that other linked polymorphisms are responsible for the association, Studies on the protein suggest inter-individual variation in the amount of MUC7 in saliva. A combination of bioinformatics and experimental tools were used to search for single nucleotide repeats (SNPs). Twenty-three SNPs were found of which 15 had not been reported before, including several in the promoter region and the first intron, which could be functionally significant. The pattern of allelic association and haplotypes are now being studied in healthy individuals and in patient groups.

Homocysteine is embryotoxic but does not cause neural tube defects in mouse embryos

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Periconceptional supplementation with folic acid has been shown in clinical trials to reduce the recurrence of neural tube defects (NTD) in humans by up to 70%. The mechanism of the prevention is not known but is unlikely to be a simple correction of folate deficiency. In humans, elevated homocysteine levels have been detected in maternal serum in pregnancies affected by NTD, leading to the suggestion that excess homocysteine may be directly causal. In addition, exposure of chick embryos to exogenous homocysteine has been reported to cause NTD. To test the hypothesis that high homocysteine levels may directly cause NTD, mouse embryos were cultured in the presence of homocysteine thiolactone (Hcy) throughout the period of cranial neural tube closure. Embryos cultured in the presence of Hcy at concentrations of 500 μM or greater were growth-retarded, and developed blisters and abnormal somites. Despite this general toxic effect of Hcy exposure there was no increase in the incidence of NTD. Incorporation of [^{3}H]thymidine was also measured as an indicator of abnormalities in the folate/methylation cycles, but no increase in incorporation was detected. These observations suggest that increased levels of homocysteine are not a direct cause of NTD but may indicate a disturbance in folate-related metabolism associated with an increased risk of NTD.

Characterization of the Kumba allele of *Zic2*

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The mouse *Zic2* gene encodes a 55·3 kDa C2H2 zinc finger transcription factor, homologous to the *Drosophila* pair-rule gene odd-paired. Evidence from studies in *Xenopus* have implicated *Zic* gene family members in neural development. The Kumba ($Zic2^{Ku}$) allele was recovered from a genetic screen for dominant, ENU-induced mouse mutants. The mutation is a T to A transversion, which changes a cysteine in one of the zinc finger domains to a serine, presumably abolishing the function of that domain. $Zic2^{Ku/+}$ mice exhibit a looped tail, occasionally accompanied by spina bifida. Additionally, many $Zic2^{Ku/+}$ mice have a ventral spot. On the C3H background, the mutation shows incomplete penetrance, with one-third of heterozygotes appearing normal. Mice that are homozygous for the mutation die during mid-gestation. To further investigate the developmental defect associated with this allele of *Zic2*, we have characterized the homozygous phenotype at 9·5 days pot-coitum. At this stage, visual analysis of $Zic2^{Ku/Ku}$ embryos reveals delayed neural tube closure, incomplete embryonic turning, incorrect heart morphology and a deformed forebrain and branchial arches. Analysis with molecular markers has revealed additional defects including aberrant neural crest differentiation/migration.

The role of Oct-4 in early post-implantation development

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The POU domain transcription factor Oct-4 is essential for establishment of the pluripotent lineage in the mouse embryo (Nichols *et al.* (1998). *Cell* **95**, 379–391). In normal mouse embryos Oct-4 expression is maintained in the epiblast, becoming restricted to the primordial germ cells. Using conditional gene deletion driven by lineage-specific Cre recombinase transgenes we have revealed a requirement for Oct-4 during epiblast expansion. Several phenotypes have been generated ranging from trophoblast nodules to embryos that have gastrulated but are reduced in size and somewhat disorganized. These abnormalities are currently being characterized and we are investigating how the timing of the loss of Oct-4 correlates with subsequent behaviour of the cells, whether they are directed along an inappropriate lineage or undergo apoptosis. In addition to the early deformities, we have observed an exencephalic phenotype in one of the transgenic lines when the Cre is maternally inherited. Since Oct-4 expression has not been detected in the anterior neurectoderm of embryos after gastrulation it is unlikely that this failure in neural tube closure is a direct effect of loss of Oct-4. Target genes that may be involved in acquisition of this phenotype are being sought using *in situ* hybridization.

Arylamine *N*-acetyltransferase and folate catabolism

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Arylamine *N*-acetyltransferase (NAT) is an enzyme capable of acetylating the folate catabolite *p*-aminobenzoylglutamate (*p*-abaglu). Its expression early in embryonic development, both in murine embryonic stem cells and human blastocysts, coupled with the essential role of folate during development, has led to the hypothesis that NAT has a role in the maintenance of folate homeostasis during development. To investigate the relationship between NAT and folate metabolism we have generated mice with a null allele of NAT2 by targeted disruption of the Nat2 locus. A lack of NAT2 is not developmentally detrimental, although we have demonstrated that in liver homogenates, mouse NAT2 is responsible for the acetylation of *p*-abaglu. Expression of NAT2 throughout gestation is being monitored using a β-galactosidase reporter gene, incorporated into the targeted allele, which is under the control of the Nat2 promoter. We have confirmed previous immunohistochemical data, showing that NAT2 expression is widespread from at least embryonic day 9·5, including expression in the neural tube, heart and developing eye. In addition we have demonstrated that NAT2 is expressed ubiquitously in the skin.

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The regulation mechanism of the *Dlk1-Gtl2* imprinted cluster on mouse chromosome 12

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The imprinted domain on mouse distal chromosome 12 contains several imprinted genes, including the paternally expressed protein-coding genes *Dlk1* and *Dio3*, and the maternally expressed non-coding RNAs *Gtl2* and *Rian*/*Meg8*. The reciprocally imprinted *Dlk1* and *Gtl2* are located 90 kb apart and share many epigenetic features with the *Igf2-H19* locus on mouse chromosome 7F. In addition, the chromosome 12 domain contains three clusters of imprinted small nucleolar RNA (snoRNA) genes derived from a primary transcript that includes *Rian*/*Meg8* and is reminiscent of the imprinted cluster on the murine orthologue of the human Prader–Willi/Angelman locus on mouse chromosome 7C. Here we conduct a targeted deletion of a germline-derived intergenic differentially methylated region (IG-DMR) located between *Dlk1* and *Gtl2*. Upon maternal transmission, deletion of the unmethylated IG-DMR causes bi-directional loss of imprinting of multiple genes in the domain. However, when deletion of the methylated allele was transmitted through the paternal germline, no significant differences in expression were observed. These results indicate that the IG-DMR is an imprinting control centre for multiple imprinted genes on the maternal allele only. This is similar to the function of the Prader–Willi imprinting centre (PWS-IC) which, on the unmethylated chromosome, regulates a large cluster of imprinted genes.

The use of methylation-sensitive representational difference analysis to identify novel differentially methylated regions/imprinted genes on mouse proximal chromosome 11

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Mice with uniparental duplications of proximal chromosome 11 exhibit reciprocal growth phenotypes. Fetuses with paternal duplications of proximal chromosome 11 [PatDp(prox11)] show overgrowth while those with maternal duplications [MatDp (prox11)] are growth-retarded. To date, two imprinted genes are known to be located on proximal chromosome 11. *Meg1*/*Grb10* is a maternally expressed imprinted gene and *U2af1-rs1* is a paternally expressed gene. *U2af1-rs1* maps 15 cM from *Meg1*/*Grb10* and is separated by conserved regions showing homology to different human chromosomes. *U2AF1-RS1* maps to human chromosome 5 and is not imprinted. *GRB10* maps to human chromosome 7p12, a region associated with Silver–Russell syndrome. As the two human homologues map to different chromosomes it is thought that these genes in the mouse are located within two separate imprinted domains. In order to identify novel imprinted genes that may form uncharacterized imprinted clusters, a screen for the differentially methylated regions (DMRs) that are characteristic of imprinted loci was performed using methylation-sensitive representational difference analysis (Me-RDA). DNA from E15.5 MatDp(prox11) and PatDp(prox11) embryos from T40(11;7)Ad intercrosses were separately digested with methylation-sensitive restriction enzymes *Hpa*II and *Hin*6I, and amplicons produced by polymerase chain reaction. The MatDp(prox11) amplicons have initially been used as the Driver and the PatDP(prox11) amplicons were used as the Tester in two rounds of subtractive hybridization to recover unmethylated fragments of paternal origin. Currently this screen has isolated clones from the *U2af1-rs1* and *Meg1*/*Grb10* loci. Candidate loci isolated from this screen are currently being tested to determine methylation and imprinting status.

The imprinted region on human chromosome 7q32 extends to the *carboxypeptidase A* gene cluster

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Identification of segmental maternal uniparental disomy (mUPD) of 7q31 to the telomere in a patient with Silver–Russell syndrome (SRS) emphasizes this domain as a candidate region for the syndrome. Since mUPD7 has been found in approximately 10% of SRS patients, the causative gene is expected to behave in an imprinted way. The human homologue (*MEST*) of mesoderm-specific transcript (*Mest*) is imprinted and along with *CIT1* (*COPG2* antisense) and *MESTIT1* (*MEST* intronic transcript 1) forms an imprinting cluster at chromosome 7q32. The testis-specific protein A14 (*TSGA14*) gene which lies in a centromeric direction to *MEST* is biallelically expressed. Since biallelically expressed genes can occur within a cluster of imprinted genes, the search for further imprinted genes was continued beyond *TSGA14* to the carboxypeptidase A (*CPA*) group of genes. Single nucleotide polymorphism (SNP) imprinting analysis of *CPA4* has revealed predominantly preferential expression of the maternal allele. We also report methylation analysis of a CpG island 650 bp upstream of the first coding exon of *CPA4* as well as imprinting analysis of *Cpa4* in the mouse.

Growth regulation by *Grb10*

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Grb10 is a maternally expressed gene that encodes an intracellular signalling molecule of the SH2-domain-containing adapter family. In the mouse, *Grb10* is located on proximal chromosome 11. Mice with uniparental disomies (UPD) of this region exhibit reciprocal growth phenotypes, with overgrowth resulting from paternal UPD and growth retardation resulting from maternal UPD. The equivalent human chromosomal region (7p11.2–p13) is associated with Silver–Russell syndrome, with features that include maternal UPD and growth retardation. Thus, Grb10 is a candidate growth inhibitory protein and is likely to act by interacting with phosphorylated tyrosine kinase receptors. In order to assess Grb10 function *in vivo* we have generated mice with a disrupted *Grb10* gene. A *LacZ* reporter gene insertion shows that *Grb10* is imprinted at most, but not all, of its sites of expression. Following maternal transmission of the mutation both the fetus and placenta exhibit overgrowth, with disproportionate effects on organ size. Grb10 has been shown to be capable of interacting with the insulin and insulin-like growth factor (IGF) type I receptors in *in vitro* assays. We find that the ontogeny of the growth effects in *Grb10* mutants approximately mirrors that of IGF action during development. Despite this, we present evidence that Grb10 acts, at least in part, via a pathway that is independent of the IGFs.

Trophoblast stem cells: an *in vitro* model for imprinted X inactivation

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Imprinted X inactivation takes place in the extra-embryonic tissues of mouse embryos and it involves the preferential silencing of the paternal X chromosome. Past studies have highlighted various interesting features of this process such as replication timing, DNA methylation and the regulation by imprinted sense/antisense genes. Random X inactivation, has been well characterized, in part due to the availability of an *in vitro* model system – differentiating embryonic stem (ES) cells. Studies on imprinted X inactivation, however, have relied on material from early embryos. In this study, we present for the first time trophoblast stem (TS) cells as an *in vitro* system to study the processes involved in imprinted X inactivation. These cells demonstrate imprinted *Xist* expression leading to preferential paternal X inactivation. The inactive X in these cells has several of the same epigenetic modifications as described for the inactive X in the ES cell system. In addition the Polycomb group protein complex Eed/Enx-1 was found to stably associate with the inactive X in interphase and

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throughout mitosis, therefore suggesting that they play a direct role in the maintenance of imprinted X inactivation. This provides the first demonstration of mitotic stability of Polycomb group protein complexes with known target loci.

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Investigation of the role of $p57^{Kip2}$ in mouse embryonic growth

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Cdkn1c is a maternally expressed gene lying within an imprinted cluster on the distal portion of mouse chromosome 7. It encodes the cyclin-dependent kinase inhibitor $p57^{Kip2}$, which is widely expressed during development and is thought to be involved in the control of cell proliferation and differentiation. *CDKN1C* has been implicated in the global regulation of embryonic growth following the discovery of mutations in patients suffering from the human overgrowth disorder Beckwith–Wiedemann syndrome. A mouse *cdkn1c* knockout model supports this hypothesis, exhibiting a phenotype which shares many features with the human syndrome. We have analysed cell proliferation in mouse embryos and primary embryonic fibroblasts lacking a functional $p57^{Kip2}$ gene. Our initial findings suggest that cells lacking $p57^{Kip2}$ derived from E14.5 knockout embryos are relatively unchanged in their serum growth dependence, but show marked insensitivity to density-dependent growth inhibition. Assessment of cell cycle parameters by fluorescence-activated cell sorting (FACS) in whole embryos from E10.5 to E15.5 provided no conclusive evidence for increased S-phase occupancy, but did show an increase in the number of cells in the embryo with a sub-G_1 DNA content, consistent with increased levels of apoptosis. The implications of these findings for the normal role of $p57^{Kip2}$ will be discussed.

Combined effects of insulin-like growth factor 2 excess and tumour suppressor loss in overgrowth and cancer

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Insulin-like growth factor 2 (IGF2) is implicated in overgrowth diseases and tumour formation in humans and transgenic animals. We have investigated whether, along with the effects of IGF2, inactivation of one or more tumour suppressor genes is involved in the transition from overgrowth to cancer. Transgenic mice, overexpressing IGF2 under the control of a keratin gene promoter, show overgrowth of the skin and colon but do not develop tumours. Excess IGF2 appears to change the spectrum of tumours seen in the *p53* knockout mice from mainly thymic lymphomas to include skin papillomas. The *IGF2* gene is located on chromosome 7 in mouse, in close proximity to the cyclin-dependent kinase (cdk) inhibitor $p57^{KIP2}$. Mutation/mis-regulation of both these genes occur in the overgrowth disorder Beckwith–Wiedemann syndrome (BWS). We have attempted to create transgenic mice overexpressing IGF2 and lacking functional $p57^{KIP2}$ but our efforts to assess potential tumour incidence in these mice have been hampered by poor animal survival, despite inbreeding to the 129 strain background. Additional experiments have concentrated on examining the effects of overexpression of IGF2 combined with the lack of another cdk inhibitor, $p27^{KIP1}$, which belongs to the same family as $p57^{KIP2}$.

Muscle-specific expression of *Igf-2* and GFP in dystrophic mice

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Muscular dystrophies (MD) cause severe disruption of skeletal muscle function in children. We have shown *Igf-2* inhibits MD skeletal muscle apoptosis and stabilizes dystrophic phenotype [1, 2]. *Igf-2* may thus be suitable for therapeutic use in MD, delivered directly to skeletal muscles. To test this we constructed a transgene (MCK-*Igf2*-IRES-*Gfp*) where *Igf-2* and *Gfp* (green fluorescent protein) genes are expressed under the control of the muscle creatine kinase (mCK) promoter. Transfection of MCK-*Igf2*-IRES-*Gfp* into skeletal muscle stem cells induces both IGF-2 and GFP in fusing myotubes under differentiation permissive conditions. We recently generated

transgenic mice by pronuclear injection of MCK-*Igf2*-IRES-*Gfp*. Preliminary data suggest that MCK-*Igf2*-IRES-*Gfp* embryos develop to full term and express the transgene in fused muscle fibres. We have noted no gross morphological abnormalities and mice do not appear enlarged compared with non-transgenic litter mates. We are establishing founder lines in order to breed these mice onto the *mdx* dystrophic background.

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Promoter competition at the *Mrf4/Myf5* locus

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Vertebrate myogenesis is controlled by the myogenic regulatory factors (MRFs): Myf5, Mrf4, myogenin and MyoD. We have previously shown that transcriptional regulation of *Myf5* is different from that of other MRFs, and described proximal and distal (up to 140 kb upstream) enhancers/regions that direct expression at different times and anatomical locations throughout development, while the linked *Mrf4* gene requires at least four elements one of which may be shared with *Myf5*. We have now started to unravel the basis of the specificity of this multitude of enhancers for their respective promoters. We show that in the absence of the *Myf5* minimal promoter (195APZΔmp5), some of the usually *Myf5*-specific distal elements direct *Mrf4* expression, suggesting that in the wild-type context both promoters compete for these enhancers. Surprisingly, *Myf5* expression is not abolished in 195APZΔmp5 and most enhancers activate transcription through an as yet unknown second promoter. Intriguingly, the intergenic early epaxial enhancer can neither interact with this alternative promoter, nor direct *Mrf4* expression, suggesting that the promoters may not compete for the proximal enhancers. However, deletion of the intergenic branchial arch element in the 195APZΔmp5 context abolishes normal *Myf5* and deviant *Mrf4* arch expression, suggesting a more subtle mechanism of promoter-enhancer specificity.

SHOX and limb development

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SHOX is a member of the homeobox-containing gene family and highly conserved amongst different species. SHOX has been shown to be causative for idiopathic short stature. It is located in the pseudoautosomal region (PAR1) and haploinsufficiency of SHOX has been implicated in short stature and additional skeletal malformations frequently observed in Turner syndrome. To explore the role of SHOX in limb development we are studying chick embryos. We performed *in situ* hybridization analysis of SHOX expression at different stages in limb development and find that a central region of the early limb bud expresses SHOX and around this is a rim of non-expressing cells. At later stages, SHOX expression is first restricted to the proximal two-thirds of the limb bud and then is expressed in proximal limb bud and digital rays with stronger expression dorsally. To investigate how SHOX expression is controlled we have carried out experimental manipulations on chicken limb buds. We found that both removing the apical ridge and implanting noggin beads extends SHOX expression to the distal limb bud mesenchyme. We have constructed a SHOX-RCAS virus and explore the effects of overexpressing SHOX in chicken limb development.

The role of endoglin in cardiovascular development

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The identification of endoglin and ALK1 as the genes in which mutations cause hereditary haemorrhagic telangiectasia (HHT) identified two key genes in the process of blood vessel formation. The occurrence of arteriovenous malformations in HHT patients and high endoglin expression in normal developing heart valves pointed to other roles for endoglin in cardiovascular development [1]. This was confirmed in the endoglin knockout mouse, in which we, and others, showed that lack of endoglin expression causes failure of angiogenesis, best seen in the yolk sac, as well as defects in endocardial cushion formation [2–4]. The heterozygous endoglin knockout mouse also provides a valuable model of human HHT disease; and

a recent detailed analysis of the vasculature has revealed subtle defects in the structure of the blood vessel wall that cause vascular fragility and bleeding. These studies point to a central role for endoglin in both the formation and maintenance of the cardiovasculature.

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Molecular mechanisms underlying tracheo-oesophageal development

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Morphological and cell differentiation studies indicate that the respiratory epithelium is first specified in the ventral endoderm of the post-pharyngeal foregut, then occupies the ventral half of the foregut during a phase of rapid longitudinal growth and finally becomes a separate structure (trachea) with the separation proceeding in a caudal to cranial direction. The molecular mechanisms that underlie the process of separation are not understood. *In situ* hybridization experiments show a ventral-to-dorsal switch in the endodermal expression of the morphogen *Shh* that is propagated in a cranial direction just ahead of tracheo-oesophageal separation. Endodermal cells undergoing programmed cell death (PCD) localize at the site of separation and the PCD pattern follows, to an extent, the *Shh* expression pattern. Furthermore, inhibition of PCD appears to interfere with the separation process. We suggest that PCD marks the site of tracheo-oesophageal separation and is thus required for its initiation. The precise relationship between PCD and the expression of foregut patterning genes (e.g. *Shh*) remains to be elucidated.

Expression and transcriptional regulation of the *FBLN3* gene causing dominant drusen ocular phenotype

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Drusen deposits in the retina are found in an important set of blinding inherited conditions, including age-related macular degeneration (AMD), a complex trait accounting for 50% of blind registration in the developed world. A single R345W mutation in the *FBLN3* gene has been causally associated with a proportion of autosomal dominant drusen mapping to 2p16. In our patient cohort without R345W mutation, no other *FBLN3* coding region mutations were identified. Using *in situ* hybridization and reverse transcription polymerase chain reaction (RT-PCR), *FBLN3* expression was localized to the outer and inner nuclear layers of the retina and also to the retinal pigment epithelium (RPE). Western blotting detected a 45 kDa *in vivo* and 64·5 kDa *in vitro* fibulin-3 protein product, suggesting that there is post-translational modification. Sequencing of the *FBLN3* upstream region identified a putative estrogen responsive element (ERE) and three Sp1 sites. To evaluate whether the ERE was active, RPE cells were incubated with 17β-estradiol, which resulted in down-regulation of the *FBLN3* mRNA and protein levels. Promoter deletion constructs also showed that the ERE down-regulated reporter gene expression. Furthermore, these assays have also implicated Sp1 binding sites as up-regulatory elements of the gene. These studies have contributed to characterizing a novel gene involved in human disease.

Gene dosage in Pelizaeus–Merzbacher disease

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Dosage of certain myelin genes is crucial during development as changes in copy number can cause abnormalities in myelin formation and/or maintenance in both the central and peripheral nervous system. The proteolipid protein (*PLP1*) gene is a dosage-sensitive gene whose product PLP is a major structural component of central nervous system (CNS) myelin.

A large genomic duplication that includes the entire *PLP1* gene is responsible for the majority of patients with Pelizaeus–Merzbacher disease (PMD). PMD is a rare X-linked disorder, characterized by an absence of myelination in the CNS. Alterations in *PLP1* dosage may cause apoptosis in the case of overexpression, and disruption of the interactions between oligodendrocytes and axons. We have devised an efficient screening test for *PLP1* gene dosage using multiplex amplifiable probe hybridization (MAPH). MAPH is a relatively new technique for assaying copy number simultaneously at several loci, with a higher throughput than existing diagnostic methods such as interphase fluorescent *in situ* hybridization. The high frequency of gene duplication as a cause of PMD means that gene dosage evaluation is the first approach for molecular diagnosis and highlights that the stoichiometry of myelin proteins is important in normal structure and function.

Investigation of the role of Wilms' tumour suppressor gene 1 (WT1) isoforms in genito-urinary development

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The Wilms' tumour suppressor gene, *WT1*, encodes a zinc finger transcription factor essential for the development of the urogenital system. Alternative splicing within the gene causes an insertion or deletion of three amino acids creating two classes of isoform: WT1 + KTS and WT1-KTS. Mutation of the isoforms, and disruption of their ratios, are linked to human disorders of the urogenital system. These isoforms are thought to have distinct roles within the nucleus. We have generated transgenic lines of mice expressing a *WT1-KTS* transgene, and bred them with *WT1* knockout animals to study the effects of the WT1-KTS isoform on genito-urinary development in isolation from the other isoforms. Preliminary data shows there may be an effect on animals expressing the *WT1-KTS* transgene and heterozygous for *WT1*. Using green fluorescent protein fusion constructs in cell culture, we demonstrate that the dynamic subnuclear localization of the two isoforms results, in part, from the presence of a nucleolar localization signal within the zinc finger regions. Combining transgenic *in vitro* techniques with *in vivo* cell culture work, our findings help elucidate the mechanisms by which the WT1 isoforms are distributed in the nucleus, and suggest a model demonstrating how their separate functions are maintained.

Detection of *Tsga10* transcripts in mouse and rat testis

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Spermatogenesis converts a stem cell population of spermatogonia into a highly differentiated population of sperm in the testis. In the course of this pathway cells pass from a maintenance state through mitotic divisions, meiosis and finally terminal differentiation. Surprisingly little information is available about any of the genes which have a role in this pathway. We have previously shown by differential display (DD) RT-PCR that a novel gene, *TSGA10*, is expressed in human testis but not in a variety of non-spermatogenic tissues. Hence, *TSGA10* is a testis-specific gene, which maps to 2q11.2. In this project we have used RT-PCR to investigate the timing of expression of *Tsga10* in rat testis. *In situ* hybridization was used to identify the location of the cells containing *Tsga10* transcripts in cryostat sections of mature mouse testis and other organs. RT-PCR had shown that *Tsga10* was expressed in testis once spermatocytes have progressed to the late pachytene stage. *Tsga10* mRNA transcripts show a pronounced expression from secondary spermatogonia to later spermatogenic cells including those closer to the lumen of the seminiferous tubules. Also *TSGA10* seems to be expressed at a lower level in some parts of the brain. Our results are the first to demonstrate that the transcripts for *Tsga10* are present in spermatogenic cells and that they mostly exist in meiotic and post-meiotic cells.

Mouse germline–soma interactions in the genital ridge

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During embryogenesis primordial germ cells (PGCs) have the potential to enter either spermatogenesis or oogenesis. In a female genital ridge, or in a non-gonadal environment, PGCs develop as meiotic oocytes. However, male gonadal somatic cells inhibit PGCs from entering meiosis and direct them to a spermatogenic fate. We have examined the ability of PGCs from male and female embryos to respond to the masculinizing environment of the male genital ridge, defining a temporal window during which PGCs retain a bipotential fate. To help understand how PGCs respond to the male gonadal environment we have identified molecular differences between

male PGCs that are committed to spermatogenesis and bipotential female PGCs. Our results suggest that one way in which PGCs respond to this masculinizing environment is to synthesize prostaglandin D_2. We show that this signalling molecule can partially masculinize female embryonic gonads in culture, probably by inducing female supporting cells to differentiate into Sertoli cells. In the developing testis, prostaglandin D_2 may act as a paracrine factor to induce Sertoli cell differentiation. Thus part of the PGCs' response to the male gonadal environment is to generate a masculinizing feedback loop to ensure male differentiation of the surrounding gonadal somatic cells.

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