Chapter 10

The NDUFS4 Knockout Mouse: A Dual Threat Model of Childhood Mitochondrial Disease and Normative Aging

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Abstract

Mice missing the Complex I subunit NADH:Ubiquinone Oxidoreductase Fe-S Protein 4 (NDUFS4) of the electron transport chain are a leading model of the severe mitochondrial disease Leigh syndrome. These mice have enabled a better understanding of mitochondrial dysfunction in human disease, as well as in the discovery of interventions that can potentially suppress mitochondrial disease manifestations. In addition, increasing evidence suggests significant overlap between interventions that increase survival in NDUFS4 knockout mice and that extend life span during normative aging. This chapter discusses the practical aspects of handling and studying these mice, which can be challenging due to their severe disease phenotype. Common procedures such as breeding, genotyping, weaning, or treating these transgenic mice are also discussed.

Key words Mitochondrial disease, Leigh syndrome, Mitochondrial dysfunction, Aging, NDUFS4, Complex I, Electron transport chain, mTOR, Rapamycin, Hypoxia, NAD

1 Introduction

The most significant risk factor in acquiring a mortality-causing disease is age. Mitochondrial dysfunction is recognized as a hallmark of biological aging and is strongly associated with many diseases of normative aging, including Alzheimer's disease, heart disease, diabetes, and cancer [1, 2]. This has led to considerable efforts being devoted to better understanding, treating, and preventing mitochondrial dysfunction. In addition, hereditary mutations in mitochondrial and nuclear DNA leading to inactive or dysfunctional mitochondrial enzymes, transport proteins, structural proteins, and/or chaperones often lead to severe mitochondrial diseases known as mitochondriopathies [3]. These Mendelian diseases are prevalent in 1 in ~5000 births and often cause premature death in the first few years of life. There are many similarities in the etiology, pathogenesis, and pathology of aging-related

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mitochondrial dysfunction and pediatric mitochondriopathies. These unique similarities may enable a better understanding of normative aging by studying childhood mitochondrial disease, and vice versa.

The *NDUFS4* knockout (NKO) mouse is a leading model of severe mitochondrial disease [4]. NDUFS4 encodes a subunit of Complex I of the electron transport chain. Loss of NDUFS4 manifests in neurometabolic disease which recapitulates many symptoms of the pediatric disease Leigh syndrome, including metabolic dyshomeostasis, retarded growth, decreased motor coordination, neurological lesions of the brain stem, cerebellum, and vestibular nuclei, and premature death [5]. These mice have been invaluable in the mechanistic understanding of the basic biology underlying mitochondrial dysfunction and have been used to discover novel interventions to treat these disorders.

We hypothesized interventions shown to delay the onset of diseases of normative aging and extend life span in wild-type mice may similarly increase survival and prevent disease progression in NKO mice. Consistent with this prediction, we reported that the drug rapamycin, a specific inhibitor of mTOR, could more than double survival of NKO mice and greatly delay the onset of disease symptoms [6, 7]. Since this report, three other longevity-promoting interventions have also been shown to enhance survival in NKO mice: the NAD+ boosting compound nicotinamide mononucleotide [8], genetic inhibition of ribosomal protein S6 kinase beta-1 (S6k1) [9] and maintaining the animals in a hypoxic environment (11% O_2) [10].

Based on its utility for both mechanistic and interventional studies and early indications of translational impact for human mitochondrial disease, at least in the case of rapamycin [11, 12], several labs have begun using the NKO mouse. This chapter will discuss best practices in working with these mice, tips for maintaining a healthy colony, and suggestions for how to avoid common pitfalls.

2 Materials

2.1 Genotyping Supplies

1. Scissors.
2. Vetbond veterinary glue.
3. 1.5 mL microcentrifuge tubes.
4. Microcentrifuge tube rack.
5. Ear tags and ear tag applicator.
6. Ice.
7. Phire Tissue Direct PCR Master Mix (Thermo Fisher Cat #F170L).

8. Agarose gels, 2% in TAE or TBE.
9. GeneRuler 1 kb Plus (Thermo Fisher Cat #SM1331).
10. Primers: wild-type forward (5′-AGT CAG CAA CAT TTT GGC AGT-3′,) KO forward (5′-AGG GGA CTG GAC TAA CAG CA-3′,) common reverse (5′-GAG CTT GCC TAG GAG GAG GT-3′).

2.2 Reagents and Materials for Injections

1. 31G 3/100 mL insulin syringes.
2. Tween-80.
3. PEG-400.
4. Rapamycin stock solution, 120 mg/mL in DMSO.
5. 0.2 μm syringe filters, either syringe filters or vacuum filtration units.
6. Laminar flow hood.
7. 1.5 mL microcentrifuge tubes, sterile.

2.3 Special Diets and Medicated Water

1. Agar powder.
2. PicoLab® 20 or other chow.
3. Food processor.
4. Bowl mixer.
5. Saran wrap.
6. Aluminum foil.
7. Single-use Hydropac® water pouch.
8. Single-use disposable valve.
9. Silicon patch.
10. Syringe.
11. Syringe needle (>22 G needle).
12. 0.2 μm syringe filter.

2.4 Tissue Collection

1. 1 L Liquid nitrogen dewar.
2. Scissors.
3. Forceps.
4. 1.5 mL microcentrifuge tubes or aluminum foil.
5. 31G needles.
6. 1 mL syringes.
7. Serum separators or plasma collection tubes.

2.5 Perfusions for Histology

1. 10% neutral buffered formalin (Sigma HT501128).
2. 1× PBS without calcium or magnesium.
3. 70% EtOH.

4. Ratchet-lock hemostat with curved serrated jaws (5.5″).
5. 30 mL syringes.
6. 27G butterfly needle (12″).
7. Three-way stopcock with male luer lock.
8. Automated low-volume syringe pump.

2.6 Anesthesia

1. Ketamine.
2. Xylazine.
3. 31G 1 mL insulin syringe.

3 Methods

3.1 Preparation and Storage of Rapamycin for Injection

1. Mix 5 mL of Tween-80 and 5 mL of PEG-400 in 89 mL of water with rapid stirring.
2. Using a P-1000 micropipettor, add 1 mL of rapamycin stock solution (120 mg/mL in DMSO) dropwise while continuing to stir rapidly (*see* **Note 1**). The final concentration is 1.2 mg/mL.
3. Let the solution stir rapidly for 30 min to allow the rapamycin to dissolve completely.
4. During this time, label 100 sterile microcentrifuge tubes in a laminar flow hood.
5. Filter the rapamycin solution inside a laminar flow hood (*see* **Note 2**).
6. Aliquot rapamycin solution in the labeled tubes.
7. Store tubes at −20 °C until needed.
8. Partially used tubes can be returned to −20 °C, if they have been opened under a laminar flow hood and handled with proper sterile techniques.

3.2 Special Diet Preparation

1. Weigh out ~1 kg of facility chow and add to a blender. Our facility uses PicoLab® 20 (LabDiet 5058); however, other chows or synthetic diets can be used (*see* **Note 3**).
2. Weigh out drug and add to chow. Liquids can be weighed or dissolved in 200 mL water and added to chow in **step 7**.
3. Grind the chow and drug in a blender to mix until it forms a coarse powder.
4. Transfer the powder to a bowl mixer (e.g., KitchenAid) and connect the paddle attachment.
5. Mix the powdered chow on low using a bowl cover to prevent aerosolization of powder.

6. Prepare molten agar by heating 3 g of agar powder in 300 mL of deionized water. This is commonly achieved by slowly heating the suspension in a microwave and carefully swirling until the agar is fully dissolved into solution.
7. After allowing the molten agar to cool slightly, slowly pour into the mixing chow to ensure homogenization.
8. Add an additional 200 mL of deionized water into the mixing chow/agar mixture. Allow to mix until a uniform mixture with good plasticity is obtained.
9. Take ~25 g of chow mixture (approximately a small handful) and form into a cylindrical pellet by rolling between the palms of your hand and using the fingers of your opposite hand to form the edges. Small pellets tend to dry out and harden too quickly.
10. Put pellet onto saran wrap and repeat forming pellets until no chow remains. We commonly put ~10 pellets per piece of saran wrap.
11. Cover the pellets in Saran wrap and wrap in aluminum foil. Be sure to label the foil with the drug, mill date, and expiration date (within 1 year of mill date).
12. Store pellets at −20 °C until needed. We generally provide 2–3 pellets per cage and replace food after ~4 days.

3.3 Medicated Water Preparation

1. For solids, weigh out desired amount of drug and fully dissolve in minimal volume of liquid. Water is ideal as vehicle to dissolve the drug. For liquids either weigh or measure volume of drug directly into a syringe. You can also dissolve the liquid drug into solution.
2. Put a silicon patch onto the top side of a single-use Hydropac® pouch and press firmly to seal the patch. If using water bottles, then dissolve drug directly in water bottle. Water bottles, however, are more prone to leaking and microbial contamination.
3. Withdraw drug solution into syringe then connect a 0.2 μm filter and small gauge needle onto the syringe.
4. Inject the drug solution into the Hydropac® pouch through the middle of the silicon patch with a small gauge needle. Ensure to remove an equal volume of air from the pouch through the syringe to maintain proper pressure within the pouch.
5. Label the pouch with drug, dose, preparation date, and any other necessary identifier information.
6. If not freshly prepared before use, then store the water pouch in refrigerator until needed. Light-sensitive drugs can be stored in aluminum foil. Freshly prepared medicated water is preferred.

7. Provide medicated water to cage by piercing the pouch with a single-use disposable valve connected to the cage. It is advised to replace water pouch and valve weekly. Sweeteners (e.g., Splenda) can be used for drugs with bad taste.

3.4 Breeding

1. In a fresh cage, set up one *NDUFS4*$^{+/-}$ sire and dam pair. Mice should be at least 50 days old.
2. Set up additional cages as needed. When planning for more than five breeder pairs, staggering groups of breeders by 1 week is helpful to avoid genotyping and weaning high numbers of pups and ensure a steady flow of new litters over time.
3. After 20 days, check cages regularly for new pups and log date of birth and litter size. Daily checkup ensures best estimation of birth date, though frequencies as low as twice a week are acceptable.
4. Retire and replace breeder pairs if they do not produce new litters for three consecutive months, have stillbirth litters or litters with neonatal mortality three times in a row, or produce litters with no NKO animals three times in a row.

3.5 Genotyping

1. Tag pups to be genotyped with ear tags. If pups are too small to be tagged, you can label tails with sharpie markers, ear punch, or tattoo. Put pups in a fresh cage bottom as you tag them.
2. Label microcentrifuge tubes with the tag numbers or sharpie colors used.
3. Scruff one pup and cut a ~ 0.5 mm section off the tip of their tail and place in the labeled microcentrifuge tube (*see* **Note 4**).
4. Stop bleeding by dipping the bleeding edge of the tail in a small drop of Vetbond tissue glue.
5. Return pup to its cage.
6. Repeat with remaining pups.
7. Extract DNA from the tail tips and assemble PCR mix as recommended by the genotyping kit manufacturer.
8. Perform PCR amplification using thermocycler settings and primer sequences as described in the Genotyping Protocols from Jackson Laboratories (Stock No.: 027058).
9. Run PCR products on a 2% agarose gel at 150 V for 20–30 min. KO mice will show a band at 400 bp, wild-type mice at 201 bp, heterozygous mice will show both bands (*see* Fig. 1).

3.6 Weaning

1. At 21 days postnatal, weigh pups and wean all pups weighing more than 6.5 g.

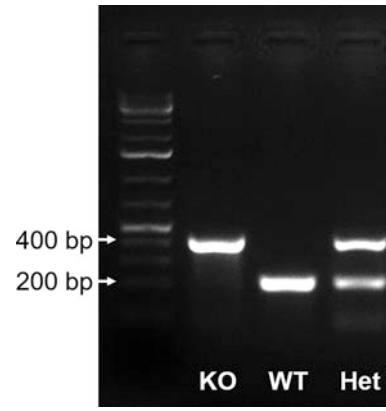

Fig. 1 SYBR-Safe stained agarose gel using products of PCR reaction of extracted DNA from tail snips for genotyping of Ndufs4 mice. The NKO mice solely show the top band at ~400 bp, while WT mice display the bottom band at ~200 bp. PCR products that show both bands are from mice heterozygous for Ndufs4

2. Ensure that NKO mice have at least one *NDUFS4*$^{+/-}$ or *NDUFS4*$^{+/+}$ companion in the cage to aid with thermoregulation. We have not observed changes in disease progression when cohousing >1 NKO and/or >1 companion.
3. NKO mice are unable to reproduce so they may be housed with companions of the opposite sex if no suitable same sex mouse is available. It is advised to pair same-sex companions; however, we rarely see aggression between cohoused mice of opposite sexes.
4. Mice smaller than 6.5 g at day 21 are unlikely to survive weaning, especially if on experimental treatments that attenuate growth, such as rapamycin. Mice below 6.5 g may be kept in the breeder cage for an additional 7 days.
5. NKO mice should be continuously provided hydrogel throughout course of study. Wet chow should be used in place of hydrogel for mice on medicated water.

3.7 Common Drug Treatment Regimens

1. Injections can start at postnatal day 10. If comparing injection and diet/water-delivered treatments, start both treatments upon weaning (postnatal day 21–28). Although possible, we have not performed diet/water-delivered treatments during gestation or prior to weaning.
2. Inject 6.66 μL/g of mouse body weight. A 1.2 mg/mL stock solution of rapamycin corresponds to a dose of 8 mg/kg.
3. Alternate injection sites across the midline to prevent systemic inflammation on one side.
4. Replace experimental diets and water bottles twice a week, e.g., on Mondays and Fridays.

3.8 End Point Criteria and Euthanasia

1. Weigh mice daily and evaluate if NKO mice reach end point (*see* **Notes 5** and **6**).
2. For mice that reach end point, euthanize via CO_2 asphyxiation followed by a proper secondary method. NKO mice in a severe disease state are believed to be hyperoxic [10], and require extended times for euthanasia. It may take up to ~10 min for NKO mice to be euthanized via CO_2.

3.9 Life Span Study Design

We commonly use a minimum of 12 NKO mice (6 female and 6 male) for life span studies and other studies. This number should provide sufficient power to detect a 20% change in life span considering a life span of approximately 50 ± 10 days for untreated NKO controls. We typically use an equal number of males and females, as treatments may show sex-specific differences; however, larger cohorts are likely required to make definitive conclusions.

3.10 Tissue Collection

1. The night before tissue collection, move mice to a new cage bottom and remove all food from the feeder rack.
2. On collection day, add fresh chow upon the beginning of the light cycle in the animal room.
3. Refeed animals for 3 h (*see* **Note 7**).
4. Sacrifice mice by cervical dislocation.
5. Cut skin at the level of the throat with scissors. Collect pooling blood with a 1 mL syringe and store in a serum or plasma separator tube.
6. Pull up the skin and make an incision at the level of the abdomen. Pull skin toward the hindlimbs and the head to deglove the carcass.
7. Cut through the peritoneum to expose internal organs. Cut on both sides of the sternum to expose the thorax.
8. Remove heart and lungs.
9. If desired, collect blood pooling in the thoracic cavity.
10. Collect desired tissues in labeled 1.5 mL tubes, vented with a syringe needle, or in aluminum foil and flash freeze in liquid nitrogen.

3.11 Perfusions

1. Anesthetize mice with a cocktail of ketamine (260 mg/kg) and xylazine (17.6 mg/kg) in saline injecting 20 μL/g mouse body weight or according to your IACUC recommended guidelines. It generally takes ~20 min until mice are properly anesthetized.
2. Evaluate if mouse is anesthetized by performing a toe pinch on both hindlimbs. If mouse responds in any way (often a jerk), wait until mouse no longer responds to toe pinch.

3. Connect a syringe filled with cold PBS and cold NBF (filtered) to a three-way stopcock buffer valve, connect a butterfly needle to the third end of the buffer valve. Push NBF through the needle followed by PBS. It is critical to remove any bubbles throughout the line. Connect the syringes to syringe pumps.
4. After mouse is fully anesthetized, pull up the skin and make an incision at the level of the upper abdomen. Pull skin toward the hindlimbs and the head to deglove the anesthetized mouse.
5. Cut through the peritoneum to expose internal organs. Expose the thorax by making an incision in the diaphragm and cut across the diaphragm to expose the heart. Take care to not nick the lungs, heart, or liver.
6. If needed, detach the heart from the sternum. This is achieved by disengaging the pericardium ligaments from the heart.
7. Insert the butterfly needle via the apex of the heart directly into the left ventricle. Due to the small size of NKO mice, the needle is often inserted into the left atrium. If needed pull back the needle so that the tip is in the left ventricle.
8. Clamp the needle in the heart using a ratchet-lock hemostat.
9. Turn on the syringe pump at a low flow rate to push PBS through the buffer valve and butterfly needle into the heart. Due to the small size of NKO mice, it is recommended to keep the flow rate $\leq$2 mL/min to prevent artifacts from high fluid pressures. Gravity perfusions can also be used.
10. Immediately cut the superior vena cava to allow for exsanguination.
11. Perfuse the mouse with PBS until liver loses color (often $>$30 mL for a $>$10 g mouse).
12. Turn off the PBS syringe pump, switch the buffer valve to NBF, and turn on the syringe pump to perform fixation using a low flow rate. Commonly $>$30 mL of NBF is necessary for sufficient fixation.

4 Notes

1. Rapamycin is hydrophobic and may form clumps that are hard to dissolve if added at a single time to the vehicle solution. Vigorous stirring and slow, dropwise addition of the stock solution would ensure full solubilization in ~30 min.
2. We use two 50 mL Steriflip filter tubes (cat. SCGP00525, Millipore-Sigma) to filter the injectable solution. Vacuum cup filters are also a good option. Syringe filters may be used but tend to create excessive pressure and may require great strength or break, especially for viscous vehicles.

3. The median survival of NKO mice in our colony is ~55 days of age using PicoLab® 20 chow. Disease progression is dependent on the composition of the chow, as synthetic diets (e.g., AIN-93G) can display severe neurobehavioral symptoms (e.g., clasping) beginning at 30 days of age and median life spans of ~40 days (*see* Table 1). The origin of this discrepancy, however, is unclear.

   We commonly use a minimum of 12 NKO mice (6 female and 6 male) for life span studies and other studies. Both NKO and companion mice are simultaneously treated. Studies that focus on small or sex-specific effects require higher animal numbers.

4. Tail tips tend to stick to the scissors. A good strategy is to leave the scissors in a microcentrifuge tube in tube rack while tending to the mouse's tail and returning it to its cage. Sometimes tail snips can fall off scissors. It is good to keep paper towels on hood surface beneath your workspace in case tail snip falls.

5. NKO mice display retarded growth and are usually smaller than littermates. NKO mice will often show loss of fur (alopecia) on their torso or thinning and rough coats around postnatal day 21 [13]. NKO behavior and physiology are largely similar to healthy mice until ~30 days postnatal when symptoms of encephalomyopathy manifest [5]. Clasping is the most noticeable neuropathological phenotype, in which NKO mice will curl their body toward their hindlimbs when suspended by the tail. Onset of clasping occurs in NKO mice between 35 and 53 days postnatal, with a median of ~41 days postnatal [6]. Neurological lesions begin to be observed by MRI imaging or other means at this time. The onset of clasping generally correlates with life span; however, it may no longer be noticeable when mice become too weak to display the phenotype. In early stages of neuropathic symptoms, mice also often display "helicoptering" behavior when suspended by the tail as well as circling behavior. As the disease progresses further mice exhibit decreased grip strength and balance, a loss of body fat, and decreases in core body temperature [6, 10]. NKO mice also have decreased motor coordination, lethargy, gait abnormalities with leg splaying and decreased hindlimb motility, difficulties in righting themselves, and severe ataxia [5]. NKO mice do not readily respond to stimulations such as light, sound, or prodding in these advanced stages of disease and may become blind and deaf [5].

6. Most animals will begin losing weight upon onset of clasping. The maximum weight is at ~38 days postnatal before rapid weight loss. However, rebounds in weight gain are possible, and mice may survive weeks after losing 20% of maximum

weight. Towards the end of their life span, weight loss is often more pronounced and is a good indicator of imminent death.

We have developed five surrogate end points to natural death for survival studies. These include:

First, the inability to eat or drink, including signs of dehydration such as dry flaky skin, shrunken look, segmented tail, thin bony paws. Second, severe lethargy, as indicated by lack of response such as reluctance to move when gently prodded or lifted. Third, severe balance or gait disturbance. Fourth, rapid weight loss and loss of over 30% of the maximum recorded body weight. Fifth, body condition score lower than 2.

Most commonly, mice falling under point 2 or 4 will die in the following 48 h and should be promptly euthanized and scored as dead. However, body weight may fall below the 30% threshold and rebound for weeks. In the absence of any of the other factors, a 24-h waiting period may be allowed prior to euthanasia, if body weight falls under 30% of the maximum. Conversely, some animals will show dramatic weight loss (over 1 g) over the course of 1–2 days and will die before falling below the 30% threshold.

7. Treatments are commonly initiated at either 10 days or 21 days of age. It is our impression that initiation of treatment at earlier stages leads to larger magnitude effects, and this has been our experience for rapamycin treatment by i.p. injection at 8 mg/kg/day. However, this may not be the case for all treatments. Rapamycin reduces rate of NKO growth most often requiring weaning at 28 days postnatal instead of the customary 21 days. Other drugs may be toxic at younger ages. Treatments using medicated food or medicated water normally begin at weaning, and thus other delivery methods, such as i.p. injection, are generally preferred.

   NKO mice show neuropathic symptoms of disease at ~35 days of age, and thus a 30-day old time point is appropriate for any studies evaluating initiation of neurodegeneration. The 50-day old time point is appropriate for any studies evaluating pathophysiology during severe mitochondrial disease. Studies that utilize time points later than 50 days may be complicated from artificial selection of long-lived NKO mice as high mortality is observed in NKO mice at these ages.

   For cross-sectional studies, we suggest collecting tissues at two different age points. The first age point should be when most mice are asymptomatic or just beginning to show disease phenotypes (postnatal day 30–35). The second age point should be when vehicle-treated and untreated mice show evident signs of disease (postnatal day 45–50). Late time points (e.g., postnatal day 60) can be considered when determining

Table 1
Diet composition impacts disease progression and survival in NKO mice

Diet	PicoLab® 20	AIN-93G	A14040402
Clasping onset (days)	42.4	34.9	38.5
Clasping range (days)	36–52	31–39	35–45
Mean maximum weight (g)	14.4	10.3	8.2
Median survival (days)	56.0	40.0	41.5
Mean survival (days)	58.2	41.7	45.2
Survival range (days)	40–84	33–56	39–69
N	24	12	11

the efficacy of a successful treatment on pathological lesions and specific molecular features. However, less than half of untreated NKO mice will survive to that age, so careful experimental planning is advised.

When collecting tissues for immunoblotting, we employ a fast/refeed regimen as described in the **Methods** section. Fasting overnight and refeeding for ~3 h leads to similar metabolic responses between mice and sufficient activation of nutrient-sensitive signaling pathways. It is critical to maintain this collection window within 1.5 h of commencement between cohorts.

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