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Phenotyping of Genetically Altered Mice (7-May-2004)

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Introduction

Genetically altered mice provide superb models of human physiology and disease. They allow us to evaluate the effects of single altered genes in the context of whole organism and provide tremendous insight into gene function. However, they can provide research results that are frequently unexpected, confusing or simply uninformative. The comparative pathologist is required to assess phenotypic impact of single gene alterations on complex molecular pathways. The effects of genetic background and the variability inherent in the gene construct used to create the animals frequently confound this assessment. Finally, findings must be integrated with published information to draw conclusions and design new experiments.

The aim of each phenotyping project is unique, however several common features can usually be identified. In most phenotyping studies, the intention is to not only identify the nature of the lesions, but also to assess how such lesions relate to deviation of normal gene expression and cellular physiology. This review will delineate the approach taken during a typical phenotypic assessment, with particular emphasis on evaluation of embryonic and neurologic phenotypes.

When initiating a phenotypic examination, it is important to collect as much information about the experiment as possible. These include: a) the aim and design of the experiment, b) the known physiology of the target gene and the methods used to manipulate its expression, and c) potential sources of phenotypic variability. The latter factor can easily obscure subtle phenotypes. A critical assessment of these factors is necessary to accurately ascribe altered function of the gene target to the observed phenotypic change. On a more practical level, these factors determine the number and age of animals that must be examined, as well as which tissues should be included in the screening panel. Sources of phenotypic variability include the following:

Method of Genetic Manipulation - Understanding the technology used in the experiment is necessary in order to identify potential factors which may confound the phenotype. Detailed descriptions of methodologies used to create genetically altered animals can be found in Williams and Wagner [1]. The following discussion will address only pitfalls associated with the more commonly used methods to generate transgenic and knockout mice.
There are two basic approaches to manipulate the mouse genome. The first method is used to create **transgenic** animals and employs random chromosomal integration of foreign DNA following injection into fertilized oocytes. The resulting offspring are screened to identify those animals in which stable chromosomal integration of the foreign DNA has occurred. In addition to the target gene, the transgenic construct contains a transcriptional regulatory region which directs both expression level and tissue specificity of the inserted gene. Depending on the aim of the experiment, the target protein may be overexpressed (excessive amounts of normal protein expressed in tissues which normally express it), or ectopically expressed (a normal

protein is expressed in tissues which do not normally express it). Alternatively, the transgene may be modified to create a "gain of function" mutant (where the protein is constitutively expressed) or a "loss of function" mutant (where the protein interacts with its partners in a dominant negative fashion). The nature of the transgenic manipulation will determine the extent to which individual tissues are examined.

Because of the random nature of transgene insertion after pronuclear injection, each resultant founder contains the transgene at a different site in the genome [2]. This **position effect** can profoundly affect the expression of both the transgene and endogenous genes whose regulatory elements may be disrupted by the insertion event. Several factors may influence the resultant phenotype. The foreign DNA usually integrates as linear arrays, resulting in variable levels of gene dosage. The site of chromosomal integration may affect the regulatory function of the transcriptional element contained within the construct. These factors result in variable expression levels of the transgene in different founder lines. In addition, random integration of the transgene may disrupt endogenous genes (insertional mutagenesis) thus further confounding phenotype. Consequently, it is essential that lines from several (at least two) different founders be examined before a conclusion relating a specific phenotype to transgene expression is made [1,3]. To assess dose-response relationships between transgene expression and phenotype, it is also important to assess lines of mice which express the transgene at different levels.

The uncertainties of random integration may be circumvented by the more challenging technology used to create **knockout** mice. Using homologous recombination, the coding region of a specific endogenous gene can be interrupted to eliminate gene expression ("knockout") or replaced with a modified variant of the gene ("knock-in"). The foreign DNA is inserted into cultured embryonic stem (ES) cells, followed by identification of clones that have the correct mutation and then injection of these clones into mouse blastocysts. If chimeric mice have integrated the foreign DNA into their germ line, they can pass it along to their progeny to establish a colony of genetically altered animals. Although gene expression may be more precisely controlled with this method, it is possible to destroy transcriptional control elements controlling expression of a neighboring gene, thus creating varying phenotypes [4].

More sophisticated methods of genetic manipulation are accompanied by their own particular pitfalls. These methods include Cre-Lox technology to create conditional mutants and drug-regulated transgene expression.

Genetic Background - Most commonly used ES cell lines are derived from several 129 substrains [5]. Chimeric animals are typically bred to C57BL/6 mice, producing genetically similar F1 animals sharing similar chromosomal complements of 129 and C57BL/6 strains. However, when these animals are crossed to generate F2 animals homozygous for the target locus, the offspring has a random mix of 129 and C57BL/6 chromosomal DNA. Consequently, phenotypic variability may be caused by alleles which are not linked to the target locus. The increasing use of double/triple knockout combinations, inducible transgenes and cell-specific knockouts provide additional opportunities for creating a genetic background so mixed that results cannot be replicated by other investigators. Ideally, the genetic background of control and experimental animals should be identical, with exception of the target locus. In cases where background effects are likely to be important, the target locus is best propagated in **congenic** strains by successive back-crossing to one inbred strain. After breeding parental strains, F1 progeny are bred back to one parental strain (usually C57BL/6). F2 progeny from this mating are then similarly bred back to the parental strain until, after 6 back-cross breedings (a process which generally takes two years), the resultant offsprings are 99% similar to the chosen strain, with the exception of the region surrounding the target locus. This strategy also provides the opportunity to place the target locus on a number of genetic backgrounds to assess the effects of strain-specific modifier loci [3]. However, circumstances frequently dictate that phenotype of genetically altered animals be evaluated long before congenic strains can be generated. In this case, wild-type and heterozygous litter-mates from the same breedings are used as controls. To ensure that the range of phenotypes possible due to genetic recombination is assessed, large numbers of animals should be examined.

Compensation - When a gene of interest is disrupted, a compensatory process may take over the function of the missing gene and mask its phenotypic outcome [6-8]. Such compensation is typically identified by the altered or increased expression of related genes in the presence of a relatively normal phenotype.

Epigenetic Phenomena - Environmental phenomena such as stress and food composition can have substantial effects on phenotype. In particular, behavioral phenotypes, or those phenotypes such as obesity which are affected by behavior and feeding can be particularly affected [9,10].

Infectious Disease Status - If animals have been produced at the research facility, information regarding the health status of the room in which they live will be available. In general, those phenotypes which affect the immune system are most likely to suffer potential confounding effects of a prevalent but subclinical infectious disease. Not infrequently, Helicobacteriosis will present as a clinical disease (rectal prolapse), particularly in animals prone to inflammatory bowel disease [11] as the result of ablation of components of their immune system.

Strain-specific or Age-related Background Pathology - Strain-specific anatomy and pathology are described and referenced in several excellent texts [12-15]. In addition, on-line resources such as the Mouse Phenome Database, the database of Inbred Strain Characteristics, and the Mouse Tumor Biology Database provide searchable databases of strain-specific anatomy and pathology (see Table 3). The latter is complemented by a recent text on murine tumor classification [16].

The Initial Anatomic Phenotyping Screen

Selection of Mutant and Control Animals - Two issues bear special consideration when selecting animals for morphologic evaluation - the age at which animals are evaluated and their genetic background. The window during which the phenotype is expressed will determine the optimal age for comprehensive examination. One particular possibility which should always be assessed is whether prenatal death of mutant animals is occurring. Disproportionate reduction in the number of homozygous mutant animals compared to numbers of heterozygous and wild-type siblings is usually the first indication of this problem. Identifying the time of fetal death requires euthanasia of pregnant dams at successive stages of pregnancy to determine the time at which embryos are lost. Good reviews detailing the evaluation of embryonic death and perinatal mortality can be found in Brayton [15] and Ward [14]. If clinical abnormalities are present in postnatal animals, the investigator is in the best position to detail the onset and progression of such signs. Numerous protocols for the ante-mortem physiologic assessment of mutant mice exist. These are succinctly reviewed in Rao and Verkman [17]. For progressive conditions, animals in early, mid and late stages of the condition should be chosen for histologic analysis. Not uncommonly, no clinical abnormalities are noted at all. In this case, baseline phenotyping can be performed in young (8 - 12 weeks) and older (12 - 15 months) adult animals.

The effects of genetic background on phenotype are well documented, and can be substantive enough to obscure subtle phenotypes originating from the induced genetic alteration. Ideal candidates for phenotypic evaluation would be back-crossed -/-, +/- and +/+ litter-mates from at least the 5th generation. These animals would be approximately 95% genetically similar, and would differ only in the region surrounding the target gene. In practice, the constraints of time, cost and potential loss of the phenotype from multiple back-crosses result in presentation of animals of more mixed background for examination. This does not pose particular problems if the phenotype attributable to the target gene is an obvious one. However, the more subtle the phenotype, the greater the number of animals that are needed to extract a significant result from the experiment. At a minimum, two age-matched, sex-matched individuals of each genotype (at each time point, and in the case of transgenic animals, from each founder line) are needed to obtain baseline histologic data.

Gross Necropsy and Fixation - The pathologic evaluation of mutant mice relies on methods common to veterinary diagnostic pathology. The necessary technology and procedures used to perform basic assessment of mutant mice have been comprehensively described in several reviews and books [14,15,18-20]. Specific organ systems have been reviewed by Car and Eng [21], and Sundberg [19].

Following euthanasia, blood is collected by cardiac puncture for clinical pathology. In the initial screen, this comprises a complete blood count and clinical chemistry. Bone marrow smears should be taken at this time, as well as a blood smear. Urine may be collected prior to euthanasia (mice will often urinate on a piece of parafilm if handled) or by cystocentesis after euthanasia. Normal murine clinical pathology values may differ from laboratory to laboratory, and should be established by submitting blood from several normal mice prior to evaluating actual cases. A list of markers used to identify individual cellular components of blood may be found in Car and Eng [21].

After exsanguination, the mouse is weighed. It is placed in dorsal recumbency and a standard necropsy performed. For an on-line demonstration of a mouse necropsy see http://www.eulep.org/Necropsy_of_the_Mouse/. Organs are removed and weighed (usually limited to major organs), and organ weights expressed as percentage of total body weight. Grossly evident pathology is noted and photographed (with ruler and identification label) at this stage. This is also the point at which tissues may be collected for molecular techniques.

The entire mouse may be fixed in 10% formalin, 4% paraformaldehyde, Bouin's solution or Feketes acid alcohol. 10% formalin is adequate for initial screening, and Bouin's solution and Feketes acid alcohol are usually used for the nervous system. All except Bouin's solution require decalcification of bony tissues prior to embedding. Bouin's solution will decalcify as it fixes, but care must be taken not to overfix the tissue (in the case of embryos and neonates), and the fixative must be rinsed with water until it runs clear prior to placing tissues in 70% ethanol for storage. Recipes for Feketes acid alcohol and Bouin's solution are given below.

Feketes acid alcohol	Bouin's solution
700 ml/L 100% ethanol 32 ml/L 37% formalin 40 ml/L glacial acetic acid de-ionized water to 1 L	160 ml 37% formalin 80 ml glacial acetic acid 1700 ml saturated picric acid

Fixation by intracardiac perfusion is recommended for tissues which autolyse rapidly, such as nervous tissue or endocrine tissue. After a surgical depth of anesthesia is attained (absence of withdrawal reflex when the foot is firmly pinched with forceps), the mouse is pinned in dorsal recumbency. The chest is opened, and the right atrium removed with scissors. A 21 - 23 ga butterfly needle is placed in the left ventricle, and 5 - 20 ml of saline flushed in over the course of about a minute. Thereafter, 30 - 50 ml of fixative is flushed in until the body becomes stiff. The skull is then opened, and the abdomen opened along the midline before the mouse is immersed in fixative for another 7 - 10 days.

Histologic Evaluation - A list of tissues typically examined during the initial screen is given below. Often, this may be adequate. However, in many cases, specific organ systems are suspected to contain more subtle lesions. In these cases, detailed evaluation of individual organs systems can be done. These most common include serial sectioning (usually embryos or organs with complex 3D structure such as the heart and brain), or immunohistochemistry using paraffin-embedded or frozen sections. Additional animals at different ages may also be required.

Tissues evaluated during initial screen.

a. Head - nasal passages, teeth, skin, vomeronasal organ, eyes, inner, middle and outer ear, tongue, salivary glands, brain, pituitary gland, bone.
b. Heart (longitudinal section), lung (whole lung), trachea and thyroid glands.
c. Liver, gall bladder, adrenal glands and kidneys (in transverse and longitudinal section).
d. Stomach, duodenum, jejunum, pancreas, mesenteric lymph nodes.
e. Ileum, cecum and colon, rectum.
f. Urinary bladder, testes, accessory sex glands, preputial gland (in males) or uterus, ovaries, clitoral gland and mammary gland (in females).
g. Cervical lymphoglandular complex (cervical lymph node, submandibular, submaxillary and parotid lymph nodes), diaphragm.
h. Hind and forelimbs and sternum (skeletal muscle, bone marrow, peripheral nerve, skin, joints)

Embryos

Collection of embryos at specific developmental stages - Matings between fertile males and spontaneously cycling females are usually set up in the late afternoon or early evening. Females in proestrus can be selected by vaginal inspection [22]. Approximately half of the females selected this way will mate that night. Consequently, a relatively large number of matings need to be set up in order to obtain the required number of timed pregnant females. Alternatively, females can be superovulated using intraperitoneal pregnant mares serum gonadotropin (PMSG, typical dose 5 IU) followed 48 hours later by human chorionic gonadotropin (hCG, typical dose 5 IU). Ovulation occurs approximately 12 hours later. Depending on the dose administered, ranging from 2.5 IU (physiologic) to 10 IU (high), large numbers of embryos may implant and result in artifactual changes from overcrowding [23].

Observation of a vaginal plug is required to accurately determine the developmental stage of embryos. In mice kept in a standard 12 hour light:dark cycle, it is assumed that mating occurs at the mid-dark point, at approximately 2 am. If a vaginal plug is identified the next morning, embryos will be assumed to be E 0.5 (embryonic day) or 0.5 dpc (days post-coitus) old [24]. Implantation usually occurs at E 4.5 and the duration of pregnancy is 19.5 - 21 days. Before implantation, embryos may be retrieved by flushing the oviduct and uterus with phosphate buffered saline. Between E 4.5 and E 8.5, it is best to isolate the embryo within its intact decidual swelling to avoid damaging it. After E 8.5, the embryo can be dissected from the uterus and its yolk sac. It can be retained within the amnion, but considerable care should be taken to avoid damage.

Fixation, embedding and orientation - Embryos may be fixed in Bouin's solution, 10% formalin or 4% paraformaldehyde. Particularly with Bouin's solution, the tissue will become brittle if placed in fixative for too long. Embryos with a crown-rump length of 2 mm require only 1 hour in fixative, while those with a crown-rump length of approximately 15 mm can be placed in fixative for up to 24 hours. After removal from fixative, embryos may be placed in 70% ethanol for long-term storage at room temperature. Before embedding, the embryos are dehydrated through graded stages of alcohol, prior to being placed in a 1:1 mixture of 100% ethanol: benzene (see Kaufmann for detailed procedures [25]). The addition of a few drops of eosin at the 90% ethanol stage will stain the embryo pink and facilitate its visualization during embedding. Embryos older than E 5.5 can be relatively easily oriented, as the head and tail can be easily visualized, and they tend to fall on their sides in the wax block. Younger embryos within their decidual swellings can be sectioned in the transverse plane by using the decidual swelling to orient the embryo. Attempting to section in the longitudinal plane often produces sections of intermediate obliquity. A large number of specimens may be required to obtain useful sections of specimens under E 5.5. For most purposes, embryos are examined by collecting serial sections cut at 5 - 8 μm in the sagittal and transverse planes. Transverse sections are generally done through the majority of the embryo, and provide the most morphologic information. Sagittal sections are usually restricted to a few sections obtained on either side of the midline, as these become less informative the further from the median plane they are. Coronal sections are usually reserved for examining the cephalic region of E 14.5 - E 15.5 embryos.

Histologic Interpretation and Staging - The most commonly used staging system is that of Theiler [26,27]. This system has been adopted by recent standard texts on mouse embryology [28,29]. A table correlating the Theiler system with embryonic age, size and morphologic features can be found at http://genex.hgu.mrc.ac.uk/. The Atlas of Mouse Development [28] provides the most comprehensive illustration of each of the Theiler stages. Each Theiler stage, up to about E 11.5 (Theiler stage 20) lasts for about 12 hours. As tissues develop so rapidly at these stages, a precise identification of embryo age may be difficult. Ageing of embryos is easier after E 12, when each Theiler stage encompasses about 24 hours. Ageing can also be done by examining the sequence of long bone ossification in whole embryos or tissue sections. This method is best used after E 15.5 [30] when ossification centers are present. The pathologist should be aware of intrinsic variations in normal embryonal development. Within the same litter, developmental maturity can vary by 6 - 12 hours. In addition, the studies of Theiler and Kaufman have used F1 hybrids between C57BL X CBA mice, and variations in the developmental velocity of other strains are likely to be present.

Figure 1. Hematoxylin and eosin stained sections of wild-type (A) and mutant (B) liver at E 12.5. The mutant animal has a very small, blood-filled liver, with poorly formed trabeculae (arrow). Bar = 50 microns. - To view this image in full size go to the IVIS website at www.ivis.org . -

Hematoxylin and eosin staining is sufficient for initial screening. Further analyses frequently make use of the spectrum of techniques traditionally used in light microscopy, e.g., special stains, histochemistry [31], immunohistochemistry and *in situ* hybridization [32,33]. In these cases, additional embryos which have been fixed and processed according to the needs of the specific protocol are usually required. Evaluation of the embryonal skeleton is best done using alcian blue-alizarin red staining of whole cleared embryos [30].

Neuroanatomical Evaluation

Clinical Evaluation of the Live Mouse - Forebrain lesions rarely induce neurologic signs and require behavioral testing to detect. Clinical examination should precede behavioral testing as a host of unrelated factors can cause profound artifactual deficits on behavioral tests. Strain-specific background pathology (e.g., retinal degeneration in C3H and related mice, or callosal defects in BALB/c, 129 and other mice) may affect behavioral test results. C57BL/6 mice tend to display "normal" hyperactive behaviors compared to other strains. Locomotor abnormalities and muscle weakness may also result from abnormalities of the cardiovascular and hematologic systems, or from generalized malaise due to renal or hepatic disease. Clinically apparent neurologic lesions generally result from lesions in the brain stem, cerebellum, vestibular system, spinal cord, nerve or muscle. Table 1 lists tests and clinical signs which may be used to localize lesions to specific regions of the nervous system. Table 2 lists the most common behavioral tests used to assess more subtle motor, sensory or cognitive capacities.

Table 1. Clinical and neurologic evaluation of the mouse

Test	Normal Response	System tested
Body condition	No visible bony protuberances, no obesity	General health
Respiration	Barely visible	Cardiopulmonary, musculoskeletal, hematopoeitic systems
Eyelids	Wide open, clean, no excessive lacrimation	Ocular disease, central nervous system
Teeth and tongue	Teeth are aligned, tongue moves and is pink and smooth, no excessive salivation	Abnormalities can cause emaciation and death
Skin and coat	Pink mucosae, smooth skin and intact coat	Cardiopulmonary, hematologic and dermatological systems
Body tone	Slight resistance when squeezed gently	General health, nervous, musculoskeletal, behavioral systems
Abdominal palpation	No masses, slight resistance	Intrabdominal disease, nervous, musculoskeletal, behavioral systems
Limb tone/grasping strength	Detectable tone, strong grasp on cage wire	Nervous, musculoskeletal, behavioral systems
Spontaneous activity	Grooming, moderate movement and exploration	General health, neuromuscular system, anxiety, fear
Observation of body position	Sitting or standing	General health, neuromuscular system
Tremors	None	Central nervous system
Circling/ataxia	None	Vestibular or cerebellar disease
Quadri/paraplegia/tail position/gait	Normal gait with horizontally extended tail	Central nervous system, musculoskeletal system
Seizures	No	Central nervous system
Stereotypies	No	Central nervous system, behavioral
Trunk and limb position when lifted by tail	Trunk curls, limbs splayed	Central nervous system, behavioral
Visual placing	Forearms extend to meet cage floor when mouse is lowered by tail	Visual system
Forelimb wheelbarrow	Forearms move normally when hind limbs elevated by tail	Central nervous and musculoskeletal systems
Eye blink and ear twitch reflexes	Single eye blink, ear retraction	Cranial nerves V and VII
Toe pinch	Retraction	Central nervous and musculoskeletal systems
Righting reflex	Immediately rights when placed supine	Central nervous and musculoskeletal systems
Induced biting	Can be normal	Behavioral
Spontaneous biting	Abnormal	Behavioral
Vocalization when restrained	Can be normal	Behavioral

Table 2: An overview of behavioral tests in mice. Adapted from Crawley and Paylor [43]

Test	Function tested
Open field test	Locomotor activity, anxiety and habituation
Rotarod	Motor coordination, balance, ataxia
Hindpaw footprint	Motor coordination, balance, ataxia
Acoustic startle and prepulse inhibition	Test of hearing as well as hippocampal function
Morris water task	Spatial learning
Acetic acid test, formalin test, hot plate test, foot shock test, tail flick test, tail pressure test	Pain tolerance
Contextual and cued fear conditioning	Memory skills, fear levels
Dark/light avoidance; passive/active avoidance test; elevated maze (plus or zero)	Anxiety
Direct observation or resident intruder tests - time taken to initiate first attack	Aggression
Forced swim test - increased time floating or not moving	Indication of behavioral despair

Tissue Collection - Perfusion (see above) or immersion fixation can be used. The former is desirable particularly if the inner ear is to be examined. Decalcification will occur over a period of about 6 - 10 days (adult mice) with Bouin's solution. Other fixatives require 24 - 48 hours of decalcification. As removal of the central nervous system prior to fixation results in significant artifact, the central nervous system (CNS) is usually fixed *in situ*, followed by sectioning of the decalcified tissues. This has the advantage of being able to evaluate the CNS in relation to its surrounding structures. In general, paraffin embedded tissue is used to obtain an overview of the pathology present - additional techniques such as electron microscopy or morphologic techniques requiring cryosections are performed once the anatomic location of a lesion has been established.

Histologic Examination - Detecting subtle lesions may require serial sectioning, however, it is best to try to identify which region of the brain is likely to contain the lesions before progressing to serial sections. Pathologists are generally most familiar with cross (or coronal) sections of the brain - the nomenclature used by Paxinos and Franklin [34] is referred to most often when identifying the localization of the section. Using this nomenclature, Bregma positions refer to the level at which the coronal section has been taken, with Bregma 0 mm representing the baseline reference value (located just caudal to the eyes). Positive Bregma values correspond to regions rostral to Bregma 0 mm, and negative values to locations caudal to Bregma 0 mm. Initial examination of the brain can be done using 6 - 8 sections representative of the following key regions:

a. Bregma 3.6 mm: eye and olfactory bulb
b. Bregma 0 mm: internal and external capsules, basal ganglia, sensory and motor cortex
c. Bregma -1 mm to -3.8 mm: hippocampus, amygdala, hypothalamus, thalamus, cortex, corpus callosum
d. Bregma -6.3 mm: cerebellum, vestibular nuclei, cerebellar nuclei, inner ear
e. Bregma -8 mm: caudal cerebellum, medulla.

Figure 2. Peripapillary retinal dysplasia in a caspase-3 knockout mouse (B). Neuroretinal folds (arrows) are noted around the optic nerve in the mutant animal, but not in the wild-type control mouse (A). Bar = 50 microns, coronal sections at Bregma 3.6 mm - To view this image in full size go to the IVIS website at www.ivis.org . -

Several sections, each of the cervical, thoracic and lumbar spine should be taken, as well as a sagittal section of a hind limb to visualize skeletal muscle and peripheral nerve. The inner ear should be examined in three axes - coronal, horizontal and paramedian. Many lesions affecting the hair cells may only be detectable using scanning or transmission electron microscopy. Hematoxylin and eosin staining will allow visualization of most lesions - additional special stains, immunohistochemical stains and histochemical stains can be done. More recently, magnetic resonance imaging has been used to characterize neurodevelopmental phenotypes in live mice [35].

Cardiovascular and Skeletal Phenotyping

In vivo cardiac phenotyping in mice is complicated by the small size of the heart and its rapid rate (approximately 500 bpm). Large variations in normal heart rate are reported (200 - 700 bpm). In addition, anesthesia results in significant reduction in heart rate, blood pressure and body temperature thus complicating evaluation of the unconscious animal. Conversely, restraint of the conscious animal results in artifactual elevation of heart rate and blood pressure. Despite these challenges, numerous non-invasive technologies such as echocardiography, electrocardiography, tail cuffs and radiotelemetry have been developed to evaluate live mice [36-39]. Subclinical phenotypes may reveal abnormalities following physiologic challenge stimuli - these include pharmacologic, dietary, exercise, and emotional challenges. As with other systems, combining mutant strains to generate double mutant animals may also reveal an underlying phenotype [40].

Skeletal phenotyping can be accomplished using an array of *in vivo* and post-mortem techniques. Observation of whole animal characteristics (abnormal skeletal features) can be done directly or with radiography. Alcian blue-alizarin red staining to visualize bone and cartilage is best for embryos. More complicated *in vivo* imaging techniques such as MRI, computed tomography or DEXA are used to evaluate bone density and quality. A review of advanced imaging techniques in live mice can be found in Hoit [41]. In post-mortem tissue, histologic morphometry plays a crucial role. Finally, mechanical tests of bone strength can be done by evaluating resistance to torsion, compression and bending [42].

Information Resources

Following completion of the initial phenotyping panel, the data should be assessed in the light of the experimental design. This requires integration of current knowledge of the cellular process in which the target gene is involved, and comparison with described related mouse phenotypes.

Information Sources on Gene and Phenotype - Collective analysis of numerous mutant mouse studies will eventually provide a more comprehensive overview of cellular physiology and pathobiology in the whole organism. As sequencing of the mouse genome nears its completion, mutant mouse studies provide the best opportunity for generating a functional correlate of descriptive genomics (i.e., structure of genes, and their associated mRNA and protein sequences). Most veterinary pathologists are trained to categorize disease according to organ, system or etiology. Because mutant mice are generated by genetic manipulation, and the resulting pathology frequently affects multiple organ systems, this approach is ill-suited to traditional analysis. Currently, no comprehensive resource exists which correlates structure and function of genes to their cognate cellular pathways and mutant phenotypes. However, extensive data exist for each of these disciplines independently, so it falls to the pathologist and investigator to integrate them. Table 3 provides a recent list of the most comprehensive resources. As few central databases exist, the pathologist must collate published information on similar or related mutants. The Entrez site (http://www.ncbi.nlm.nih.gov/Entrez/) hosted by National Center for Biotechnology Information (NCBI) is most suited to this purpose.

Table 3: Information Resources for Phenotyping - Pathology and Morphology

Resource	Location	Description of Contents
General Histology and Strain-Specific Pathology		
HistoBank	http://histology.nih.gov/	Mouse and human pathology images
Inbred Strain Characteristics	http://www.informatics.jax.org/external/festing/search_form.cgi	Strain-specific physiology and pathology
Mouse Phenome Database	http://aretha.jax.org/pub-cgi/phenome	Searchable database of strain-specific physiology and anatomy
Histology Atlas of the Mouse Mammary Gland	http://mammary.nih.gov/atlas/	Mammary development and pathology
The Mouse and Rat Research Home Page	http://www.tu-bs.de/institute/ibb/cell-biol/rodent_page.html	Broad array of genetics, anatomy and pathology links
The Visible Mouse	http://ccm.ucdavis.edu/tvmouse/	Mouse anatomy by system
Guide to the Necropsy of the Mouse	http://www.eulep.org/Necropsy_of_the_Mouse/	A step by step guide through a mouse necropsy

Table 3: Information Resources for Phenotyping - Pathology and Morphology		
Resource	**Location**	**Description of Contents**
Cancer Biology		
Mouse Models of Human Cancers Consortium	http://emice.nci.nih.gov/mouse_models/	Mouse models of human cancer
Mouse Tumor Biology Database	http://tumor.informatics.jax.org	Search by tumor, strain or tissue
Technical information		
Immunohistochemistry	http://web.ncifcrf.gov/rtp/lasp/phl/immuno/	Antibodies known to work in mouse tissue
The Antibody Resource Page	http://www.antibodyresource.com/	Searchable index of sources of antibodies
Neuroanatomy and Neuropathology		
The Molecular and Biological Basis of Genetic Deafness	http://www.ihr.mrc.ac.uk/ hereditary/MutantsTable.shtml	Mouse models of inherited deafness
High Resolution Mouse Brain Atlas	http://www.hms.harvard.edu/research/brain/	Interactive atlas of coronal sections
Digital Anatomist Project	http://www9.biostr.washington.edu/da.html	Interactive atlases, predominantly neuroanatomy
Development		
Embryo Images Online	http://www.med.unc.edu/embryo_images/unit-welcome/welcome_htms/akgs.htm	Scanning EM tutorial by organ system
UNSW Molecular Development	http://anatomy.med.unsw.edu.au/ cbl/embryo/OtherEmb/Mouse.htm	Molecular information, signaling pathways, inmages
The Kidney Development Database	http://golgi.ana.ed.ac.uk/kidhome.html	Anatomy, genes and animal models related to renal devleopment
The Glandular Organ Development Database	http://www.ana.ed.ac.uk/anatomy/ database/orghome.html	Lung, salivary gland, mammary gland, pancreas, prostate development
Tooth Development	http://bite-it.helsinki.fi/	Gene expression in developing teeth

Gene/Protein Structure and Function - In order to critically evaluate phenotype, the pathologist should be aware of the gene structure, expression pattern of the gene and protein in the embryo and the adult, and the role in cellular physiology. By the time a genetically altered animal is created, a fair amount of information is available about the gene. On-line resources are listed in Table 4. The Unigene site provides the gene sequence and structure, protein sequence, and associated literature. The site can be searched according to organism, and is useful for comparing mouse and human genes. In addition to search by gene, LocusLink allows searches by organ system or cellular process. The latter searches provide a list of functionally related genes and literature. Knowing when and in which tissue the gene is normally expressed is important to select the correct age animals for examination, interpret the resulting phenotype and select the correct repertoire of tissues for detailed examination. Examination of the images in appropriate research articles usually provides the best description of gene expression. In addition, multiple databases provide comprehensive lists of gene expression by tissue, organism and disease/physiologic state (see Table 4). The WWW Virtual Library of Cell Biology (http://vlib.org/Science/Cell_Biology/index.shtml) provides numerous links to sites related to most aspects of cellular function.

Table 4: Information Resources for Phenotyping - Genomics		
Resource	**Location**	**Content of Site**
Gene Structure and Function		
Unigene	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene	Gene, protein sequences

Table 4: Information Resources for Phenotyping - Genomics		
Resource	**Location**	**Content of Site**
LocusLink	http://www.ncbi.nlm.nih.gov/LocusLink/	Functionally related genes
Gene Expression		
SAGE	http://www.ncbi.nlm.nih.gov/SAGE/	Differentially expressed genes in different tissues/states
CGAP	http://www.ncbi.nlm.nih.gov/ncicgap/	Tissue specific expression
Body Map	http://bodymap.ims.u-tokyo.ac.jp/	Mouse/human gene expression
Mouse Genome Informatics	http://www.informatics.jax.org	RNA/protein expression of selected genes
Cell Biology		
The WWW Virtual Library of Cell Biology	http://vlib.org/Science/Cell_Biology/databases.shtml	Multiple links to cellular processes
Genes and Mouse Phenotype		
Mouse Genome Informatics	http://www.informatics.jax.org	Known mouse phenotypes
Jackson Lab Mouse Models Lists	http://jaxmice.jax.org/jaxmice-cgi/jaxmicedb.cgi	Organized by body system or cellular process
BioMedNet	http://research.bmn.com/mkmd	Search for genetically altered mice by gene
Genes and Human Disease		
OMIM	http://www.ncbi.nlm.nih.gov/omim	Human disease: Gene, processes, mouse mutants

Genes and Mouse Phenotypes - The most comprehensive on-line resource linking gene to mouse phenotype is the knockout mouse portion of BioMedNet (Table 4). This site retrieves literature citations following searches by gene, gene function, cellular process, organ system or pathology. The mouse model lists provided by the Jackson Laboratory are organized according to categories of organ system and physiologic processes and provide a good starting point to look for mutant mice falling under these categories. Published descriptions of organ specific mutant mouse pathology can be found in several texts.

Linking Mouse and Human Phenotypes - The OMIM website (Table 4) is the most comprehensive source of human gene defects and associated phenotypes. A search by gene or phenotype typically produces a substantial list of results, which usually describe known information on a gene or the phenotype(s) of murine counterparts.

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