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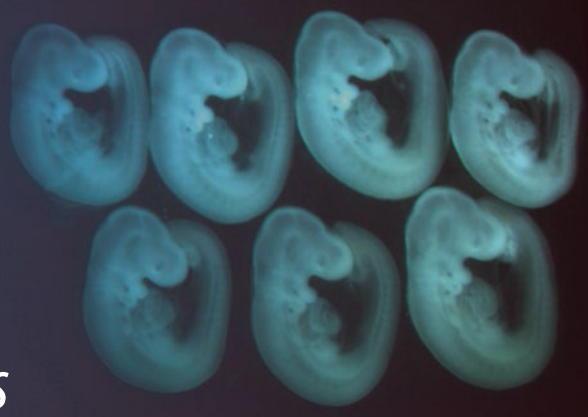

Jason M. Hansen
Louise M. Winn *Editors*

Developmental Toxicology

Methods and Protocols

Second Edition

Humana Press

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School of Life and Medical Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

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Second Edition

Edited by

Jason M. Hansen

Department of Physiology and Developmental Biology, Brigham Young University, Provo, UT, USA

Louise M. Winn

Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada

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Editors
Jason M. Hansen
Department of Physiology and Developmental Biology
Brigham Young University
Provo, UT, USA

Louise M. Winn
Biomedical and Molecular Sciences
Queen's University
Kingston, ON, Canada

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Preface

Developmental toxicology is a unique, integrated field, containing concepts from developmental biology, embryology, nutrition, genetics, cellular and molecular biology, and general toxicology. Assimilation of these diverse disciplines provides a specific means to evaluate alterations to normal developmental patterning and signaling and to assess mechanisms of congenital malformations, changes to neurobehavioral status, and increased embryonic death. Amazingly, nearly approximately 3% of all live births manifest with a structural or functional birth defect, but this statistic, although alarming, may likely be an underestimation of the overarching developmental problem at hand as many in utero effects may manifest later in life, supporting the fetal basis of disease. The connection between in utero exposures and postnatal, adult health consequences has become an emphasized focus to thereby further our understanding of embryonic and fetal exposure effects. These studies become paramount to more fully understanding other causes of many diseases that are more prevalent in later life, while not specifically apparent at birth. As the notion of the fetal basis of disease is developed, characterizing contributing developmental factors and mechanisms as a result of prenatal exposure becomes increasingly important.

Several methodological approaches can be extremely informative for our understanding of developmental toxicant mechanisms, characterization of developmental outcomes, and development of potential interventions that are clinically relevant. Due to the unique nature of development itself, these approaches can be modified to capture specific aspects of development, including proliferation, differentiation, apoptosis, migration, and morphology. Many of these approaches are found in this volume. We regret that not all developmental toxicology methods are fully represented here but feel that many of the popular, staple and newer, state-of-the-art approaches found in this volume will be beneficial and provide readers with techniques for tackling important developmental toxicology questions. Here, we focus on numerous cellular models (induced pluripotent stem cells, neural crest culture, etc.), some less frequently used but important animal models (chick, zebrafish), in vitro approaches using whole embryos (rat, mouse, and rabbit), and specific outcome methodologies to assess changes on the morphological to molecular level. We anticipate that as our knowledge of development and developmental toxicology progresses, new, exciting methods will emerge to support more investigation into specific mechanisms and outcomes with developmental exposures.

Provo, UT, USA — *Jason M. Hansen*
Kingston, ON, Canada — *Louise M. Winn*

Contents

Contributors

Barbara D. Abbott • *National Health and Environmental Effects Research Laboratory, Toxicity Assessment Division, Office of Research and Development, US Environmental Protection Agency, Research Triangle Park, NC, USA*

Joslynn G. Affleck • *Department of Biology, Vancouver Island University, Nanaimo, BC, Canada*

Jonathan K. Alder • *Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA*

Joe A. Arosh • *Department of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, USA*

Kathryn Bambino • *Department of Environmental Medicine and Public Health, Icahn School of Medicine at Mount Sinai, New York, NY, USA*

Sakhila K. Banu • *Department of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, USA*

Jeffery R. Barrow • *Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, Provo, UT, USA*

Cecilia Berg • *Department of Environmental Toxicology, The Center for Reproductive Biology in Uppsala (CRU), Uppsala University, Uppsala, Sweden*

Shama Bhatia • *Department of Pharmaceutical Sciences and Centre for Pharmaceutical Oncology, University of Toronto, Toronto, ON, Canada*

Jaime Chu • *Department of Pediatrics, Icahn School of Medicine at Mount Sinai, New York, NY, USA*

Nathan Cook • *Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, Provo, UT, USA*

Danielle M. Drake • *Department of Pharmaceutical Sciences and Centre for Pharmaceutical Oncology, University of Toronto, Toronto, ON, Canada*

Charles A. Easley • *Department of Environmental Health Science, University of Georgia College of Public Health, Athens, GA, USA*

Miho K. Furue • *Laboratory of Stem Cell Cultures, National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Ibaraki, Japan*

Korance Goodwin • *Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, Provo, UT, USA*

Michelle Graf • *Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, Provo, UT, USA*

Barbara F. Hales • *Department of Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada*

Jason M. Hansen • *Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, Provo, UT, USA*

Craig Harris • *Department of Environmental Health Sciences, School of Public Health, University of Michigan, Ann Arbor, MI, USA*

Jonathon T. Hill • *Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, Provo, UT, USA*

Patricia B. Hoyer • *Department of Physiology, University of Arizona, Tucson, AZ, USA*
Chunwei Huang • *Department of Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada*
Timothy Leishman • *Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, Provo, UT, USA*
Rebecca D. Maciver • *Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada*
Jason Matthews • *Department of Pharmacology and Toxicology, University of Toronto, Toronto, ON, Canada; Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway*
Omar J. Mohammed • *Faculty of Medicine and Health Sciences, School of Life Sciences, Medical School, QMC, University of Nottingham, Nottingham, UK*
Joshua Morrison • *Department of Pediatrics, Icahn School of Medicine at Mount Sinai, New York, NY, USA*
Terence R. S. Ozolinš • *Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada*
France-Hélène Paradis • *Department of Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada*
Ted B. Piorczynski • *Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, Provo, UT, USA*
Margaret K. Pratten • *Faculty of Medicine and Health Sciences, School of Life Sciences, Medical School, QMC, University of Nottingham, Nottingham, UK*
John M. Rogers • *Toxicity Assessment Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, United States Environmental Protection Agency, Research Triangle Park, NC, USA*
Micah M. Ross • *Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, Provo, UT, USA*
Robert E. Seegmiller • *Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, Provo, UT, USA*
Aaron M. Shapiro • *Department of Pharmaceutical Sciences, University of Toronto, Toronto, ON, Canada; British Columbia Provincial Toxicology Centre, Vancouver, BC, Canada*
Jone A. Stanley • *Department of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, USA*
Michael R. Stark • *Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, Provo, UT, USA*
Mika Suga • *Laboratory of Stem Cell Cultures, National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Ibaraki, Japan*
Arminda Suli • *Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, Provo, UT, USA*
Emily W. Y. Tung • *Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada*
Virginia K. Walker • *Department of Biology, Queen's University, Kingston, ON, Canada*
Brendon K. Warner • *Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, Provo, UT, USA*

Peter G. Wells • *Department of Pharmaceutical Sciences and Centre for Pharmaceutical Oncology, University of Toronto, Toronto, ON, Canada; Department of Pharmacology and Toxicology, University of Toronto, Toronto, ON, Canada*

Louise M. Winn • *Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada; School of Environmental Studies, Queen's University, Kingston, ON, Canada*

Han Yan • *Department of Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada*

Robert M. Zucker • *Toxicity Assessment Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, US Environmental Protection Agency, Research Triangle Park, NC, USA*

Chapter 1

In Vitro Models in Developmental Toxicology

Louise M. Winn

Abstract

Developmental toxicity associated with exposure to exogenous compounds such as drugs and environmental chemicals can be assessed using a variety of different in vitro models, each with their own advantages and disadvantages. These models include cultured cells (Chapters 3–6), organ and tissue cultures (Chapters 7 and 8), and whole embryo cultures (Chapters 13–15) and typically support the guiding principles of the three Rs: replace, reduce, and refine. These models can be used in early chemical screens and have helped further our understanding into the mechanisms associated with developmental toxicity following exposure to many chemicals.

Key words Cell culture, Tissue culture, Organ culture, Whole embryo culture, In vitro methods, Development, Toxicant

1 Introduction

In normal embryonic development, cells proliferate extensively, differentiate, and undergo cell death, all in a tightly controlled, spatial, temporal, and tissue-specific fashion. Therefore, exposures to drugs or environmental chemicals that result in alterations in these processes during development have the potential to cause developmental toxicity. This toxicity can range from embryo death to abnormal development resulting in structural defects such as cleft lip and palate and biochemical and functional malformations, including long-term neurological deficits. There are several in vitro models that can be used to assess this toxicity, each with their own advantages and disadvantages, which should be carefully considered when deciding how to approach answering specific experimental questions [1, 2]. These models all include some type of biological constituent that can undergo physiological activities that allows the investigator to study processes such as differentiation, proliferation, and cell death and include cultured cells (Chapters 3–6), organ and tissue cultures (Chapters 7 and 8), and whole embryo cultures (Chapters 13–15).

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Generally speaking, in vitro studies are less expensive, require less time to complete, can be more flexible in terms of avenues of investigation, and require the use of fewer animals when compared to in vivo animal studies. In vitro models also impart the investigator the advantage of having precise control over toxicant exposure conditions, including concentration, timing of exposure, and length of exposure. Timing of exposure can be significant given the dynamic nature of mammalian development and the known principle of critical periods of susceptibility in teratogenesis. Therefore, overall in vitro models usually support the guiding principles of the three Rs: replace, reduce, and refine [3]. In addition, in vitro models break down the physiological complexity of the whole system, which has its advantages but also a clear disadvantage of not replicating the interrelationships that exist in the body between the various organs and tissues. Furthermore, these models also remove any maternal factors, including nutritional and metabolic pathways that may influence toxicity outcome. Therefore, choice of what specific in vitro model to adopt will depend on the goal of the study and whether the model includes the necessary molecular pathways and biological processes to provide meaningful answers.

2 Cell Cultures

2.1 Embryonic Stem Cell Cultures

Elucidating the mechanisms governing developmental toxicity can be aided by the use of in vitro cell culture models. One example is the use of cultured primary murine embryonic stem cells, which are derived from the inner cells of the blastocyte and therefore pluripotent. This gives the investigator the ability to manipulate the cells so that they differentiate into a variety of different cell types depending upon the components of the culture media, for example, cardiomyocytes or osteoblasts [4–6]. The impacts of exposure to a potential developmental toxicant can then be assessed with respect to effects on differentiation and cell death, including the molecular and biochemical pathways governing these processes. In addition, cell-specific toxicity can also be evaluated which can be highly relevant given the known cell-specific susceptibility versus resistance to various toxicants. In these types of investigations, species differences in the way a particular toxicant is metabolized can be overcome by including the appropriate metabolizing system or specific metabolites themselves.

Stem cells can also be generated through the reprogramming of somatic cells into pluripotent stem cells (Chapter 4). For example, Palmer et al. [7] have developed a human-induced pluripotent stem (iPS) cell-based assay to assess changes in metabolism to predict the developmental toxicity potential of a given toxicant (devTOX *quick*Predict). This assay specifically determines changes in

the levels ornithine and cystine, which are metabolic biomarkers, over a broad dose-response range [7].

2.2 Primary Cell Cultures

A variety of different embryonic primary cells can also be cultured including neural crest cells (Chapter 5). These cells can also be induced to differentiate, proliferate, and migrate to form important embryonic cellular structures including neurons and glia [8, 9]. Isolated embryonic fibroblasts (Chapter 3), cardiomyocytes, and dorsal root ganglia are some other examples of primary cell cultures that have been widely used to study developmental toxicity. We have also isolated primary liver cells from gestational day 14 fetal mice to specifically study mechanisms of developmental hematotoxicity induced by toxicants such as benzene [10]. The fetal liver is of interest because, during gestational days 12–14.5 in the mouse, the liver contains the highest population of hematopoietic stem cells and is the primary site of hematopoiesis prior to the site of hematopoiesis switching to the spleen followed by the bone marrow as the fetus matures [11].

2.3 Immortalized Cell Cultures

The use of cultured immortalized cells in vitro can also be a beneficial in vitro tool. For example, we have used the immortalized cell line P19 in our laboratory to study the mechanisms of toxicity of the anticonvulsant drug valproic acid [12]. The P19 cell line is derived from a gestational day 7.5 mouse embryo transplanted from the uterus into the testes of another mouse resulting in the formation of an embryonal carcinoma [13]. These pluripotent P19 cells have been used as an early model of embryonic development when cultured in their undifferentiated state, although they can be induced chemically to differentiate into cardiovascular and neuronal cell types [13]. P19 cells have been used extensively in developmental toxicology studies, especially in the context of valproic acid-mediated transcriptional alterations [14, 15]. Previous studies have evaluated both short- (<6 h) and long-term (24 h) valproic acid exposures in P19 cells and mouse embryos and have demonstrated a high correlation of gene expression changes using microarray analysis [14, 15]. Furthermore, many of the genes similarly altered in both P19 cells and mouse embryos following valproic acid exposure have also been implicated in neural tube defects. Although there may be concerns over the use of embryonal carcinoma cells in terms of relevance to embryonic development, a subsequent study has demonstrated a high concordance between the observed transcriptional changes following valproic acid exposure in mouse embryonic stem cells, P19 cells, and mouse embryos [16]. It is however important to recognize the limitations of using immortalized cells given that they typically lack significant biotransformation capacity and cell population diversity and acquire mutations resulting from immortalization [17].

3 Micromass and Organ Cultures

Micromass culture systems from species such as mouse, rat, and chicken can be created by seeding a high density of primary cells isolated from various embryonic structures such as limbs (Chapter 6), brain, and palate during the early stages of gestation. During culture the cells will organize themselves and come together and form micromasses. The effects of potential developmental toxicants can then be evaluated for their impact on a variety of endpoints such as cell-to-cell communication, cell division, differentiation, and so on.

Additionally, whole embryonic organs such as limbs (Chapter 7), ovaries (Chapter 8), palate, and eyes can be microdissected from several species and grown in culture. Since these are intact systems that grow and differentiate to a significant degree of intricacy, they are considered to embody an intermediate level of complexity (Chapter 7). Once isolated these cultures can be exposed to potential toxicants, either as a single or repeated dose, for various lengths of time depending upon the endpoints assessed in the study. Endpoints can include morphological considerations and biochemical measurements including impacts on mRNA and protein expression.

4 Whole Embryo Cultures

Rodent embryo cultures (Chapters 13 and 14) first developed in the new laboratory in 1978 have been routinely used in several laboratories to study the mechanism of action of various developmental toxicants [18–22]. This approach has also expanded to include rabbit embryo culture (Chapter 15) and chick embryo cultures. These models offer several advantages over in vivo studies including (1) the removal of potential maternal and paternal confounding factors; (2) visualization and selection of embryos that are within a defined developmental stage, which may decrease variability seen in vivo where littermates may be at different stages of development; and (3) the ability to have direct control over chemical concentrations, including toxicants, probes, and potential embryoprotective additives.

5 Summary

In conclusion, in vitro approaches offer additional tools to further our understanding of developmental toxicology. Some of these models are described in detail in the following chapters, specifically giving detailed applicability to each model with respect to predicting

potential developmental toxicity. Future directions will likely involve integrated testing strategies with the aim of reducing the number of experimental animals while increasing the accuracy and predictability of potential toxicity associated with new drugs and chemicals.

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Chapter 2

Use of Primary Mouse Embryonic Fibroblasts in Developmental Toxicity Assessments

Jason M. Hansen and Ted B. Piorczynski

Abstract

Mouse embryonic fibroblasts (MEFs) are commonly collected as a means to maintain the culture and growth of embryonic stem cells (ESCs). However, their utility can extend well beyond their use exclusively in ESC culture. With collection from various transgenic mouse models, use of MEFs may serve as a more simplistic means to reconstitute in vivo/in utero toxicological assessments in an in vitro format for evaluation of function of specific proteins during toxic insults. The ease of collection, rapid growth kinetics, and large-scale expansion to perform multiple, high-throughput experiments are just some of the advantages of MEF use. Here, we describe procedures for successful MEF isolation and culture. As an example of MEF utility, we use MEFs collected form wild-type (WT) and Nrf2 knockout mice. After collection, MEFs were pretreated with the Nrf2 activator, dithiol-3-thione (D3T; 10 μM) for 12 h, and then treated with either hydrogen peroxide (0–2000 μM) or mercury (0–100 μM) for another 24 h. Viability was measured via MTT assay after 24 h of treatment.

Key words Mouse embryonic fibroblasts, MEF, Nrf2, Transgenic

1 Introduction

Mouse embryonic fibroblasts (MEFs) are easily collected from embryos on gestational day (GD) 14.5–15.5. These cells are commonly used as feeder layers in the maintenance of embryonic stem cells (ESCs) and are preferential as a substrate as they help to maintain ESC pluripotency, enhance plating efficiency, and support ESC growth and survival [1]. However, MEF use can be expanded beyond their use as merely a feeder layer and a support cell in ESC culturing methodologies.

Previous studies have used MEFs as an "embryonic" model for better understanding the mechanism of action for specific toxicants. For example, fumonisin B1 (FB1) is a mycotoxin found in corn, and exposure to FB1 is correlated to the manifestation of neural tube defects (NTDs) in humans and in animal models [2–4]. Results showed that FB1 inhibits ceramide synthase causing sphingosine

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kinase-mediated production of sphinganine-1-phosphate (SaP1) from sphinganine [5]. SaP1 is an inhibitor of histone deacetylases (HDACs), supporting the hypothesis that FB1-induced SaP1 accumulation and subsequent HDAC inhibition are potential contributors to NTD pathologies.

Use of MEFs can also be advantageous to evaluate protein function. To evaluate cadmium (Cd) toxicity, MEFs were isolated from wild-type and Nrf2 knockout (KO) mice. Data show that Cd causes a significant increase in reactive oxygen species (ROS) generation in wild-type MEFs, but the Cd effect was exacerbated in Nrf2 KO MEFs, which were more highly sensitized to Cd-induced cell death [6]. Nrf2 is a transcription factor that is regulated through ubiquitination and proteasomal degradation during unstressed periods but is largely responsible for the upregulation of antioxidant and phase II detoxification genes during episodes of oxidative stress. In wild-type MEFs, the expression of the cytoprotective genes, NADPH/quinone oxidoreductase-1 (NQO1) and heme oxygenase-1 (HO1), was increased but not in Nrf2 KO MEFs, suggesting that Cd instigates an antioxidant response through Nrf2 activities capable of conferring protection from cell death. In another study, MEFs lacking the polyubiquitin (Ubc) gene demonstrated increase Nrf2-related gene expression with arsenite treatment, but cell death was not decreased [7]. These data suggest that arsenite toxicity in Ubc-deficient MEFs is not due to the disruption of the Nrf2 pathway but rather was demonstrated to be a result of the accumulation of misfolded proteins yielding an unfolded protein response.

In some cases, MEF methods are advantageous as knockout MEFs can be collected from mice where the deletion does not yield viable offspring. For example, the p65 subunit of NF-kappaB deletion is embryonic lethal, where on GD 15–16 massive hepatic apoptosis is observed [8], but MEFs are still isolatable. MEFs lacking p65 (−/−) were exposed to the teratogen, methotrexate [9]. p65 −/− MEFs were more susceptible to methotrexate-induced cell death than wild-type MEFs, suggesting a protective role of NF-kappaB during embryonic exposures.

Another benefit of MEF use is that they retain some differentiation capacities. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a polyaromatic hydrocarbon that can cause cleft palate, dental abnormalities, hydronephrosis, and reproductive organ malformations during development [10–13]. Using TCDD, MEFs were pretreated and then differentiated into adipocytes. MEFs demonstrated markers of adipogenesis, but with TCDD pretreatments, adipogenesis was significantly attenuated [14]. In c-Src-deficient MEFs, TCDD was unable to decrease the expression of these

important adipogenic regulators, suggesting a role of c-Src in TCDD-mediated disruption of adipogenic differentiation.

In the above studies (and many others not listed here), MEFs have proven to be a beneficial tool, and their use has some unique advantages for the assessment of developmental toxicity and the study of mechanisms of birth defects. These advantages include the ease of MEF collection, the use of a primary cell model, the means to study differentiation, and the collection of cells from specific transgenic animal models. Here, we describe MEF isolation procedures and evaluate transgenic MEFs responses to toxicants to demonstrate their usefulness.

2 Materials

All reagents and equipment are readily available from commercial sources.

2.1 Embryo Removal and Mouse Embryonic Fibroblast Isolation

1. Gestational day (GD) 14.5–15.5 time-mated, primigravida pregnant mice, 8–12 weeks old (*see* **Note 1**). C57Bl6 mice are generally used for MEF isolation (*see* **Note 2**).
2. Forceps.
3. Microforceps.
4. Dissection scissors.
5. Hank's Balanced Salt Solution.
6. 10 cm sterile petri dishes.
7. Phosphate-buffered saline (PBS).
8. Razor blades.
9. 70% ethanol.
10. Dissection microscope.
11. Trypsin (0.25%) with EDTA.
12. Dulbecco's Modified Eagles Medium (DMEM) with high glucose, phenol red, and glutamine.
13. Fetal bovine serum (FBS).
14. Water bath.
15. Pasteur pipettes with rubber bulbs (modified; *see* **Note 3**).
16. Cell strainers (40 μm).
17. 15–50 mL centrifuge tube.
18. Centrifuge.
19. Hemocytometer.
20. Cell culture incubator.

3 Methods

3.1 Mouse Breeding

Primigravida female C57Bl6 mice (8–12 weeks old) are housed with a C57Bl6 male at a ratio of 2:1 from 1800 h to 0600 h the following day. At 0600 h, a probe is used to check for the presence of a vaginal plug as evidence of mating. If positive, the dam is removed and placed in a cage to be singly housed.

3.2 Euthanasia

Mice are placed in a container and gassed with 100% CO_2 for 10 min. Mice are monitored for loss of consciousness and are removed when unresponsive to a toe pinch.

3.3 Laparotomy and Uterus Removal

1. Mice are placed ventral side up and are swabbed generously with 70% ethanol to disinfect the area and wet the fur. A full-length midline abdominal incision is made with scissors from the urethral opening to the sternum to expose the abdominal cavity. A second incision can be made along the body wall one-third the way along the midline incision for better abdominal cavity viewing.
2. The gravid uterus is then removed by pinching the cervix tightly with a pair of forceps and cutting between the cervix and vagina. At the same time, lift the cervix to pull the uterus upward until the ovaries are visible. The uterus is bicornate. A final cut can be made just beyond the ovaries to free the uterus, leaving the ovaries attached to the uterus. The uterus is placed in Hank's Balanced Salt Solution (*see* **Note 4**) (Fig. 1).

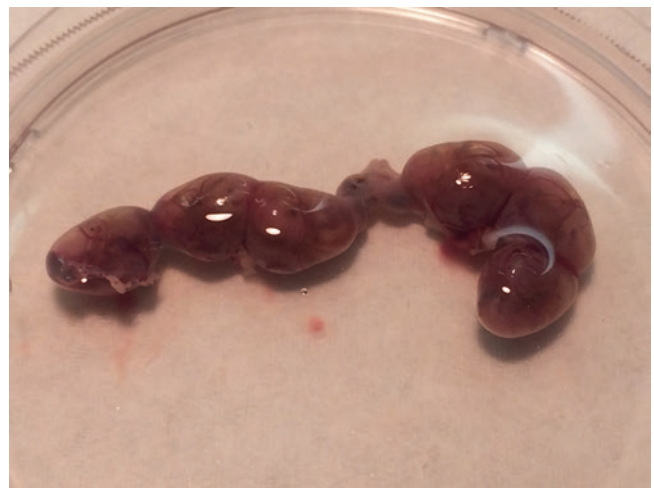

Fig. 1 Gravid uterus on gestational day 15. The uterus contains numerous conceptuses

3.4 Removal of Embryos

1. The gravid uterus is washed with fresh HBSS and then placed in a petri dish with a silicone-covered bottom. The dish is filled with HBSS. The uterus is pinned just below the ovaries with needles to the silicone. The uterus should be stretched as this will aid in making incisions through the myometrium but not overstretched.
2. Starting at one ovary, a small incision is made that is large enough to insert a prong of the dissection scissors. As additional cuts are made along the original incision, the incision will move toward the cervix in a straight line. This will expose the embryos.
3. Embryos are removed by gently scraping the embryos out of the uterus by blunt dissection. Embryos are then freed into the HBSS (Fig. 2).

3.5 Embryo Preparation

1. Embryos are carefully prepared by removing all extraembryonic membranes and exogenous tissues (e.g., placenta) (Fig. 3). They are placed into a new petri dish with fresh HBSS (*see* **Note 5**).
2. The head is removed with dissection scissors, and the internal organs can be removed through blunt dissection with forceps (Fig. 4). Wash embryos thoroughly to assure removal of all internal organs. Discard head and internal organs (*see* **Note 6**) (Fig. 5).
3. Remove the remaining trunk to a dry petri dish.
4. Using a sterile, new razor blade, mince the embryos in a sterile environment (Fig. 6). Mince continuously for approximately 10 min and avoid any large chunks of tissue. The consistency should be like a viscous liquid.

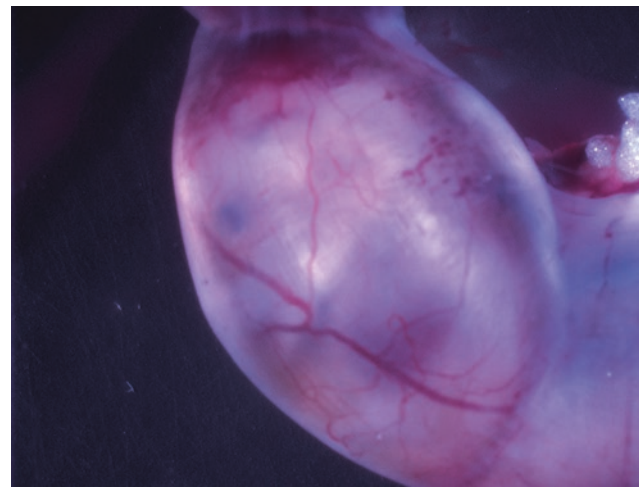

Fig. 2 Gestational day 15 mouse embryo after removal from the uterus. Extraembryonic membranes are still intact

Fig. 3 Gestational day 15 mouse embryo following removal from the uterus and the removal of extraembryonic membranes

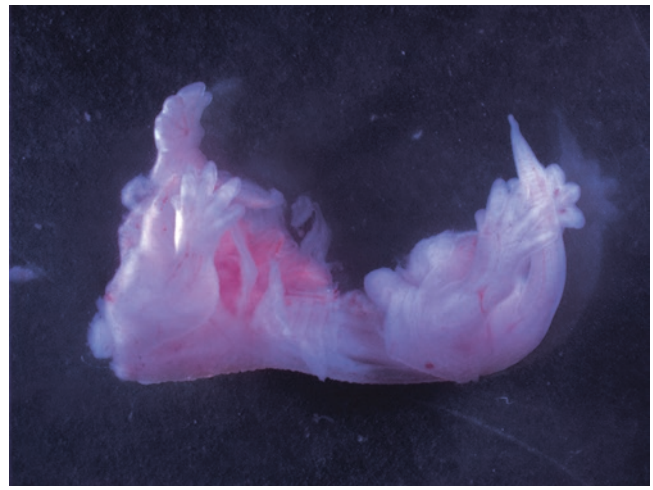

Fig. 4 Gestational day 15 mouse embryo following evisceration (liver and intestines) and removal of the head. Hepatic and neuronal cells can interfere with mouse embryonic fibroblast grown and take over a culture. Removal of these tissues allows for the optimization of fibroblast isolation

3.6 Mouse Embryo Fibroblast Isolation

1. Collect minced embryos with a pipette and place in a 50 mL centrifuge tube (*see* **Note 7**).
2. Add approximately 5 mL of 0.25% trypsin with EDTA for every embryo. Vortex briefly to break up isolate.
3. Place in water bath at 37 °C for approximately 45–60 min with agitation every 10–15 min.

Fig. 5 Complete dissection of gestational day 15 embryo with the liver, intestines, and head removed

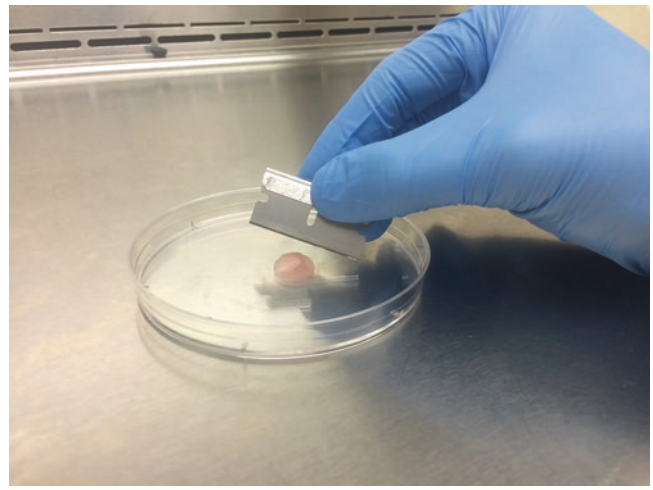

Fig. 6 Embryo mincing using a sterile razor blade. Embryos are minced thoroughly in a sterile plastic petri dish performed in a sterile culture hood. Mince for 10 min and avoid chunks until tissue has the consistency of a viscous liquid as shown here

4. After trypsinization, use a modified Pasteur pipette (*see* **Note 3**) and pipette the solution up and down several times to promote disassociation.
5. Add 1 mL of FBS for every embryo in the solution to stop trypsinization and mix gently.
6. Using a cell strainer (40 μm), strain the cell containing solution into a new 50 mL centrifuge tube. This removes partially dissociated material.

7. Centrifuge the collection for 5 min at 1200 × *g* at room temperature.
8. Resuspend pelleted cells in growth media (DMEM with 10% FBS and antibiotics).
9. Seed cells into a 100 mm tissue culture plate and place into a humidified incubator at 37 °C with 5% CO_2 (*see* **Note 8**). This is passage 0 (*see* **Note 9**).
10. Replace media the following day.

3.7 Maintenance of Isolated Mouse Embryonic Fibroblasts

1. Cultured cells should grow rapidly and reach confluence with 48–72 h (Fig. 7).
2. Passages should be at a 1:4 dilution (every plate should be split into four plates of equal size) following trypsinization with 0.25% trypsin with EDTA (*see* **Note 10**).

3.8 Example of MEF Use

Mouse embryonic fibroblasts were collected from wild-type and Nrf2 knockout (−/−) (B6.129X1-*Nfe2l2*^{tm1Ywk}/J) mice from Jackson Laboratories as outlined in Subheading 3 above. Nrf2 is a transcription factor that regulates the antioxidant response. Under periods of oxidative stress, Nrf2 is activated and upregulates many antioxidant and phase II detoxification genes [15]. The purpose of this small experiment is to determine if Nrf2 activation prior to oxidant exposure (hydrogen peroxide [H_2O_2] and mercury chloride [Hg]) yields protection against MEF death. On passage 2, MEFs were plated in a 96-well plate and, after 24 h, were confluent. Confluent cultures were treated overnight with an Nrf2 inducer, dithiole-3-thione (10 μM; D3T) [16]. The following morning, MEFs were treated with 0–2000 μM of H_2O_2 or

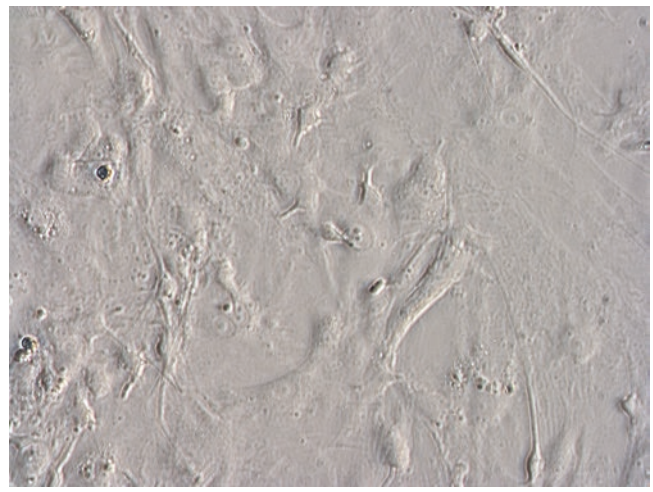

Fig. 7 After plating, mouse embryonic fibroblasts will adhere to the plate. They can be expanded after initial plating

0–100 μM of Hg for 24 h. After 24 h of exposure, MEF viability was assessed via MTT assay as described elsewhere [17].

Data in wild-type MEFs show that both H_2O_2 and Hg decrease cell viability in a dose-dependent manner (Fig. 8a, b, respectively). Interestingly, pretreatment with D3T increased cellular viability at similar concentrations that were toxic, suggesting that pre-activation of an Nrf2-mediated antioxidant response provides increase protection from either H_2O_2 or Hg. To verify the role of Nrf2, Nrf2 KO MEFs were treated in identical fashion. As expected, non-pretreated Nrf2 KO MEFs demonstrated increased toxicity in a dose-dependent manner similar to what was observed

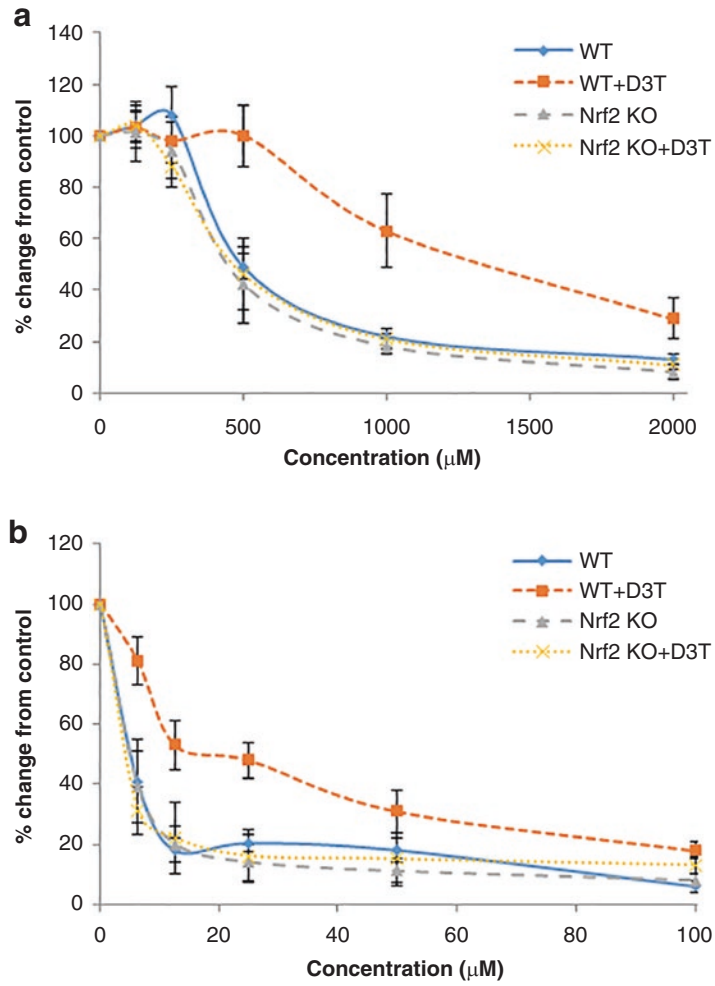

Fig. 8 Comparison between mouse embryonic fibroblasts collected from wild-type (WT) and Nrf2 knockout (Nrf2 KO) gestational day 15 mice. Using a Nrf2 inducer, D3T, to promote an antioxidant response, fibroblasts were then treated with increasing concentrations of either (**a**) hydrogen peroxide or (**b**) mercury chloride (Hg) for 24 h after which cell viability was assessed. Treatment with D3T showed protection in WT fibroblasts but not in Nrf2 KO fibroblasts