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Structural-Proliferative Units and Igf2

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We know a great deal about molecular pathways involved in the growth and proliferation of individual cells, but much less about how entire organs actually grow, and almost nothing about how they know when to stop growing. Insulin-like growth factor II (Igf2) is a potent molecule. Complete absence of Igf2 results in a proportionate dwarf mouse, while tissue-specific over-expression in the 'Blast' transgenic line causes overgrowth of skin and colon. This is one of very few molecular tools available to tweak the growth of individual organs. We have been trying to ascertain the mechanisms by which Igf2 may regulate growth of skin and colon. Our working model for these and other epithelial organs is that of the Structural-Proliferative Unit (SPU), derived from a small pool of stem cells. A well-defined example is the colon crypt. We have compared the size, number and density of skin and gut SPUs between Igf2KO, wild-type and Blast mice, as well as looking at proliferative and apoptotic indices and expression of various molecules known to be involved in growth and proliferation.

Heparin-binding VEGF isoforms provide spatially restricted cues for blood vessel patterning during embryogenesis

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We study the mechanisms that transform nascent blood vessels into complex patterned networks. Our analysis of mice engineered to express single isoforms of vascular endothelial growth factor (VEGF) demonstrates that the balanced expression of VEGF isoforms regulates the choice between the alternate fates of growth in microvessel diameter versus growth in branching complexity. Microvessel networks of mouse embryos expressing only the VEGF120 isoform consisted of larger vessels with fewer branchpoints when compared with wild-type littermates. At the cellular level, branching mutants largely lacked filopodia, which in wild-type littermates formed at the front of growing vessels and extended towards the VEGF source. Since VEGF isoforms differ in their ability to bind heparan sulphate proteoglycans in the extracellular matrix of cultured cells, we reasoned that the lack of endothelial cell polarity could be due to the abnormal localization of VEGF. Consistent with this idea, secreted VEGF protein normally formed a steep gradient surrounding the VEGF-expressing midline region of the hindbrain, but dispersed away from this area in mutants containing VEGF120 only. We conclude that VEGF isoforms, through their differential association with the extracellular matrix, act in concert to form a concentration gradient around producing cells. This gradient polarizes and thereby guides endothelial cells at the leading edge of expanding vessel networks, providing a mechanism to control vascular patterning.

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 dpc. 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.

Folate homeostasis and neuronal specification: investigating brain development from different angles

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Arylamine *N*-acetyltransferase (Nat) is an enzyme capable of acetylating the folate catabolite *p*-aminobenzoylglutamate. Its expression during embryonic development, both in murine ES cells and in human blastocysts, coupled with the apparent protective effect of folate against neural tube defects has led to the hypothesis that Nat has a role in the maintenance of folate homeostasis during development. We are conducting parallel studies to investigate the developmental consequences of both the ablation and over-expression of Nat in transgenic mice. Analysis of these transgenics should aid our understanding of the possible developmental role for Nat, in particular in the development of the brain. To further our understanding of mammalian brain development, we are also investigating the developmental role of Wnt7b, a member of the Wnt gene family of secretory proteins. The complex developmental expression pattern of Wnt7b suggests it has an important role in embryonic development, particularly in the development of the central nervous system. Mice homozygous for a null allele of Wnt7b die at mid-gestation due to a lack of fusion between the allantois and the chorion, complicating the study of Wnt7b in brain development. We plan to use a neuronal culture system to investigate a possible role for Wnt7b in neuronal specification.

The developmental basis of anterior segment abnormalities in small eye mice

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Aniridia is a genetic disease that results from heterozygous null mutations in *PAX6*, which is widely expressed during eye development. *PAX6*$^{+/-}$ patients show defects of the anterior segment of the eye, including iris hypoplasia, corneal stem cell deficiencies, corneal opacities and a persistent lens corneal bridge, as well as cataracts and long-term ocular degeneration often leading to blindness. The small eye mouse is an excellent model of human aniridia. In order to understand the developmental basis of the mutant phenotype, chimeric mice were made by aggregating *Pax6*$^{+/-}$ and wild-type embryos. These showed that there are primary developmental defects in the heterozygous lens. Eye size and defects of the iris and cornea were corrected in chimeras that are largely mutant but in which the lens was wild type. These abnormalities may therefore be secondary consequences of the defect in the lens. This implies that any attempts at corrective therapy for aniridia should centre on the repair of the lens. Recent data suggest that *Pax6*$^{+/-}$ corneal stem cells can function almost normally in the presence of a wild type lens.

Allocation of tetraploid cells in mouse chimeric tetraploid ↔ diploid blastocysts

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In mid-gestation (E12.5) mouse tetraploid ↔ diploid chimeras the tetraploid cells contribute well to the derivatives of the trophectoderm and primitive en-

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doderm but are usually excluded from the fetus and other epiblast derivatives. Previous studies have shown that tetraploid cells are present in the inner cell mass (ICM) of early chimeric blastocysts but are largely excluded from the epiblast lineage by E7.5. It is possible that tetraploid cells are excluded from the epiblast at the late blastocyst stage by preferential allocation to the primitive endoderm during the differentiation of the ICM into the epiblast and the primitive endoderm. Alternatively tetraploid cells could be present initially in the epiblast but lost before E7.5. We are using confocal microscopy to analyse tetraploid↔diploid chimeric blastocysts in which either the tetraploid or diploid cells express a tau-tagged green fluorescent protein (tau-GFP) transgene. Analysis of early and late tetraploid↔diploid chimeric blastocysts demonstrates that tetraploid cells are present in the epiblast region of the inner cell mass. Tetraploid cells present in the epiblast region must then be lost before E7.5 through cell selection against tetraploid cells.

How the cell cycle co-ordinates neural tube closure

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During embryogenesis, the precursor to the brain and spinal cord forms through the directed shaping, folding and fusion of a sheet of neurectoderm to form the neural tube. The cellular mechanisms orchestrating this fundamental morphogenetic process are not understood. Studies of the two specific sites of bending in the neurectoderm during neurulation, reveal localized areas of interkinetic nuclear migration and proliferation. Within the midline bending area of the upper to mid-spinal region, significant numbers of nuclei lie basally and are slower to incorporate the S-phase label, [^{3}H]thymidine. In contrast, in the dorsal bending area of the mid- to lower spinal region, nuclei are more evenly distributed and exhibit a more rapid incorporation rate. We suggest that there is an increased proliferation rate in the dorsal area and that this is co-ordinated by the increased activity of the cyclin D-Rb cell cycle pathway. Specifically, we find that cyclin D1 is induced and maintained by the surface ectoderm overlying the neurectoderm at the site of dorsal bending. This interaction is an example of how the cell cycle regulates morphogenesis during development.

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BMP4 induces T-box gene expression during mammalian eye development

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The molecular mechanisms involved in morphogenesis and differentiation of the developing eye are not fully understood. Several studies indicate that members of the T-box family of transcription factors play important roles in eye development. The *Drosophila* T-box gene, *omb*, is essential for visual system development, and over-expression of *Tbx5* in the chick eye disrupts retino-tectal connections. In the mammalian eye, we found that *Tbx2*, *Tbx3* and *Tbx5* were expressed in the dorsal aspect of the optic cup and later in specific retinal neurones, suggesting roles in dorso-ventral patterning and neuronal differentiation. Patterning of the eye by asymmetric gene expression is critical for morphogenesis. The signalling molecule BMP4 is implicated in this process, and like the T-box genes it is dorsally expressed. We used a mouse embryo culture system to examine BMP4 as a potential regulator of T-box gene expression. BMP4-soaked beads were implanted in the embryonic eye at stages covering the transition from optic vesicle to optic cup. Ectopic BMP4 signalling in the eye induced *Tbx5* and *Tbx2* expression in early (E9.5) embryo cultures, but only *Tbx2* was induced in late (E11.5) cultures. These results show that the T-box genes are downstream targets of BMP4 signalling in the developing mouse eye, and indicate that closely related T-box genes respond differently to BMP4 signalling.

The role of Notch signalling in embryonic and tumour vasculature development

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Delta-Notch signalling regulates cell-fate choices in a variety of tissues during development. *Delta4* (*Dl4*) is a putative Notch ligand expressed primarily in arterial endothelium during mouse embryogenesis. Expression begins at 8 days post-coitum, earlier than Ephrin B2. *Dl4* is thus the earliest reported arterial endothelium

marker. Subsequent expression is restricted to smaller vessels and capillaries and is reduced in most adult tissues. However, expression is intense in the vasculature of xenograft human tumours in the mouse and in endogenous human tumours. Furthermore, *Dl4* expression is induced by hypoxia and VEGF. These data implicate *Dl4* and the Notch signalling pathway in angiogenesis and suggest possible new targets for anti-angiogenic tumour therapy.

IGF2 function in skeletal muscle differentiation

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The peptide growth factor insulin-like growth factor 2 (IGF2) is predominantly found in embryonic tissues and has been shown to have a dose-dependent effect on embryonic size, which is controlled by genomic imprinting [1]. Its function at the tissue-specific level is less well understood. In adult tissues and *in vitro* cell cultures, IGF2 and the related peptides IGF1 and insulin have been shown to act on skeletal muscle cells to promote their differentiation [2]. IGF2 is also a survival factor for skeletal muscle stem cells [3,4]. We have carried out immunostaining on mouse embryos undergoing myogenesis (skeletal muscle development) and find IGF2 localized to the cytoplasm of newly formed secondary myotubes. The identity of these fibres is confirmed by staining with fibre-specific myosins. IGF2 is evident in developing muscle fibres at E13.5 and this peaks around E15.5. IGF2 is present in muscle fibres in later stages but is more diffuse and by birth has largely disappeared. Using mRNA *in situ* hybridization we show that *Igf2* is locally re-expressed in regenerating skeletal muscle fibres in juvenile and adult skeletal muscles and IGF2 protein and developmental myosin are transiently synthesized at high levels in these muscle fibres at an early stage. These data suggest an important and early role for IGF2 in the genesis of skeletal muscle fibre formation and indicate that it is required for normal muscle fibre formation in both embryos and adult mice.

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Production of transgenic mice with increased *Foxn1* gene dosage: complete rescue of the *nude* mutant phenotype by a wild-type *Foxn1* transgene and potential uses in identification of Foxn1 target genes

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The gene mutated at the *nude* locus encodes the winged-helix transcription factor Foxn1. Animals homozygous for the *nude* mutation exhibit defects in both hair follicle and thymus development, which appear to be due, at least in part, to the reduced keratinization of the hair rudiments and the thymic epithelium. Transgenic mice were produced that carried a 110 kb genomic DNA fragment encompassing the wild-type *Foxn1* genomic locus. In the hair follicles, transgenic mice with increased *Foxn1* gene dosage exhibited increased *Foxn1* expression that was restricted correctly to the nascent, post-mitotic cells of the differentiating hair cortex and hair cuticle lineages. When animals were bred that were homozygous for the *nude* mutation at the endogenous *Foxn*1 locus and that also carried the 110 kb *Foxn1* transgene, they developed both a complete coat of hair and a normal population of peripheral blood T lymphocytes. Thus, the 110 kb *Foxn1* transgene fully rescues the *nude* phenotype and therefore contains sufficient *cis*-acting regulatory information to determine reliable and appropriate tissue-specific expression of the *Foxn1* gene. Comparative gene expression profiling between matched tissues from transgenic animals with increased *Foxn1* gene dosage and *nude* mutants may be an efficient route to the identification of *Foxn1* target genes.

Defining the role of PD-ECGF/TP in development and tumourigenesis

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Human PD-ECGF, also known as thymidine phosphorylase (TP), has been shown to stimulate endothelial mitogenesis and chemotaxis *in vitro* and angiogenesis *in vivo*. The mechanism for the angiogenic role remains unknown, but as PD-ECGF/TP is upregulated in a variety of different tumours, it will be

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important to examine whether it plays a critical role in pathological angiogenesis. We are investigating the structure and expression of mouse PD-ECGF/TP and are looking to define the role it plays in mouse development and tumourigenesis. The mouse gene was isolated from a BAC library and characterized by sequencing. The genomic structure shows strong similarity of organization with the human gene. However, there does not appear to be an obvious polyadenylation signal in the mouse sequence and the mouse gene is followed by a second gene, SCO2. Analysing the cDNA structure of PD-ECGF/TP by RT-PCR, 5′ RACE-PCR and Northern analysis indicates the generation of multiple transcripts from the locus. To target a mutation to the mouse gene, a replacement-type gene targeting construct was used for homologous recombination in ES cells. An in-frame lacZ-fusion was used to generate a loss of function allele and facilitate our understanding of the pattern of expression throughout mouse development and tumourigenesis.

Zic4 is expressed in the developing mouse nervous system

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Mouse *Zic* genes are the homologues of the pair-rule gene, odd-paired (*opa*). In *Drosophila*, pair-rule genes are essential for the parasegment subdivision of the embryo by regulating the timely activation of wingless (*wg*) in odd parasegments and engraved (*en*) in all parasegments. *Zic* genes encode 2H2C-like transcription factors and comprise a family of four members, *Zic1–4*. In parallel with *Drosophila*, mammalian *Zic* genes may regulate expression of *Wnt* genes: for example, *Wnt3a* is reported to be down-regulated in *Zic2* knockdown mice. Here we report the expression pattern of the *Zic4* mRNA during mouse embryogenesis by *in situ* hybridization. *Zic4* mRNA transcripts are first ubiquitously detected at E9. At E10.5 the expression becomes restricted to the dorsal part of the spinal neural tube, excluding the roof plate, and to the dorsal sclerotome which comes to surround the dorsal neural tube. *Zic4* mRNA transcripts are also detected in the dorsal midline (roof plate) of the midbrain and forebrain neural tube with a strong, expanded domain of expression at the diencephalon–telencephalon boundary. At E12.5 the expression becomes restricted to regions of the nervous system, including the telencephalon, diencephalon and dorsal root ganglia. *Zic4* is also expressed in the developing cerebellum at this stage. Therefore, *Zic4* has an expression pattern that is distinct from, but partly overlapping with, other members of the *Zic* family.

Gene targeting of *Nesp* and *Gnasxl* in the imprinting cluster on mouse distal chromosome 2 using a self-excision Cre-loxP strategy

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A screen (Me-RDA) for new imprinted genes resulted in the identification of *Gnasxl* and *Nesp* on mouse distal chromosome 2. *Nesp* and *Gnasxl* are oppositely methylated on the paternal and maternal allele, respectively. They are located upstream of *Gnas* ($G_s$alpha-subunit of trimeric G-proteins), which is imprinted only in some tissues. *Gnasxl* encodes an extra long version of $G_s$alpha, while *Nesp* is a chromogranin of secretory vesicles. Mice with maternal duplication of distal chromosome 2 show long, flat-sided bodies, arched backs, hypoactivity, failure to suckle and death on P1. PatDp.dist2 mice have short square bodies, broad flat backs, oedema, hyperactivity and survival for several days. To determine the contributions of *Gnasxl* and *Nesp* to these phenotypes, we produced specific knock-outs. Homologous recombination in yeast was used to generate the targeting constructs introducing small deletions in the open reading frames. The targeting strategy includes a tACE-Cre cassette for self-excision of selection markers in the male germline. Offspring from male *Nesp* chimeras have a correctly recombined targeted locus. *Gnasxl* targeting was performed in an XX–ES cell line and chimeras have been produced.

A new maternally expressed transcript within the *Gnas* imprinting cluster

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The *Gnas* cluster on chromosome (Chr) 2 of the mouse produces a number of imprinted and alternatively spliced transcripts. On the sense strand these include *Nesp*, which is maternally expressed, *Gnasxl* and *Gnas* exon1a, which are paternally expressed. All three splice into exon 2 of *Gnas* itself and act as alternative first exons for *Gnas*. *Gnas* itself is bi-

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allelically expressed in most tissues. Genomically *Gnas* lies at one end of the cluster with *Gnas* exon 1a, *Gnasxl* and *Nesp* 2 kb, 30 kb and 45 kb upstream, respectively. Analysis of the 15 kb genomic sequence between *Nesp* and *Gnasxl* has revealed a number of ESTs. Their imprinting status has been analysed in mice carrying either two maternal copies and no paternal copies (MatDp) or two paternal copies and no maternal copies (PatDp) of distal Chr 2. A transcript was found in the MatDp(dist2) mice which was not present in the PatDp(dist2) mice. Preliminary analysis suggests this new maternal transcript links the groups of ESTs lying between *Nesp* and *Gnasxl*. One of the ESTs links to exon 2 of *Gnas* and thus would appear to represent a new alternative first exon of *Gnas*.

Epigenetic analysis of the *Dlk1-Gtl2* imprinted domain on mouse chromosome 12: implications for imprinting control from comparison with *Igf2-H19*

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Dlk1 and *Gtl2* are reciprocally imprinted genes located 80 kb apart on mouse chromosome 12. Similarities between this domain and that of the well-characterized *Igf2-H19* locus have been previously noted. Comparative genomic and epigenetic analysis of these two domains might help identify allele-specific epigenetic regulatory elements and common features involved in aspects of imprinting control. We have conducted a complete methylation analysis of the *Dlk1-Gtl2* domain on both parental alleles in the mouse. Like the *Igf2-H19* domain, areas of differential methylation are hypermethylated on the paternal allele and hypomethylated on the maternal allele. Three differentially methylated regions (DMR), each with different epigenetic characteristics, have been identified. One DMR is intergenic, contains tandem repeats and inherits its paternal methylation mark from the germline. An intronic DMR contains a conserved putative CTCF binding domain which we show can specifically bind CTCF. All three DMRs have both unique and common features compared with those identified in the *Igf2-H19* domain. These studies suggest that aspects of Dlk1-Gtl2 regulation are similar to that of Igf2-H19 while others may be different.

Mono-allelic expression of a variegating transgene locus in the mouse

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In contrast to plants and insects, mammals do not appear as predisposed to gene silencing associated with *trans*-sensing of homologous sequences. Exceptions include mono-allelic autosomal gene expression, imprinting and X-chromosome inactivation. We now add to this by establishing the existence of mono-allelic expression of a variegating transgene in mice. To study possible *trans*-effects on expression we compared hemizygous and homozygous mice for a transgene displaying position effect variegation in the mammary gland. These mice carry approximately 25 copies of an ovine β-lactoglobulin transgene integrated peri-centromerically into chromosome 15 [1]. In mice homozygous for the transgene, even though the transgene copy number was doubled, the expression level was not. Using FISH we demonstrate that this is due to neither enhanced variegation nor a reduction in the activity of both transgene loci. Rather, using primary transcript and chromosomal transgene FISH, we demonstrate that in cells expressing the transgene only one allele is active in homozygous mice. A separate line of transgenic mice carrying a similar β-lactoglobulin transgene did not display mono-allelic expression, indicating that the mammary environment was not limiting for transgene expression. Our data suggest successive silencing events occur at this locus and that these probably differ mechanistically.

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Histone acetylation and X chromosome inactivation

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In mammals equal dosage of X-linked gene products between males (X) and females (XX) is achieved by transcriptional silencing of most of the genes on one of the two X chromosomes in female somatic cells. The inactive X chromosome (X_i) can be distinguished from its active counterpart (X_a) in several ways, one of which involves the underacetylation of core histones. Female mouse embryonic stem (ES) cells have two X_as and when induced to differentiate in culture inactivate one at random. By taking ES cells at different stages of differentiation we have shown that all four core histones are deacetylated concurrently during a time period 3–5 days after induction to differentiate. Furthermore it was demonstrated that addition of the histone deacetylase inhibitor trichostatin A (TSA) early in differentiation prevents the appearance of an underacetylated X chromosome. However, once the cells had differentiated for 3 days the establishment of underacetylated core histones on the X_i was TSA-insensitive. We have used chromatin immunoprecipitation (ChIP) to study the relative levels of histone acetylation of the four core histones in male and female ES cell lines along the X chromosome. In support of the immunofluorescence results, X-linked coding regions demonstrated a drop in acetylation of all four core histones following 7 days of differentiation that was specific to female cells. Interestingly, undifferentiated female cells exhibited significantly higher levels of histone acetylation along X-linked coding regions than female autosomal genes or genes on the male X chromosome. In view of the sex-specific hyperacetylation of all four core histones along the female X chromosome we have unveiled an epigenetic mark that may prove integral to the process of X chromosome inactivation.

Molecular and cytogenetic studies of spreading of X inactivation in five X; autosome translocations

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We have used allele-specific RT-PCR and late-replication studies to determine the spread of X inactivation through autosomal chromatin in five unbalanced X; autosome translocations. In the first case, 46,X,der(X)t(X;10)(q26.3;q23.3), four genes spread over 25 Mb of autosomal DNA were inactive on the der(X;10). In contrast to this spread of inactivation and the patients' normal phenotype, no spreading of late replication was observed. Similar long-range, but incomplete, inactivation was also observed in both the second 46,X,der(X)t(X;11)(q26.1;p11.2) and third 46,X,der(X)t(X;7)(q27.3;q22.3) cases, with gene inactivation accompanied by CpG island methylation. Consistent with transcription and phenotype data, there was a spreading of late replication through most of the translocated segments of autosome. Two further cases, both of which involve similar regions of 6p, were also studied. In both cases long-range inactivation was observed but the pattern of gene silencing was discontinuous. Observations of late replication were discordant between the two der(X;6) chromosomes, with a lack of spreading of late replication in one case, and a discontinuous pattern in the second. Observations of H4 acetylation and XIST RNA also showed a similar distribution in this latter case. We conclude that long-range silencing of autosomal genes by X inactivation can occur without many of the features normally associated with the inactive X.

Novel imprinted genes on mouse chromosome 15 identified by methylation-sensitive representational difference analysis

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Methylation-sensitive representational difference analysis was applied to parthenogenetic and normally fertilized mouse embryos in a genome-wide screen for regions of maternal-chromosome-specific methylation. Regions of parental-allele-specific epigenetic modification such as this are associated with genes regulated by genomic imprinting and expressed from only one parental chromosome. Amongst the many loci identified in this screen, two separate regions of imprinted methylation were found on distal chromosome 15. This is the first evidence of genomic imprinting on this chromosome. These differentially methylated regions display characteristic sequence properties of imprinted genes: they are located at CpG islands, and GC-rich tandem repeats are present at one locus. Transcripts have been identified at each of these loci and are currently being investigated. One of the loci under investigation was mapped between D15Mit67 at 40·9 cM and D15Mit105 at 47·9 cM using radiation hybrids. Transcribed from this locus is

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a non-coding, unspliced RNA of unknown function. The transcript is highly expressed in the brain, particularly in the hippocampus, and is also highly expressed in adrenal glands. This novel imprinted gene is expressed exclusively or predominantly from the paternal allele in all tissues examined. The function of this transcript will be investigated using gene targeting approaches.

Comprehensive aneuploidy screening in human embryos using comparative genomic hybridization: implications for developmental genetics and report of first clinical application

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Less than half of all human conceptions result in a live birth, most failing to survive beyond the first few days of life. Many factors negatively influence embryo survival, but one of the most important is chromosomal abnormality. Cytogenetic analyses have revealed that more than 50% of preimplantation embryos generated using *in vitro* fertilization (IVF) contain aneuploid cells. Some infertility centres have now introduced aneuploidy screening via preimplantation genetic diagnosis (PGD) in an effort to identify and exclude such embryos from transfer. This practice has been shown to have a positive influence on IVF outcome for certain groups of patients. Unfortunately, current PGD protocols do not allow every chromosome to be analysed and therefore a proportion of abnormal embryos remain undetected. We report the first clinical application of a method, based on comparative genomic hybridization (CGH), which overcomes these problems and allows a full analysis of chromosome imbalance in polar bodies, oocytes and single embryo cells (blastomeres). This technique has already begun to answer some unresolved questions of developmental genetics (e.g. the true frequency of aneuploidy and mosaicism in human embryos), and in the longer term may offer the hope of an improvement in IVF success rates.

Identification of the gene mutated in the *loop-tail* mouse: a model for severe neural tube defects

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The *loop-tail* mutant mouse provides a model for the severe form of human neural tube defect, craniorachischisis. Homozygous *Lp/Lp* embryos fail to initiate neural tube closure and consequently exhibit an open neural tube from the midbrain throughout the spine. Genetic and physical mapping localized *Lp* to a 1·2 cM (450 kb) region on distal chromosome 1, a region homologous to human 1q22–q23. Comparative sequence analysis of candidate genes identified a mutation within Kiaa1215 (renamed *Lpp1*), encoding a novel transmembrane protein related to *Drosophila* van gogh. The single nucleotide substitution causes an amino acid substitution near the carboxyl terminus of the Lpp1 protein. *In situ* hybridization analysis reveals specific expression of *Lpp1* in numerous embryonic domains, closely correlating with the regions of developmental defects in *Lp* mice. *Lpp1* is first expressed in the developing neural tube at the time and site of initiation of neurulation, and may play a role in regulating formation of the floor plate. At later stages, *Lpp1* is expressed in the developing heart, ear and eyelids, tissues which exhibit defects in the *Lp* mutant. *Lpp1* therefore plays important roles in the development of several embryonic structures.

The homeobox gene *Emx2* is responsible for hearing defects in the deaf mouse mutant pardon

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Pardon mutants (*Pdo/+*) were induced by ENU mutagenesis and lack a Preyer reflex (ear flick)

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following the delivery of a 20 kHz 90 dB SPL tone burst. Dissection of the middle ear has revealed morphological defects of the ossicles which disrupt the ossicular chain resulting in a conductive hearing loss. Physiological recordings of cochlear responses of Pardon mutants show very raised thresholds which could not be accounted for by a conductive hearing loss alone. Analysis of the surface of the organ of Corti by scanning electron microscopy has revealed an increase in numbers of outer hair cells. *Pdo*/+ mutants appear to be healthy and fertile but *Pdo*/*Pdo* mutants die shortly after birth. The reason for their demise requires further investigation. *Pdo* was mapped to mouse chromosome 19 between markers *D19Mit137* and *D19Mit6*. The homeobox gene, *Emx2*, was located in this region and was considered a strong candidate for *Pdo* due to its expression in the branchial arches and the otic vesicle. Sequence analysis of the *Emx2* gene in *Pdo* mutants revealed a missense mutation causing a non-conservative amino acid change in the homeodomain.

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Towards cloning and characterization of the *curly tail* gene

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The *curly tail* (*ct*) mouse is a mouse model for defective neurulation, exhibiting strong similarities with defects observed in humans. The *curly tail* phenotype is not rescued by folic acid treatment but inositol and retinoic acid treatments have been proven effective. The *ct* gene has recently been mapped precisely to an interval of 0·3 cM on mouse chromosome 4 by using a BALB/c–*curly tail* congenic strain. Several candidate genes in this interval were selected on the basis of their established or putative roles in proliferation, since the earliest identified *ct* abnormality is a proliferation defect of tail bud cells. We have been focusing our attention on Wnt4, Cdc42, Idb3 and Cappb1. These genes were analysed for changes in mRNA expression levels by wholemount embryo *in situ* hybridization at the stage of posterior neuropore closure. In addition, these genes were screened for mutations by cDNA sequencing. So far, none of these genes appears to be affected in the *curly tail* mice. Recently, an additional candidate gene was found to be located very near to the polymorphic marker most closely associated with the *curly tail* phenotype. This gene, perlecan, encodes a heparan sulfate proteoglycan and is currently under investigation.

A single locus controls expression of the autoantigen, gp70, in BXSB mice which are prone to the development of the complex trait systemic lupus erythematosus

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Retroviral envelope glycoprotein, gp70, is present in the sera of immunologically normal and autoimmune-prone strains of mice. However, only lupus-prone mice spontaneously develop anti-gp70 antibodies and these have been implicated in the development of nephritis. We investigated the genetic factors that affect the production of both free serum gp70 and anti-gp70 antibodies in the lupus-prone BXSB mouse strain, by analysing $(BXSB \times (C57BL/10 \times BXSB)F_1)$ and $(C57BL/10 \times (C57BL/10 \times BXSB)F_1)$ backcross male mice. Production of gp70 mapped to a single major locus located on chromosome 13 (Bxs6) with a maximum log likelihood of the odds (LOD) of 36·7 ($P = 1{\cdot}6 \times 10^{-38}$). The level of anti-gp70 antibodies was highly dependent on Bxs6-directed gp70 production, and high-titre autoantibody production occurred only when serum gp70 levels were greater than a threshold value of approximately 4·0 μg/ml. A remarkable association was observed between high levels of anti-gp70 antibodies and severe nephritis in Bxs6 homozygote mice. A further mapping study has identified a novel BXSB interval on chromosome 9, Gp1, associated specifically with the production of anti-gp70 antibodies. This work has demonstrated that a single locus is responsible for a specific subset of autoantibody production in a complex trait.

QTL analysis identifies multiple behavioural dimensions in ethological tests of anxiety in inbred strains of mice

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The interpretation of ethological tests of anxiety-related behaviours in rodents, such as the open-field arena and elevated plus maze, is problematic, as it is not clear that different tests measure the same thing or whether anxiety is a unitary phenomenon. We have addressed these questions by asking whether behaviours in five ethological tests of anxiety are under the influence of a common set of genes. Using over 1600 F2 intercross mice we demonstrate that separate, but overlapping, genetic effects can be detected that influence different behavioural dimensions in the open field, elevated plus maze, square maze, light dark box and mirror chamber. We identify a locus on chromosome 4 that influences activity regardless of threat to the animal, indicating that it controls general locomotor activity. We find loci on chromosomes 1 and 15 that operate in all five tests of anxiety, but can be differentiated by their action on behaviour in threatening and non-threatening environments and whether habituation to an aversive environment alters their influence. We conclude that the locus on chromosome 15 acts primarily on avoidance behaviour and the locus on chromosome 1 influences exploration.

Identifying the genes underlying eye phenotype mutations

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We have been screening for novel eye and vision *N*-ethyl-*N*-nitrosourea (ENU)-induced mouse mutants. We chose to concentrate on the eye because it is a tractable developmental system and because of the value of creating new models of human eye disease. Using such a phenotype-driven approach where no assumption is made about gene function we expect to identify novel genes and pathways. We screened 6500 potentially mutant mice for vision defects using a visual tracking drum and physically examined the eyes of about 6000 using a slit-lamp biomicroscope to detect anterior segment defects and an indirect ophthalmoscope to detect retinal defects. We found 51 mutant phenotypes of which 25 are inherited. The screen has yielded dominant mutations and new recessive mutant alleles of the *Pde6b* gene. The inherited mutations affect all parts of the eye. Of the mutations mapped so far, some are located near candidate genes. We have probable mutant alleles of *Pde6b* (seven), *Pax6* (four), *Mitf* (two), *Egfr* (one), *Rtg4* (one) and *Pax2* (one). Amongst the others are novel mutations with specific effects on the cornea, the iris or the retina and others that affect multiple systems. Strategies to identify candidate genes and elucidate the underlying mutations will be discussed.

Mapping, isolation and characterization of gene(s) involved in ethanol preferences: pilot study

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Ethanol preference has been known for several decades to differ greatly between C57BL/6 (high drinker), C3H and BALB/c (reported as low drinkers) inbred mouse strains. *N*-ethyl-*N*-nitrosourea (ENU) has the highest mutation rate of any germ-line mutagen tested in the mouse, allowing phenotype-driven approaches to isolate mutations in any gene of interest. Its ability to produce single base pair mutations *in vivo* allows for a detailed analysis of a gene's normal function and the physiological consequences when mutated, making it ideal for modelling human disease. For mapping a gene(s) that affects ethanol preference we will undertake a genome-wide, phenotype-driven screen for dominant mutations in the mouse. We will treat male mice with ENU, will mate them and will screen F1 hybrid progeny for new mutant phenotypes. The purpose of the pilot study in this project was to characterize the C57BL/6, C3H, BALB/c and (C57BL/6 × BALB/c) inbred strains of mice for voluntary alcohol consumption. The inbred strains were screened for levels of alcohol intake by receiving 2 weeks of free choice between distilled water and

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10% ethanol. A wide and continuous distribution of values for alcohol consumption and preference was obtained. As anticipated from results obtained by other investigators, C57BL/6 mice exhibited a high degree of alcohol preference. Statistical analysis of results indicated that the difference in alcohol consumption between C57BL/6 males and females is statistically significant but is not significant between C3H males and females. On the other hand, we expected that C3H mice would exhibit a low degree of alcohol preference; there are significant differences between C3H females and C57BL/6 females but not between males of the two strains. However, there are not significant differences between C3H (males plus females) versus C57BL/6 (males plus females). We also screened mice using the SHIRPA protocol, three times (before, during and after ethanol consumption). The results show that behaviour remains similar within each strain through the test; however, slight differences from the beginning between strains were observed where C57BL/6 were more aggressive and more hyperactive than C3H mice. We are in the process of standardizing the technique to measure ethanol levels in blood using gas chromatography, with promising results.

Induction of germline mutation at mouse tandem repeat DNA loci by chemical mutagens

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Earlier studies have shown that Expanded Simple Tandem Repeat Loci (ESTR, previously known as minisatellites) provide a useful system for monitoring radiation-induced mutation in mice. However, to date little is known about germline mutation induction at mouse ESTR loci by chemicals. To evaluate mutation induction at these loci, CBA/Ca male mice were exposed to two alkylating agents, ethylnitrosourea (ENU) or isopropyl methanesulphonate (iPMS), subsequently bred with untreated C57BL/6J females, and their offspring were profiled using two ESTR probes, Ms6-hm and Hm-2. A substantially elevated paternal mutation rate was found after exposure of pre-meiotic spermatogonia, whereas the frequency of ESTR mutation after post-meiotic exposure of spermatids was slightly increased. For both chemicals, a non-linear dose–response curve for paternal mutation induced at pre-meiotic stages was found, with a sharp linear increase at low-dose exposure and a plateau at higher doses. Most importantly, high frequencies of spontaneous and induced mutations at ESTR loci permitted us to evaluate mutation induction at doses well below those detected with previously used genetic systems. The mechanisms of mutation induction at ESTR loci and potential applications of this system for monitoring environmentally-induced germline mutation will be discussed.

Of mice, men and pufferfish: three-way comparative sequence analysis studies

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The UK mouse sequencing consortium was initiated in October 1999 with the remit of underpinning the UK mouse genomics effort by obtaining 50 Mb of genomic sequence, targeting four regions which have been the subject of intensive genomic and biological investigation in the UK:

- The WAGR-homologous region on mouse chromosome 2
- The brown deletion complex on mouse chromosome 4
- The Del(13)Svea36H chromosome 13 deletion
- Dmd-Ar region on chromosome X.

The project complements ENU mutagenesis programmes under way at Harwell and HGU that are focused on the recovery and characterization of mutations from the chromosome 2, 4 and 13 deletion regions. A principal aim of the project is the discovery of novel genes and features in the targeted regions.

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The other main rationale is to construct complete transcript maps with accurate gene models derived from bioinformatic and experimental analysis. Obtaining the finished genomic sequence of these three regions will greatly improve the efficiency of mutation scanning and the identification of genes underlying mutations of interest. The MRC mouse consortium is working with the Ensembl project (http://mouse.ensembl.org/) to provide initial automatic annotation of its sequence. A project update will be presented as well as three-way comparative sequence analysis of the WAGR region in fugu, mouse and man. Analysis of early results indicates the presence of novel genes and aid to assign function and understand the regulation of known genes.
Project web-site: http://mrcseq.har.mrc.ac.uk

Hemifacial microsomia: progress in understanding the genetic basis of a complex malformation syndrome

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Hemifacial microsomia (HFM) is a common facial malformation affecting first and second branchial arch derivatives. Principal features include facial asymmetry, hypoplastic ears often associated with preauricular skin tags, and hearing impairment. The phenotype is extremely variable and may be associated with additional craniofacial, renal and vertebral defects. HFM exhibits a complex aetiology potentially involving both genetic and non-genetic factors. The majority of cases are sporadic, but there is substantial evidence for genetic involvement in this condition, including rare familial cases exhibiting a defined mode of inheritance. As an approach towards identifying molecular pathways involved in ear and facial development, we have ascertained both familial and sporadic cases of HFM. Linkage was previously demonstrated in one of these families to 14q32, a locus harbouring the goosecoid gene. A second family, in which autosomal recessive HFM is associated with acro-osteolysis and mullerian anomalies, has been mapped to 2q35. This region contains two members of the WNT protein family. Screening of these genes is in progress. It is hoped that the identification of the disease-causing genes in these families will suggest further candidates for study, particularly in the many sporadic cases of HFM, therefore enhancing our understanding of this complex and heterogeneous condition.

Oculo-oto-dental syndrome: first linkage study

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Oculo-oto-dental syndrome is a rare but unique autosomal dominant disease. The condition results in ocular coloboma, progressive, high-frequency sensorineural deafness and globodontia. It is the only genetic disease known to result in pathologically enlarged teeth. We have identified a large, four-generation British family segregating this condition and have undertaken a genome-wide linkage analysis to localize the disease gene. Two-point analysis showed significant linkage to marker locus *D20S836* with a maximum lod score (Z_{max}) of 3·31 ($\theta = 0{\cdot}00$). Haplotype analysis identified critical recombinants and has localized the disease gene to a 7 cM region flanked by marker loci *D20S108* and *D20S159*. Bioinformatic assessment has identified a number of candidate genes. The most likely candidate within this localization is the *EYA2* gene that is expressed in the eye, ear and craniofacial mesenchyme. No *EYA2* mutation has been found by direct sequencing of the entire coding region. The identification of the disease gene will not only be relevant to the study of neurological disease but will also highlight an exceptional gene involved in the development of human dentition.

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Genetic studies in vesicoureteric reflux

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Primary non-syndromic vesicoureteric reflux (VUR) is a genetically heterogeneous disorder that causes up to 15% of end-stage renal failure in children and adults. We previously reported evidence for linkage to chromosome 1 in autosomal dominant families; however, a subset clearly did not map to chromosome 1. We report here on the genetic localization of a unique pedigree in which the variable phenotype includes reflux nephropathy, VUR and bladder abnormalities. An initial genome screen performed on the first and second generation members of the pedigree led to the discovery of a large region suggestive of linkage on chromosome 3; successive haplotype analysis of the whole genome proved the model of inheritance to be autosomal dominant and the region on chromosome 3 to be the only possible region of linkage. When recently ascertained third generation members were included in the study, not only did the region of linkage reduce significantly, but it was also shown that the disease-causing haplotype was incompletely penetrant. The results suggest that a gene responsible for urinary and bladder development lies in the region of 3q13 with the possible involvement of modifier genes or environmental factors.

The T-box transcription factor gene *TBX22* is mutated in X-linked cleft palate and ankyloglossia

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Cleft palate (CP) has an incidence in humans of approximately 1/1500. As a consequence of deformity, an affected child will have problems with feeding, speech, hearing, dentition and psychological development. CP most commonly occurs sporadically and displays a multifactorial mode of inheritance. The complex interactions between environmental and genetic factors have made it difficult to identify the genetic defects responsible for clefting. However, several families from different ethnic origins have been reported in which cleft palate displays an X-linked, semi-dominant mode of inheritance. Ankyloglossia (tongue-tie) has also been reported in most of these families and, along with a submucous cleft and bifid or absent uvula, this has become an important diagnostic criterion for X-linked cleft palate (CPX, MIM 303400). Linkage data performed on five well-characterized families localizes the gene responsible for CPX to Xq21, between the markers *PGK1* and *DXS1217*. Here we show that CPX is caused by mutations in the recently described T-box transcription factor *TBX22*. We have demonstrated that TBX22 is a major gene determinant crucial to human palatogenesis and the spectrum of nonsense, splice site, frame shift and missense mutations suggest that the cleft phenotype may result from a complete loss of TBX22 function.

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