Published in final edited form as:

Methods Mol Biol. 2016 ; 1438: 199–224. doi:10.1007/978-1-4939-3661-8_12.

Skin Diseases in Laboratory Mice: Approaches to Drug Target Identification and Efficacy Screening

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Abstract

A large variety of mouse models for human skin, hair, and nail diseases are readily available from investigators and vendors worldwide. Mouse skin is a simple organ to observe lesions and their response to therapy, but identifying and monitoring the progress of treatments of mouse skin diseases can still be challenging. This chapter provides an overview on how to use the laboratory mouse as a preclinical tool to evaluate efficacy of new compounds or test potential new uses for compounds approved for use for treating an unrelated disease. Basic approaches to handling mice, applying compounds, and quantifying effects of the treatment are presented.

Keywords

skin; alopecia areata; atopic dermatitis; chronic proliferative dermatitis; full thickness skin grafts; hair; xenograft

1. Introduction

Domestic animals have been incredibly useful for discovering novel approaches to combat major disease problems in humans for centuries. For example, the most notorious infectious disease of all time with a prominent skin lesion was small pox. Descriptions by Barron [1] remind us of the severity of the skin disease, even though the viral infection involved many other organs. Jenner's controversial work, first published in 1798, based on interspecies transmission of cow pox or, more likely, horse pox, from its natural host to human caretakers who subsequently were immune to small pox infection. This observation led to the development of vaccines (derived from the Latin word *vaccinus*, i.e. relating to, or derived from a cow)[2–4]. Today, we use laboratory mice as a leading biomedical tool, and have amassed a variety of molecular methods to analyze mice with spontaneous or genetically

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engineered diseases that closely resemble human diseases. These small mammals can effectively be used for preclinical trials for new drugs, identify potential new uses for approved drugs, or using large scale gene arrays, RNAseq and alike, and other technologies in combination with sophisticated molecular pathway analysis software to predict the best model for drug testing or drugs to test on a particular mouse model.

The mouse skin represents a somewhat unique organ system to work on. For example with the simple topical application of drugs, effects can easily be seen without special manipulation to rapidly determine if the compound is efficacious. However, with mice they can simply lick the compound off and change the intended treatment from a topical to a systemic effect. As such, education in the use of mouse skin as a model system is of the utmost importance.

There are many reviews on what constitutes good mouse models for human diseases [5,6], as well as models for specific diseases[7–15]. It is beyond the scope of this chapter to discuss the pros and cons of each of these potential models, especially since a recent scan of the public literature and databases revealed hundreds of genetically engineered and spontaneous mouse mutations that have skin disease phenotypes (Mouse Genome Informatics, MGI; <http://www.informatics.jax.org/>) many of which may serve as models for specific human diseases [15]. Furthermore, we will not describe the usefulness of the mouse to study wound healing, for this the reader is referred to other reviews on the topic [16–18].

In this chapter, we will focus on the use of several specific models illustrating how mouse models for cutaneous drug studies can be effectively utilized to address primary questions and methodologies which will also be applicable to most, if not all mouse skin models. Many so-called single gene based diseases, including many of the epidermolysis bullosa blistering diseases [19], can be successfully reproduced using mouse models. Yet, even these vary due to background modifier genes [20]. However, similar modifier genes in the mouse genome [20] can be found as modifiers of the homologous human disease (Roopenian, Sproule, and Sundberg unpublished data), such that while difficult, it is still possible to model even specific subsets of human diseases. Some complex diseases, such as psoriasis, may never be adequately mimicked by mouse models as they lack some of the key anatomic structures or response features found in human patients (Table 1) [13,21]. However, other complex genetic diseases, like alopecia areata, a cell mediated autoimmune disease that involves the classical lymphocyte co-stimulatory cascade mechanism found in many cell mediated autoimmune diseases, [22,23] can be modeled in mouse and used for screening compounds with a variety of applications [24,25]. Furthermore, many human diseases are actually groups of very similar diseases lumped together based on similar clinical phenotypic features. Now that the molecular bases for many human skin diseases are being unraveled, it is possible to more accurately match them with the specific mouse homologue(s). These mouse models are extremely useful when common molecular targets are the focus of preclinical testing, even where these are not always easy to define.

If the goal is to alter proliferative, scaly skin disease, albeit psoriasis or ichthyosis, panels of mutant mice with this basic phenotype are readily available [26] (<https://www.jax.org/jax-mice-and-services>) and should be tested and results compared. For many of these mouse

models the specific mutated gene and genetic lesion within that gene is known. Furthermore, many of these models have transcriptome studies completed that can be used to search for specific drug targets that may be dysregulated. If the drug works in one model but not another and the gene is known, this can focus on the best homologous human disease to target. [27,23] If the target is known, for example as with filaggrin (*Flg*) or the closely linked transmembrane protein 79 (*Tmem79*), which are risk factors for atopic dermatitis [28–39], then a mouse with double mutations in *Flg* and *Tmem79*, such as the flaky tail-matted mutant mouse (*Flg^{fl}Tmem79^{ma}*) can be used for testing targeted drugs [40,41].

Gene expression profiling is an approach to both identify dysregulated genes as a potential target for drug treatment and monitor changes in response to drug treatment. If the drug under investigation is known to up or down regulate a specific protein, then knowing which mutant mice have down or up regulation of transcripts coding for these proteins are logical for initial preclinical screening. Large sets of gene expression profiling data are available through public repositories such as the Gene Expression Omnibus at NIH (<http://www.ncbi.nlm.nih.gov/geo/>) and the ArrayExpress at EBI (<http://www.ebi.ac.uk/microarray-as/ae/>). Such databases can be searched for the targeted protein to find candidate mouse models.

References for websites and repositories worldwide for mice models can be found in the “*Genetically Engineered Mice Handbook*” [42]. Another good source of information on mouse strains is the International Mouse Strain Resource (IMSR) which is available online at MGI (<http://www.findmice.org>). There are also a number of repository databases that provide summaries on the mouse models and their potential uses. Traditional textbooks continue to serve a useful purpose with detailed images of normal anatomy [43] as well as gross and histologic lesions seen in mutant mice [44,42]. In addition, there are specialized website databases, such as the Mouse Tumor Biology Database (<http://tumor.informatics.jax.org/mtbwi/index.do>) [45–48], which focuses on cancer models, or more generalized mouse pathology databases such as the European Mouse Pathology Consortium website (<http://www.pathbase.net/>), whereby one can also access the more specific skin pathology database, see <http://skinbase.org> [49–51].

With some disease models, even after obtaining them from commercial or collaborative sources it can be difficult to continuously maintain these animals and their desired features, and may be even difficult to generate sufficient sample material for detailed analysis. This can be due to mutant mice not thriving, and/or dying at a young age or developing severe lesions making managing and maintaining a colony difficult. One approach to overcome this problem is to maintain the colony by heterozygous breeding and/or to use full thickness skin grafts, a simple surgical approach, to expand such limited mouse resources [52–54]. Heterozygous mice may be more useful if the mutation causing the problem is semi-dominant. Such mice will develop the phenotype less severely than homozygous mice. Skin grafting can functionally expand from one to many mice. For example, C3H/HeJ mice develop alopecia areata as they age. As with humans, the spontaneous disease will wax and wane [55]. One can perform full thickness skin grafts from one mouse with extensive alopecia areata (alopecia universalis) from one mouse to up to ten young recipients. As C3H/HeJ mice are highly inbred and therefore histocompatible, immunologically intact mice

can be used [54,56]. This can also be done for neonatal lethal mutant mice [53] or mice that can be difficult to maintain [57]. Perhaps of greater importance, the most appropriate animal model may not be the mouse but the human. In that case, making mouse xenografts in which human skin [58,59] is grafted onto immunodeficient mice can become the model of choice [60–62]. However, this limits analysis of the immune system if such is a driver of the disease.

In this chapter we will cover approaches to test compounds on mouse skin and skin disease models in order to identify potential anatomic, metabolic, and genetic changes that correspond to those seen in comparable human diseases. This type of information has proven very useful in identifying and repurposing FDA approved drugs which are currently available to treat different diseases but with a similar pathogenesis basis. Quantifying changes, as well as selecting the most appropriate changes to measure can be daunting, yet it is a critical aspect of these types of studies. Here we will show and discuss a variety of approaches and detail how these can all be done.

2. Material

2.1. Application of drugs

Gavage 20 or 22 gauge feeding tube with 2mm ball (Instech Laboratories, Inc., Plymouth Meeting, PA)

Cherry syrup (can be purchased from local pharmacies anywhere)

Sulfatrim (Schein Pharmaceutical, Florham, NJ) The final concentration is ~ 1 mg/ml (10 mls Sulfatrim {240 mg/5 mls}/480 mls water).

Elizabethan collars (Harvard Apparatus, Holliston, Massachusetts)

Hilltop Chambers® (Hill Top Research, Miami, Ohio)

3M™ Coban™ wrap (3M™, any supplier of surgical bandaging)

Wound clips (9mm wound clips; Stoelting Co, Wood Dale, IL)

Nonstick pads, non-adhering dressing (Johnson & Johnson)

ALZET® Osmotic Pumps (Durect Corp., Cupertino, CA)

Silastic tubing (Dow-Corning Corp., Midland, MI)

Custom-made pelleted implants (Brookwood Pharmaceuticals; <http://www.brookwoodpharma.com/drug-loaded-implants.html>)

2.2. Quantification of Drug Response

2.2.1. Hair Regrowth/Repigmentation Evaluation

- 1 Isoflurane
- 2 Tribromoethanol
- 3 Stick-on type rulers (crime-scene.com/ecpi/references.shtml)

- 4 DermLite (San Juan Capistrano, CA) (<http://www.dermlite.com/>)
- 5 TrichoScan Software (Tricholog, Freiburg, Germany) (www.tricholog.de)

2.2.2. Histopathology

- 1 10% neutral buffered formaldehyde, Fekete's acid-alcohol-formalin, Bouin's solution, or other tissue fixative (Table 1)
- 2 70% ethanol for storage of tissues after fixation
- 3 Hematoxylin/Eosin stain
- 4 Toluidine blue stain
- 5 Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU)
- 6 Cleaved caspase-3 specific antibody: cleaved caspase-3 (Asp175) antibody (Cell Signaling, cat # 9661, Danvers, MA)
- 7 Cleaved caspase-9 specific antibody (Novus Biologicals cat# NB100-56118, Littleton, CO)
- 8 SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA)
- 9 MembraneSlides (Zeiss, Oberkochen, Germany).

2.2.3. Scanning electron microscopy

- 1 Fixation buffer: 2.5% glutaraldehyde in 0.1 M cacodylate buffer
- 2 Nylon Mesh (Sefar Filtration, Inc., Depew, NY)
- 3 Hitachi S3000N VP Scanning Electron Microscope (Hitachi Science Systems, Japan)
- 4 EDAX X-ray microanalysis system (Mahwah, New Jersey)

2.2.4. Transmission electron microscopy

- 1 Extraction buffer: 0.1 mM sodium phosphate buffer (pH 7.9), 2% SDS, 10 mM dithiothreitol
- 2 Karnovsky's fixative: 16% paraformaldehyde solution, 50% glutaraldehyde, 0.2 M sodium phosphate buffer
- 3 1% osmium tetroxide
- 4 Dehydration solutions: Specimens are transferred through graded solvents (50–60% in distilled water) up to 100% in the solvent. Solvents such as ethanol, methanol, or acetone can be used.
- 5 Epoxy resin combination: Araldite, Embed 812 (Electron Microscopy Sciences, Hatfield, PA)
- 6 TEM JEOL JEM-1230 (JEOL)

2.2.5 Laser capture microdissection

- 1 Laser Capture Microdissection system, either Infrared or Ultraviolet based systems available; e.g. ArcturusXT LCM (ThermoFisher Scientific, Grand Island, NY), PALM Zeiss UV LCM (Zeiss, Oberkochen, Germany)
- 2 Fixative: 70% Ethanol or Formalin
- 3 Staining Solutions: Cresol Violet
- 4 LCM Caps

2.3. Mice

Chronic proliferative dermatitis (*Sharpin^{cpdm}/Sharpin^{cpdm}*, JAX 007599) mouse

Flaky tail, matted (*Flg^{fl}, Flg^{fl}, Tmem79^{ma}/Tmem79^{ma}*, JAX 00281) mouse

Immunodeficient mice: e.g. NSG (JAX 00557)

3. Methods

3.1. Application of drugs

There are various ways to administer drugs to rodents in order to test for efficacy and toxicity. The most common approaches are summarized below with references to provide more specific details on how to conduct these types of studies [63].

3.1.1. Oral Routes—Mice can be dosed using a gavage tube or mixing the drug with cherry syrup to allow for measured doses to be delivered to the rodents. Mice should receive sulfamethoxazole in their drinking water for 1 week after surgery, to minimize the risk of infection. Immunodeficient mice receive sulfatrim (final concentration is ~ 1 mg/ml (10 mls Sulfatrim [240 mg/5 mls]/480 ml water) in their drinking water to minimize complications from *Pneumocystis* spp. infections.

3.1.2. Topical Routes—Although topical applications are the most practical for human patients, it can be problematic as mice depending upon the vehicle, may lick and groom the test compounds off themselves or others in the same box.

Compounds in volatile vehicles (such as acetone) can be applied with a micropipette, allowed to spread over a marked area (site can be tattooed to ensure the compound is repeatedly applied to the same site and spread over the same unit area of skin) and allowed to dry [64]. Small amounts can be applied repeatedly allowing for evaporation between applications to maintain volume in a defined area.

Aqueous or ointment vehicles are more problematic primarily because they cannot be easily contained. We have tried a variety of stick on bubble chambers, compression bandages, and “Elizabethan collars” and rodent jackets for mice that are available from various vendors with variable results. Elizabethan collars, also known as E-collars, are made of plastic, the neck openings are lined with padding, and they close with a Velcro fastener preventing the animal grooming itself (*see* Note 1). Hill Top® Chambers are molded plastic chambers that

are flexible and conform to the skin. Within the chamber is a pad which holds the test sample. The chambers are secured to the mouse using Coban™ wrap, or other self-adhering bandages, which is secured by 9 mm wound clips. The compounds/drugs are applied with a pipette under the bandages (*see* Note 2).

Bandaging the drug application site can be a simple and very effective alternative. A nonstick type pad is placed over the shaved site and a small custom cut vest-shaped elastic bandage is used to hold the pad in place [56]. The compound is applied under the bandage daily and the bandages are changed weekly and untoward effects are noted, e.g. ulcers, swelling, etc. [65].

3.1.3. Injectable Routes—Several routes of administration are available. Frequency will depend on route, volume, and tolerance of the compound, all depending upon Institutional Animal Care and Use Committee (IACUC) approval.

1. Intravenous injections: The ventral coccygeal (tail) vein is a common site for intravenous injections. This is located on the ventral side of the mouse tail, running along the midline. The mouse can be placed in a restraint device with the tail protruding. This can be a plastic box or tube with a slot the width of the tail at one end. The mouse is allowed to enter the device while the tail is held. Warming the mouse or its tail will help increase the flow of blood. Small gauge (30 g) needles are used on syringes through the skin and into the vein. Albino strains are easier to venipuncture than pigmented strains as the red vessel can be seen easily more clearly through the finely haired skin of the tail. Mouse body skin usually lacks interfollicular epidermal pigmentation but this is not the case for the tail skin which also has a very thick epidermis. There are also several alternative but less commonly used sites for intravenous injections including either the femoral or jugular veins, as well as various veins that can be surgically exposed for injection [66,67].

2. Subcutaneous injections: The mouse body skin is very loosely attached to the underlying fascia and musculature and can be lifted easily. A small gauge needle can then be inserted into the tent of skin over the back and neck once it is raised.

3. Intradermal injections: It is commonly believed that due to the extremely thin dermis and epidermis of the mouse body skin, injections into the “dermis” are the same as subcutaneous. This can be verified using labeled cells such as those ubiquitously expressing green fluorescent protein (GFP) and tissue sections examined directly by immunofluorescence or indirectly using a GFP-specific antibody and immunohistochemistry [68]. However, it is possible to stretch the dorsal or ventral skin and, using a very small gauge needle, position it within the layers of the skin instead of through the skin to produce small blisters with the injected material. Recently, dissolving polymer microneedle patches, 25 mm² patches housing an array of approximately one hundred 650 µm long needles

¹Elizabethan collars and rodent jackets have not been found to be very effective. In our hands all mice managed to slip out of them within a 24 h period making these collars and jackets unreliable.

²Bandages and chambers in our studies were on for 5 days and off for 2 days to allow for any irritation or inflammation of the skin caused by the wound clip to heal.

capable of penetrating 200 μm into the skin, have been developed that would allow for the delivery of compounds to the dermis of mice [69].

4. Intramuscular injections: Generally, intramuscular injections are made with small gauge needles into the epaxial muscles on either side of the lumbar vertebral column or the quadriceps femoris muscle on the ventral side of either rear leg. Due to the small size of mice, very small volumes (e.g. 0.05 mL) should be injected.

5. Intralesional injections: Small gauge needles can be used on syringes to inject drugs into a neoplasm or other raised abnormality affecting a defined area of the skin. The volume used will depend upon the size and number of lesions being treated and what the Institutional Animal Care and Use Committee will allow.

6. Intraperitoneal injections: The mouse is manually restrained with the head and body tilted downward. A small gauge needle is inserted into the caudal left abdominal quarter, thereby avoiding injection into the cecum on the right side.

7. Retro-orbital injections: The mouse is anesthetized and placed in the lateral recumbence position. Gentle downward pressure is applied to skin dorsal and ventral to the eye. A small gauge needle is placed through the conjunctiva at the medial canthus to the base of the eye and a small volume (150 μL) is injected.

8. Unusual injections: Although rarely used, protocols are available for intracardiac, intrathecal, intrathoracic, and intrarectal injections [66,67].

3.1.4. Osmotic pumps—ALZET® Osmotic Pumps are miniature, infusion pumps for the continuous dosing of drugs to mice. These minipumps can be surgically implanted intraperitoneally or subcutaneously to transport drugs from 1 day to 6 weeks. Longer durations may be accomplished through serial implantations of pumps. These minipumps provide a convenient and reliable method for controlled agent delivery *in vivo*. The doses are constant and accurate and the variables are minimized thereby producing constant results. By using the minipumps the stress and handling is reduced for the mice and this is especially true where daily doses would be required.

3.1.5. Slow-release subcutaneous implants—Silastic capsules, consisting of 10 mm silastic tubing packed with drug or hormone with sealed ends (insertion of 3 mm glass beads) can be implanted through a small incision over the dorsal thoracic midline into the subcutaneous fat [70,71]. These capsules enable a slow release of encapsulated drug the rate of which is dependent upon formulation. A number of companies will formulate drugs into various pelleted implants and these companies can be found online (e.g. SurModics, <http://www.surmodics.com/>).

3.2. Quantification of Drug Response

Determination of efficacy of a drug in the mouse models appears to be superficially simple, i.e. the treatment leads to the mouse resolving the abnormal clinical phenotype and does not die due to the treatment. However, to test this unequivocally or to determine a dose response

curve is often, in practice, technically difficult. Although obvious, it is of key importance that the specific goal/s of the study be clearly defined. With skin this can be as simple as hair growth promotion, or even just hair growth on a bald mouse. With wound healing, resolution of scaly skin diseases, etc. needs to be quantified to various degrees. Also it is necessary that one understands the biological and anatomic differences between mice and humans to appreciate the drug effects (Table 2). This begins with the fact that mice have a prolonged telogen and humans have a prolonged anagen hair cycle, their hair cycles in a wave pattern instead of the human mosaic pattern. Also mouse truncal skin lacks interfollicular pigmentation that is seen in human epidermis. This translates to the fact that mice have pink skin naturally [72], and observed pigment is only seen in actively growing, late anagen stage hair follicles where the hair bulb and shaft are heavily pigmented (Fig. 1). These observations are well known criteria for monitoring induction and patterning of the hair cycle in pigmented mice. Scoring systems by grey tone intensity are used to monitor hair cycle in the commonly used production strains, C57BL/6J (black) and C3H/HeJ (agouti) for these types of studies [73]. Changing in color from unpigmented (pink) to pigmented (grey to black) indicates hair growth, onset of anagen, and therefore initiation of the hair cycle, which may be the goal of the study.

3.2.1. Hair Regrowth/Repigmentation Evaluation—While a number of magnification and photography tools are available to dermatologists to visualize and record changes in human skin, these can be more problematic in mice because their relatively small body makes finding flat sites difficult to find, especially when they are very active. To avoid, minimize, or altogether circumvent these issues a number of approaches can be taken.

1. Gross photography: How does one stop a very active mouse from moving? This can be simply done at the end of a study by euthanasia using Institutional Animal Care and Use Committee (IACUC) approved protocols. Protocols recommended by the American Veterinary Medical Association are available and are regularly reviewed and revised [74] (<https://www.avma.org/KB/Policies/Documents/euthanasia.pdf>). Currently, the most commonly used and IACUC approved euthanasia method is carbon dioxide gas asphyxiation. This has the advantage that the mouse dies quickly and a complete necropsy of all tissues can be performed.

Alternative survival methods include anesthesia by inhalation (isoflurane) or injectable (tribromoethanol) anesthetics which can be used to temporarily immobilize the mice for examination and/or photography [75,76]. While isoflurane is commonly used for repeated anesthetics, tribromoethanol is contraindicated [75]. Another approach for partial temporary immobilization of mice includes the use a 50–50% O₂/CO₂ mixture in a sealed container (see Note 3). The use of a restraint device where mice are placed into a transparent device in which there is a black concave area for them to insert their heads [77]. The side walls of this device are adjustable to keep the mice from moving laterally. The mice are also naturally frightened by bright lights, e.g. flood lamps and will stand still without any anesthesia. Of course this last method will not work in strains that are blind, which is a major issue since

³The mice do rapidly recover from these treatments, so the photographer needs to be ready and fast when the mouse is removed from the container.

many of the commonly used inbred strains carrying mutations causing skin diseases, such as C3H/HeJ and FVB/NJ, also carry the retinal degeneration 1 (e.g. *Pde6b^{rd1}*, phosphodiesterase 6B, cGMP, rod receptor, beta polypeptide) mutation [78]. One surprise with the decision to use C57BL/6N substrains for the international knockout mouse project was that, unlike C57BL/6J mice, these mice carried another single gene mutation (in the *Crb1^{rd8}* gene) that is linked with blindness. Some participants in the project corrected this by genetic engineering [79], emphasizing the importance of knowing the peculiarities of not only the strain but the substrain used. Another approach is to evaluate efficacy on the ventral abdomen of the mice. It is common practice to handle and restrain mice by picking them up by pinching lightly the skin behind their ears and grabbing the tail with the small finger of the same hand. This restrains, immobilizes, and stretches the ventral skin so a photograph can be taken by an assistant.

Many journals prefer to see photographs of live, unrestrained mice and many institutions are implementing policies that discourage the publication of pictures depicting euthanized animals. This can also be accomplished by photographing groups of mice unrestrained in their cages. Individual mice can be placed on the bottom of inverted beakers with a colored background (cloth or art paper).

For whole mouse photographs, a high quality single lens reflex type digital camera provides ease of use while generating high quality photographs. A regular 50–55 mm macro lens is adequate for whole body images but a 100 mm macro lens allows closer evaluation of the skin surface. Repeat photographs at regular time intervals can be taken of the same area if the skin is tattooed at the start of the project. A fixed ruler should be placed in the field at the same height as the area of skin being photographed as a fixed internal standard for comparison and morphometric analyses. The animal identification number and date can be written on the ruler, especially if disposable, stick-on type rulers are used. Many modern cameras permit data to be added directly to the images, including date and time (see Note 4). Several companies sell devices that are designed specifically to photograph human skin in a narrow field with or without magnification. These may have associated software to enable specific types of quantitative analyses to be done in a standardized manner. DermLite has been used for evaluating melanomas and other skin lesions in humans and has the potential for fine analysis of drug response in a variety of mouse skin disease models [80–82]. This unit attaches to a hand held camera. Tricholog uses a small cylindrical camera that lays directly on the skin surface but the image is visualized and focused when attached by a cable to a portable computer. The TrichoScan software allows counting and measuring hair shaft size.

3.2.2. Histopathology—Histopathology of mouse skin is preferably done by a board certified veterinary anatomic pathologist with experience interpreting mouse skin. There are many examples of mistakes when investigators use “do-it-yourself-pathology” [83]. Especial attention needs to be exercised as misinterpretations of mouse specialized organs that humans do not have occur; for example the preputial and clitoral glands which are modified

⁴Remember to keep individual identification information (labels with rulers, case number, etc.) off the actual site of interest. This way, the data markers can be cropped out of the images used in the final report.

sebaceous glands in the genital regions, are often misdiagnosed as sebaceous gland tumors [84] or even teratomas [85,86]. For pathologists experienced with a specific model, normality vs. disease can usually be quickly seen. This is particularly important when strain specific background diseases confuse the interpretation of results, especially for the skin diseases [87,88]. These evaluations can be done on routinely fixed and processed hematoxylin and eosin (H&E) stained paraffin sections (5–6 μm). Quantification or semi-quantitative data on specific structures are often useful to assess drug responses and may need to be developed. Many pathologists use very simple but effective methods based on disease severity. The commonly used adjectives can also have numbers added (normal, 0; mild, 1; moderate, 2; severe, 3; and extreme, 4). Once calibrated or defined, e.g. by a set of images, these can be used effectively when multiple criteria are used to generate a score per criteria as well as a total score. Data are summarized in spreadsheets for all criteria and can be sorted quickly for analysis. This approach can be used to evaluate all organ systems under review [89–91,88].

Valid quantitative scores can be generated by counting the number of mitotic figures, specific cell types (e.g. mast cells using a toluidine blue stain), per high power field or whatever microscopic field is most appropriate. The area of the field can be obtained easily by measuring the diameter with a micrometer ($A = \pi r^2$). Image analysis programs, such as NIH Image (<http://rsb.info.nih.gov/nih-image/>), ImageJ (<http://rsb.info.nih.gov/ij/>), or a calibrated ocular micrometer can be used to rapidly generate quantitative data. It is critical to only choose areas of the slide where the entire lengths of hair follicles are present to serve as an internal standard for orientation. This is extremely important for the epidermis because it is very thin in normal mice. Routine measurements can include the interfollicular epidermal thickness (and each layer, if appropriate), length of the hair follicle, dermal thickness, hypodermal fat layer thickness which normally varies with each hair cycle [92,93,26] (Fig. 2,3).

Proliferation (mitotic) rates or cell death (apoptosis of keratinocytes in the epidermis) are other useful criteria in looking at compound effects on skin. Both can be evaluated using a simple H&E stained paraffin section (Fig. 3). Mitotic figures, one criterium for proliferation rate, can be seen in the basal cell layer. We found that hematoxylin alone provides optimal contrast to visualize mitotic figures. In traditional H&E stained slides apoptotic keratinocytes are brightly eosinophilic with dark blue to black shrunken nuclei (pycnotic or karyorrhectic nuclei). Proliferation rates can be quantified by injecting mice with 50 $\mu\text{g/g}$ body weight of bromodeoxyuridine (BrdU) intraperitoneally 2 hours before necropsy (time interval is critical and can vary based on age and organ under investigation; i.e. normal cell division rates in the tissue under investigation) and then labeling the tissues with an antibody against BrdU by immunohistochemistry or immunofluorescence (Fig. 4). The numbers of positive cells (which are nuclear labeled by BrdU incorporation during the S phase of the cell cycle) per millimeter of skin, per 1000 basal cells, or other quantitative criteria can be used [94]. Alternatively, activated caspase 3 or 9 specific antibodies can be used to stain and identify cells undergoing apoptosis [95] (Fig. 5). Lastly, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is another approach to evaluate apoptosis, but this method is not specific to apoptosis and can also indicate cells undergoing necrosis.

The hair cycle can be roughly graded at the gross level in shaved or alopecic mice by mapping color changes in the skin of pigmented mice from pink (follicles in telogen), to increasing darkening of areas of skin as follicles proceed into the later stages of anagen. The reason for this is described above as illustrated (Fig. 1,6). At the microscopic level this can be done two ways. The major hair cycle stages (anagen, catagen, or telogen) can be estimated (as a percentage of the total skin section) to demonstrate major shifts associated with a mutant mouse phenotype or drug treatment [96]. Alternatively, if very subtle differences between the test and control groups need to be identified, scoring hairs based on the finely divided grading of all stages and sub-stages of the hair cycle is a complicated but functional approach. This system, developed by the Paus laboratory [97], divides anagen into six stages, catagen into eight stages and telogen into one stage (Table 3). Although exogen as a stage has been discussed, available defining histologic criteria for the purpose of scoring are very limited [98–101].

All of the types of collected data listed above can be stored in databases for summary and analysis. One such database, the Mouse Disease Information System (MoDIS), is freely available online (<https://www.jax.org/research-and-faculty/tools/mouse-disease-information-system>). It will automatically spell organ names and diseases processes as word strings are typed which helps in maintaining nomenclature. This database allows the recording of semi-quantitative histological scores and can be modified easily for specific tasks. More importantly, when used online and linked to Pathbase (<http://www.pathbase.net/>), it is possible to confirm the definition of terms used and search for photomicrographs to serve as a virtual second opinion [90,91].

3.2.3. Scanning electron microscopy—To evaluate structural integrity of the hair surfaces, scanning electron microscopy (SEM) provides a three dimensional view of the structure (Fig. 7). The phenotype of many mouse mutations affecting the skin and hair can be due to marked abnormalities of the hair shaft structure [9]. Resolution of these abnormalities can best be seen using SEM. A concurrent qualitative or semi-quantitative follow up can be done, when the SEM is appropriately equipped to perform element analysis on the hair shafts. Sulfur levels are higher in hair shafts and nails than epidermis of the skin due to the presence of the hard (hair and nail) keratins and keratin associated proteins which are often proteins containing high to ultrahigh sulfur containing amino acids [102]. Hair defects are often due to low sulfur levels, e.g. forms of trichothiodystrophy. Therefore, response to treatment can be validated for hair structural mutants by demonstrating normal hair shafts with normal levels of sulfur.

To perform these assays, it is necessary to collect mouse hair samples. This is easily done from adults where hair follicles are in prolonged telogen and exogen stage. Hair shafts are easily removed without pain to the mouse simply by plucking. Because of the hair cycle stage, it is rare that damage occurs to the shafts no matter how defective they are. However, as human hair is usually in anagen and tightly attached to the scalp, human hairs have to be removed with hemostats to grip the hair which can damage the hair shafts. As this raises concerns with physicians, an alternative approach utilizes 1 cm² biopsies removed from the anatomic site of interest, laid flat on a firm nylon mesh, and fixed routinely in a glutaraldehyde based fixative. Hair should be removed from the same location on each

mouse and the same hair shaft types should be compared at the same location on each hair within a study.

We routinely examine hairs from the dorsal, interscapular region of the trunk. We found sulfur level variations in normal and mutant hairs from three commonly used inbred strains along the length of the hairs and by hair shaft types not significantly detected in normal mice hairs [103].

For SEM, hair shafts are mounted with double-stick tape on aluminium stubs, sputter-coated with a 4 nm layer of gold, and examined at 20kV at a working distance of approximately 15 mm in our hands on a Hitachi S3000N VP Scanning Electron Microscope [104].

For sulfur content assessment by weight of hair shafts we suggest using an EDAX X-ray microanalysis system. Samples should be examined for an average of at least 300 live seconds to ensure a comprehensive reading is obtained [102].

3.2.4. Transmission electron microscopy—To evaluate the cellular structure within hair shafts, the shafts can be processed for transmission electron microscopy (TEM). Due to hair's hard consistency in plastic blocks, evaluation may be impossible as the hairs are easily lost during sectioning. To avoid this hair shafts must first be extracted before sectioning:

- 1 Ten or more hair shafts per mouse are incubated in 2 mL Extraction buffer at room temperature
- 2 When the hair became swollen (2–2.5 h), in comparison to a sample incubated in parallel without dithiothreitol, it is immersed in Karnovsky's fixative.
- 3 Hair is post-fixed with osmium tetroxide overnight at 4 °C.
- 4 Hair is dehydrated in graded series of ethanol.
- 5 Hair is embedded in an epoxy resin combination.
- 6 Blocks are orientated visually to produce longitudinal and cross sections of the hair shafts.
- 7 80 nm sections are prepared.
- 8 Sections are then examined by a high performance high contrast TEM (JEOL JEM-1230) [103,105–107].

These TEM studies can be further evaluated by proteomic analysis of hairs to determine specific changes due to various treatments [105–107].

3.2.5 Laser Capture Microdissection—To evaluate single cell or isolated structural effects in tissue at the protein, RNA or DNA level laser capture microdissection (LCM) can be utilized on tissue sections. LCM allows for fixed, sectioned, and stained samples to be examined under a microscope and have either single cells or small homogenous regions of tissue dissected from the larger sample for analysis [108].

Tissue samples must be fixed though a variety of fixatives with best fit for the following staining protocol and end product, e.g. DNA, RNA, lipid or protein. Commonly 70% ethanol fixation yields better results than most other fixatives but alternatives such as Tellyesniczky/Fekete (Telly's) Fixative, Formalin, or Bouin's fixative may be used [108–111]. In general tissue samples are sectioned before subsequent staining. Frozen sections can be sectioned using a cryostat, while paraffin embedded sections may be sectioned on a standard microtome at 5µm. Sections can be placed on SuperFrost + slides, slides coated with poly-L-lysine, or membrane coated slides. (MembraneSlide).

Tissue sections may then be stained either by standard histological stains, such as Hematoxylin or Cresyl Violet, or immunohistologically labelled using antibodies specific to a target of interest. Staining of the sections allows easy visualization of the region of interest while dissecting samples [108–111].

The sections may now be placed on the laser capture microdissection platform. There are two dominant platforms on the market that utilize either Infrared (ArcturusXT, Applied Biosystems) or Ultraviolet (PALM MicroBeam, Carl Zeiss Microscopy) lasers to microdissect tissue sections. The infrared platform uses a cap placed over the region of interest. A transfer film is bonded to the top of the cap. After determining the cells and regions to be dissected, the Infrared laser interacts with the transfer film to spot-weld the film to the tissue of interest and lifts the dissected material away from the remainder of the slide. The ultraviolet platform also uses a cap, but instead of physically interacting with the tissue the cap is only used as a trap. The Ultraviolet laser is used to cut and then float the dissected tissue away from the original section and into the waiting cap.

Lastly, tissue from either platform can be used to extract DNA, RNA, or protein depending on the type of assay needed.

3.3. Alopecia Areata

Alopecia areata is a relatively common autoimmune skin disease that affects humans, mice, rats, horses, dogs, and cattle. There is even a feather form of the disease in chickens [25,11,112–114]. It occurs spontaneously however, mice have a low frequency of this disease. Full thickness skin grafts from affected C3H/HeJ mice provided a reproducible and predictable model of alopecia areata [54,115,56]. This mouse model has been used to test drugs effective for human alopecia areata applying all methods discussed in this chapter [24].

3.4. Chronic Proliferative Dermatitis

The chronic proliferative dermatitis mutant mouse is one of a number of mouse models proposed for psoriasis. This spontaneous mutant was recently shown to be caused by a mutation in the *Sharpin* gene [116]. This mouse model was used to screen recombinant human cytokines in which recombinant interleukin 12 (IL12) but not interleukin 11 (IL11) effectively corrected the skin disease [27].

Acknowledgments

This work was supported by grants from the National Institutes of Health (R01 AR049288, R01 AR056635, R01 CA089713, R01 AR055225, R21 AR063781), Cicatricial Alopecia Research Foundation (CARF), DEBRA International for development of the junctional epidermolysis bullosa mouse model, and The National Alopecia Areata Foundation (NAAF) for support of the alopecia areata mouse model development. The Jackson Laboratory Shared Scientific Services were supported in part by a Basic Cancer Center Core Grant from the National Cancer Institute (CA34196, to The Jackson Laboratory).

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Figure 1.

Hair cycle related skin pigmentation. Hair cycle stage can be estimated by skin color in pigmented (A, C3H/HeJ) but not lightly pigmented or albino (B, BALB/cByJ) mice. Note the light colored (in live mice this is pink vs. grey to black) skin correlates histologically with telogen while dark skin is due to hair follicles in late anagen. Unlike humans most of the skin pigmentation in mice is in the hair follicles during the actively growing, anagen stage.

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Figure 2.

Quantitative measurements of skin. Routine measurements of skin, in late anagen in this figure, include interfollicular epidermal thickness (circled area), hair follicle length (L), dermal thickness (D), hypodermal fat layer thickness (H, normally varies with the hair cycle), and full thickness (FT) from the surface of the stratum corneum to the top of the panniculus carnosus muscle.

Figure 3.

Epidermal measurements, mitotic figures, and apoptotic keratinocytes in a chronic proliferative dermatitis mutant (*Sharpin^{cpdm}/Sharpin^{cpdm}*) mouse. Routine H&E stained paraffin histologic sections can be used to determine proliferation rates based on mitotic index (number of mitotic figures, circled in the figure, in the stratum basale per 1000 cells) or the presence and numbers of apoptotic epidermal keratinocytes (dotted arrows) when present. Epidermal thickness can be measured at high dry magnification (40 X) to include the Malpighian, living cell, layer (M), the stratum corneum thickness (SC), or the full thickness of the epidermis (M+SC).

Figure 4.

Bromodeoxyuridine (BRDU) labeling of cells in “S” phase. Routine paraffin sections from mice injected 1 hour prior to euthanasia with BRDU have brown nuclei when labeled by immunohistochemistry using diaminobenzidine as a chromogen. These cells were synthesizing DNA at the time of necropsy and therefore incorporated the label. Counting the number of positive nuclei in basal cells per 1000 basal cells yields the proliferation rate. The boxed area in A is enlarged in B to illustrate the large number of positive (dark) nuclei in the skin of an adult chronic proliferative dermatitis mutant mouse (*Sharpin^{cpdm}/Sharpin^{cpdm}*).

Figure 5. Determination of apoptosis. Apoptotic keratinocytes can be confirmed using cleaved caspase 3 specific antibodies using immunohistochemistry. The boxed area in A is enlarged in B to illustrate the large number of positive (dark) cells (arrows) in the skin of an adult chronic proliferative dermatitis mutant mouse (*Sharpin^{cpdm}/Sharpin^{cpdm}*).

Figure 6.

Estimating changes in hair cycle. A simple means to estimate changes in hair cycle in a semi-quantitative manner is to estimate the percent of hair follicles in anagen, catagen, or telogen in histologic sections, entering the data into an Excel type spreadsheet, and graph results. The section of skin from the mouse in A is all in telogen. By contrast, the second section (B) is approximately 50% in late anagen, 45% in catagen, and 5% in telogen. These data can be graphically presented in various types of bar graphs (C, D).

Figure 7.

Scanning electron microscopy reveals details of hair shafts. Normal hairs from an adult C57BL/6J examined as a whole mount (A) illustrates density of mouse hairs and the nature of the normal skin surface. Manually plucked hairs illustrate the structural differences between some of the hair shaft types (B). Higher magnification of boxed area in B reveals the regular cuticular scale patterns on these hair shafts (C). These approaches illustrate details of hair shaft structure and density.

Table 1

Formulations of Commonly Used Fixative for Mouse Tissues

<i>Tellyesniczky/Fekete' solution</i>	100 mL 70% EtOH (undenatured, USP) 5 mL Glacial acetic acid 10 mL 37–40% Formalin
<i>Bouin's Solution</i>	85 mL, Sat. Ag. Picric Acid 5 mL Glacial acetic acid 10 mL 37–40% Formalin
<i>10% Neutral Buffered Formalin</i>	100 mL 37–40% Formalin 900 mL dH ₂ O 4 g, Sodium phosphate-monobasic 6.5 g Sodium phosphate-dibasic

Table 2

Differences between human and mouse skin

CRITERIA	HUMAN	MOUSE
Hair cycle pattern	Mosaic pattern	Wave pattern
Predominant cycle stage	Anagen	Telogen
Hair follicle size	Large	Small
Hair types: head/trunk	Terminal and vellus	Guard, auchene, awl, and zigzag
Unique hair types	No homologous structure	Vibrissae
Relative hair density	Low	High
Epidermal thickness	High	Low
Rete ridges	Prominent	Non-existent
Interfollicular epidermal pigmentation	Yes	No
Hair bulb/shaft pigmentation	Yes	Yes

Table 3

Stages of the mouse hair cycle [97,98]

ANAGEN	CATAGEN	TELOGEN	EXOGEN
I	I	One stage although more complicated based on hypodermal fat thickness	Not defined Anatomically
II	II		
IIIa, b, c	III		
IV	IV		
V	V		
VI	VI		
	VII		
	VIII		