Pathology Principles and Practices for Analysis of Animal Models

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Abstract

Over 60% of NIH extramural funding involves animal models, and approximately 80% to 90% of these are mouse models of human disease. It is critical to translational research that animal models are accurately characterized and validated as models of human disease. Pathology analysis, including histopathology, is essential to animal model studies by providing morphologic context to in vivo, molecular, and biochemical data; however, there are many considerations when incorporating pathology endpoints into an animal study. Mice, and in particular genetically modified models, present unique considerations because these modifications are affected by background strain genetics, husbandry, and experimental conditions. Comparative pathologists recognize normal pathobiology and unique phenotypes that animals, including genetically modified models, may present. Beyond pathology, comparative pathologists with research experience offer expertise in animal model development, experimental design, optimal specimen collection and handling, data interpretation, and reporting. Critical pathology considerations in the design and use of translational studies involving animals are discussed, with an emphasis on mouse models.

Key words: animal models; genetically modified; mice; pathology; phenotype; preclinical; scoring; translational research

INTRODUCTION

To use a comparative pathologist or not to use a comparative pathologist: Why is this important for my model? Accurate characterization and validation of models of human conditions are mission critical to biomedical research.¹ Universities, research foundations, and pharmaceutical companies have a growing need for animal models that provide a means of characterizing physiologic interactions from basic science to translational medicine.² Common models utilized include immunodeficient, chemically induced, surgically induced, and genetically engineered models of the particular entity or disease being studied. By far, the most common models utilized are genetically engineered mouse (GEM) models. Recently, preclinical efficacy studies and

biomarker identification have greatly increased the number of models utilized. Histopathological evaluation is often an endpoint of translational research conducted in these models, and it is highly dependent on the expertise of comparative pathologists with the knowledge of and extensive experience and training in the pathobiology of rodent and nonrodent models.³ Thus, there is an increased demand for those who can understand the unique biology of various animal species and can accurately characterize the models generated and utilized. As such, comparative pathologists have a great impact on the validation of animal models of human disease.^{4,5} With the background, knowledge, experience, and skill set to accurately evaluate animal models of human disease, comparative pathologists are an integral part of

the research team. Beyond pathology, a comparative pathologist can provide expertise in animal model development, experimental design, optimal sample collection, and data interpretation. A discussion of the various animal models is beyond the scope of this manuscript. However, we will discuss the importance of including a comparative pathologist, and specific pathology considerations at various stages of translational research incorporating animal models, with an emphasis on mouse models of human disease.

EXPERIMENTAL DESIGN: MODEL DEVELOPMENT (EXPERIMENTAL PLAN)

Prior to the initiation of a study involving animal models, the development of a detailed experimental plan is highly recommended. The inclusion of a comparative pathologist is critical to studies including histopathological analysis of the model as an endpoint. For the pathologist to best support the goals of the project, it is best for the research group to meet with the pathologist to discuss the ultimate goals of the study and assist in the development of the experimental plan. Flaws in research design are often the basis for model failure and failure to predict therapeutic response in the human disease being modeled. The earlier a pathologist is involved in the project, the more impactful the pathologist may be in providing guidance with study design and identification of potential pathology endpoints. Beyond pathology, the pathologist may advise the investigator on selection of the most appropriate animal model, number of animals, and ages and genders to evaluate. The inclusion of appropriate controls is critical to understand the genetic background and husbandry effects on the model. For rodent models and specifically genetically engineered models, a pathologist can recommend a preferred background strain as well as strains that should be avoided due to the high incidence of background lesions in tissues of interest to investigative staff. In the case of genetically engineered models, there may not be a known or anticipated phenotype; thus, recommendations regarding control groups and number of animals to be evaluated are valuable for sample size calculations and grant budget preparation. Further guidelines may be discussed with a statistician, and additional references are available in this issue and in the literature (Everitt et al. 2018 [this issue]).⁶⁻⁸ At this time, it is also ideal to discuss pathology endpoints, scope of necropsy, tissue and/or fluid samples to be collected, and whether samples are needed for collaborators and/or additional assays.

Each type of model has its pros and cons, and an understanding of the proposed or studied model will assist the pathologist in determining pathology endpoints. Details regarding any experimental manipulation including administered xenobiotics or surgical procedures performed should include clear methods as well as details such as: type of xenobiotic and vehicle, mechanism of action, pharmacokinetic data, duration, route, dose and frequency, surgical and sham procedures, etc. Essential details of neoplasia induction and anticipated metastasis include initiation and promotion protocols for chemical carcinogenesis, or the species and cell of origin as well as route of injection for xenograft models. For example, subcutaneous xenograft tumor models are easy to monitor and provide easy access for treatment; however, they may fail to grow or may grow too quickly and often fail to recapitulate the human disease. Orthotopic transplant models will metastasize more readily so collection of the lungs, liver, regional lymph nodes, and skeleton will need to be included in the necropsy plan,

depending on the cell line.⁹ Chemical induction of tumors recapitulates the human neoplastic disease secondary to mutagenic agents; however, tumor formation or latent period may be extensive so pathology endpoints may need to be adjusted.

Reproducibility and transparency are key components of any scientific experiment and allow for the generation of consistent data within the same laboratory or permit the reproducibility of published work performed at other sites. Further information on transparency and reproducibility may be found in additional resources and within this issue (Everitt et al. 2018 [this issue]).² In short, although more animals may be needed and additional samples may need to be collected, an increased awareness of and attention to rigor and transparency will lead to reduction of artifacts and errors and efficient use of resources, and will allow for consistency, reproducibility, and, ultimately, translatability of the proposed model.¹⁰ For study consistency and reproducibility, the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines were developed by the National Centre for the Replacement, Refinement and Reduction of Animals in Research to provide guidance and an online Experimental Design Assistant tool for the design, analysis, and publication/reporting of research utilizing animal models.⁸ The inclusion of reportable information includes detailed methodology regarding experimental procedures conducted, control groups, animal species, age, sex, strain, and housing and husbandry details (Everitt et al. 2018 [this issue]).^{8,10,11} This information is essential for accurate reporting in manuscripts and grant applications and has been adopted as essential information by many journals. As such, involving a comparative pathologist who may assist in thoughtful study design in the beginning of a proposed study can help prevent pitfalls later.

EARLY CONSIDERATIONS

Animal Model

It is important to understand the overarching goal of the studied or proposed animal model for accurate evaluation and interpretation. Meeting with the study pathologist is essential to exchange relevant information about the human disease to be modeled, model design, previous studies, target organs, relevant literature, and observed or anticipated phenotypes. Knowledge of study design and animal history will help confirm that the appropriate model has been selected, guide the pathologist to perform a detailed and comprehensive analysis of the model, and aid in the identification of subtle phenotypes. Any ancillary data that is already known or has been collected, including data on morbidity or mortality, clinical pathology, gene or protein expression, and flow cytometry, should be discussed. Pathology endpoints may be determined based on previous findings, published reports, and natural history. For rodent models, essential model details include: species, age(s), background strain, sex, immune status, diet, and housing. Comparative pathologists and pathology support resources routinely accept samples and work with a spectrum of animal models that includes nonrodent models. Although discussing every species and type of animal model is beyond the scope of this piece, Table 1 provides a list of selected key pathology reference texts for common animal models, other than the mouse, used in translational research.

Genetically Engineered Models

Genetically engineered models are often used to study the molecular and genetic mechanisms of human diseases and

Table 1 Selected Key Pathology Reference Texts for Preclinical Studies Involving Non-Mouse Species

Title	Editor(s)	Species Covered
Background Lesions in Laboratory Animals: A Color Atlas	E. F. McInnes	NHP, rats, dogs, mice, hamsters, GP, minipigs, rabbits
Biology and Diseases of the Ferret (3 rd Ed.)	J. G. Fox, R. P. Marini	Ferrets
Boorman's Pathology of the Rat: Reference and Atlas (2 nd Ed.)	A. W. Suttie	Rats
Comparative Anatomy and Histology: A Mouse, Rat, and Human Atlas (2 nd Ed.)	P. M. Treuting, S. M. Dintzis, K. S. Montine	Mice, rats, humans
Flynn's Parasites of Laboratory Animals	D. G. Baker	Fish, amphibians, reptiles, birds, rats, mice, hamsters, gerbils, GP, rabbits, ferrets, dogs, cats, pigs, sheep, goats, NHP
Haschek and Rousseaux's Handbook of Toxicologic Pathology (3 rd Ed., Vols. 1–3)	W. M. Haschek, C. G. Rousseaux, M. A. Wallig	Mice, rats, dogs, NHP, minipigs, fish, amphibians, reptiles, birds
Laboratory Animal Medicine (2 nd Ed.)	J. G. Fox, L. C. Anderson, F. M. Loew, F. W. Quimby	Mice, rats, hamsters, GP, other rodents, woodchucks, rabbits, dogs, cats, ferrets, ruminants, pigs, NHP, amphibians, reptiles, zebrafish, other fish
Natural Pathogens of Laboratory Animals: Their Effects on Research	D. G. Baker	Rats, mice, gerbils, hamsters, GP, rabbits, ferrets, cats, dogs, pigs, NHP
Nonhuman Primates in Biomedical Research: Diseases (Vol. 2)	C. R. Abee, K. Mansfield, S. Tardif, T. Morris	NHP
Pathology for Toxicologists: Principles and Practices of Laboratory Animal Pathology for Study Personnel	E. McInnes	Rats, mice, dogs, minipigs, NHP, rabbits
Pathology of Laboratory Rodents and Rabbits (4 th Ed.)	S. W. Barthold, S. M. Griffey, D. H. Percy	Mice, rats, hamsters, gerbils, GP, rabbits
The Laboratory Primate	S. Wolfe-Coote	NHP
The Laboratory Rat	G. J. Krinke	Rats

GP, guinea pigs; NHP, nonhuman primates.

ultimately the treatment or mitigation of disease. The most common GEM are rodents that have a genetic alteration associated with a human disease. GEM models are evaluated for specific phenotype(s) or characteristics of the disease being modeled. Phenotyping studies involving GEM models should be planned in advance, and the hypothesis or question often dictates the number of animals and can influence the analysis and reported data.¹² Integral model details include source, background strain(s) of mouse and/or embryonic stem cells, any genetic manipulations, and genetic quality control measures. Descriptions of genetic modifications are essential for pathologic evaluation and include: specific gene of interest, type of genetically engineered construct, tissue specificity of the model, type of promoter, and whether the model is conditional.^{12–14} An understanding of the generation of models and specifics regarding the proposed or evaluated model aids in the pathologic evaluation. Technical approaches for making mouse models have been described in the literature.¹³ Conditional mutagenesis techniques must be described and brought to the attention of the study pathologist because tissue-specific Cre technology may have widespread affects or affects in tissues other than the model target tissue.^{14,15} Unwanted effects of GEM technology that are not related to the engineered phenotype include alteration of the normal flora and microbiome through the antibiotic effects of tetracycline-based systems and inducible systems utilizing tamoxifen in corn oil.¹⁴ Aberrant or unexpected phenotypes may also develop in GEM secondary to random integration of transgenes.¹⁴ Thus, as will be discussed further, controls should be included in any pathologic evaluation for accurate interpretation.

Background Strain

Differences in background strain may have an impact on model development. There is a large amount of information on the pathological, behavioral, immunological, and physiological differences between inbred and noninbred strains that should be taken into consideration when choosing a background strain and that may impact the pathologic endpoints (Brayton et al. 2018 [this issue]).^{16–18} In the case of rodent models and, in particular, GEM, background strain and substrain have been shown to influence the development of target lesions, including neoplasia.¹⁴ Examples include the difference in immunologic response to pathogens in infectious disease studies^{14,16,19} and susceptibility to developing tumors in models of carcinogenesis.^{20,21} Additionally, some strains are more resistant to tumor development whereas other strains are predisposed to tumor development,²¹ and the strain can also vary along the spectrum of tumor phenotypes in established models.^{18,21,22} Background strains (inbred and noninbred) are also predisposed to many spontaneous lesions, which are considered normal findings for the respective strain but may be misinterpreted as a model phenotype or may cause unexpected fatalities (Brayton et al. [this issue] 2018).^{17,22,23} These common spontaneous lesions are referred to as “background lesions.” A comparative pathologist with the knowledge of background lesions and expected strain phenotype(s) is invaluable for the recognition of phenotypes secondary to genetic manipulation versus those associated with background strain.

Thus, a thorough genetic history is essential for the study pathologist, and in addition to mouse strain and substrain information should include breeding history and backcross

generation. Inbred mouse strains are not genetically identical and often vary by source.^{12,23} The specific vendor or source of the background strain is critical for accurate comparison because vendor substrains have often genetically drifted from the original parent strain.^{24,25} These differences may impact model interpretation. Thus, C57BL/6J mice obtained from The Jackson Laboratory are different from C57BL/6N mice obtained from the National Institutes of Health. In the event models are acquired via collaborators, the original vendor information and backcross data are necessary to fully understand the influence of background strain and substrain, its impact on the model phenotype, and its relevance to the human disease being studied.^{20,26} Specific vendor information and correct nomenclature for mice, genes, and any genetic manipulations should be used to fully describe the model and for accurate interpretation of model data. Recommendations for correct nomenclature are described in this issue and elsewhere (Elmore et al. 2018 [this issue]).^{26–28} Beneficial differences between strains may evolve from modern models such as the clustered regularly interspaced short palindromic repeats (CRISPR) that can generate mutations on selected genetic background(s). Therefore, researchers and pathologists should work together early in designing studies and choosing the most appropriate background strain and model for the intended study.

Human Samples and Cell Lines

Human tumor cell line xenografts and patient-derived tumor samples are often used for in vivo human tumor studies. The benefit of such models is that primary patient samples are used and are thus directly relevant to the human disease; however, successful engraftment requires the use of immunocompromised animals. Some of the commonly used immunodeficient models are highly immunocompromised and may require special housing and specific pathogen-free conditions because they are predisposed to pathogenic infections, opportunistic infections, and commensal microorganism complications such as intestinal dysbiosis.⁹ Popular immunocompromised models include NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ), NOG (NOD.Cg-Prkdcscid Il2rgtm1Sug/JicTac), and NRG (NOD.Cg-Rag1tm1Mom Il2rgtm1Wjl/SzJ) mice with severely compromised immune systems.²⁹ Traditional specific pathogen free (SPF) conditions may not be appropriate for these severely immunocompromised background strains. Biological materials may harbor adventitious pathogens and, thus, when introduced into mice as part of an experimental procedure, the pathogens are capable of infecting and propagating in the mouse and in some cases are capable of being transmitted to humans (e.g., lymphocytic choriomeningitis). Testing/screening of cell lines and tumors should be performed to confirm that biologics are free of infectious agents that may infect rodents and/or humans and are originating from the appropriate host species.³⁰ Health considerations include pathogens that require animal biosafety level 2 housing and biosafety level 2 laboratory, and possible mouse pathogens from the human donor such as lymphocytic choriomeningitis. Infectious agents of human and murine health concern such as Epstein Barr Virus and *Corynebacterium bovis* can be transmitted through patient-derived tumor samples.³¹ The same consideration for elimination of exposure to humans and nonexperimental animals must be given to any infectious agents that may be part of an infectious disease model such as experimental models of bacterial, fungal, parasitic, and viral infections.

Husbandry Considerations

Husbandry considerations and key information for study interpretation include: vivarium location, health status, caging, bedding, housing density, dark-light cycles, temperature, humidity, diet, and type of water. Changes in husbandry and environmental conditions may impact the microbiome, predispose to opportunistic infections, and, ultimately, may alter or change the documented model phenotype.^{12,17}

Commercial diets vary widely in formulation and ingredients, and although nutritionally complete, may be affected by processing, method of diet handling (i.e., irradiation or autoclave), storage, and facility-specific feeding procedures. Such variables may influence nutrient composition, consumption, research endpoints, phenotype, and even survival.^{18,32}

Another consideration in study design is housing density or the number of mice per cage. Pathology phenotype can be impacted by the type of housing and housing density. Wounds and early injury or death may be related to aggression between male mice; however, single housing or lower density cages increase the budget and may bias pathology findings and lead to single-sex studies.³³ This may be particularly problematic in long-term aging or survival studies. Thus, concurrent littermate controls from the same facility living under the same conditions are essential to understanding the environmental impacts on models; both sexes should be included in studies to assess sex-specific responses.

Infectious Agents

Genetic, experimental, and environmental factors may impact rodent models, including genetically modified models. An important consideration for pathologists and the investigative team is the identification of infectious disease outbreaks and facility microbes that may alter phenotypes and have a large impact on study results.^{33–35} Most serious infectious agents have become less prevalent with the advent of SPF requirements; however, it is important to understand the impact of emerging agents and “acceptable” commensal organisms. It is essential for the study pathologist to be aware of the respective institution’s and facility’s SPF conditions and list of excluded agents as well as the type of barrier for the entire facility and each room, but in particular the specifics for the model being studied. Serious infectious agents may cause fatalities. Opportunistic pathogens include but are not limited to *Helicobacter* spp., *Pneumocystis murina*, *Pasteurella multocida*, *Staphylococcus xylosum*, and *Klebsiella oxytoca*. With the rise in use of immunodeficient background strains, many facilities have seen an increase in complications secondary to opportunistic agents. Less serious pathogens that are not fatal may impact immune responses, generation of specific disease phenotypes, and tumor development. For example, *Helicobacter* spp. have been known to alter the frequency and type of tumors in gastrointestinal (GI) neoplasia models. Depending on the background strain and immune status of mice, *Helicobacter* spp. have been shown to induce severe typhlocolitis, hepatitis, and carcinomas in the intestine and liver.^{22,34} Most facilities use sentinel mice for pathogen monitoring and health surveillance. As part of the experimental design, it is essential for the pathologist to be aware of the institution’s type of barrier/housing facilities, the list of acceptable and unacceptable pathogens, sentinel strain and sex, and sentinel health report. Communication with the facility’s laboratory animal veterinary staff is key for 2-way communication about antemortem health concerns and

postmortem diagnoses of unexpected pathogens as well as for understanding impact on model phenotype.

Controls

Controls are an essential part of any experiment and should be routinely included as part of the experimental design. Positive and negative control groups should be represented. In the case of toxicologic studies, positive control groups are included to ensure a response is detectable whereas negative controls, which are spared from treatment, are included because they are not expected to develop treatment-related lesions. In the case of compound or chemical administration, a vehicle control should be included. When evaluating GEM models, inclusion of age- and sex-matched controls of the same genetic background, ideally littermates, is critical to differentiate between the phenotype of a specific mutation and background strain pathology.¹² Appropriate controls should also come from the same environment to account for the impact of environmental variables such as diet, housing, and husbandry.¹⁹ Concurrent controls should ideally be littermates to account for similar husbandry conditions such as vivarium location, health status, caging, bedding, housing density, dark-light cycles, temperature, humidity, diet, and type of water.

For GEM models, an increased number of mice at various ages (serial evaluation by age) may need to be evaluated to fully understand the GEM and its phenotype(s), which is important for model validation. As a general guideline, recommendations include 5 to 10 mice per group with both sexes represented; however, statistical power should be considered in sample size calculations to ensure reproducibility. Concurrent controls at appropriate ages are necessary for the evaluation of phenotype because background lesion incidence varies with age and may complicate the interpretation of experimental groups.^{20,21,32} The inevitable loss of animals secondary to congenital disease, background strain-related lesions, tumor progression, and other unexpected deaths should be considered when planning a study. This is critical for long-term aging studies and when evaluating older cohorts.³² Specific GEM of cancer may progress and vary with age where the progression to malignancy may occur only in older animals and primary tumors may occur in organ systems other than the targeted organ system; therefore, concurrent controls are essential to help discern metastases from primary tumors in other organ systems.

PATHOLOGY CONSIDERATIONS: SUBMISSION OF SAMPLES

Necropsy and Histopathology

The reporting of lesions and pathologic evaluation of animal models is based on systematic gross examination and collection of tissues at necropsy with subsequent reporting of histopathologic lesions.¹⁰ There are many variables to consider prior to performing a necropsy and tissue collection. It is recommended that a standard, systematic approach based on published recommendations be utilized. A standard approach will improve the quality, reproducibility, and usefulness of pathology data. It is also critical to have a necropsy plan and protocol in place that best defines the study. Although variables such as types of tissues, measurements, fixatives, orientation, and sectioning may differ based on the aims of the study, it cannot be overemphasized to “expect the unexpected.” Given this, and the small size of mice, a “snout to tail” evaluation should be conducted and, at the very least, all tissues saved in fixative until the study has concluded and the

results are published. The study pathologist may assist in formulating a plan and may provide resources for performing the necropsies or can assist and/or train the investigative team performing the necropsies. The plan should include: extent of samples to be collected and evaluated, whether fresh or frozen tissues are needed for additional assays, anticipated lesions to be evaluated/scored, and additional assays to include such as special histochemical and/or immunohistochemical stains. All required instruments and supplies should be identified, and unique identifiers for each animal should be established and used to properly label all samples. A complete necropsy protocol with a checklist of tissues and samples to be collected should be established. A necropsy plan and utilization of best practices will help minimize variability and promote reproducibility.

Budget Considerations

Histopathological assessment requires appropriate funding and a commitment to maintaining available funds for pathologic endpoints and analysis. Failure to plan for pathology needs may significantly impact the interpretation of final molecular and functional data.³² This is particularly important for long-term studies. Routine morphologic assessment is comprehensive and cost-effective when a specific budget is allocated for histopathologic evaluation. Planning will help facilitate tissue needs for specialized assays. A lack of planning and a lack of appropriate budgeting may lead to “do it yourself” (DIY) pathology, which runs the risk of bias, uninterpretable samples, misinterpretation, and ultimately more expense. Involving a pathologist in the budget plan may help alleviate unnecessary costs later and ensure appropriate funds are available for histopathological analysis.

Endpoint Considerations

Study endpoints should be planned to account for type of study, goals of study, and number of mice in specific cohorts. The study pathologist may assist in planning based on previous/known model data and known background strain lesions. Clinical presentation and evaluation criteria should be established with the facility laboratory animal veterinary team and may impact endpoints. For phenotypic evaluation of GEM models, endpoints may be determined based on presentation of model phenotype.

Endpoint criteria are especially critical for long-term and aging studies where extra consideration may be necessary when defining study endpoints. Aging mice often exhibit normal age-related clinical signs that are indicative of disease in younger animals, such as decreased body condition, increased respiratory rate, and pallor.³⁵ Longevity or longitudinal studies assess lifespan and causes or contributing causes of death.¹⁷ Longitudinal studies are often employed for long-term aging studies. Assessment of lesion burden and phenotypes at specific timepoints or cross-sectional studies are employed and described in the toxicology literature with the aim of evaluating compound or chemical toxicity, carcinogenicity, or drug safety.¹⁷ Cross-sectional studies may also be beneficial in carcinogenesis studies where neoplastic burden is assessed at specific timepoints. Example timepoints include evaluations at 3, 6, 12, 18, and 24 months, but will vary based on study goals and design.

Hematology

In preliminary discussions and when making the necropsy plan, a complete blood cell count with white blood cell

differential and serum biochemistry screen should be considered. Both tests are recommended to complement phenotyping and toxicology studies. It is paramount that sex- and age-matched control mice are submitted for comparison because the reference intervals from the laboratory performing the tests may not necessarily be established according to specific background strains and/or could be derived from mice on a different genetic background or exposed to a different environment and/or diet from the mice submitted for evaluation. To this point, it is recommended that 5 to 6 mice per group with appropriate controls are tested for identification of abnormalities or trends. Method of euthanasia,³⁶ collection site,³⁷ and method of blood collection may alter values. For consistency of results, it is recommended that the method and site of collection as well as the specific analyzers and medical technologist reviewing blood smears remain unchanged for the duration of the study.³⁸ In the event blood collection will involve different treatment groups, it is advised to stagger animals for collection (i.e., one animal from each group, then repeat) because this will minimize diurnal effects. Therefore, minimizing variables by applying consistent handling, collection times, sites and methods, and use of specific analyzers are imperative to ensuring accurate hematologic analysis and interpretation (Barnhart et al. [this issue] 2018).³⁸ Similarly for bone marrow collection and cytology, consistency of site collection, slide preparation, and evaluation is recommended for best comparison within or between studies.

When included as part of a necropsy plan, the sternum, humerus, femur, and vertebrae may be collected for histopathologic evaluation of bone marrow. Tissues should be fixed immediately in neutral buffered 10% formalin and decalcified gently via a chelating agent, ethylenediaminetetraacetic acid (EDTA), to preserve cellular morphology and enable histochemical and immunohistochemical special staining. Bone marrow for cytology or flow cytometry should be collected at this time and methodology is described elsewhere.^{38–40} It is also critical to compare automated hematology, blood smear and bone marrow cytology, and flow cytometry results with *in situ* bone marrow, spleen, and liver histopathology. The spleen and liver are active sites for hematopoiesis throughout the life of the mouse, and exuberant extramedullary hematopoiesis is recognized in response to anemia and other disease. A complete hematologic assessment should therefore include evaluation of peripheral blood, bone marrow, spleen, and liver.³⁸

Molecular Pathology Techniques (Immunolabelling and In Situ Hybridization)

Should tissue sections be intended to undergo immunohistochemical staining, it is important to understand the requirements for optimum fixation. Some antibodies will only work in frozen tissue versus formalin-fixed paraffin-embedded tissues. With modern antigen retrieval techniques, the majority of antigens may be detected in formalin-fixed tissues; however, this may vary by species and for some antibodies. In the case of xenograft models, careful consideration of the model is necessary to determine whether selection of a human-directed or mouse-directed primary antibody is appropriate for the question at hand. It is also essential to prepare positive control tissue for antibody optimization. Reviewing appropriate immunohistochemical protocols and appropriate primary antibodies should also be included in the necropsy plan and collection of control tissues during the necropsy if warranted. This may include positive tissue for expression of a specific antigen, but also as a

means to confirm genotype and tissue expression relevant to the model. Thus, it is best to plan and prepare to ensure tissues are collected in the appropriate manner for later use, including any potential molecular assays and immunostaining.^{41,42} Similarly, *in situ* hybridization can be applied to fresh, frozen, or fixed tissue to identify DNA and RNA of specific gene(s) of interest. It is best to prepare samples appropriately for optimal results. Further information on immunohistochemistry may be found elsewhere^{41,42} and in this issue (Boyd et al. [this issue] 2018).

NECROPSY, FIXATION, AND TRIMMING CONSIDERATIONS FOR MOUSE MODELS

Complete Necropsy (Autopsy) for Research

A necropsy is an autopsy for animals, whereby a postmortem analysis of an animal or animals is performed to examine and collect organs and tissues. There are several methods of performing a proper necropsy. The goals of the study and specific research questions will help drive the specific protocol employed; however, it is recommended that a standard and systematic approach based on published and reviewed recommendations is utilized.^{43–45} Similar recommendations are relevant to nonrodent models that may be encountered in academic institutions. It should be stressed that necropsy protocols may be dictated by the type of study, that is, toxicity studies that utilize specific standard operating procedures. Regardless of type of model, it is advisable to collect and evaluate a complete set of tissues when evaluating a new model. A systematic approach based on published recommendations and study needs will ensure nothing is overlooked and improve reproducibility and utility of the data. Evaluating a complete set of tissues and including the evaluation of age- and sex-matched controls of the same genetic background, ideally littermates, is important to differentiate between a true phenotype and spontaneous background strain pathology. In an effort to minimize variability and bias, randomization of animal groups and the order in which they are necropsied is recommended. If necropsy and tissue harvest involves large groups, a subset of each of the groups should be processed each day. As part of the protocol, a necropsy checklist may be developed based on the research questions, target organs, and model. It is critical to triage tissue collection according to need and ancillary testing procedures. Although necropsy protocols may vary based on the needs of the study, it is imperative to stress that all tissues should be properly collected, appropriately fixed, and saved for future needs.

Customized necropsy and tissue collection techniques may be employed for disease-specific models and unique anatomic considerations. Examples include the Swiss roll technique for GI models,⁴⁶ separation of individual prostate lobes^{47,48} and/or *en bloc* preparation of the male reproductive tract for rodent models of prostate cancer or other male reproductive system diseases,⁴⁹ and mammary whole mounts for rodent mammary models.⁵⁰

As part of the necropsy protocol, a standardized trimming and embedding protocol is needed for consistency across the study. The RENI guides (<https://reni.item.fraunhofer.de/reni/trimming/>) provide recommendations on proper necropsy, trimming, orientation, and number of sections per organ for rodent models.^{51–53} Examples of standard, complete tissue sets for histologic evaluation may be found in the literature.²²

The type and method of fixation as well as the length of time in fixative are variables that can affect the morphology and utility of tissues. The most commonly employed fixatives are aldehydes such as glutaraldehyde, paraformaldehyde, and formaldehyde. Commercially available formulations of neutral buffered 10% formalin (i.e., 4% formaldehyde containing methanol as a stabilizing agent) and freshly prepared 4% paraformaldehyde (i.e., methanol-free 4% formaldehyde) are commonly used fixatives for standard morphologic analysis. Immersion of tissues in neutral buffered 10% formalin is the methodology and fixative of choice in routine bioassays; however, for special studies and specific tissues, other methods and fixatives may be employed. A good example is the use of whole animal perfusion by directly perfusing fixative through the circulatory system. This technique is particularly beneficial to the central nervous system. Whole body perfusion fixation will help eliminate many common artifacts seen with immersion fixation and introduced upon removal of the brain. Whole body perfusion is also beneficial for other organ systems because the technique directly perfuses fixative through the circulatory system, which enables useful, detailed, and near fixation artifact-free data and therefore may save money in the long run. The specific choice of fixative may vary based on the type of tissue. For example, Bouin's fixative is excellent for preservation of embryos, and Bouin's and modified Davidson's fixatives are recommended for eyes and enable optimal identification of germ cells in the testes.⁵⁴ When immunohistochemistry is anticipated, the use of Bouin's should be avoided because it may alter antigens. Due to their toxicity and corrosiveness, care should be taken when using all fixatives and special care should be taken with potentially explosive fixatives such as Bouin's, which contains picric acid, necessitating special handling and storage considerations. Bouin's also fixes tissues quickly so tissues must be removed after a limited period of time and rinsed to avoid becoming hard and brittle.

For optimal fixation, the volume of fixative should be greater than 10 times the tissue volume, fixation should take place at room temperature, and the size of the tissue should be no more than 2 to 3 mm thick and no larger than 15 to 20 mm wide.⁴³ It is recommended to consult with the pathologist reviewing the tissues, as well as the laboratory processing the tissues, for a discussion of optimum fixation selection and timing and to avoid irreversible damage and tissue artifacts.

Necropsy (Autopsy) for Phenotypic Characterization

Phenotyping of models has become one of the most important methods of characterizing genetically engineered models of human disease. Important considerations include budget, animal selection, and number of animals to evaluate. The aims of the study, animal availability, and resources may dictate a phenotyping protocol. Because many factors may influence phenotype, consistency is key when developing a protocol and comparing groups. The development of such a phenotyping necropsy protocol should be undertaken in consultation with or by a comparative pathologist. Pathology evaluations are used adjunctively to other tests when validating genetically modified models of human disease so involving a comparative pathologist early and discussing a panel of appropriate assays or tests will help when planning and budgeting. There are existing academic and commercial resources that provide comparative pathology and phenotyping pathology services. Most are directed by or involve a comparative pathologist for consultation and evaluation of the collected tissues.

Many factors, including those discussed above, may influence phenotype. Genetically modified mice are often developed on mixed backgrounds then bred for the genotype of interest or bred to other genetically modified mice carrying other mutations. As such, the genetic effects of multiple backgrounds and individual mutations must be considered. Examination of concurrent age-, strain-/substrain-, and sex-matched littermates is critical for characterization of subtle phenotypes. It cannot be overstated that concurrent controls should ideally be littermates to account for similar husbandry conditions such as vivarium location, health status, caging, bedding, housing density, dark-light cycles, temperature, humidity, diet, and type of water.^{10,12,14,22,24}

Similar to other standard necropsy protocols, phenotyping necropsy and tissue procurement protocols require a standardized methodology for tissue collection, trimming, and evaluation. Submission of live animals is ideal, because phenotyping necropsy protocols often involve photography, weighing of the carcass and all tissues, standard collection of tissues from "snout to tail," and collection of body fluids and blood for hematology assays such as complete blood cell count and serum biochemistry screen. As the carcass and most of the tissues are weighed, it is important to remember to routinely obtain carcass weights *after* blood collection, and organ weights and collection of tissue for molecular assays and freezing *before* perfusion or immersion in fixative. Additional tests such as imaging and clinical pathology assays are often performed to complement histopathological data. To this point, it is recommended that 5 to 6 mice per sex, age, genotype, and experimental group, including appropriate controls, are tested for identification of abnormalities or trends.¹² Group sizes may vary from 2 to 4 to 10 mice per group depending on variability of the phenotype and statistical analyses.¹² As such, phenotyping protocols may be expensive if many groups or genotypes are evaluated. To limit the number of animals analyzed, reduce costs, and maximize tissues evaluated, many tissues are grouped together based on density and size in a standard set of slides. An example of a phenotyping tissue trimming and embedding protocol is available in the literature.⁵⁵ It is of utmost importance for the pathologist to be involved early as part of the pathology and experimental plan and/or to ensure all specific details of the model are communicated prior to the date of scheduled euthanasia. Phenotypes may be subtle so the pathologist should be armed with the appropriate data so as not to overlook subtle lesions.

Necropsy (Autopsy) of Developing Rodents

Various lethal phenotypes of embryos and postnatal mice have been reported that have necessitated the methodical evaluation of embryos, often working in reverse from embryologic day 18, to determine the cause of embryologic or perinatal mortality.⁵⁶ Necropsy strategies for embryos and postnatal mice are similar to those techniques that have already been described for adult mice. Key points include a familiarity with the anatomy and biology of developing mice and the careful attention to detail needed to perform necropsies on a smaller scale. In the case of embryos, collection and examination of the placenta is crucial and must be included. When evaluating embryos at timed stages of pregnancy, the dam, including the uterus should also be examined. Resources are available that provide important details of the basic biology of embryos and the pathology techniques used for their analysis.⁵⁷

Anatomic Considerations and Avoiding Tissue Artifacts

Proper tissue collection and standardized tissue trimming are essential to maintain consistency in a study, to avoid costly artifacts, and for accurate histological assessment. As previously mentioned, the methodology and choice of fixative is critical for all tissues, but it is especially important to avoid tissue-specific artifacts. For example, the process of removing the brain from the skull and immersion fixation in neutral buffered 10% formalin may impart “dark neuron” artifacts to the brain. For optimal morphologic preservation with reduction in artifacts, whole body intravascular perfusion is the best methodology. For routine necropsy, the brain may be left inside the skull with the skull cap (calvarium) removed for immersion fixation or the brain may be left in the skull for fixation, decalcification, and coronal sectioning of the entire head.⁴³ When the central nervous system is the focus of the investigation, a modified protocol that includes additional coronal sections of the brain is recommended.⁵⁸

Decalcification is a necessary step in the processing and sectioning of bone. Proper care and selection of decalcification agents must be considered for specific evaluations such as coronal sections of the whole head and in situ bone marrow sections. Although a variety of decalcification methods, including formic acid-based solutions, will enable sectioning of bone and preserve cellular morphology of various tissues, it is recommended that bones be fixed immediately in neutral buffered 10% formalin and decalcified gently via a chelating agent such as EDTA to ensure the application of the widest array of histochemical and immunohistochemical special staining. Acidic decal solutions yield more rapid decalcification with possible degradation of fragile epitopes, whereas chelating solutions (e.g., EDTA) are slower but offer better antigen preservation. See further references^{41,42} and this issue (Boyd et al. [this issue] 2018) for additional information.

The collection and insufflation of the lungs should be performed routinely at necropsy. This is especially critical for pulmonary disease models as insufflation with fixative is performed to avoid artifacts such as underinflation. Underinflation precludes accurate evaluation because the collapsed alveoli and septae may be misinterpreted as interstitial pneumonia. This is especially important in inhalation studies as well as pulmonary neoplasia and metastasis studies. The lungs may be insufflated via whole body perfusion or instillation via the trachea. Whole body perfusion is performed by injection of the right ventricle with formalin and transecting the abdominal aorta to allow for blood to exit the vasculature.⁵⁹ Alternatively, the lungs can be insufflated by instillation via the trachea with a given volume of formalin, or even RNA preservation solution or media when indicated for ancillary testing. Care should be taken with both techniques to avoid overinflation. Overinflation of the lungs with fixative may result in artifactual separation of perivascular and peribronchiolar collagen fibers, mimicking perivascular interstitial edema, or damage to the septal walls, which may be misinterpreted and preclude evaluation of emphysema models.^{43,58} It is therefore recommended to insufflate to a consistent and specific measured pressure for pulmonary studies and pulmonary disease models such as emphysema models to avoid high pressure inflation, and inflate slowly to physiologic inspiration.

As previously mentioned, small and large intestinal gut rolls may be used for examination of the entire intestine in 1 to 2 slides.⁴³ This technique is good for a comprehensive evaluation of the entire gut; however, it may not be optimal for GI neoplasia models due to the predisposition of artifacts and tangential

sections that may lead to misinterpretation. For GI neoplasia models, the entire GI tract may be opened from stomach to anus and flattened on paper for identification, counts, and photo-documentation of grossly visible lesions. The gut may then be fixed flat and either rolled or divided into representative sections for histopathologic analysis. Large tumor lesions may be separated and cut in half with normal tissue on each end and embedded into one block or, alternatively, cut into transverse sections for evaluation.⁴⁶

With any model of neoplasia, it is critical to plan for preparation of step and/or serial sections to be utilized for future immunohistochemical and/or histochemical special staining as well as confirmation of tumor morphology and invasion for accurate reporting and model interpretation.

HISTOPATHOLOGY: PATHOLOGIST'S EVALUATION

The histopathologic evaluation of tissues is a crucial part of the evaluation and validation of animal models. Standard histopathologic evaluation in rodent models is often a descriptive-based evaluation of morphology. In translational models, communication is key and involving a comparative pathologist with knowledge of normal anatomy and physiology as well as knowledge of spontaneous lesions in various laboratory animal species is critical for accurate interpretation and reporting of the pathological data. A comparative pathologist understands and uses the internationally accepted nomenclature for lesions, strains, and the genes of interest, ensuring that studies are accurately reported for publication. References for lesion classification include the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) project. The INHAND project devised criteria for the harmonization of nomenclature across documents and databases for both proliferative and nonproliferative lesions in mice and rats in toxicologic pathology.⁶⁰ The INHAND publications also have a complimentary nomenclature online reference site, goRENI (<https://www.goreni.org/>), that provides online and interactive access to diagnostic criteria and images. Further information and references may be found in this issue (Elmore et al. 2018 [this issue]).

In the evaluation of animal models of neoplasia and carcinogenesis, the biologic potential of the various organ system models may be assessed via histological and cytological grading schemes. For example, GEM of epithelial carcinogenesis often exhibit a multistage progression or continuum through preneoplastic/precancerous lesions to malignancy. This multistage progression is observed in the pancreas, prostate, mammary gland, female tubular genitalia, kidney, GI tract, lung, liver, and skin.^{61,62} The National Cancer Institute Mouse Models of Human Cancers Consortium established organ-specific pathology consensus papers on diagnostic criteria and terminology for mouse models of human cancer.⁶³ Each consensus paper compares the microanatomy between species as well as the standardized terminology for each tumor type and grade within a specific system.^{47,48,50,64–67}

As clinical trials are increasing and the effectiveness of new chemotherapeutic drugs and other treatment modalities is studied, it has become necessary to devise numeric-based scoring systems that measure treatment efficacy and outcomes for specific diseases (Meyerholz et al. 2018 [this issue]).^{68–69} Depending on the goals of the study, the pathologist may devise a specific semiquantitative scoring scheme based on

recommendations in the literature and on the biology of the specific model. Semiquantitative scoring systems assign numeric grades and yield ordinal data. Semiquantification allows initial group comparisons; however, quantitative scoring based on actual measured values may be performed via microscopy or via digital image analysis. Image analysis via software systems is the gold standard for quantitative scoring. Further information and references may be found in this issue (Aeffner et al. 2018 [this issue]).⁷⁰

Pathologists are often asked to review and report studies “blinded,” also referred to as “masked.” The concealment of group allocation to various people involved with a preclinical animal study is intended to prevent biased conclusions regarding experimental group effects. However, absolute concealment can negatively impact the quality of information that the pathologist provides and prolong the time it takes to complete the evaluation; therefore, a practical tiered-approach is recommended.⁷¹ In new studies, phenotypic characterization of new models, or treatment studies, it is paramount for the pathologist to be armed with background information regarding the generation of models, specifics regarding the proposed or evaluated model, genotypes, and controls. This provides the pathologist with the information needed so as not to overlook subtle phenotypes or changes and prevent diagnostic drift that may occur without reference to baseline controls. Once lesions are identified, a pathologist may conduct another evaluation, masked to slide and group identifiers, to ensure accurate interpretation and identification of lesions. Ongoing studies with known lesions or phenotypes may be read blinded to experimental groups then re-read unblinded.⁷¹ Once all groups within a study have been read and evaluated/scored, the pathologist may re-read unblinded when making final interpretations to ensure consistency.

Reporting

The goal of reporting histopathologic findings is to clearly communicate results and foster intra- and inter-study reproducibility and translation of models to human disease. Histopathological data provide morphologic descriptions and include diagnostic criteria, consensus terminology, lesion scoring, clear protocols, methods of assessment and scoring, and interpretative comments and representative photographs.

The role of the pathologist continues post-reporting. Ideally, the pathologist will meet with the research team to review findings and discuss next steps, which may include ancillary tests based on pathological or phenotypic findings and/or pathologic examination of additional cohorts. Beyond pathological analysis and reporting, the pathologist is essential for the preparation of manuscripts, abstracts, and grants. In addition to confirming the context of the pathologic findings, the pathologist will provide details on associated methods, ensure usage of proper nomenclature and inclusion of other essential information (i.e., the ARRIVE guidelines), prepare and assemble figures, and conduct an overall critical review of the entire document.^{3,8,10} This extended involvement as a critical member of the research team warrants inclusion of the comparative pathologist as co-author on publications and co-investigator on extramural funding applications.

DIY PATHOLOGY

Animal models often recapitulate aspects of the human diseases they are designed to model. As such, it is tempting to

overinterpret or “mold” results to fit with the study hypothesis. It is important to remember that laboratory animal species differ from the humans for which they are often modeled and thus, also form unique lesions, often according to species-specific mechanisms. Upon consideration that many mouse models in particular are genetically modified and develop unique phenotypes that are influenced by strain genetics, husbandry, and experimental conditions, care should be taken to avoid the misinterpretation of models. Unfortunately, there are many published examples, often in high-impact scientific journals, of misidentification of normal rodent tissues and misinterpretation of common artifacts by individuals who do not possess the extensive training and experience of comparative pathologists.^{3,5,72}

Examples of misinterpretation or questionable pathology in the literature include misidentification of normal rodent anatomic structures as lesions, reporting of artifacts as neoplasms, and use of incorrect or unconventional terminology and diagnoses.³ Incorrect interpretations of immunohistochemical staining are also very common, especially in the absence of appropriate positive and negative controls. Incomplete reporting of the pathology protocol and experimental design is also problematic. With the advent of the ARRIVE guidelines, several journals and funding agencies have required and are committed to ensuring the inclusion of appropriate information in the methods.^{3,10}

Inadequate numbers of comparative pathologists at biomedical research institutions and insufficient budgeting for pathology services in funding budgets has led to “DIY” pathology studies that have resulted in erroneous conclusions.^{3,72} Consultation with a comparative pathologist is critical to assist in the validation of the mouse model by differentiating between a true phenotype and normal or background lesions. Studies are typically enhanced by seeking this guidance at the beginning of a study so the pathologist may positively influence the selection, development, and analysis of animal models and thereby prevent common pitfalls and subsequent misinterpretation of anatomic and pathologic findings.

ACCESSING A COMPARATIVE PATHOLOGIST

Academic research institutions may be fortunate to have comparative pathology and/or phenotyping resources with pathologists who may assist with pathology needs; however, there are institutions and smaller research facilities that may not have direct access to an experienced pathologist. Researchers may choose to use pathologists associated with external pathology resources or consult with a pathologist via telepathology. Many current pathology and phenotyping resources extend services to outside clients. Comparative pathologists may be found in academia, commercial diagnostic laboratories, industry via a contract research organization or biopharmaceutical company, as well as independent consultants, but as of yet, there is no central registry or resource for finding experienced pathologists.⁶⁸ Pathologists may be accessed via social media outlets such as LinkedIn.⁷³ The Comparative Pathologist Consortium on LinkedIn is a social network and resource for comparative pathologists. Other resources include veterinary and comparative pathology departments in universities as well as organizations that certify veterinary pathologists i.e. the American College of Veterinary Pathologists (www.acvp.org) and professional societies that include veterinary pathologists i.e. The Society of Toxicologic Pathology (www.toxpath.org).

CONCLUSIONS

Animal models are invaluable for elucidating the mechanisms of human disease and the effectiveness of therapeutic interventions. It is therefore critical to translational research that animal models are accurately characterized and validated as models of human disease. Mouse models are some of the most commonly utilized models of human disease, and pathology is an important endpoint in the analysis and verification of these models. Although care must be taken at each step of the development and evaluation of these models in translational research, there are many important considerations throughout all stages of the pathological analysis. Pathologists can assist with development and adoption of consistent pathology protocols, standardized terminology and reporting, and validation of these models. Importantly, beyond pathology, comparative pathologists also have the comprehensive training to provide expertise in animal model development, experimental design, optimal sample collection, and data interpretation, thus serving as essential contributors on the research team.

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